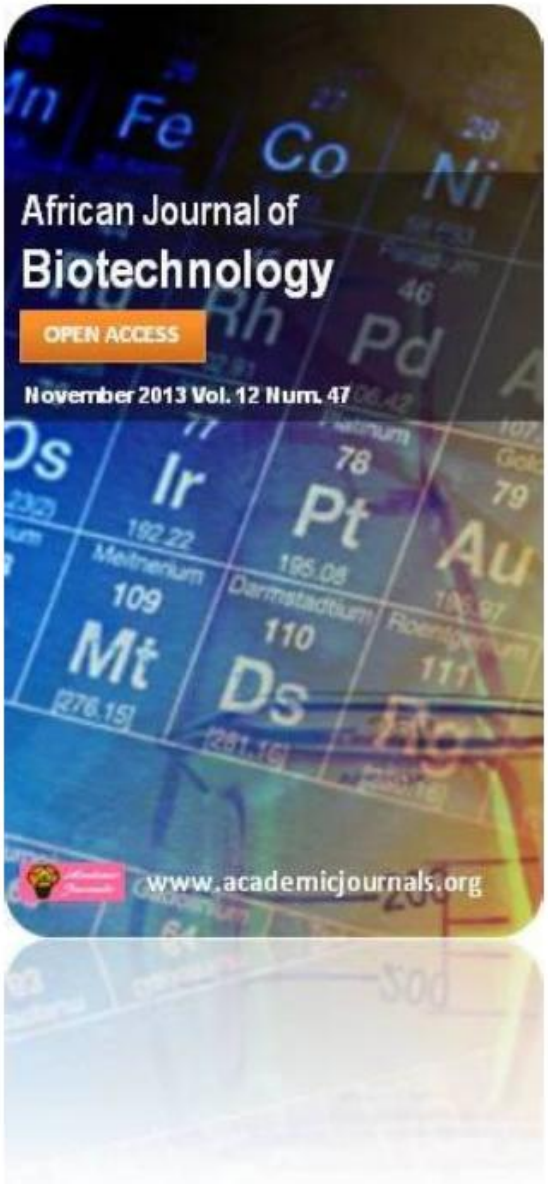


African Journal of Biotechnology

November 2013 - Vol. 12 Num. 47

Submit manuscripts: www.ms.academicjournals.org

Editorial Office: ajb@academicjournals.org

URL: www.academicjournals.org

academicJournals

ABOUT AJB

The **African Journal of Biotechnology (AJB)** (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Submission of Manuscript

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajb@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajb@academicjournals.org.

Editor-In-Chief

George Nkem Ude, Ph.D
Plant Breeder & Molecular Biologist
Department of Natural Sciences
Crawford Building, Rm 003A
Bowie State University
14000 Jericho Park Road
Bowie, MD 20715, USA

Editor

N. John Tonukari, Ph.D
Department of Biochemistry
Delta State University
PMB 1
Abraka, Nigeria

Associate Editors

Prof. Dr. AE Aboulata
Plant Path. Res. Inst., ARC, POBox 12619, Giza, Egypt
30 D, El-Karama St., Alf Maskan, P.O. Box 1567,
Ain Shams, Cairo,
Egypt

Dr. S.K Das
Department of Applied Chemistry
and Biotechnology, University of Fukui,
Japan

Prof. Okoh, A. I.
Applied and Environmental Microbiology Research
Group (AEMREG),
Department of Biochemistry and Microbiology,
University of Fort Hare.
P/Bag X1314 Alice 5700,
South Africa

Dr. Ismail TURKOGLU
Department of Biology Education,
Education Faculty, Fırat University,
Elazığ,
Turkey

Prof T.K.Raja, PhD FRSC (UK)
Department of Biotechnology
PSG COLLEGE OF TECHNOLOGY (Autonomous)
(Affiliated to Anna University)
Coimbatore-641004, Tamilnadu,
INDIA.

Dr. George Edward Mamati
Horticulture Department,
Jomo Kenyatta University of Agriculture
and Technology,
P. O. Box 62000-00200,
Nairobi, Kenya.

Dr. Gitonga
Kenya Agricultural Research Institute,
National Horticultural Research Center,
P.O Box 220,
Thika, Kenya.

Editorial Board

Prof. Sagadevan G. Mundree
Department of Molecular and Cell Biology
University of Cape Town
Private Bag Rondebosch 7701
South Africa

Dr. Martin Fregene
Centro Internacional de Agricultura Tropical (CIAT)
Km 17 Cali-Palmira Recta
AA6713, Cali, Colombia

Prof. O. A. Ogunseitan
Laboratory for Molecular Ecology
Department of Environmental Analysis and Design
University of California,
Irvine, CA 92697-7070. USA

Dr. Ibrahima Ndoye
UCAD, Faculte des Sciences et Techniques
Departement de Biologie Vegetale
BP 5005, Dakar, Senegal.
Laboratoire Commun de Microbiologie
IRD/ISRA/UCAD
BP 1386, Dakar

Dr. Bamidele A. Iwalokun
Biochemistry Department
Lagos State University
P.M.B. 1087. Apapa – Lagos, Nigeria

Dr. Jacob Hodeba Mignouna
Associate Professor, Biotechnology
Virginia State University
Agricultural Research Station Box 9061
Petersburg, VA 23806, USA

Dr. Bright Ogheneovo Agindotan
Plant, Soil and Entomological Sciences Dept
University of Idaho, Moscow
ID 83843, USA

Dr. A.P. Njukeng
Département de Biologie Végétale
Faculté des Sciences
B.P. 67 Dschang
Université de Dschang
Rep. du CAMEROUN

Dr. E. Olatunde Farombi
Drug Metabolism and Toxicology Unit
Department of Biochemistry
University of Ibadan, Ibadan, Nigeria

Dr. Stephen Bakiamoh
Michigan Biotechnology Institute International
3900 Collins Road
Lansing, MI 48909, USA

Dr. N. A. Amusa
Institute of Agricultural Research and Training
Obafemi Awolowo University
Moor Plantation, P.M.B 5029, Ibadan, Nigeria

Dr. Desouky Abd-El-Haleem
Environmental Biotechnology Department &
Bioprocess Development Department,
Genetic Engineering and Biotechnology Research Institute (GEBRI),
Mubarak City for Scientific Research and Technology Applications,
New Burg-Elarab City, Alexandria, Egypt.

Dr. Simeon Oloni Kotchoni
Department of Plant Molecular Biology
Institute of Botany, Kirschallee 1,
University of Bonn, D-53115 Germany.

Dr. Eriola Betiku
German Research Centre for Biotechnology,
Biochemical Engineering Division,
Mascheroder Weg 1, D-38124,
Braunschweig, Germany

Dr. Daniel Masiga
International Centre of Insect Physiology and Ecology,
Nairobi,
Kenya

Dr. Essam A. Zaki
Genetic Engineering and Biotechnology Research Institute, GEBRI,
Research Area,
Borg El Arab, Post Code 21934, Alexandria
Egypt

Dr. Alfred Dixon
International Institute of Tropical Agriculture (IITA)
PMB 5320, Ibadan
Oyo State, Nigeria

Dr. Sankale Shompole
Dept. of Microbiology, Molecular Biology and Biochemisty,
University of Idaho, Moscow,
ID 83844, USA.

Dr. Mathew M. Abang
Germplasm Program
International Center for Agricultural Research in the Dry Areas
(ICARDA)
P.O. Box 5466, Aleppo, SYRIA.

Dr. Solomon Olawale Odemuyiwa
Pulmonary Research Group
Department of Medicine
550 Heritage Medical Research Centre
University of Alberta
Edmonton
Canada T6G 2S2

Prof. Anna-Maria Botha-Oberholster
Plant Molecular Genetics
Department of Genetics
Forestry and Agricultural Biotechnology Institute
Faculty of Agricultural and Natural Sciences
University of Pretoria
ZA-0002 Pretoria, South Africa

Dr. O. U. Ezeronye
Department of Biological Science
Michael Okpara University of Agriculture
Umudike, Abia State, Nigeria.

Dr. Joseph Hounhouigan
Maître de Conférence
Sciences et technologies des aliments
Faculté des Sciences Agronomiques
Université d'Abomey-Calavi
01 BP 526 Cotonou
République du Bénin

Prof. Christine Rey
Dept. of Molecular and Cell Biology,
University of the Witwatersand,
Private Bag 3, WITS 2050, Johannesburg, South Africa

Dr. Kamel Ahmed Abd-Elsalam
Molecular Markers Lab. (MML)
Plant Pathology Research Institute (PPathRI)
Agricultural Research Center, 9-Gamma St., Orman, 12619,
Giza, Egypt

Dr. Jones Lemchi
International Institute of Tropical Agriculture (IITA)
Onne, Nigeria

Prof. Greg Blatch
Head of Biochemistry & Senior Wellcome Trust Fellow
Department of Biochemistry, Microbiology & Biotechnology
Rhodes University
Grahamstown 6140
South Africa

Dr. Beatrice Kilel
P.O Box 1413
Manassas, VA 20108
USA

Dr. Jackie Hughes
Research-for-Development
International Institute of Tropical Agriculture (IITA)
Ibadan, Nigeria

Dr. Robert L. Brown
Southern Regional Research Center,
U.S. Department of Agriculture,
Agricultural Research Service,
New Orleans, LA 70179.

Dr. Deborah Rayfield
Physiology and Anatomy
Bowie State University
Department of Natural Sciences
Crawford Building, Room 003C
Bowie MD 20715,USA

Dr. Marlene Shehata
University of Ottawa Heart Institute
Genetics of Cardiovascular Diseases
40 Ruskin Street
K1Y-4W7, Ottawa, ON, CANADA

Dr. Hany Sayed Hafez
The American University in Cairo,
Egypt

Dr. Clement O. Adebooye
Department of Plant Science
Obafemi Awolowo University, Ile-Ife
Nigeria

Dr. Ali Demir Sezer
Marmara Üniversitesi Eczacilik Fakültesi,
Tibbiye cad. No: 49, 34668, Haydarpasa, Istanbul,
Turkey

Dr. Ali Gazanchain
P.O. Box: 91735-1148, Mashhad,
Iran.

Dr. Anant B. Patel
Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad 500007
India

Prof. Arne Elofsson
Department of Biophysics and Biochemistry
Bioinformatics at Stockholm University,
Sweden

Prof. Bahram Goliaei
Departments of Biophysics and Bioinformatics
Laboratory of Biophysics and Molecular Biology
University of Tehran, Institute of Biochemistry
and Biophysics
Iran

Dr. Nora Babudri
Dipartimento di Biologia cellulare e ambientale
Università di Perugia
Via Pascoli
Italy

Dr. S. Adesola Ajayi
Seed Science Laboratory
Department of Plant Science
Faculty of Agriculture
Obafemi Awolowo University
Ile-Ife 220005, Nigeria

Dr. Yee-Joo TAN
Department of Microbiology
Yong Loo Lin School of Medicine,
National University Health System (NUHS),
National University of Singapore
MD4, 5 Science Drive 2,
Singapore 117597
Singapore

Prof. Hidetaka Hori
Laboratories of Food and Life Science,
Graduate School of Science and Technology,
Niigata University.
Niigata 950-2181,
Japan

Prof. Thomas R. DeGregori
University of Houston,
Texas 77204 5019,
USA

Dr. Wolfgang Ernst Bernhard Jelkmann
Medical Faculty, University of Lübeck,
Germany

Dr. Moktar Hamdi
Department of Biochemical Engineering,
Laboratory of Ecology and Microbial Technology
National Institute of Applied Sciences and
Technology.
BP: 676. 1080,
Tunisia

Dr. Salvador Ventura
Department de Bioquímica i Biologia Molecular
Institut de Biotecnologia i de Biomedicina
Universitat Autònoma de Barcelona
Bellaterra-08193
Spain

Dr. Claudio A. Hetz
Faculty of Medicine, University of Chile
Independencia 1027
Santiago, Chile

Prof. Felix Dapare Dakora
Research Development and Technology Promotion
Cape Peninsula University of Technology,
Room 2.8 Admin. Bldg. Keizersgracht, P.O. 652,
Cape Town 8000,
South Africa

Dr. Geremew Bultosa
Department of Food Science and Post harvest Technology
Haramaya University
Personal Box 22, Haramaya University Campus
Dire Dawa,
Ethiopia

Dr. José Eduardo Garcia
Londrina State University
Brazil

Prof. Nirbhay Kumar
Malaria Research Institute
Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health
E5144, 615 N. Wolfe Street
Baltimore, MD 21205

Prof. M. A. Awal
Department of Anatomy and Histplogy,
Bangladesh Agricultural University,
Mymensingh-2202,
Bangladesh

Prof. Christian Zwieb
Department of Molecular Biology
University of Texas Health Science Center at Tyler
11937 US Highway 271
Tyler, Texas 75708-3154
USA

Prof. Danilo López-Hernández
Instituto de Zoología Tropical, Facultad de Ciencias,
Universidad Central de Venezuela.
Institute of Research for the Development (IRD),
Montpellier,
France

Prof. Donald Arthur Cowan
Department of Biotechnology,
University of the Western Cape Bellville 7535
Cape Town,
South Africa

Dr. Ekhaise Osaro Frederick
University Of Benin, Faculty of Life Science
Department of Microbiology
P. M. B. 1154, Benin City, Edo State,
Nigeria.

Dr. Luísa Maria de Sousa Mesquita Pereira
IPATIMUP R. Dr. Roberto Frias, s/n 4200-465 Porto
Portugal

Dr. Min Lin
Animal Diseases Research Institute
Canadian Food Inspection Agency
Ottawa, Ontario,
Canada K2H 8P9

Prof. Nobuyoshi Shimizu
Department of Molecular Biology,
Center for Genomic Medicine
Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku
Tokyo 160-8582,
Japan

Dr. Adewunmi Babatunde Idowu
Department of Biological Sciences
University of Agriculture Abia
Abia State,
Nigeria

Dr. Yifan Dai
Associate Director of Research
Revivicor Inc.
100 Technology Drive, Suite 414
Pittsburgh, PA 15219
USA

Dr. Zhongming Zhao
Department of Psychiatry, PO Box 980126,
Virginia Commonwealth University School of Medicine,
Richmond, VA 23298-0126,
USA

Prof. Giuseppe Novelli
Human Genetics,
Department of Biopathology,
Tor Vergata University, Rome,
Italy

Dr. Moji Mohammadi
402-28 Upper Canada Drive
Toronto, ON, M2P 1R9 (416) 512-7795
Canada

Prof. Jean-Marc Sabatier
Directeur de Recherche Laboratoire ERT-62
Ingénierie des Peptides à Visée Thérapeutique,
Université de la Méditerranée-Ambrilia
Biopharma inc.,
Faculté de Médecine Nord, Bd Pierre Dramard,
13916,
Marseille cédex 20.
France

Dr. Fabian Hoti
PneumoCarr Project
Department of Vaccines
National Public Health Institute
Finland

Prof. Irina-Draga Caruntu
Department of Histology
Gr. T. Popa University of Medicine and Pharmacy
16, Universitatii Street, Iasi,
Romania

Dr. Dieudonné Nwaga
Soil Microbiology Laboratory,
Biotechnology Center. PO Box 812,
Plant Biology Department,
University of Yaoundé I, Yaoundé,
Cameroon

Dr. Gerardo Armando Aguado-Santacruz
Biotechnology CINVESTAV-Unidad Irapuato
Departamento Biotecnología
Km 9.6 Libramiento norte Carretera Irapuato-
León Irapuato,
Guanajuato 36500
Mexico

Dr. Abdolkaim H. Chehregani
Department of Biology
Faculty of Science
Bu-Ali Sina University
Hamedan,
Iran

Dr. Abir Adel Saad
Molecular oncology
Department of Biotechnology
Institute of graduate Studies and Research
Alexandria University,
Egypt

Dr. Azizul Baten
Department of Statistics
Shah Jalal University of Science and Technology
Sylhet-3114,
Bangladesh

Dr. Bayden R. Wood
Australian Synchrotron Program
Research Fellow and Monash Synchrotron
Research Fellow Centre for Biospectroscopy
School of Chemistry Monash University Wellington
Rd. Clayton,
3800 Victoria,
Australia

Dr. G. Reza Balali
Molecular Mycology and Plant Pthology
Department of Biology
University of Isfahan
Isfahan
Iran

Dr. Beatrice Kilel
P.O Box 1413
Manassas, VA 20108
USA

Prof. H. Sunny Sun
Institute of Molecular Medicine
National Cheng Kung University Medical College
1 University road Tainan 70101,
Taiwan

Prof. Ima Nirwana Soelaiman
Department of Pharmacology
Faculty of Medicine
Universiti Kebangsaan Malaysia
Jalan Raja Muda Abdul Aziz
50300 Kuala Lumpur,
Malaysia

Prof. Tunde Ogunsanwo
Faculty of Science,
Olabisi Onabanjo University,
Ago-Iwoye.
Nigeria

Dr. Evans C. Egwim
Federal Polytechnic,
Bida Science Laboratory Technology Department,
PMB 55, Bida, Niger State,
Nigeria

Prof. George N. Goulielmos
Medical School,
University of Crete
Voutes, 715 00 Heraklion, Crete,
Greece

Dr. Uttam Krishna
Cadila Pharmaceuticals limited ,
India 1389, Tarsad Road,
Dholka, Dist: Ahmedabad, Gujarat,
India

Prof. Mohamed Attia El-Tayeb Ibrahim
Botany Department, Faculty of Science at Qena,
South Valley University, Qena 83523,
Egypt

Dr. Nelson K. Ojijo Olang'o
Department of Food Science & Technology,
JKUAT P. O. Box 62000, 00200, Nairobi,
Kenya

Dr. Pablo Marco Veras Peixoto
University of New York NYU College of Dentistry
345 E. 24th Street, New York, NY 10010
USA

Prof. T E Cloete
University of Pretoria Department of
Microbiology and Plant Pathology,
University of Pretoria,
Pretoria,
South Africa

Prof. Djamel Saidi
Laboratoire de Physiologie de la Nutrition et de
Sécurité
Alimentaire Département de Biologie,
Faculté des Sciences,
Université d'Oran, 31000 - Algérie
Algeria

Dr. Tomohide Uno
Department of Biofunctional chemistry,
Faculty of Agriculture Nada-ku,
Kobe., Hyogo, 657-8501,
Japan

Dr. Ulises Urzúa
Faculty of Medicine,
University of Chile Independencia 1027, Santiago,
Chile

Dr. Aritua Valentine
National Agricultural Biotechnology Center,
Kawanda
Agricultural Research Institute (KARI)
P.O. Box, 7065, Kampala,
Uganda

Prof. Yee-Joo Tan
Institute of Molecular and Cell Biology 61 Biopolis
Drive,
Proteos, Singapore 138673
Singapore

Prof. Viroj Wiwanitkit
Department of Laboratory Medicine,
Faculty of Medicine, Chulalongkorn University,
Bangkok
Thailand

Dr. Thomas Silou
Universit of Brazzaville BP 389
Congo

Prof. Burtram Clinton Fielding
University of the Western Cape
Western Cape,
South Africa

Dr. Brnčić (Brncic) Mladen
Faculty of Food Technology and Biotechnology,
Pierottijeva 6,
10000 Zagreb,
Croatia.

Dr. Meltem Sesli
College of Tobacco Expertise,
Turkish Republic, Celal Bayar University 45210,
Akhisar, Manisa,
Turkey.

Dr. Idress Hamad Attitalla
Omar El-Mukhtar University,
Faculty of Science,
Botany Department,
El-Beida, Libya.

Dr. Linga R. Gutha
Washington State University at Prosser,
24106 N Bunn Road,
Prosser WA 99350-8694.

Dr Helal Ragab Moussa
Bahnay, Al-bagour, Menoufia,
Egypt.

Dr VIPUL GOHEL
DuPont Industrial Biosciences
Danisco (India) Pvt Ltd
5th Floor, Block 4B,
DLF Corporate Park
DLF Phase III
Gurgaon 122 002
Haryana (INDIA)

Dr. Sang-Han Lee
Department of Food Science & Biotechnology,
Kyungpook National University
Daegu 702-701,
Korea.

Dr. Bhaskar Dutta
DoD Biotechnology High Performance Computing Software Applications
Institute (BHSAI)
U.S. Army Medical Research and Materiel Command
2405 Whittier Drive
Frederick, MD 21702

Dr. Muhammad Akram
Faculty of Eastern Medicine and Surgery,
Hamdard Al-Majeed College of Eastern Medicine,
Hamdard University,
Karachi.

Dr. M. Muruganandam
Departtment of Biotechnology
St. Michael College of Engineering & Technology,
Kalayarkoil,
India.

Dr. Gökhan Aydin
Suleyman Demirel University,
Atabey Vocational School,
Isparta-Türkiye,

Dr. Rajib Roychowdhury
Centre for Biotechnology (CBT),
Visva Bharati,
West-Bengal,
India.

Dr Takuji Ohyama
Faculty of Agriculture, Niigata University

Dr Mehdi Vasfi Marandi
University of Tehran

Dr FÜgen DURLU-ÖZKAYA
Gazi Üniversity, Tourism Faculty, Dept. of Gastronomy and Culinary Art

Dr. Reza Yari
Islamic Azad University, Boroujerd Branch

Dr Zahra Tahmasebi Fard
Roudehen branche, Islamic Azad University

Dr Albert Magrí
Giro Technological Centre

Dr Ping ZHENG
Zhejiang University, Hangzhou, China

Dr. Kgomotso P. Sibeko
University of Pretoria

Dr Greg Spear
Rush University Medical Center

Prof. Pilar Morata
University of Malaga

Dr Jian Wu
Harbin medical university , China

Dr Hsiu-Chi Cheng
National Cheng Kung University and Hospital.

Prof. Pavel Kalac
University of South Bohemia, Czech Republic

Dr Kürsat Korkmaz
Ordu University, Faculty of Agriculture,
Department of Soil Science and Plant Nutrition

Dr. Shuyang Yu
Department of Microbiology, University of Iowa
Address: 51 newton road, 3-730B BSB bldg. Iowa City, IA, 52246, USA

Dr. Binxing Li

Dr. Mousavi Khaneghah
College of Applied Science and Technology-
Applied Food Science, Tehran, Iran.

Dr. Qing Zhou
Department of Biochemistry and Molecular
Biology,
Oregon Health and Sciences University Portland.

Dr Legesse Adane Bahiru
Department of Chemistry,
Jimma University,
Ethiopia.

Dr James John
School Of Life Sciences,
Pondicherry University,
Kalapet, Pondicherry

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJFS to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $650 handling fee. Publication of an article in the African Journal of Biotechnology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

Copyright: © 2013, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJB, whether or not advised of the possibility of damage, and on any theory of liability.
This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication. While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.

African Journal of Biotechnology

Table of Contents: Volume 12 Number 47, 20 November, 2013

ARTICLES

Research Articles

Table of Contents: Volume 12 Number 47, 20 November, 2013

academicJournals

Vol. 12(47), pp. 6636-6642, 20 November, 2013
DOI: 10.5897/AJB2013.12859
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Textile azo dye degradation by *Candida rugosa* INCQS 71011 isolated from a non-impacted area in Semi-Arid Region of Brazilian Northeast

Carlos Roberto S. do Nascimento[1,2], Marília M. Nishikawa[1], Aline B. M. Vaz[3], Carlos Augusto Rosa[3] and Manuela da Silva[2]*

[1]Fiocruz - Oswaldo Cruz Foundation, National Institute for Health Quality Control, Avenida Brasil 4365, – Manguinhos, Rio de Janeiro, RJ 21040-900, Brazil.
[2]Fiocruz - Oswaldo Cruz Foundation, National Institute for Health Quality Control, Post-graduate Program in Health Surveillance, Avenida Brasil 4365, – Manguinhos, Rio de Janeiro, RJ 21040-900, Brazil.
[3]Federal University of Minas Gerais, Microbiology Department, ICB, CP 486, Belo Horizonte, Minas Gerais, Brazil.

Accepted 17 October, 2013

The yeast *Candida rugosa*, deposited in the Collection of Reference Microorganisms on Health Surveillance from Oswaldo Cruz Foundation under accession number INCQS 71011, was isolated from a sediment sample from Caldeirão Escuridão, a pristine water reservoir in the surroundings of Serra da Capivara National Park, and was identified based on molecular, physiological and morphological characterization. In addition, it was tested regarding its capacity to degrade three textile azo dyes, namely Reactive Red 198, Reactive Red 141, and Reactive Blue 214 at a concentration of 100 mg l^{-1} during 7 days of incubation. *C. rugosa* INCQS 71011 was highly efficient towards two azo dyes tested, Reactive Red 198 and Reactive Red 141, demonstrating potential as a biological treatment agent of textile effluent. These results are pioneers for the yeast *C. rugosa*, since its degradation capacity of textile azo dyes has not yet been described. In addition, this study provides important evidence that fungi from non-impacted areas can efficiently degrade azo dyes.

Key-words: Fungi, taxonomic characterization, degradation, textile azo dyes.

INTRODUCTION

Wastewaters from textile industries are a complex mixture of several pollutants such as heavy metals, pigments, and dyes (Coulibaly et al., 2003). The potential of pollution from textile dyes has been acknowledged primarily due to possible problems towards human and environmental health (Banat et al., 1996; Meehan et al., 2000; Stolz, 2001). Effluents discharged by textile industries, if not treated properly, can contain large quantities of hazardous chemical compounds (Chen and Hwang, 1997; Aksu and Dönmez, 2003). There is a necessity to develop better treatment technologies to remove color from industrial effluents that prove technically and economically satisfying as treatment technology. Therefore, several emerging approaches such as advanced oxidation process, membrane filtration, photocatalysis, and sonication, are being proposed and tested at different stages of commercialization (Anjaneyulu et al., 2005).

*Corresponding author. E-mail: manueladasilva@fiocruz.br; Tel: +55 21 3885-1714; Fax: +55 21 2590-9741.

Abbreviations: YMA, Yeast extract-malt extract agar; **PCR,** polymerase chain reaction; **RR198,** reactive red 198; **RR141,** reactive red 141; **RB214,** reactive blue 214; **PDA,** potato dextrose agar; **PDB,** potato dextrose broth.

A number of studies have focused on some microorganisms that are able to biodegrade and biosorb dyes in wastewaters. Some bacteria, fungi, and algae are capable of decolorizing a wide range of dyes (Fu and Viraraghavan, 2001). The treatment using fungi is a promising alternative to replace or complement conventional treatment processes due to the efficiency of dye decolorization by fungi, mainly because of the fungal enzymatic apparatus that enables the degradation of these compounds and for not producing material to be discarded. Additionally, fungal treatment has a good public acceptance (Fu and Viraraghavan, 2001). However, more studies on the ability of fungi to decolorize dyes used by the textile industry are needed. Little is known about the degradation of dyes and color removal by yeasts (Singh, 2006); nonetheless, most studies show positive results. Several genera of yeast have been studied such as *Kluyveromyces*, *Schizosaccharomyces*, *Issatchenkia*, *Debaryomyces*, *Pseudozyma*, and *Saccharomyces* (Aksu and Dönmez, 2003; Ramalho et al., 2004; Yang et al., 2003, 2005; Yu and Wen, 2005; Kumari and Abraham, 2007). *Candida* species have been used in some works and good results were presented for removal of reactive dyes in concentrations ranging from 10 - 700 mg l^{-1}, nevertheless, these studies showed adsorption, besides degradation (Gönen and Aksu, 2009; Ertugrul et al., 2009) and absorption (Dönmez, 2002; Aksu and Dönmez, 2003).

Compared with bacteria and filamentous fungi, the yeasts display attractive characteristics. Despite the fact that yeasts do not always grow as fast as bacteria, they grow faster than filamentous fungi and have the ability to resist to unfavorable environments (Pajot et al., 2007). Based on this context, the isolation of fungal species from an environment with potential bioremediation has been spreading (da Silva et al., 2003; Junghanns et al., 2008; Passarini et al., 2011) and different ecosystems have been exploited for this purpose.

Brazil has ecosystems and biomes that are not yet fully known, such as the semi-arid Caatinga, an area of uncertain rainfall situated in the northeastern state of Piauí. This biome represents a valuable biodiversity reservoir, in which there is an important reserve for the preservation of its specific environment, the Serra da Capivara National Park (Pessis, 1998; Gusmão and Maia, 2006).

From the surroundings of Serra da Capivara National Park, sediment samples of pristine water reservoirs where collected and mixed fungal cultures were selected for dye degradation. From the most efficient mixed fungal culture, Caldeirão Escuridão (CE), a yeast strain (CE-9) was isolated (Nascimento et al., 2011). Therefore, the aim of the present work was to identify by phenotypic and molecular methods the yeast CE-9 as well as to test its capacity to degrade three textile azo dyes.

MATERIALS AND METHODS

Yeast isolation and preservation

The yeast strain CE-9 was isolated from a mixed fungal culture collected from Caldeirão Escuridão (CE), a natural water reservoir situated in the surroundings of the Serra da Capivara National Park, at an altitude of 423 m (xUTM - 769341, yUTM – 9022784 (Nascimento et al., 2011). The yeast is currently deposited as *Candida rugosa* INCQS 71011 in the Collection of Reference Microorganisms on Health Surveillance (Fiocruz-CMRVS) at the National Institute for Quality Control in Health (INCQS) from Oswaldo Cruz Foundation (Fiocruz). The American Type Culture Collection (ATCC) strain of *C. rugosa* ATCC 10571 was used as a reference strain. The strains are maintained lyophilized and cryopreserved at -70 and -150ºC. For routine work at laboratory scale, the selected yeasts were maintained on yeast extract-malt extract agar (YMA) slants at 4°C and subcultured at 15 day regular intervals.

Yeast identification

The isolate CE-9 was characterized by standard methods (Yarrow, 1998) and the identification followed the keys of Kurtzman and Fell (1998). Concurrently, the characterization by the Vitek®2 using the Yeast Biochemical Card (YBC) (bioMérieux, Durham, NC) was conducted. Genomic DNA of CE-9 and of the reference strain *C. rugosa* ATCC 10571 was prepared after 2 days of incubation on YMA using the methodology described by de Barros Lopes et al. (1998). The D1/D2 variable domains of the large-subunit rDNA were amplified by polymerase chain reaction (PCR) using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') according to Lachance et al. (1999). Amplification was performed as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 54°C for 25 s, and 72°C for 20 s, and a final extension at 72°C for 10 min. The amplified DNA was concentrated and cleaned (Kit Wizard Plus SV Minipreps DNA Purification System—Promega, USA). Sequencing was conducted using an ET dynamic terminator kit in a MegaBACE 1000/ automated 96 capillary DNA sequencer (GE Healthcare, Buckinghamshire, UK). The quality of each electropherogram was evaluated using Phred-Phrap software and consensus sequences were obtained by alignment of both sequence strands using CAP3 software available on the Eletropherogram quality analysis web page (http://asparagin.cenargen.embrapa.br/phph). The nucleotide sequences and other related sequences were aligned using the CLUSTALW software package (EMBL-EBI) (http://www.ebi.ac.uk/clustalw/). Phylogenetic relationships were estimated using the MEGA program Version 4.0 (Tamura et al., 2007). The phylogenetic trees were constructed using the neighbor joining (NJ) algorithm with bootstrap values calculated from 1,000 replicate runs. The Maximum Composite Likelihood model was used to estimate evolutionary distance.

Dyestuff

Three commercial reactive dyes were used: Reactive Red 198 (RR198), Reactive Red 141 (RR141), both from DyStar (Brazil) and Reactive Blue 214 (RB214) from Clariant (Brazil) with maximum wavelength of 520 nm, 548 nm and 608 nm, respectively. All of them are azo dyes and the chemical structures are shown in Figure 1. Stock solutions (5 g l^{-1} for RR198 and RR141; 4 g l^{-1} for RB 214) were filter-sterilized (Millipore filter, 0.22 µm, Millipore Corp., Bedford, USA).

Yeast growth

CE-9 was grown on potato dextrose agar (PDA) with 200 mg l^{-1} of each of the three dyes separately and incubated for 48 h. Aliquots from these cultures were transferred to sterile purified water and the turbidity was adjusted to 2 McFarland standard (6 x 10^8 CFU ml^{-1}). From this suspension, 2 ml were used to inoculate 100 ml of potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks in duplicate. The Erlenmeyer flasks were incubated under agitation for 2 days in the dark (Heinfling-Weidtmann et al., 2001; Máximo et al., 2003) on a

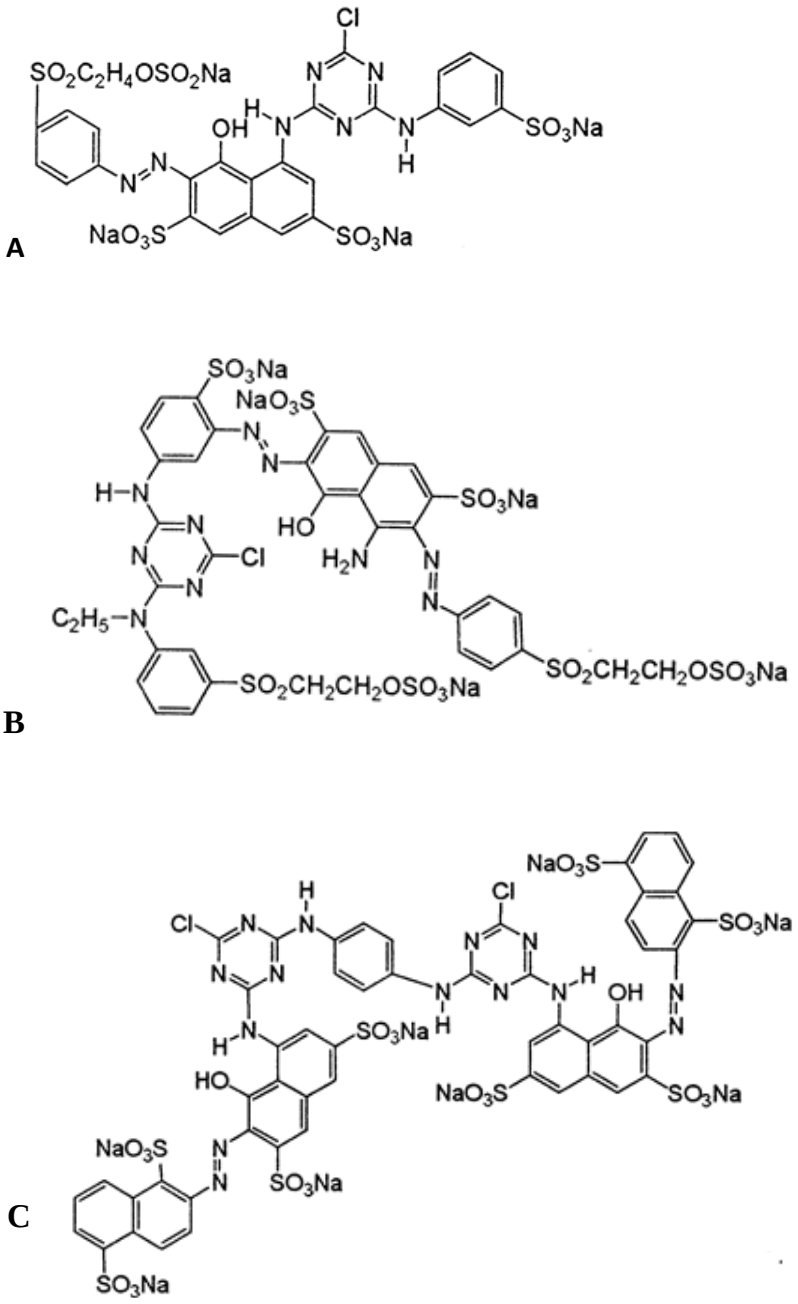


Figure 1. Chemical structures of the dyes tested: A, Reactive Red 198; B, Reactive Blue 214; C, Reactive Red 141.

rotary shaker (New Brunswich, Innova 4335, USA) at 140 rpm. After this incubation period, 100 mg l^{-1} of the dyes were added separately in all flasks. The cultures were kept incubating under the same conditions during 7 days. Control experiments were conducted by means of incubating PDB medium without yeast and with the same concentrations of the dye tested as well as with PDB without either dye or yeast.

Evaluation of dye decolorization

The evaluation of dye decolorization by CE-9 was conducted during the 7-day incubation at the following times: 24, 48, 72, 96, 120, 144 and 168 h. After centrifugation at 10,000 rpm for 10 min of 2 ml aliquots of the cultures, 0.5 ml of supernatant was diluted 10 times in purified water in order to be analyzed using spectrophotometer UV/VIS (Shimadzu - UV-1601, Japan). The culture medium with dye and without inoculum was used as a negative control whereas medium without either dye or inoculum was used as blank. The spectra were scanned between 200 to 700 nm of absorbance (Máximo et al., 2003) in order to analyze dye disappearance. Decolorization activity (%) was calculated for the two flasks (duplicate) of each culture according to the formula below, considering the maximum wavelength of 520 nm for RR198, 548 nm for RR141, and 608 nm for RB214.

Decolorization activity (%) = (A-B)/A x 100

Table 1. Physiological results of isolate CE-9 identified as *C. rugosa.*

Compound	Result
Assimilation of carbon compounds	
Glucose	+
D-galactose	+
L-sorbose	-
D-glucosamine	-
D-ribose	-
D-xylose	+
L-arabinose	-
L-rhamanose	-
Sucrose	-
Maltose	-
Trehalose	-
α-methyl-D- glucoside	-
Cellobiose	-
Salicin	-
Melibiose	-
Lactose	-
Raffinose	-
Melezitose	-
Glycerol	+
Erythritol	-
Ribitol	-
Galactitol	-
D-mannitol	+
D-glucitol	+
Inositol	-
D-gluconate	-
DL-Lactate	+
Succinate	+
Citrate	-
Assimilation of nitrogen compounds	
KNO^3	-
KNO^2	-
Others	
Vitamin-free	-
Cycloheximide 0,1 %	-
Cycloheximide 1 %	-
Urease	-
Amyloid compounds	-
Fermentation	
D-glucose	-
D-galactose	-
Maltose	-
Sucrose	-
α-α-trehalose	-
Lactose	-
Cellobiose	-
Raffinose	-

Where, A is the Initial absorbance and B is the observed absorbance (Özsoy et al., 2005). All assays were conducted in duplicate and results were expressed as the mean values.

RESULTS

Phenotypic characterization

The macro-morphological observations on YMA medium after 2 days at 25°C of the isolate CE-9 demonstrated a butyrous texture, colored white to cream. From the micro-morphological analysis, cells were elongate, single and in pairs and pseudo-hyphae were produced. These are morphological characteristics typical of *C. rugosa*. In Table 1, the physiological results obtained from CE-9 were present. The yeast was negative for urea hydrolysis and formation of extracellular amyloid compounds. It did not ferment carbohydrates, it did not grow in the absence of vitamins, it was sensitive to cycloheximide, it did not assimilate the $NaNO_2$ and KNO_3 as nitrogen source, but assimilated the following carbon compounds: glucose, D-galactose, D-xylose, glycerol, D-mannitol, D-glucitol, lactic acid, and succinic acid. The other carbon compounds used in the test were not assimilated by the yeast. Hence, the isolate was identified biochemically and physiologically as *C. rugosa*.

The automated method of identification using Vitek®2 was also used for the biochemical characterization whereas the YBC was applied to confirm the conventional method. As the result, the identification was 97% of probability for *C. rugosa*. The D1/D2 sequence of strain *C. rugosa* ATCC 10571 has 527 nucleotides, where 24 and 19 correspond to the nucleotide primers NL1 and NL4, respectively. Thus, the sequence to be obtained from isolate CE-9 should be 484 nucleotides.

Forward and reverse strands of D1/D2 domain of isolate CE-9 were sequenced using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). Consensus sequence had 447 nucleotides and was aligned with the sequences of *C. rugosa* ATCC 10571. Manual editing of the sequence was performed based on the chromatograms obtained and no differences between the sequences were observed. When performing blast, it had 99% of identity with only one Gap of difference. Therefore, these results confirm the phenotypic characterization, leading to the identification of the isolate CE-9 as the species *C. rugosa*.

Decolorization

C. rugosa INCQS 71011 decolorized 78.46% of RR198 at 100 mg l^{-1} dye concentration after 24 h of incubation and reached 100% decolorization of this same dye after 48 h of incubation (Table 2), respectively. Regarding RR141 dye, decolorization reached 75.60% after 24 h of incubation, and complete decolorization was achieved within 144 h of incubation (Table 2). In both cases, there was no adsorption by the biomass but degradation, as demonstrated by the results of spectrophotometric analyses

Table 2. Rate of decolorization of the dyes RR198, RR141 and RB214 by *C. rugosa* INCQS 71011 during 24 – 168h of incubation.

Sample	Decolorization (%)						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Treatment of RR198	78.46	100	100	100	100	100	100
Treatment of RR141	75.60	86.46	88.86	88.58	89.39	100	100
Treatment of RB214	50.20	59.21	69.01	70.43	73.82	73.91	76.05

that showed the disappearance of the characteristic peak of those two dyes (Figures 2a and 2b). The other dye tested, RB214, was less efficiently decolorized by *C. rugosa* INCQS 71011. In the first 24h incubation, decolorization achieved 50%. From 48 to 144 h, decolorization increased progressively, however slowly; and at the end of the incubation period (168 h), the decolorization rate was 76.05% (Table 2). The adsorption to the biomass just occurred with this dye, which decreased up to the end of the incubation period (data not shown), while the characteristic peak of this dye was transformed into a new peak (Figure 2c).

DISCUSSION

In some investigations, positive results for textile dye decolorization were obtained using fungi isolated from non-impacted areas (Pajot et al., 2011; Martorell et al., 2012), as similarly obtained in the present study. The ascomycetous yeast *C. rugosa* INCQS 71011 showed a very good result mainly in the decolorization of RR198, a monoazo, decolorizing 100% of the dye at a concentration of 100 mg l^{-1} after 48 h, while within 24 h the decolorization was 75.60%. Regarding RR141, the results were also satisfactory 75.20% was decolorized after 24 h incubation and the complete decolorization was achieved within 144 h incubation. For RB214, however, the decolorization took longer, 76.05% was decolorized at the end of the incubation period (168 h) (Table 2). The decolorization of a number of simple azo dyes derived from 2-naphtholaminoazobenzene and p-N,N-dimethylaminoazobenzene in concentrations of 10-50 mg l^{-1} were tested in liquid aerated batch cultures using a strain of the yeast *Candida zeylanoides* UM2, by which method a 90% adsorption was obtained from the best result after seven days of incubation (Martins et al., 1999). This same strain was used by Ramalho et al. (2005) which, after changing some parameters, such as medium, for example, *C. zeylanoides* UM2 showed 100% efficiency of decolorization without traces of absorption after 60 h.

Dye degradation occurs when the biomass remains with its original color and the medium becomes decolorized (Chen et al., 2003; Yu and Wen, 2005). It can be monitored during cultivation of the fungus in liquid medium through the ratio between two characteristic wavelengths of the dye. Adsorption occurs when the characteristic peak of absorption of the dye remains constant during the incubation period, while degradation occurs when this peak disappears completely or a new peak appears (Glenn and Gold, 1983). In the present study, *C. rugosa* 71011 succeeded in promoting the disappearance of the characteristic peaks of absorption of visible light from both dyes, namely RR198 (520 and 370 nm) and RR141 (548 and 522 nm) (Figures 2a and 2b), with no dye adsorption by the biomass, thus typifying this fungal activity as the degradation of these dyes.

Degradation was also determined by Yang et al. (2003) when using *Candida tropicalis* Y2-0814 and *Debaryomyces polymorphus* Y1-0813 to decolorize 100 mg l^{-1} of seven synthetic dyes. Within 16-48 h of incubition the color disappeared completely without residual color on the biomass in the case of only one of these dyes, the Reactive Black 5. The metabolic profile species-specific, such as enzymatic activity (Ramalho et al., 2005; Martorell et al., 2012), media culture (Ramalho et al., 2002; Kaushik and Malik, 2010) and the structure complexity of the dye (Fu and Viraraghavan, 2001; Bergsten-Torralba et al., 2009) can greatly influence the final response of fungal degradation. In the present work, *C. rugosa* INCQS 71011 was much more efficient degrading RR198, a single azo class dye, than it was towards RR 141, a double azo class dye, which took longer to be degraded and towards RB 241, another double azo class dye, which was not completely degraded under the conditions established in this study.

The present results demonstrated the efficiency of *C. rugosa* INCQS 71011 to degrade textile azo dyes and indicates its potential as a biological treatment agent of textile effluent, despite its origin, a non-impacted area in Semi-Arid Northeastern Brazil. To our knowledge this is the first report of a strain of *C. rugosa* with the ability to degrade textile azo dyes. Further studies on the optimization conditions for this yeast to degrade azo dyes, nonetheless, are needed.

ACKNOWLEDGMENTS

The present work was supported by a grant from Fiocruz/CNPq (PAPES V - 403647/2008-8). The authors are indebted to DyStar and Clariant industries (SP, Brazil) for donating the dyes used in the present investigation.

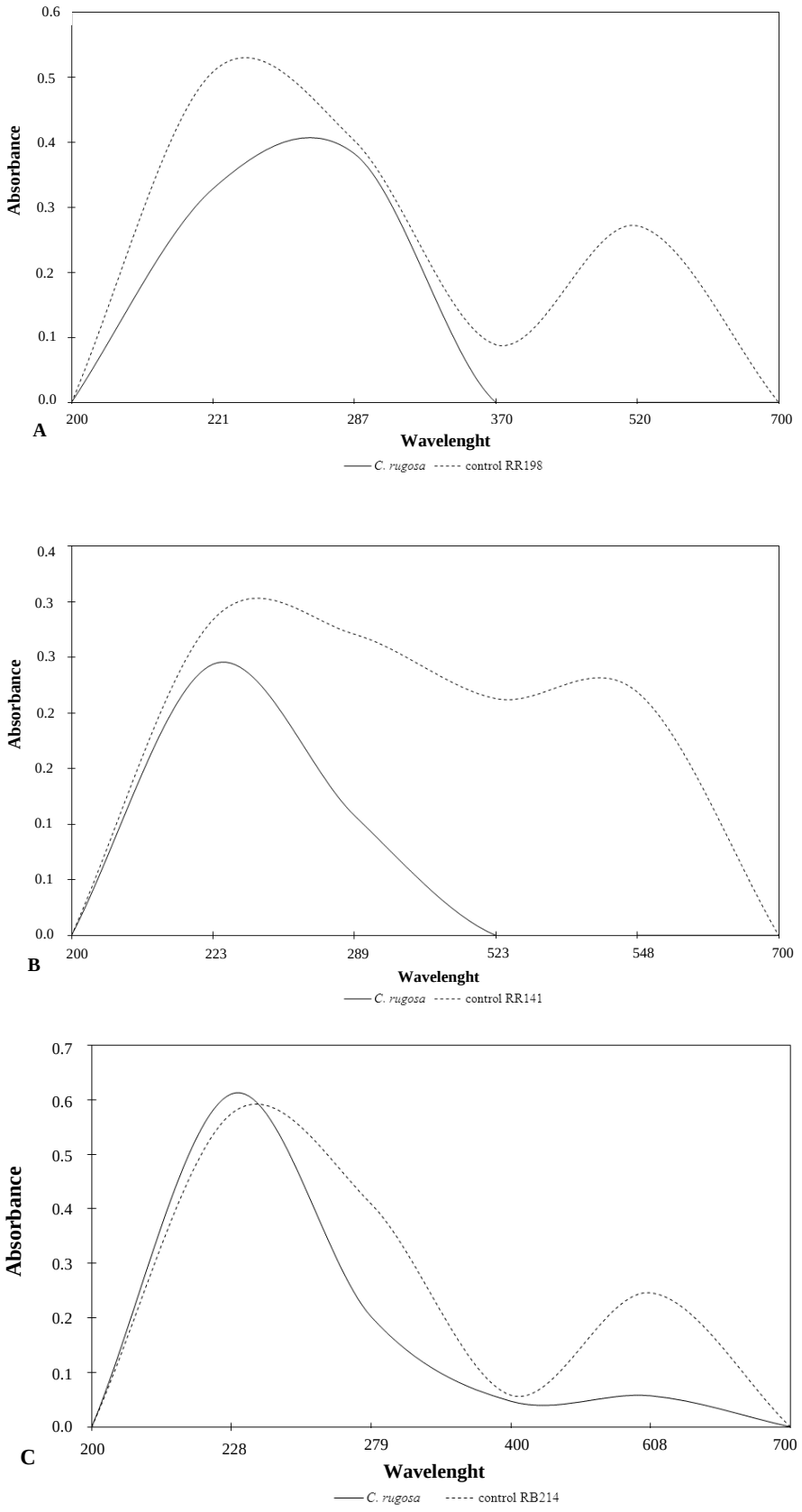


Figure 2. Espectra resulting from the dyes RR 198 (a), RR 141 (b) and RB 214 (c) degradation by the *C. rugosa* INCQS 71011.

REFERENCES

Aksu Z, Dönmez G (2003). A comparative study on the biosorption characteristics of some yeasts for Remazol Blue reactive dye. Chemosphere 50: 1075-1083.

Anjaneyulu Y, Chary NS, Raj DSS (2005). Decolourization of industrial effluents – available methods and emerging technologies – a review. Rev. Environ. Sci. Biotechnol. 4: 245-273.

Banat IM, Nigam P, Singh D, Marchant R (1996). Microbial decolorization of textile-dye-containing effluents: a review. Bioresour. Technol. 58: 217-227.

Bergsten-Torralba LR, Nishikawa M, Baptista D, Magalhães DP, da Silva M (2009). Decolorization of different textile dyes by *Penicillium simplicissimum* and toxicity evaluation after fungal treatment. Braz. J. Microbiol. 40: 808-817.

Chen K-C, Wu J-Y, Liou D-J, Hwang S-CJ (2003). Decolorization of the textile dyes by newly isolated bacterial strains. J. Biotechnol. 101: 57-68.

Chen M, Huang C-L (1997). Industrial workers' health and environmental pollution under the new international division of labor: the Taiwan experience. Am. J. Public. Health 87: 1223-1231.

Coulibaly L, Gourene G, Agathos SN (2003). Utilization of fungi for biotreatment of raw wastewaters. Afr. J. Biotechnol. 2: 620-630.

da Silva M, Cerniglia CE, Pothuluri JV, Canhos VP, Esposito E (2003). Screening filamentous fungi isolated from estuarine sediments for the ability to oxidize polycyclic aromatic hydrocarbons. World J. Microbiol. Biotechnol. 19: 399-405.

de Barros Lopes M, Soden A, Martens AL, Henschke PA, Langridge P (1998). Differentiation and species identification on yeasts using PCR. Int. J. Syst. Bacteriol. 48: 279-286.

Dönmez G (2002). Bioaccumulation of the reactive textile dyes by *Candida tropicalis* growing in molasses medium. Enzyme Microb. Technol. 30: 363-366.

Ertugrul S, San NO, Dönmez G (2009). Treatment of dye (Remazol Blue) and heavy metals using yeast cells with the purpose of managing polluted textile wastewaters. Ecol. Eng. 35: 128-134.

Fu Y, Viraraghavan T (2001). Fungal decolorization of dye wastewaters: a review. Bioresour. Technol. 79: 251-262.

Glenn JK, Gold MH (1983). Decolorization of several polymeric dyes by the lignin-degrading Basidiomycete *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. 45: 1741-1747.

Gönen F, Aksu Z (2009). Single and binary dye and heavy metal bioaccumulation properties of *Candida tropicalis*: Use of response surface methodology (RSM) for the estimation of removal yields. J. Hazard Mater. 172: 1512-1519.

Gusmão LFP, Maia LC (2006). Diversidade e caracterização dos fungos do semi-árido brasileiro. APNE, Recife, Brazil.

Heinfling-Weidtmann A, Reemtsma T, Storm T, Szewzk U (2001). Sulfophthalimide as major metabolite formed from sulfonated phthalocyanine dyes by the white-rot fungus *Bjerkandera adusta*. FEMS Microbiol. Lett. 203: 179- 183.

Junghanns C, Krauss G, Schlosser D (2008). Potential of aquatic fungi derived from diverse freshwater environments to decolourise synthetic azo and anthraquinone dyes. Bioresour. Technol. 99: 1225–1235.

Kaushik P, Malik A (2010). Effect of nutritional conditions on dye removal from textile effluent by *Aspergillus lentulus*. World J. Microbiol. Biotechnol. 26: 1957-1964.

Kumari K, Abraham TE (2007). Biosorption of anionic textile dyes by nonviable biomass of fungi and yeast. Bioresour. Technol. 98: 1704-1710.

Kurtzman CP, Fell JW (1998). The yeast: a taxonomic study. Elsevier, Amsterdam, Netherlands.

Lachance MA, Bowles JM, Starmer WT, Barker JSF (1999). *Kodamaea kakaduensis* and *Candida tolerans*, two new ascomycetous yeast species from Australian Hibiscus flowers. Can. J. Microbiol. 45: 172-177.

Martins MAM, Cardoso MH, Queiroz MJ, Ramalho MT, Campos AMO (1999). Biodegradation of azo dyes by the yeast *Candida zeylanoides* in batch aerated cultures. Chemosphere 38: 2455-2460.

Martorell MM, Pajot HF, de Figueroa LIC (2012). Dye-decolourizing yeasts isolated from Las Yungas rainforest. Dye assimilation and removal used as selection criteria. Int. Biodeterior. Biodegrad. 66: 25–32.

Máximo C, Amorim MTP, Costa-Ferreira M (2003). Biotransformation of industrial reactive azo dyes by *Geotrichum* sp. CCMI 1019. Enzyme Microb. Technol. 32: 145-151.

Meehan C, Bannat IM, McMullan, Nigam P, Smyth F, Marchant R (2000). Decolorization of Remazol Black-B using a thermotolerant yeast, *Kluyveromyces marxianus* IBM3. Environ. Int. 26: 75-79.

Nascimento C, Magalhães DP, Brandão M, Santos AB, Chame M, Baptista D, Nishikawa M, da Silva M (2011). Degradation and Detoxification of Three Textile Azo Dyes by Mixed Fungal Cultures from Semi-Arid Region of Brazilian Northeast. Braz. Arch. Biol. Technol. 54: 621-628.

Özsoy HD, Ünyayar A, Mazmanci MA (2005). Decolourisation of reactive textile dyes Drimarene Blue X3LR and Remazol Brilhant Blue R by *Funalia* trogii ATCC 200800. Biodegradation 16: 195-204.

Pajot HF, de Figueroa LIC, Farina JI (2007). Dye-decolorizing activity in isolated yeasts from the ecoregion of Las Yungas (Tucumán, Argentina). Enzyme Microb. Technol. 40: 1503-1511.

Pajot HF, Delgado OD, de Figueroa LIC, Fariña JI (2011). Unraveling the decolourizing ability of yeast isolates from dye-polluted and virgin environments: an ecological and taxonomical overview. Antonie Leeuwenhoek 99: 443–456.

Passarini MRZ, Rodrigues MVN, da Silva M, Sette LD (2011). Marine-derived filamentous fungi and their potential application for polycyclic aromatic hydrocarbon bioremediation. Mar. Pollut. Bull. 62: 364-370.

Pessis AM (1998). Parque Nacional Serra da Capivara. Perfil Sócio-econômico. Área de Preservação permanente. Municípios de Coronel José Dias e São Raimundo Nonato. FUMDHAM/SUDENE, Recife, Brazil.

Ramalho PA, Scholze H, Cardoso MH, Ramalho MT, Oliveira-Campos AM (2002). Improved conditions for the aerobic reductive decolourisation of azo dyes by *Candida zeylanoides*. Enzyme Microb. Technol. 31: 848-854.

Ramalho PA, Cardoso MH, Cavaco-Paulo A, Ramalho MT (2004). Characterization of azo reduction activity in a novel Ascomycete yeast strain. Appl. Environ. Microbiol. 70: 2279-2288.

Ramalho PA, Paiva S, Cavaco-Paulo A, Casal M, Cardoso MH, Ramalho MT (2005). Azo reductase activity of intact *Saccharomyces cerevisiae* cells is dependent on the Fre1p component of plasma membrane ferric reductase. Appl. Environ. Microbiol. 71: 3882-3888.

Singh H (2006). Mycoremediation: fungal bioremediation. Wiley-Interscience, New Jersey, USA.

Stolz A (2001). Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol. 56: 69-80.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599.

Yang Q, Yang M, Pritsch K, Yediler A, Hagn A, Schloter M, Kettrup A (2003). Decolorization of synthetic dyes and production of manganese-dependent peroxidase by new fungal isolates. Biotechnol. Lett. 25: 709–713.

Yang Q, Yediler A, Yang M, Kettrup A (2005). Decolorization of an azo dye, Reactive Black 5 and MnP production by yeast isolate: *Debaryomyces polymorphus*. Biochem. Eng. J. 24: 249-253.

Yarrow D (1998). Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman CP, Fell JW (ed) The Yeasts, a Taxonomic Study, 4th ed. Elsevier, Amsterdam, Netherlands, pp. 77-100.

Yu Z, Wen X (2005). Screening and identification of yeasts for decolorizing synthetic dyes in industrial wastewater. Int. Biodeterior. Biodegrad. 56: 109-114.

academicJournals

Vol. 12(47), pp. 6574-6582, 20 November, 2013
DOI: 10.5897/AJB11.2882
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

A comparative genetic diversity analysis in mungbean (*Vigna radiata* L.) using inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP)

Renu Singh[1]*, Adriaan W. van Heusden[1] and Ram C. Yadav[2]

[1]Laboratory of Plant Breeding, Plant Research International (PRI), Wageningen UR, Wageningen, Netherlands.
[2]Department of Biotechnology and Molecular Biology, CCS HAU, Hisar, Haryana, 125004, India.

Accepted 7 December, 2011

Amplified fragment length polymorphism (AFLP) and inter-simple sequence repeat (ISSR) markers were used to study the DNA polymorphism in elite mungbean genotypes. A total of nine AFLP primer combination and 22 ISSR primers were used. Amplification of genomic DNA of the 30 genotypes, using AFLP analysis, yielded 300 fragments that could be scored, of which 192 were polymorphic, with an average of 21.3 polymorphic fragments per primer. Number of amplified fragments with AFLP primers ranged from 29 (E-AAC: M-CAG) to 10 (E-ACG: M-CAT). Percentage polymorphism ranged from 46.3% (E-AAC: M-CCA) to a maximum of 100% (E-AAC: M-CAC), with an average of 64%. The 22 ISSR primers used in the study produced 108 bands across 30 genotypes, of which 68 were polymorphic. The number of amplified bands varied from two UBC820) to ten URP 6F). The average numbers of bands per primer and polymorphic bands per primer were 4.9 and 3.1, respectively. Percentage polymorphism ranged from 25% (UBC844) to 85% (UBC846, UBC864, UBC895), with an average percentage polymorphism of 58.3% across all the genotypes. AFLP markers were more efficient than the ISSR assay, as they detected 64% polymorphic DNA markers in *Vigna radiata* as compared to 58.3% for ISSR markers. The Mantel test between the two Jaccard's similarity matrices gave r = 0.19, showing low correlation between AFLP- and ISSR-based similarities. Clustering of genotypes within groups was not similar when AFLP and ISSR derived dendrograms were compared.

Key words: AFLP, ISSR, *Vigna radiata* (mung bean), marker index, unweighted pair-group method with arithmetic averages (UPGMA).

INTRODUCTION

Mungbean is a widely grown food grain legume in the developing world. It is cultivated almost in all the four seasons in one state or the other state of India. It is thought to have originated in the Indian subcontinent (de Condolle, 1884; Vavilov, 1926) with maximum diversity in the upper Western Ghats and Deccan hills and secondary centre of diversity in Indo-Gangetic plains. From the standpoint of production, (19.7 mt), field pea(10.4 mt), chickpea (9.7 mt), cowpea (5.7 mt), lentil (3.6 mt) and pigeon pea (3.5 mt) are the most important

*Corresponding author. E-mail: renu.singh@wur.nl, renuboora@gmail.com. Tel: +91-9466860066, +31- 683290784.

(FAO, 2010). Out of the 16 essential nutrients, seven are classified as micronutrients or trace elements. Out of these seven micronutrients, iron and zinc play very important and vital role in animals, humans and plants health and development. Beside this, it also improves the soil fertility by fixing atmospheric nitrogen into available form with the help of *Rhizobia* species present in the nodules of its roots (Ashraf et al., 2003). The major constraint in pulses improvement is presence of limited genetic diversity in primary gene pool. In pulses, the morphological characterization of accessions belonging to cultivated species reveals only ample genetic variability for a trait other than genetic diversity. The problem of narrow genetic base of the cultivated germplasm of pulses develop from using only few genotypes with a high degree of relatedness repeatedly as parents in crossing programmes for the development of new cultivars (Kumar et al., 2011). Pedigree analysis of released varieties of mungbean revealed that only the top three to ten ancestors contributed 30 to 79% to the genetic base (Katiyar et al., 2007, 2008).

Therefore, an assessment of the genetic diversity of pulses is an important first step in a program to improve crop yield along with the traditional molecular makers which provide valuable information and can be used in a number of ways in crop improvement programme. Inter simple sequence repeat (ISSR) analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites, using a single simple sequence repeat (SSR) motifs (di-, tri-, tetra-, or penta-nucleotides) containing primers anchored at the 3′ or 5′ end by two to four arbitrary, often degenerate nucleotides (Souframanien and Gopalakrishnan, 2004). The primers used in our analysis were anchored at 3′ end to ensure that perfect annealing of the primer occurs at the 3′ end of the microsatellite motif, thus obviating internal priming and smear formation. The anchor also allows only a subset of the targeted inter-repeat regions to be amplified, thereby reducing the high number of PCR. The sequence of repeats and anchored nucleotides were randomly selected and had the advantage of analysing multiple loci in a single reaction. Thus, ISSR offers several advantages and this technique is already used in many crop plants (Ajibade et al., 2000; Ranade et al., 2000).

Further, AFLP (amplified fragment length polymorphism) which combines the advantage of RFLP and PCR, has a high multiplex ratio (number of loci observed in a single assay) and have high reproducibility and thus prove very useful in genetic diversity analysis. AFLP's were extensively used in genetic characterization of germplasm/ cultivars of black gram, soybean, wild bean etc. (Gupta and Gopalakrishna, 2009). Different molecular marker combinations were previously used in grain legumes (Gupta and Gopalakrishna, 2008, 2009). Therefore, the objective of the present study was to evaluate and compare the genetic diversity among 30 elite genotypes selected on basis of their performance in previous year experiments of known origin, using ISSR and AFLP markers.

MATERIALS AND METHODS

Plant material and DNA extraction

Table 1 lists the green gram genotypes used in the present study. Materials were collected from pulses station, CCS HAU, Hisar, India. The material was selected on the basis of contrast micronutrient content and agronomic performance in experiment conducted in the previous years (work un-published). Young leaves from three to five weeks-old seedlings were immediately stored at -80°C until the total genomic DNA was extracted. The molecular analysis was carried out at the laboratory of plant breeding, plant research international, Wageningen University.

The stored leaf tissue from each individual was ground to a fine powder using two grinding beads in a Shatter-box and total genomic DNA was extracted using 96 well plate automated DNA isolation machine. In the buffer solution, RNAse and proteinase K were added to get DNA free from these impurities. The estimates were confirmed by ethidium bromide staining of the gels after electrophoresis in 0.8% agarose gel at 100 V for about 45 min in TBE (Tris boric acid ethylene diamine tetra-acetic acid) buffer using known DNA concentration standards.

PCR optimization and primer selection

Varying concentrations of template DNA (10 to 20 ng), Taq DNA polymerase (0.5 to 2 U) and $MgCl_2$ salt (0 to 5 mM) were used to optimize reaction conditions of the polymerase chain reaction (PCR). Four randomly selected cultivars, namely ML-803, MH-125, ML-5, ML-735, were chosen for primer survey. Out of the different primers combination, only nine of AFLP and twenty two of ISSR were found suitable and used to analyse all 30 genotypes.

AFLP amplification

Li-Cor AFLP kit was used according to manufacturer recommenddations. According to the kit, 100 ng of pure DNA was digested with EcoR I and MSe I restriction enzymes. The enzyme adaptors were ligated to the digested DNA. The pre-amplification product was analysed on 1% agarose gel in 0.5XTBE buffer, run at 80 to 100 V for about 35 to 45 min. Selective amplification of restriction fragments was conducted using primers with three selective nucleotide labelled IRD700/800 with dyes.

After this, the reaction product was mixed with an equal volume 10 µl of formamide –loading buffer (98% formamide, 10 mM EDTA, pH 8.0 and 0.1% Bromo-phenol blue). The total mixture was carefully vortexed and heated for 5 min at 94°C in denaturation hot-block and then quickly cooled to ice). Out of the total 10 µl, 8 µl is loaded on a 6% denaturing polyacrylamide gel 1XTBE buffer. Li-Cor 4300 S DNA analyser machine was used to image, analyse and screen markers.

ISSR amplification

ISSR amplification reactions were carried out in 25-µl volume containing 50 ng template DNA, 0.5 U Taq DNA polymerase, 10

Table 1. List of mungbean (*Vigna radiata* L.) along with their origin, and micronutrients (Fe and Zn) used in the study.

Code No.	Variety	Origin	Code No.	Variety	Origin
1	ML-803	Ludhiana (Punjab)	16	ML-818	Ludhiana (Punjab)
2	MH-125	Hissar (Haryana)	17	ML-406	Ludhiana (Punjab)
3	ML-5	Ludhiana (Punjab)	18	2KM-151	Pant Nagar (Uttrakand)
4	ML-735	Ludhiana (Punjab)	19	2KM 155	-
5	2KM 112	IARI (New Delhi)	20	2KM-138	Hissar (Haryana)
6	ML-1108	Ludhiana (Punjab)	21	MH3-18	Hissar (Haryana)
7	MI-3580	Ludhiana (Punjab)	22	MH-124	Hissar (Haryana)
8	ML-839	Ludhiana (Punjab)	23	ASHA	Hissar (Haryana)
9	L-24-2	Ludhiana (Punjab)	24	MH-215	Hissar (Haryana)
10	MH-421	Hissar (Haryana)	25	SMH-99-DULL B	Hissar (Haryana)
11	2KM-139	Hissar (Haryana)	26	PDM-9-249	Kanpur (Uttar Pradesh)
12	2KM 135	(Rajasthan	27	ML-759	Ludhiana (Punjab)
13	SMH-99-2	Hissar (Haryana)	28	M 395	Ludhiana (Punjab)
14	2 KM-107	-	29	PMB-14	Ludhiana (Punjab)
15	BG-39	Bangladesh	30	ML-506	Ludhiana (Punjab)

mM dNTP, 10 µM primer, in 1× reaction buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$. Amplification was performed in an Eppendorf Master cycler gradient. Amplification conditions were one cycle at 94°C for 4 min, and 94°C for 30 s, 55°C for 45 s, followed by 72°C for 2 min. In the subsequent 35 cycles, annealing temperature was maintained at 50°C, followed by one cycle of 7 min at 72°C. Amplified products were loaded on 2% agarose gel and separated in 1× TBE buffer at 75 V. The gels were visualized under UV after staining with ethidium bromide and documented using gel documentation.

Statistical procedure

Gel images were scored using the QUANTAR software for the presence (1) and absence (0) of an amplification product across the lanes for each of the primers combinations. Data were statistically analysed by the software program NTSYSpc 2.01b (Numerical Taxonomy and Multivariate Analysis System, Applied Biostatistics Inc., 1986 to 1997) (Rohlf, 1990). The SIMQUAL program was used to calculate the Jaccard's coefficient, a common estimator of genetic identity and was calculated as follows:

Jaccard's coefficient = $N_{AB} / (N_{AB} + N_A + N_B)$

Where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated. Similarity matrices were utilized to construct the un-weighted pair group method with arithmetic average (UPGMA) dendrogram. The Jaccard's similarity coefficients matrix was subjected to principal coordinates analysis for three-dimensional plot to depict the relationships among the varieties.

In order to characterize the capacity of each primer to detect polymorphic loci among the genotypes, marker index for AFLP and ISSR markers were calculated. It is the sum total of the polymorphism information content (PIC) values of all the markers produced by a particular primer. PIC value was calculated as follows:

PIC = *1-Σpi 2,*

Where, *pi* is the frequency of the i^{th} allele (Smith et al., 1997). While marker index (MI) is calculated as PIC value multiples with the number of polymorphic bands.

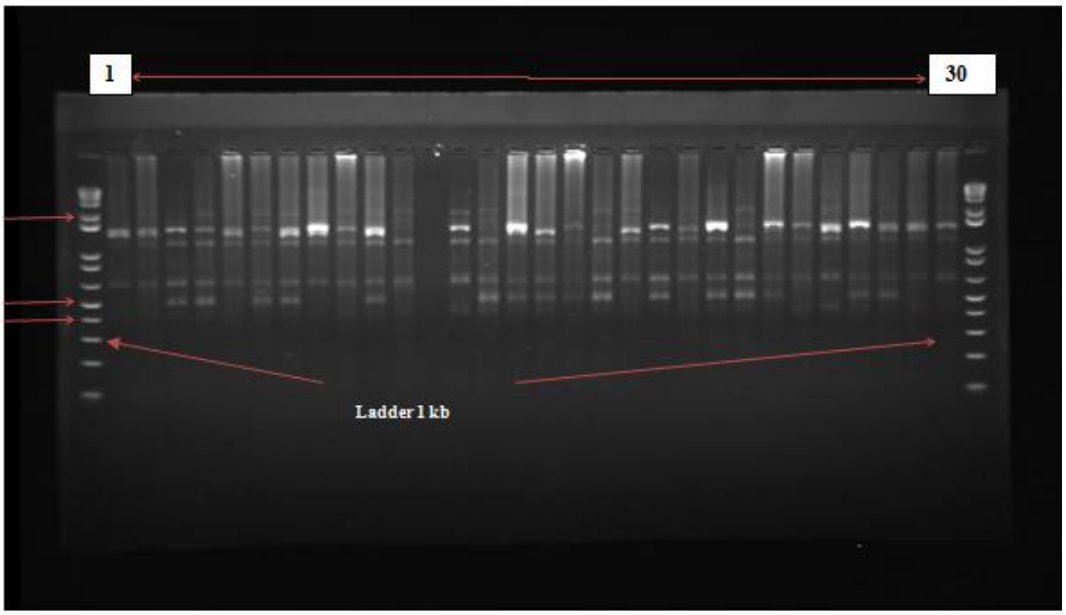
RESULTS

The criteria for selecting the primer pairs were a) high-quality of amplification, b) polymorphism among the cultivars used for primer survey. Figures 1a and b are representatives of AFLP and ISSR pattern obtained in the present study.

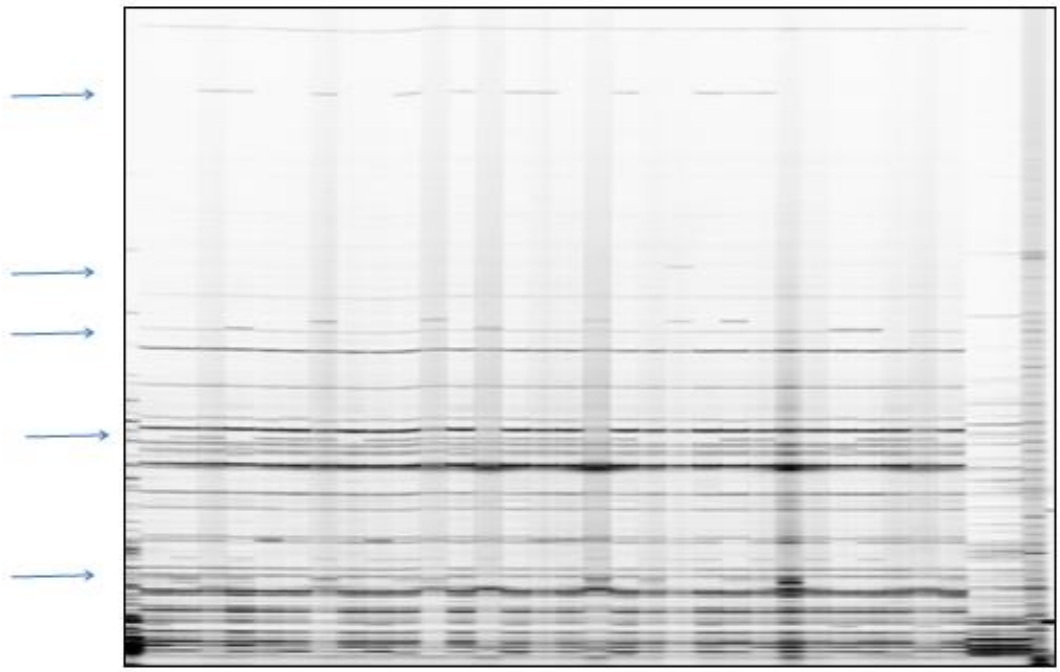
ISSR analysis

All the 23 ISSR markers were successfully amplified across the 30 mungbean genotypes but only 22 markers were polymorphic (Table 2). The 22 makers collectively yielded 108 amplification products with about bands bands per primer. ISSR primers gave 58.3% polymorphism and the PIC value ranged from 0.09 to 0.71 with an average of 0.46 (Figure 2a).

The UPGMA distributed the 30 genotypes into five main clusters, clusters with Dice similarity coefficient ranging from 0.65 to 0.85 (Figure 3a). Cluster I comprised of six genotypes (ML-803, MH-125, BG-39, 2KM 155, MH3-18 and MH-215).



a



b

Figure 1. (a) PCR amplification pattern for ISSR markers (UBC 821) in thirty green gram genotypes (Lane 1 and 32 is marker λ DNA marker and Lanes 2 to 31 green gram genotypes as listed in Table 1). (b) AFLP fingerprint generated by AFLP pair E-ACG:M-CAT in thirty green gram genotypes. The arrows indicate the polymorphic marker fragments.

Cluster II is the largest group among all the clusters with 12 genotypes (ML-5, ASHA, PDM-9-249, MH-124, SMH-99-DULL B, PMB-14, ML-506, MH-421, ML-406, 2KM-139, 2 KM-107 and 2KM-151) while cluster III comprised of only six (ML-1108, ML-759, MI- 3580, ML-839, M 395 and M 395) genotypes. 2KM112 and L-24-2

Table 2. List of primers and their sequences along with some of the characteristics of the amplification products obtained by selected twenty two ISSR primers in thirty genotypes of mungbean (*V. radiata* L.).

S/No.	Marker	Primer sequence (ISSR)	Total no. of amplification product	No. of polymorphic product	Percentage of polymorphism	PIC value of primer
1	UBC820	GTGTGTGTGTGTGTGTC	2	1	50	0.458
2	UBC836	AGAGAGAGAGAGAGAGYA	6	4	66.6	0.085
3	UBC821	GTGTGTGTGTGTGTGTA	5	4	80	0.144
4	UBC844	CTCTCTCTCTCTCTCTRC	4	1	25	0.194
5	IS 61	GAGAGAGAGAGAGAGAT	4	2	50	0.511
6	IS 65	AGAGAGAGAGAGAGAGT	4	2	50	0.523
7	UBC811	GAGAGAGAGAGAGAGAC	3	1	33.3	0.689
8	IS 63	AGAGAGAGAGAGAGAGC	3	1	33.3	0.611
9	UBC849	GTGTGTGTGTGTGTGTYA	4	2	50	0.465
10	UBC855	ACACACACACACACACYT	3	1	33.3	0.548
11	UBC857	ACACACACACACACACYG	7	4	57.1	0.635
12	UBC848	CACACACACACACACARG	7	5	71.4	0.462
13	UBC846	GAGAGAGAGAGAGAGAA	5	4	85.7	0.464
14	UBC864	ATGATGATGATGATGATG	7	6	85.7	0.433
15	UBC880	GGAGAGGAGAGGAGA	6	4	66.6	0.327
16	UBC812	GAGAGAGAGAGAGAGAA	3	1	33.3	0.537
17	UBC862	AGCAGCAGCAGCAGCAGC	3	1	33.3	0.704
18	URP 6F	GGCAAGCTGGTGGGAGGTAC	10	8	80	0.594
19	UBC835	AGAGAGAGAGAGAGAGYC	5	4	96	0.525
20	UBC859	TGTGTGTGTGTGTGTGRC	4	2	50	0.421
21	UBC895	AGGTCGCGGCCGCNNNNNNAT	7	6	85.7	0.419
22	URP 13R	TACATCGCAAGTGACACACC	6	4	66.6	0.463
		Total	108	68	1283	10.21
		Average	4.91	3.09	58.31	0.464

formed the fourth cluster and cluster V comprised of three genotypes (ML-5, 2KM 135, and SMH-99-2).

One genotype namely, ML 818 forms an out-group by not falling in any cluster. Principal component analysis (PCA) also resolved the ML-803 and BG-39 as a separate unit in cluster analysis (Figure 4a).

AFLP analysis

Nine AFLP primer combinations used to evaluate the genetic diversity among the 30 mungbean genotypes produced 300 scorable amplification products (Table 3). The number of amplified fragment per primer combination ranged from 13 (E-ACG: M-CAT) to 54 (E-AAC: M-CCA) with an average of 33.3 fragments per primer combination. The maximum polymorphism was shown by E-AAC: M-CAC (100% polymorphic) while the

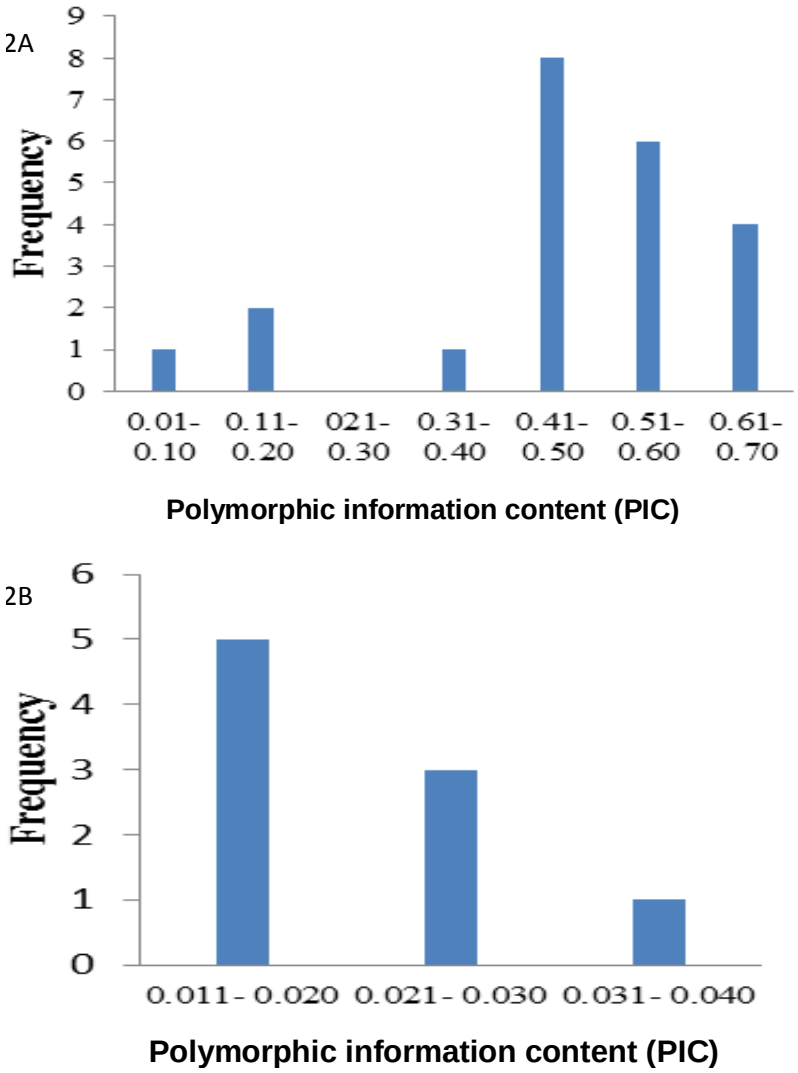


Figure 2. Polymorphic information content score for (a) 22 ISSR markers and (b) 9 AFLP primer combinations in thirty mungbean genotypes.

minimum polymorphism (46.3%) was shown by E-AAC: M-CCA. The PIC of individual AFLP markers varies from 0.01 to 0.04 (Figure 2b). Jaccard's similarity coefficient values were calculated from the data and Dice similarity coefficient ranged from 0.59 to 0.78 with an average of0.69. Further these were used to construct a UPGMA dendrogram (Figure 3b) and three-dimensional plot.

The UPGMA analysis distributed the 30 genotypes into main four clusters. Cluster I comprised of five genotypes (ML-803, BG-39, MH3-18, SMH-99-DULL B, PMB-14) and the cluster being the biggest cluster carried 16 genotypes (MH-125, 2KM 155, MH-215, ML-839, M 395, ML-5, 2KM 135, MH-124, MH-421, 2 KM-107, 2KM-151, 2KM 112, L-24-2, ML-1108, ML-759 and SMH-99-2).

Only five genotypes (MI-3580, 2KM-139, 2KM-138, ML-406, ML-506) were there in cluster III and genotypes, ML-735, PDM-9-249 and ASHA made the last cluster IV. In the AFLP dendrogram, ML-818 again makes an out-group as in case of ISSR dendrogram. Further from the PCA, it was found that ML-803 and Asha appeared to be distinct from other genotypes as in cluster analysis (Figure 4b).

DISCUSSION

Genetic variation was detected among 30 genotypes of mungbean using the ISSR and AFLP marker techniques. A total of nine primer combinations were used in AFLP study and all the primers gave polymorphic bands rang-

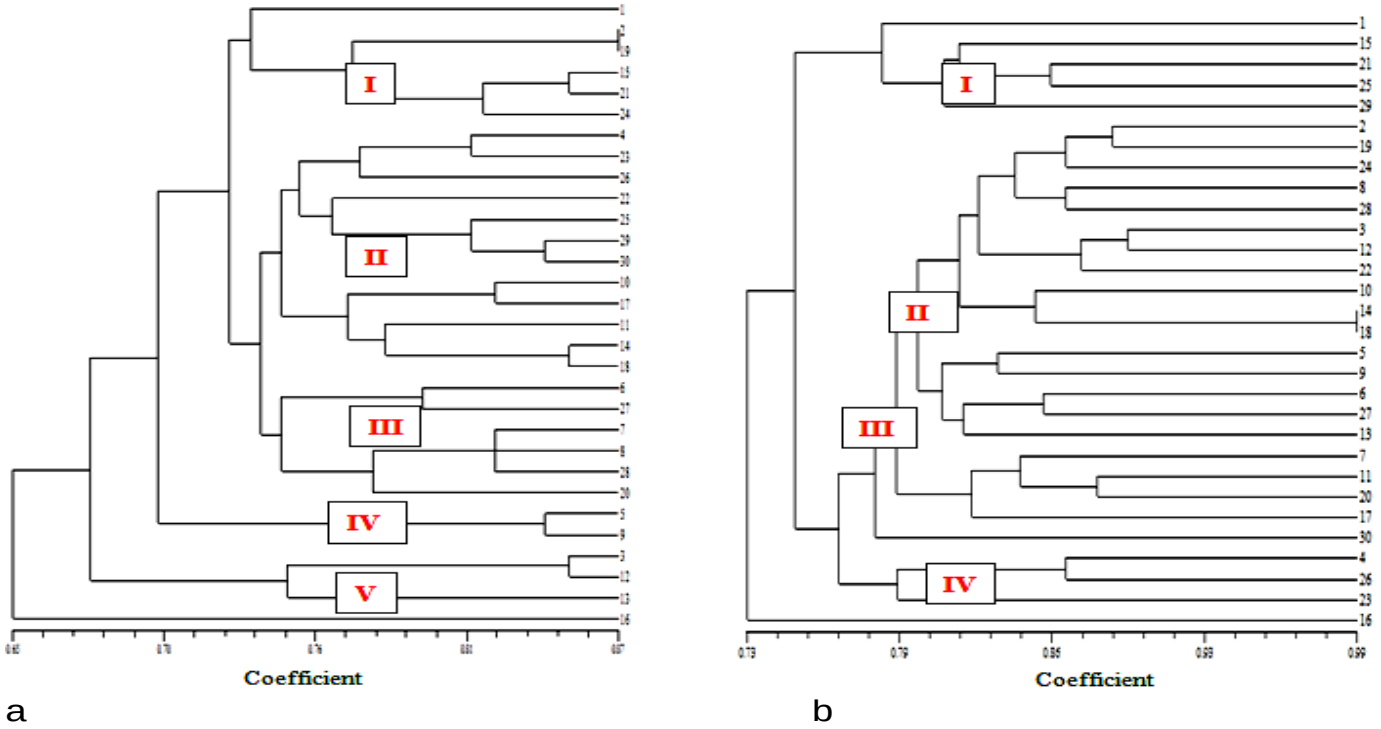


Figure 3. A UPGMA dendrogram of genetic relationships of the thirty genotypes constructed from (a) ISSR and (b) AFLP. ISSR, Inter-simple sequence repeat; AFLP, amplified fragment length polymorphism.

ing from 28 to 10 bands, while in case of ISSR markers out of the 23 primers only one did not show polymorphism while the rest showed. The results obtained in the present study shows a moderate genetic similarity for both the markers techniques (AFLP- 0.73 to 0.99; ISSR-0.65 to 0.87).

Thus, in comparison to the AFLP study ISSR responded well. This was also supported by previous studies (Russell et al., 1997; Meng and Chen, 2000). ISSR techniques have been previously used in estimating the genetic relationships in genus *Vigna* (Ajibade et al., 2000) and in several other crops (Souframanien and Gopalakrishna, 2004). Similarly, the AFLP marker techniques was also used successfully in different crops and legumes such as common bean (Tohme et al., 1996), cowpea (Coulibaly et al., 2002), pigeon pea (Panguluri et al., 2006), black gram (Gupta and Balakrishna, 2008) and azuki bean (Xu et al., 2000).

In the study, 22 polymorphic ISSR markers produced 68 alleles with an average 3.1 alleles per locus. The results are comparable with those reported earlier for other grain legume including black gram (3.4 alleles per locus; Souframanien and Gopalakrishna, 2004) and soybean (3.3 alleles per locus, Meng et al., 2001). The average PIC score of 33 ISSR markers was 0.46 with over 50% loci having PIC value > 0.5 (Figure 2a), indicating high resolving power of the ISSR markers. The markers were able to discriminate the 30 genotypes in five clusters on the basis of their performance in the field. Cluster I comprised of a short duration (<60 days), dwarf genotype with high resistance to mungbean yellow mosaic virus, while the biggest cluster II comprised of genotypes which showed moderate resistance against the MYMV virus and with medium height (65 to 70 cm). Cluster III comprise of early maturing tall (>85 cm) and moderate resistant genotypes while the smallest cluster V, which comprised of two genotypes with medium height, took average of 65 days to maturity and showed moderate resistance against the virus. As the marker is comparable to the traits shown by the genotypes in the field therefore, the ISSR markers thus would provide valuable tool for the varietal identification and germplasm maintenance in mungbean.

In AFLP, 9 AFLP primers combination generated 300 polymorphic bands with an average 21.3 bands per primer combination. As AFLP markers shows high multiplex ratios, much of the genome is covered using fewer primer combinations. Earlier the comparison of different marker techniques showed that the AFLP is the most efficient method to estimate genetic diversity compared to random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) (Powell et al., 1996;

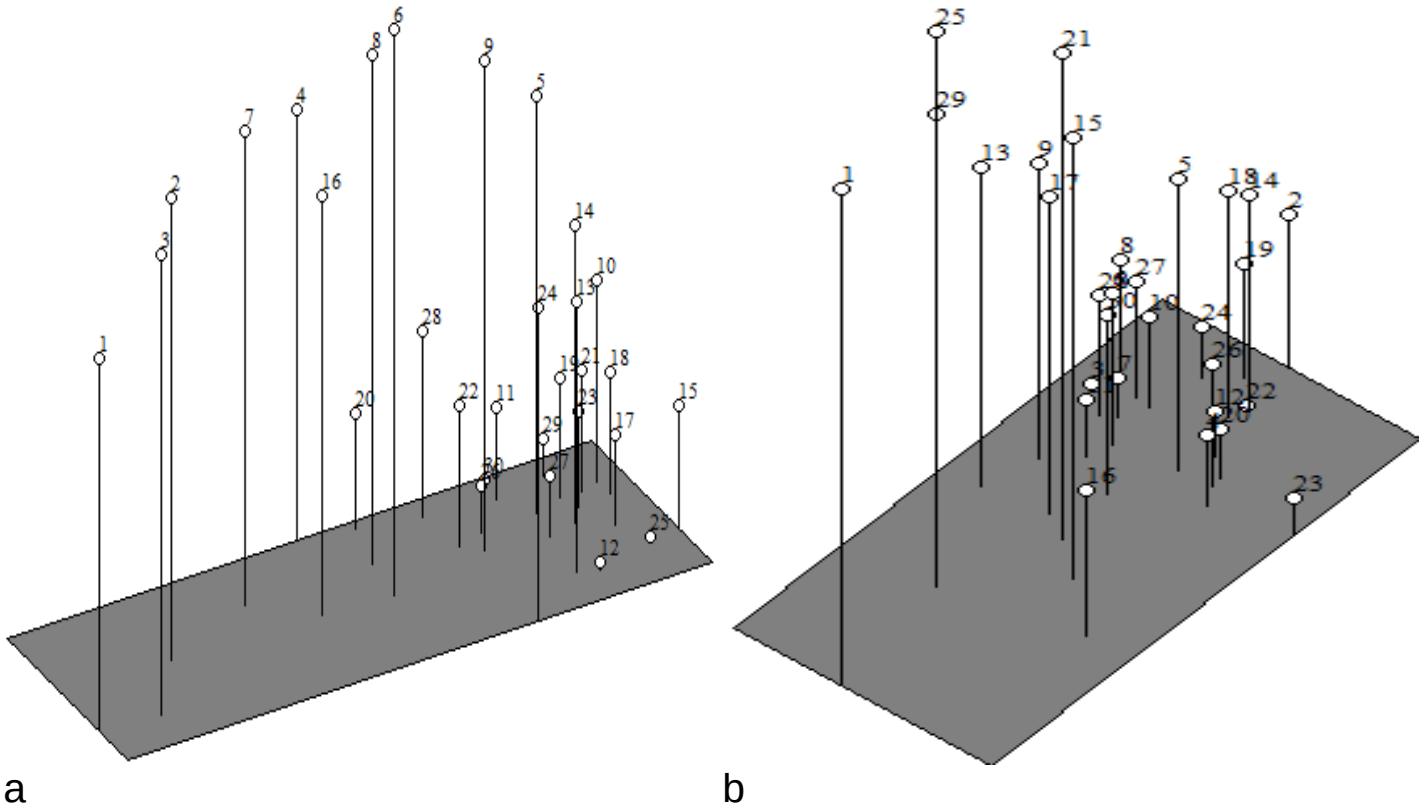


Figure 4. Principal componet analysis map showing the relationship among the mungbean genotypes based on (a) ISSR and (b) AFLP data. The number plotted represents individual genotypes and corresponds to those listed in Table 1. ISSR, Inter-simple sequence repeat; AFLP, amplified fragment length polymorphism.

Table 3. List of primers and their sequences along with some of the characteristics of the amplification products obtained by selected nine AFLP primer combination primers in 30 genotypes of mungbean (*V. radiata* L.).

S/N	Marker	Primer sequence (AFLP)	Total no. of amplification product	No. of polymorphic product	Percentage of polymorphism	PIC value of primer	Marker index
1	P32+P51	E-AAC:M-CCA	54	25	46.3	0.014	0.35
2	P35+P50	E-ACA:M-CAT	33	28	84.8	0.018	0.51
3	P35+P48	E-ACA:M-CAC	30	18	60.0	0.021	0.38
4	P37+P50	E-ACG:M-CAT	13	10	76.9	0.037	0.37
5	P35+P59	E-ACA:M-CTA	43	25	58.2	0.017	0.43
6	P35+P62	E-ACA:M-CTT	42	28	66.7	0.014	0.39
7	P32+P47	E-ACC:M-CAA	36	21	58.3	0.019	0.39
8	P32+P48	E-AAC:M-CAC	20	20	100	0.021	0.42
9	P32+P49	E-AAC:M-CAG	29	17	58.6	0.021	0.36
Total			300	192	609.8	0.182	34.9
Average			33.3	21.3	64	0.02	0.43

Gupta, 2008). To characterize the capacity of each primer to revel or detect polymorphic loci in the genotypes, primers index was calculated which varied from 0.35 (E-AAC:M-CCA) to 0.51 (E-ACA:M-CAT) with an average of 0.43. Cluster analysis from AFLP reveals that cluster II is the biggest cluster and carries mostly resis-

tance to moderate resistance with medium to tall genotypes while the smallest cluster III comprised of genotypes with early maturity (< 60 days) with medium height (65 to 75 cm) and with less or no resistance against the virus. The clustering pattern in both the markers system was comparable to some extent. In both the dendrogram, one genotypes ML-818 was out grouped which may be thought to be as a result of some contamination.

In the current study, the average PIC score for the ISSR marker (0.46) was much higher compared with AFLP marker (0.02) indicating that ISSR are more informative than the AFLP's. Cluster analysis based on the ISSR and AFLP data grouped 30 genotypes into six main clusters. However, clustering of the genotypes within the groups was not similar when both the dendrogram were compared. In PCA analysis, genotypes were resolved into four different groups in the case of the AFLP and results were congruent with the cluster analysis, but in case of ISSR, genotypes could not be resolved well in PCA.

The matrices for ISSR and AFLP marker were also compared using mantels test (Mantle, 1967) for matrix correspondence. The correlation values for the dendrogram based on ISSR and AFLP data was low (r = 0.19). A possible explanation to this low magnitude between the marker techniques is that the two marker techniques targeted different positions of the genome. The ability to resolve genetic variation among different genotypes may be more directly related to the number of polymorphism detected with each marker technique rather than a function of which technique is employed. Our results indicate the presence of moderate genetic variability among the elite mungbean genotypes. ISSR markers are useful in the assessment of the mungbean diversity and the selection of core collection to enhance the efficiency of germplasm management for use in the mungbean breeding and conservation.

REFERENCES

Ajibade SR, Weeden NF, Michite S (2000). Inter simple sequence repeat analysis of genetic relationships in the genus *Vigna*. Euphytica 111(1):47-55.

Ashraf M, Mueen-ud-din M, Warraich NH (2003.) Production efficiency of mungbean as affected by seed inoculation and NPK application. Int. J. Agric. Biol. 5:179-180.

Coulibaly S, Pasquet RS, Papa R, Gepts P (2002). AFLP analysis of the phenetic organization and genetic diversity of *Vigna unguiculata* L. Walp. reveals extensive gene flow between wild and domesticated types. Theor. Appl. Genet. 104:358-366.

De Candolle AP (1884). Origin of cultivated plants. Kegan Paul, Trench, London.

Gupta SK, Gopalakrishna T (2008). Molecular markers and their application in grain legumes breeding. J. Food Legumes. 21:1-14.

Gupta SK, Gopalakrishna T (2009). Genetic diversity analysis in blackgram (*Vigna mungo* (L.) Hepper) using AFLP and transferable microsatellite markers from azuki bean (*Vigna angularis* (Willd.) Ohwi & Ohashi). Genome.52 (2):120-129.

Katiyar PK, Dixit GP, Singh BB (2007). Ancestral relationship of greengram (Vigna radiata) advance breeding lines developed in India. Indian J. Agri. Sci. 77:579-582.

Katiyar PK, Dixit GP, Singh BB (2008). Genetic base of advanced urdbean breeding lines developed in India as revealed by pedigree analysis. Indian J. Genet. 68:324-326.

Kumar J, Arbind KC, Ramesh KS, Aditya P (2011). Towards markers-assisted selection in pulses: a review. Plant Breeding. 130:297-313.

Mantle N (1967). The detection of disease clustering and generalized regression approach. Cancer Res. 27:209-220.

Meng X, Chen W (2001). Applications of AFLP and ISSR techniques in detecting genetic diversity in the soybean brown stem rot pathogen *Phialophora gregata.* Mycol. Res. 105 (8):936-940.

Panguluri SK, Janaiah K, Govil JN, Kumar PA, Sharma PC (2006). AFLP fingerprinting in pigeonpea (*Cajanus cajan* (L.) Millsp.) and its wild relatives. Genet. Resour. Crop Evol. 53:523-531.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 2:225-238.

Ranade R, Vaidya UJ, Kotwal SA, Bhagwat A, Gopalakrishna T (2000) Hybrid seed genotyping and plant varietal identification using DNA markers. In: DAE-BRNS symposium on the use of nuclear and molecular techniques in crop improvement, 6–8 December 2000, Mumbai: 338–345.

Rohlf FJ (1990). NTSYS -pc. *Numerical taxonomy and multivariate analysis system.* Exeter Software, New York.

Russell JR, Fuller JD, Macaulay M, Hatz BG, Jahoor A, Powell W, Waugh R (1997). Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs, and RAPDs. Theor. Appl. Genet. 95:714-722b.

Smith, JSC, Chin ECL, Shu H, Smith OS, Wall SJ, Senior ML, Mitchell SE, Kresovich S, Zeigle J (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. Theor. Appl. Genet. 95(1-2):163-173.

Souframanien J, Gopalakrishna T (2004). A comparative analysis of genetic diversity in black gram genotypes using RAPD and ISSR markers. Theor. Appl. Genet.109:1687-1693.

Tohme J, Gonzalez DO, Beebe S, Duque MC (1996). AFLP analysis of gene pools of a wild bean core collection. Crop Sci, 36**:** 1375-1384.

Vavilov NI (1926). Studies on the origin of cultivated plants. Inst. Bot. Appl. et d' Amelior. des Plantes. State Press, Leningrad (In Russian and English).

Xu RQ, Tomooka N, Vaughan DA (2000). AFLP markers for characterizing the azuki bean complex. Crop Sci. 40:808-815.

academicJournals

Vol. 12(47), pp. 6591-6598, 20 November, 2013
DOI: 10.5897/AJB12.1920
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Response of cowpea genotypes to *Alectra vogelii* parasitism in Kenya

J. Karanja*, S. N. Nguluu, J Wambua and M. Gatheru

Kenya Agricultural Research Institute (KARI) – Katumani, P.O Box 340-90100, Machakos, Kenya.

Accepted 24 October, 2013

Cowpea is popular in Eastern Kenya where it is attractive to farmers because of its high economic value and the belief that it does not require many external inputs. Farmers are however discouraged to grow the crop in this region due to massive attack by a parasitic weed *Alectra vogelii* (Benth). Yield losses due to *A. vogelii* have being estimated to range from 50 to 100% in Mbeere, Kitui and Makueni districts. No single method however is available to farmers in these regions in control of the parasitic weed. Combining several control methods, as in the management of *Striga spp* in Western Kenya should be a sustainable option. Field studies were conducted in 2010 and 2011 at Kenya Agricultural Research Institute (KARI), Kiboko farm to determine the response of 143 cowpea genotypes to *Alectra* infestation. The aim for the study was to identify resistant genotypes that could be used in breeding programme. Significant differences were observed amongst cowpea genotypes in days to first *Alectra* emergence, number of *Alectra* shoots emerged at 6, 8, 10 and 12 week after planting and grain yield. Cowpea genotypes Kir/Nya-005 and Mbe/Mach-022 showed complete resistance to *Alectra* while Ken-Kunde, M66 and K80 (all commercial varieties) supported the highest number of *Alectra* shoots. Grain yield loss in the three susceptible varieties was 80, 79 and 50% respectively. On the other hand, Sia/Cia-004, Mbe/Mach-014 and Kib-006 had high grain yields despite the high number of *Alectra* shoots present. There was a strong correlation (r = -0.57) between grain yield and number of *Alectra* shoots emerged at 12 weeks after planting. A significant negative (r = -0.37) correlation was also obtained between pod number per plant and number of emerged *Alectra* shoots at 12 weeks after planting. This negative correlation proves the high accumulation dry matter in the cowpea roots at the expense of the pods thus decreasing grain yield. This information showed that there is sufficient genetic variability in the cowpea genotypes studied, which can be exploited in breeding improved cowpea varieties for resistance to *A. vogelii* in Kenya. A great progress towards developing improved cowpea variety that meets farmer's preferences with durable resistance to *A. vogelii* can be achieved if the genes from the resistant and tolerant local cowpea cultivars identified in this study could be introgressed into the adapted susceptible improved varieties. This will increase the potential impact of adoption of resistant cowpea varieties in the zones.

Key words: Cowpea, *Alectra vogelii*, Resistance/tolerance and grain yield.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. (Walp.) is an herbaceous warm-season annual crop that is similar in appearance to common bean except that leaves are generally darker green, shinier, and less pubescent. Early maturing cowpea

cowpea varieties provide the first food from the current harvest sooner than any other crop (in as few as 55 days after planting), thereby shortening the “hunger period” that often occurs just prior to harvest of the current season’s crop in farming communities in the developing world. The relatively high protein content (22%) of cowpea makes it an important supplement to the diet of many African people (Bressani, 1995) who consume cereals, roots, and tuber high in carbohydrate and low in protein. Being a fast growing crop, cowpea curbs erosion by covering the ground, fixes atmospheric nitrogen, and its decaying residues contribute to soil fertility (Carsky et al., 2002; Tarawali et al., 2002; Sanginga et al., 2003).

Cowpea is the second most important grain legume in Kenya after common beans. The area under cowpea is estimated at 1800 hectares excluding the area under the crop in home gardens (Kimiti et al., 2009). About 85% of the total area under the cowpea is in arid and semi-arid lands (ASALs) of Eastern province and 15% in the Coast, Western, and Central provinces (Kimiti et al., 2009). Despite its importance in the dry regions of Eastern Kenya, its potential, growth and yield are constrained by several abiotic and biotic factors. Among them include low soil fertility, inadequate farm inputs, noxious weeds, pest and diseases and lack of seeds during planting times. This has decreased in the yield potential of 1500 to 239 kg/ha (Kimiti et al., 2009). A parasitic weed *Alectra vogelii (*Benth) an obligate root-parasitic flowering plant of the family *Scrophulariaceae* is one of the major concerns in lowering cowpea yields. In 1929, one report estimated a 20% loss in yield for cowpea crops in Embu district Eastern Kenya (Bagnall-Oakley et al., 1991).

A. vogelii has also being observed in Bambara (*vigna subterranean* (L) Verdc.), soyabean (*Glycine max* (L) Merr.), mung bean (*Vigna radiate* (L.) Wilczek.), ground nut (*Arachi hyogaea* L.) and common bean (*Phaseolus vulgaris* L.) (Botha, 1948; Visser, 1978; Salako, 1984; Riches, 1989; Riches et al., 1992; Lagoke et al., 1993). Although *A. vogelii* is autotrophic, its photosynthetic rate is half that of host leaf on per gram dry mass basis (Gouws et al., 1980). It is said that it is not able to fix carbon at a daily rate in excess of its diurnal requirements (Harpe et al., 1981). This means *A. vogelii* depends on the host photosynthate as it induces the formation of lateral roots of the host plant (Doerr et al., 1977), also for water and nutrients. Information on cowpea yield losses resulting from *A. vogelii* infection is very scarce, but ranges from 41% (Lagoke et al., 1993) and 80 – 100% in Botswana (Riches,1989) in a highly susceptible cultivar Black-eye. In Tanzania yield losses of up to 50% have been reported (Mbwaga et *a*l., 2000). Yield losses of up to 15% have been reported in groundnut in Nigeria (Salako, 1984), while in South Africa 30 – 50% reductions in yield of bambara were reported (Beck, 1987). The negative effect of the weed in reducing the vegetative and grain yield of cowpea has been well researched (Botha, 1948; Visser et al., 1977, 1990; Okonkwo and Raghavan, 1982; Rambakudzibga et al., 2002). Reports show that yield reduction is mediated through the delayed onset of flowering, reduced number of flowers and pods, and reduced mass of pods and grain (Mugabe, 1983).

From a recent survey of the level of *A. vogelii* infestation on farmers’ field, Karanja et al. (2010) observed that more than 80% of the fields grown to cowpea in Mbeere district of Eastern Kenya were infested with *A. vogelii* leading to serious crop losses. The threat of *A. vogelii* to the crop is increasing with farmers reporting up to 100% yield loss under severe infestation in these regions (Karanja et al., 2010). It is expected that the rapid spread of this parasitic weed and the enormous yield reduction caused would constitute a severe threat to cowpea production in the region.

Despite the scourge of *Alectra* on cowpea, relatively less work has been done on its control in Kenya. Several control measures, including hand weeding, chemical control, biological control, trap crops and host plant resistance have been suggested (Boukar, 2004; Riches, 1993). Of all these methods, host plant resistance appears the most effective, economical and environmentally friendly method in controlling the *Alectra* and affordable to farmers (Rubiales et al., 2006; Mainjeni, 1999; Riches, 1989). While a number of improved, high yielding *striga/Alectra* resistant cowpea genotypes have been developed and are fast becoming popular with farmers in Nigeria, Bukinafaso, Malawi, Tanzania and South Africa (Singh et al., 2002; Kabambe et al., 2008), the same cannot be said for Kenya.

Cowpea as a crop of resource-poor households been affected by *A. vogelii,* it imposes an additional stress with which farmers, who have little capacity for investment in crop production, have to cope in an environment characterized by marginal rainfall for cropping and declining soil fertility. Cowpea has traditionally been grown in multiple cropping systems in which low populations of landraces are planted in mixtures with cereals. An increase in the importance of *A. vogelii* in the last 20 years in Kenya has often been associated with a change to sole cropping of introduced, potentially higher yielding susceptible cultivars, an increase in the area and frequency of cultivation (Farms’ own communication, Mbeere district). Based on the foregoing, it is clear that there is need to screen for *Alectra* resistance among existing local cowpea cultivars or varieties. This would aid in identifying *A. vogelii* resistant genotypes that can be exploited in breeding improved cowpea varieties for resistance to *A. vogelii* in Kenya. A great progress towards developing improved cowpea

*Corresponding author. E-mail: jakakah79@gmail.com.

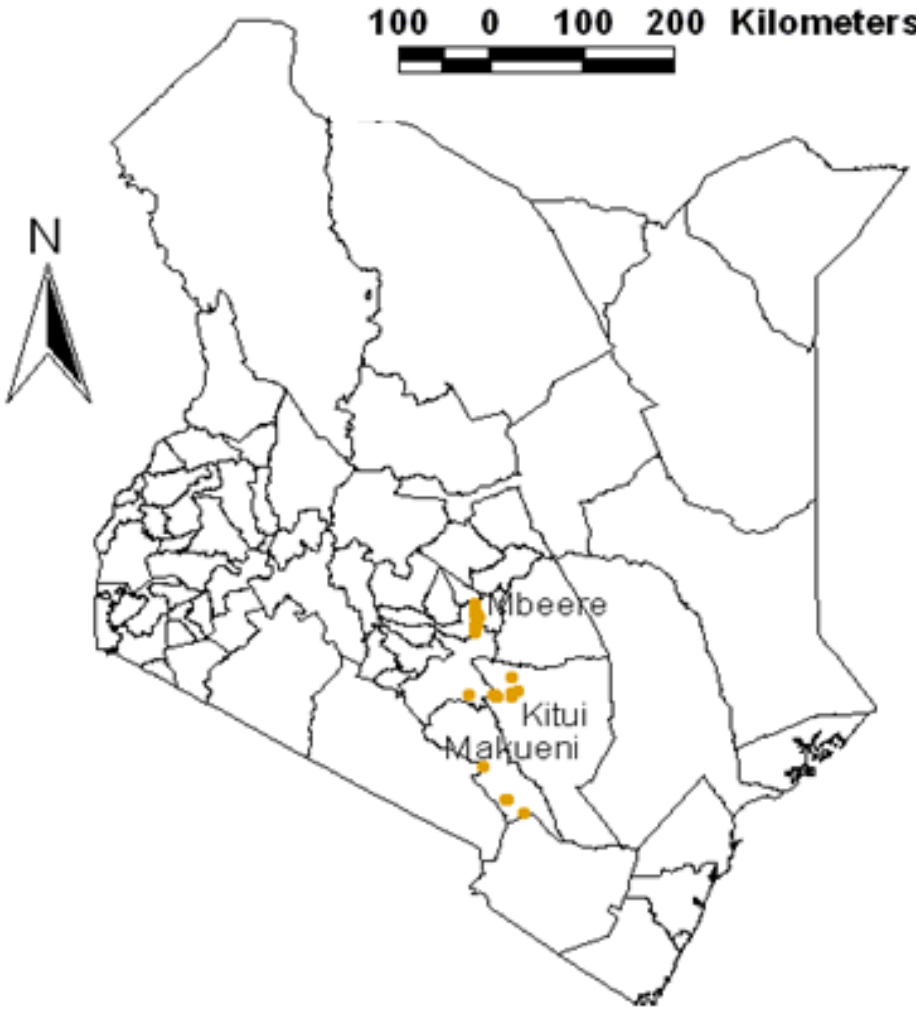


Figure 1. Map showing areas with high incidences of *Alectra vogelii* weed and point of cowpea local germplasm collection points in Eastern Kenya.

variety that meets farmer's preferences with durable resistance to *A. vogelii* can be achieved if the genes from the farmer's local cultivars are introgressed into the adapted susceptible improved varieties in the area. This would increase the potential impact of adoption of resistant cowpea varieties in the zones. The objectives of the studies in this paper were to collect and screen collected accessions of cowpea for their resistance/tolerance/ susceptibility to *A. vogelii* in Kenya.

MATERIALS AND METHODS

Pot and field evaluations were conducted at the research farm of Kenya Agricultural Research Institute, Kiboko sub-centre (11° 11'N; 7° 38'E; 686 m above sea level). The pot screening was conducted in 2009 while field trials were conducted in 2010 and 2011 under irrigation. A total of 143 cowpea genotypes were collected from Mbeere, Kitui, Makueni and Makindu (Figure 1). The 143 cowpea genotypes and two commercial checks were planted in pots. The experiment was arranged in a randomized complete block design with three replications. *Alectra* inoculum stock was obtained by thoroughly mixing 30 g of *Alectra* seeds with 500 g of sieved sand. About 10 g of *A. vogelii* from the stock (about 7,000 *Alectra vogelii* seeds) were mixed with 1.8 kg of top soil (4-10 cm) and transferred into 2 kg polythene pot. Four seeds were sown in each pot and thinned to two plants two weeks after planting. The pots were watered everyday and maintained free from any other weed other than *A. vogelii* in the course of the research through hand weeding. Observation was made on days to cowpea flowering, days to *A. vogelii* first emergence, number of emerged *Alectra* shoot at 6, 8, 10 and 12 weeks after planting (WAP) and grain yield. Analysis of variance (ANOVA) was performed on all data with the general linear model (GLM) procedure of SAS (version 8.0; SAS Institute, Cary, NC). The GLM procedure of SAS uses the method of least squares to fit data to a general linear model. The accessions were rated as resistant/tolerance and susceptible depending on number of *Alectra* plant/s emerged and grain yield.

Twenty eight cowpea accessions were selected from the pot results for further screening. The 28 cowpea genotypes and two commercial checks; M66 and K80 were further evaluated to re-validate their reaction to *A. vogelii* in 2010 at Kiboko research farm. The soils of the area are broadly classified as alfisols. The soil of the experimental site was loam with pH of 6.7. The trials were conducted under irrigation in a field that had previously been observed to be heavily infested with *A. vogelii.* The land was ploughed with disc harrow in order to get a fine tilth. Cowpea cultivars were arranged in a randomized complete block design with 3 replicates. Each plot consisted of four rows 5m long. Two seeds were sown at intra-row spacing of 0.20 m between hills and 0.75 m between rows. About 10 g of *A. vogelii* inoculum from the stock (as explained above) were added to each planting hill and thoroughly mixed before sowing to add the pool of *Alectra* seeds in the soil. The trials were always surrounded with three rows of *Alectra* susceptible check (M66). All the weeds other than *A. vogelii* were controlled through hand weeding. Cyper-methrin and dimethoate were applied with a knapsack sprayer fortnightly at the rate of 1.01/ha 4 WAP until harvest to control insect pests.

At 8 and 10 WAP, the numbers of emerged *Alectra* shoots were recorded to assess the host support for growth in the two central rows of each plot ($3.75m^2$). All the plots were harvested at maturity and grain yield was measured as the weight of threshed grain from a plot.

In 2011, three cowpea genotypes; Kir/Nya-005, Mbe/Mach-022 and Mnk/Wot-003 identified to support zero *Alectra* emergence in 2010 were further evaluated alongside three widely grown cowpea genotypes; M66, K80 and Ken-Kunde to assess their yield potential under artificial, natural *Alectra* infestation, and *Alectra*-free condition. The trials were arranged in a randomized complete block design and in three replications. Annual weeds, except *Alectra* were controlled by hoe-weeding at 2 and 5 WAP and hand pulling during the last two weeding to avoid tampering with the un-emerged *Alectra* shoots.

Data on the number of days to first *Alectra* emergence and number of emerged *Alectra* shoots were collected from the two middle rows of each plot at 6, 8, 10, and 12 WAP from the artificial and natural *Alectra* infested treatment, while number of days to cowpea flowering, number of seeds for 10 randomly selected pods and grain yield at physiological crop maturity were collected from the three treatment. Analysis of variance (ANOVA) was performed on all data with the general linear model (GLM) procedure of SAS (version 8.0; SAS Institute, Cary, NC). The GLM procedure of SAS uses the method of least squares to fit data to a general linear model. Duncan Multiple Range (DMR) Tests were performed to compare treatment means at the 5% level.

RESULTS

Cowpea germplasm collection

Cowpea germplasm were collected late in the season, 2009 from farmers in Mbeere, Kitui, Yatta, Machakos, Makueni and Makindu districts of Eastern Kenya (Figure 1). Other than Machakos district, *A. vogelii* incidences were found to have severely affected cowpea production in Mbeere, Kitui, Yatta and Makueni districts. A total of 143 cowpea landraces were collected and were found almost all of them to have mixed seed colours ranging from white to black, with cream and red colours dominating. Also they were of different seed sizes from small to large, but large was dominating. The collections were sorted according to seed colour and screened against *A. vogelii* resistance. From farmers' views, mixed seed colour was not a problem as the majority grow the crop for green vegetables and home consumption and local markets. However, those found to grow the crop for other markets reported said that traders prefer uniform seed colour.

In both pot and field trials, *Alectra* shoots emerged on susceptible cowpea genotypes 44 days after planting (Table 2). However, the genotypes varied significantly in their support for *Alectra* shoots in the year. High number of *Alectra* shoots was observed at 10 and 12 WAP during the screening period. Genotypes Kir/Nya-005, Mbe/Mach-022 and Mkn/Wot-003 exhibited zero support for *Alectra* shoots, both in pot and field screenings. Under pot experiment, 81% of the collected cowpea genotypes supported *A. vogelii* emergence while 19% showed resistance for the parasitic weed (data not shown). After prescreening the selected cowpea genotypes from the pot experiment were planted at Kiboko field to confirm their resistance against two checks (M66 and K80), Kir/Nya-005, Mbe/Mach-022 and Mkn/Wot-003 confirmed their resistance (Table 1). From Table 1, Kir/Nya-005, Mbe/Mach-022 and Mkn/Wot-003 supported no *Alectra* emergence while Mbe/Mach-004, Gac/Kar-003, Sia/Wit-004 and Ki-006 recorded the highest *Alectra* shoots at 10 WAP compared to M66 and K80. At 10 WAP, the number of emerged *Alectra* shoots ranged from 0 to 127 per 7.5 m^2 under artificial infestation.

In addition, *Alectra* infestation significantly reduced grain yield of Gac/Nge-003, Mkn/Kai-001 and Wot/Kil-002 recording 123, 125 and 200 kg/ha. However, Sia/Wit-001, Mbe/Mach-004 and Kib-021 recorded the highest yields despite the high number of *Alectra* shoots present (Table 1).

In 2011, there was significant differences in the number of emerged *Alectra* shoot per plot in both artificial and natural infestation. The results showed that Mbe/Mach-022, Mkn/Wot-003 and Kir/Nya-005 completely supported zero *Alectra* weed emergence. However, *Alectra* first emergence coincided with 50% days to host flowering in susceptible genotypes (Table 2). There was no significant difference on 50% days to cowpea flowering and number of seeds per pod under *Alectra* infestation and *Alectr*a free condition. *Alectra* infestation significantly reduced grain yield of the susceptible cowpea genotypes (Table 3). Yield losses were statistically highly significant with Ken-Kunde and M66 recording 80 and 79%, respectively. Resistant genotype Kir/Nya-005 was the most stable recording insignificant reduction of 6.5% (Figure 1).

Mkn/Wot-003 which recorded zero number of emerged *Alectra* shoots recorded grain yields reduction of 63.5% (Table 3 and Figure 2).

DISCUSSION

For seed size, large seeded types were more preferred

Table 1. Responses of cowpea genotype to *Alectra vogelii* parasitism at Kiboko, Kenya, 2010

Code	Collection points			Cowpea days	Number Emerged *Alectra* shoots		Grain yield
	Local name	Latitude (S)	Longitude (E)	to Flowering	8 WAP	10 WAP	(kg/ha)
Gac/Nge-003	-	-	-	49d	85.5	91	123b
Mkn/Kai-001	-	-	-	52bdc	55	68	125b
Wot/Kil-002	Ndamba	038^{0}07.23'	02^{0}42.052'	73.3a	11	16	200b
Kib-016	C	037^{0}43.14'	02^{0}12.946'	69.3ba	74.5	127	437.5b
Mbe/Kir-016	Ndamba	00^{0}41.686'	037^{0}40.846'	61.7bdac	13	14.5	512.5b
Sia/Cia-004	A	00^{0}35.721'	037^{0}36.554'	70.7ba	89.5	37	587.5b
Mbe/Kir-020	Nangwe	00^{0}45.146'	037^{0}38.693'	69bac	121.5	93.5	825b
Mbe/Mach-022	Kang'ao	037^{0}58.37'	02^{0}33.853'	63.7ba	0	0	825b
Mbe/Mach-012	Kamurugu	00^{0}45.038'	037^{0}38.674'	49.3d	16	22	1075b
Kir/Nya-013	Kinyuru	00^{0}40.418'	037^{0}38.389'	60.7bdac	4.5	6.5	1100b
K80	Check 1	-	-	61bdac	77	97.5	1387.5b
Mbe/Kir-016-2	KVU	00^{0}45.118'	037^{0}38.632'	68.3ba	7.5	11	1550b
M66	Check 2	-	-	67ba	76.5	109	1662.5b
Mbe/Kir-003-2	Ndune	00^{0}41.777'	037^{0}40.917'	62.7bac	17.5	63	2137.5b
Mbe/Mach-014	Ndamba	00^{0}45.038'	037^{0}38.674'	58.3bdc	6	10.5	2337.5b
Kib-010	B	037^{0}43.16'	02^{0}12.940'	68.7ba	3.5	4.5	2550b
Kir/Nya-005	Ndamba	00^{0}40.808'	037^{0}38.305'	68ba	10.5	13	2812.5b
Kib-018	-	-	-	66.3bac	50	7.5	3212.5b
Kib-006	Ndamba	037^{0}57.21'	02^{0}34.344'	68ba	14	23	367.5b
Mkn/Wot-003	A	037^{0}43.16'	02^{0}12.939'	60.7bdac	0	0	3887.5ba
Kir/Nya-004	Muthiriri	00^{0}40.808'	37^{0}38.305'	66.3bac	8	24	3925ba
Kir/Nya-010	Ndamba	00^{0}40.722'	037^{0}38.022'	65.3bac	0	0	4012.5ba
Gac/Kar-003	Ndamba	00^{0}35.562'	037^{0}31.342'	64.7bac	81.5	116	4400ba
Kir/Nya-016	Gikuu	00^{0}40.418'	37^{0}38.389'	59.3bdac	77	87	4512.5ba
Mbe/Mach-007	Ndamba	00^{0}47.051'	37^{0}39.878'	65.3bac	27	69	4812.5ba
Sia/Wit-004	Ndamba	00^{0}35.605'	37^{0}38.440'	69ba	99.5	124	4837.5ba
Sia/Cia-005	B	00^{0}35.721'	037^{0}36.554'	61.7bdac	19	37	5812.5ba
Kib-021	-	-	-	65.3bac	12.5	17.5	6237.5ba
Mbe/Mach-004	Ndamba	00^{0}46.916'	37^{0}39.548'	66bac	81	110	6362.5ba
Sia/Wit-001	Kamurugu	00^{0}35.631'	37^{0}38.549'	61.3bdac	69	91.5	10512.5ba
Mean				63.7	40.3	50.4	2875
L.s.d (P<0.05)				11.93	66.1	57.2	5796.2
Cv%				11.5	87.3	54.6	15037.5

Means followed by the same letter (s) in a column are not significantly different at 5% level of probability using Duncan multiple range test.

at the local markets than the small seed. These are good guidelines that are to be considered when breeding for *Alectra* resistance to select what the farmers and market like. This study showed significant differences exist amongst cowpea genotypes in their performance under *Alectra* infestation. These differences occurred in the number of emerged *Alectra* shoots and grain yield. Lower cowpea grain yield with number of emerged *Alectra* shoot, shows that *Alectra* infestation reduced grain yield for cowpea genotypes susceptible to *Alectra*. This is well signified by Ken-Kunde which is the highest yielder under un-infested conditions but lowest under *Alectra* infested conditions. Ken-Kunde, M66 and K80 which had high grain yield reduction (over 50%) could be regarded as being susceptible. The symptoms displayed these susceptible cultivars were that of stunted growth, chlorosis and premature defoliation as earlier reported by Magani (1994). Longe et al. (2002) observed that resultant chlorosis could be due to chlorophyll degradation which result to reduction in photosynthetic site hence, yield reduction.

A combination of high yield, growth potentials and no support for *Alectra* shoots by Kir/Nya-005, and Mbe/Mach-022 cultivars make them suitable candidates for use to improve the genetic base of existing cultivars. The

Table 2. Days to *Alectra* first emergence, emerged *Alectra* shoots at 6, 8, 10 and 12 weeks after planting of cowpea genotypes under artificial and natural field infestation of *Alectra vogelii* at Kiboko, 2011.

Genotype	Artificial					Natural				
	Days to *Alectra*	Emerged *Alectra* shoots (WAP)				Days to *Alectra*	Emerged *Alectra* shoots (WAP)			
	First emergence	6	8	10	12	First emergence	6	8	10	12
M66	40.7	2	18.3	65	70	43	0.7	6.7	56	57
K80	43	0	5	32.7	34	43.6	0.3	12.3	48	49.7
Ken-kunde	42.7	0.7	12.7	75	76.7	45.3	0	7.7	42	42.3
Mbe/Mach-022	0	0	0	0	0	0	0	0	0	0
Mkn/Wot-003	0	0	0	0	0	0	0	0	0	0
Kir/Nya-005	0	0	0	0	0	0	0	0	0	0
Mean	31.2	0.4	6.1	29.1	30.4	22	0.2	4.4	24.3	24.8
L.s.d (P<0.05)	18.7	1.14	7.7	19.5	22.6	2.9	1.0	13.1	27.6	27.3
cv%	15.1	57.3	15.7	10	12.8	7.2	328.6	162.1	62.4	60.3

Table 3. Combined analysis of 50% Days to cowpea flowering, number of pods per plant, grain yield and percentage yield reduction of six cowpea genotypes under *Alectra vogelii* infected and un-infected condition at Kiboko, 2011.

Genotype	Infected			Un-infected			Yield reduction (%)
	Days to flowering (50%)	Seeds/pod	Grain yield (kg/ha)	Days to flowering (50%)	Seeds/pod	Grain yield (kg/ha)	
M66	50.5a	13.0a	337.8b	50.0a	13.3a	1600.0a	78.9
K80	48.8ba	13.6a	711.1b	51.0a	14.0a	1511.1a	52.9
Ken-kunde	48.3ba	13.111a	400.0b	47.0b	12.4a	2044.4a	80.4
Mbe/Mach-022	48.3ba	14.0a	1511.1a	50.0ba	12.6a	1955.6a	22.7
Mkn/Wot-003	48.11b	10.5b	600.0b	50.0ba	12.6a	1644.4a	63.5
Kir/Nya-005	47.0b	12.8a	1288.9a	51.3a	12.6a	1377.8a	6.5
Mean	48.4	12.9	808.2	50.06	12.9	1688.9	50.8
L.s.d (0.05%)	2.2	1.5	349.4	1.63	1.5	608.6	
cv%	4.5	11.9	43.2	3.3	11.9	36	

Means followed by the same letter(s) in a column are not significantly different at 5% level of probability using Duncan Multiple Range Test.

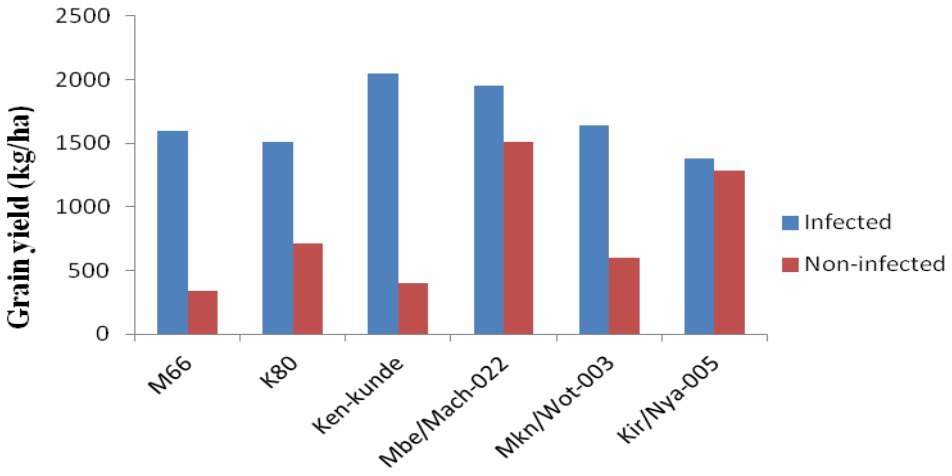


Figure 2. Effect of *Alectra* vogelii on grain yield on six cowpea genotypes compared to *Alectra* free condition.

failure of *Alectra* emergence may be due to low production of germination stimulants by these two genotypes or host-plant-parasite incompatibility whereby the initiation of haustoria, and subsequent attachment and penetration are inhibited (Okonkwo and Raghavan, 1982). This confers resistance of the genotypes as earlier reported in cereals to striga resistance (Ejeta, 1993a, b). In 1983, Mugabe showed delayed onset of flowering, reduced number of flowers and pods and reduced weight of pods and seeds in cowpea due to *Alectra* infestation. This indicates that Sia/Wit-004, Sia/Cia-005, Kib-021, Mbe/Mach-004 and Sia/Wit-001 are either moderately resistance or tolerant to *A. vogelii.* This might have been achieved through low production of germination stimulant which in one of the resistance mechanism advocated by Yohanna et al., 2010. Kurech and Alabi (2003) considered tolerance as a horizontal resistance which is polygenic in contrast to vertical resistance which breaks down faster with time. Since the tolerant genotypes can produce high yield in spite of high parasitism, it implies they have to be very efficient in the production of assimilates to support the parasites and still have enough to give high yields (Musell, 1980). Several authors (Atokple et al., 1995; Kim and Adetimirin, 1997; Adetimirin et al., 2000; Kim, 2000) have indicated that the use of moderately tolerant varieties in combination with other control measures help in the depletion of *Alectra/Striga* soil seed bank.

The decrease in the yield of varieties Mnk/Wot-003 (variety that was hardly found with any attached *Alectra*) by *Alectra* could have been partly due to the reduction in its root growth and root nodulation by the *Alectra*. This could result in inadequacy of nitrogen and nutrient absorption for adequate shoot growth and thereby reduced yield production (Dart and Mercer, 1965). It is also likely that, the seeds of *Alectra* contain certain toxins which leaked into the soil and hindered Mkn/Wot-003 root growth, but this may require further investigation as also suggested by Omoigui et al., 2007. In addition to the export of nutrients, water and metabolites from host to the parasite, *Alectra* prevents nodulation (Kurech and Alabi 2003).

Conclusion

These results could be used as a preliminary basis for choosing cowpea genotypes in encouraging farmers to grow and assembling management packages for *A. vogelii* to ensure reduced risk to farmers. Compared to Ken-Kunde, M66 and K80, it was interesting to note that the Kir/Nya-005 and Mbe/Mach-022 had zero emergences *Alectra* recorded throughout the evaluation period hence suggesting a possible absolute resistance. This information would be valuable for breeding effort to develop or select *Alectra* resistant cowpea varieties. However, there is need to evaluate the genotypes with farmers.

ACKNOWLEDGEMENT

This publication is part of the output from a project funded by the European Union through Kenya Arid and Semi-arid lands program in Kenya Agricultural Research Institute (KARI) to whom we are very grateful.

REFERENCES

Adetimirin VO, Kim SK, Aken'ova ME (2000). Expression of mature plant resistance to *striga hermonthica* in maize. Euphytica, 34:1-10.

Atokple IDK, Singh BB, Emechebe AM (1995). Genetics of resistance to *striga* and *Alectra* in cowpea. J. Heredit. 86:45-59.

Bagnall-Oakley H, Gibberd V, Nyongesa TE (1991). The incidence and control of *A. vogelii*, in Embu district, Kenya. In: Ransom, J. K., Musselman, L. J., Worsham, A. D., Parker, C. (Eds.), Proceedings of the 5th International Symposium on Parasitic Weeds, CIMMYT, Nairobi, Kenya.

Beck BDW (1987). The effect of *Alectra vogelii* Benth. On the yield of njigo beans (*Vigna subterranean* (L.) *Verdc*.). In: Chr. Weber H, Forstreuter W (Eds). Parasitic Flowering Plants. Phillips University, Marburg, Gemarny. pp.79-82.

Botha PJ (1948). The parasitism of *Alectra vogelii* with special reference to the germination of its seeds. J. South Afri. Bot. 16:49–72.

Boukar O, Kong L, Singh BB, Murdock I, Ohm HW (2004). AFLP and AFLP-derived SCAR markers associated with striga gesnerioides resistance in Cowpea {Vigna unguiculata (L.) Walp.}. Crop Science 44:1259-1264.

Bressani R (1985). Nutritive value of cowpea. In cowpea research, production, and utilization. Singh, S. R and Rachel, K. O (Eds.), Wiley and sons, Chester, Uk. pp. 353-360.

Carsky RJ, Vanlauwe B, Lyasse O (2002). Cowpea rotation as a resource management technology for cereal-based systems in the savannas of West Africa. In: Fatokun, C. A., Tarawali, S. A., Singh, B. B., Kormawa, P. M., Tamo, M. (eds.). Challenges and Opportunities for Enhancing Sustainable Cowpea Production. International Institute of Tropical Agriculture, Ibadan, Nigeria, pp.252–266.

Dart PJ, Mercer F**V (**1965**).** The effect of growth temperature, level of ammonium nitrate, and light intensity on the growth and nodulation of cowpea (Vigna *sinensis* End]. ex Hassk.). Aust. J. Agricul. Res. 16:321-345.

Doerr I, Visser JH, Albers F (1977). On the parasitism of *Alectra vogelii* (Benth.). (Scrophulariaceae) II. Origin of lateral roots in the contact area of the haustorium.

Ejeta G, Butler LG (1993a). Host plant resistance to striga. In: International crop science 1. Crop Science Society of America., Madson, WI, USA. pp.561-569.

Ejeta G, Butler LG (1993b). Host plant interactions throughout the striga life cycle, and their contributions to striga resistance. Afr. Crop Sci. J. 1(2):75-80.

Gouws J, Visser JH & Grobbelaar NZ (1980) Some aspects of the bidirectional translocation of C-14 labeled metabolites between *Alectra vogelii* (Benth) and Voandzeia subterranea (L) Thou. Zeitschrift fu¨r Pflanzenphysiologie 99, 225–233.

Harper JM (1981). Extrusion of foods. CRC Press, Inc. Boca Raton, F.L. Vol. 1: 212.

Kim SK (2000). Horizontal resistance, misunderstanding, approach and importance. In: Kim, S. K., Robinson, K., Atkinson, V. O., Adetimirin, C., and Salle, G. (ed.) Combating *striga* parasitic weeds through horizontal resistance. Preedings of the International workshop on horizontal resistance for controlling parasitic weeds held at Hotel President World Centre, Brussel, Belgium. 1997:35-53.

Kim SK, Adetimirin VO (1997). *Striga hermnthica* seed inoculum rate effects on maize hybrids tolerance and susceptibility expression. Crop Sci. 37:1066-1071.

Kabambe VH, Katunga L, Kapewa T, Ngwira AR (2008). Screening legume for integrated management of witchweeds (Alectra vogelii and Striga asiatica) in Malawi. Afr. J. Agric. Res. 3(10):708-715.

Karanja JK, Nguluu SN, Gatheru M (2010). Farm yard manure reduces the virulence of *Alectra vogelii* (Benth) on cowpea (*Vigna unguiculata)*. Paper presented at Bondo University College conference, February, 2010.

Lagoke STO, Shebayan JY, Magani I (1993). *Striga* problem and control in Nigeria. Paper presented at the Third General Workshop of Pan Africa *Striga* control Network, Harare, Zimbabwe.

Magani EI (1994). Effect of fertilizers and herbicides on the reaction of cowpea varieties (*Vigna unguiculata* L Walp) to *Alectra vogelii* Benth; Doctor of Philosophy Thesis, Department of Agronomy, Ahmadu Bello University, Zaria. pp.133-138.

Mbwaga AM, Kaswende J, Shayo E (2000). A reference manual on striga distribution and control in Tanzania. Kilosa, Tanzania: Ilonga Agricultural Research Institute.

Mugabe NR (1983). Effect of *Alectra vogelii* (Benth.) on cowpea (Vigna unguiculata (L.) Walp.) I. Some aspects on reproduction of cowpea. Zimbabwe J. Agric. Res. 21:135-147.

Mainjeni CE (1999). The host range of *Alectra vogelii* Benth. From Malawi and resistance in common bean and cowpea. MSc. Thesis, University of Bath, UK. p. 83.

Musell H (1980). Tolerance to Disease. In: Horsfall, J. and Cowling, E. B. (Ed.). How plant defend themselves. An Advanced Treatise Academic Press, New York. 5:39-52.

Okonkwo SNC, Raghavan V (1982). Studies on the germination of seeds of the root parasites. *Alectra vogelii* and *Striga gesnerioides*. I Anatomical changes in the embryo. Am. J. Bot. 69:1636-1645.

Omoigui LO, Kamara AY, Massawe FS, Ishiyaku MF, Boukar O, Alab SO, Ekeleme F (2007). Evaluation of cowpea genotypes for their reaction *to striga gesnerioides* in the dry savanna of northeast Nigeria. Africa Crop Science Conference Proceedings 8:273-278.

Rambakudzibga AM, Manschadi AM, Sauerborn J (2002). Host-parasite relations between cowpea and *Alectra vogelii.* European Weed Research Society Weed Research, 42:249-256.

Riches CR (1989). The biology and control of *Alectra vogelii* Benth (*Scrophulariaceae*) in Botswana. Ph.D. Thesis, University of Reading, UK, p.208.

Riches CR, Hamilton KA, Parker C (1992). Parasitism of grain legumes by *Alectra* species (*Scrophulariaceae*). Ann. Appl. Biol. 121:361-370.

Rubiales D, Perez D, Luque A, Fernandez-Aparica M, Sillero JC, Roman B, Kharrat M, Khalil S, Joel DM, Riches CR (2006). Screening techniques and sources of resistance to parasitic weeds in grain legumes. Euphytica 147: 187-199.

Salako E.A (1984). Observation on the effect of *A. vogelii* infestation on the yield of groundnut. Trop. Pest Manage. 30: 209-211.

Sanginga N, Dashiell K, Diels J, Vanlauwe B, Lyasse O, Carsky RJ, Tarawali S, Asafo-Adjei B, Menkir A, Schulz S, Singh BB, Chikoye D, Keatinge D, Rodomiro O (2003). Sustainable resource management coupled to resilient germplasm to provide new intensive cereal-grain legume-livestock systems in the dry savanna. Agric. Ecosyst. Environ. 100: 305–314.

Singh BB (2002). Breeding cowpea varieties for resistance to *striga gesnerioides* and *Alectra vogelii.* In Challenges and opportunities for enhancing sustainable cowpea production. Fatokun, C. A. (Ed.). IITA, Ibadan, Nigeria. pp.154-166.

Tarawali SA, Singh BB, Gupta SC, Tabo R, Harris F, Nokoe S, Fernández-Rivera S, Bationo A, Manyong VM, Makinde K, Odion EC (2002). Cowpea as a key factor for a new approach to integrated crop–livestock systems research in the dry savannas of West Africa. In: Fatokun CA, Tarawali SA, Singh BB, Kormawa PM, Tamo M (eds) Challenges and Opportunities for enhancing Sustainable Cowpea Production. International Institute of Tropical Agriculture, Ibadan, Nigeria, pp. 233–251.

Visser JH (1978). The biology of *Alectra vogelii* (Benth.). an angio-spermous root parasite. Beitrage Zur Chemischem Kommunikation in Biologie und Oekosystemen, Witzenhausen, Germany, pp.279-294.

Visser JH, Doerr I, Kollmann R (1977). On the parasitism of Alectra vogelii (Benth.) (Schrophulariaceae) I. Early development of the primary haustorium and initiation of the stem. Zeitschrift fur Pflanzephysiologie 84:213-222.

Visser JH, Doerr I, Kollmann R (1990). Compatibility of *Alectra vogelii* with different leguminous host species. J. Plant Physiol. 135:737-745.

Yohanna MK, Olussoji O, Balarabe T, Shebayan J, Lagoke STO (2010). Genotype Effect on the Reaction of Groundnuts to *Alectra vogelii* (Benth.) Infestation in a Sub-humid Environment. J. Am. Sci. 6:10.

academicJournals

Vol. 12(47), pp. 6625-6631, 20 November, 2013
DOI: 10.5897/AJB2013.12123
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Hydrolytic and synthetic activities of esterases produced by *Bacillus* sp. A60 isolated from an oil contaminated soil

Flavia del Valle Loto[1], Cintia Mariana Romero[1,2], Alfonso Emanuel Carrizo[1], Mario Domingo Baigori[1,2] and Licia María Pera[1]*

[1]PROIMI-CONICET, Av. Belgrano y Pasaje Caseros, T4001 MVB, Tucumán, Argentina.
[2]Facultad de Bioquímica, Química y Farmacia, Batalla de Ayacucho 471, T4000INI, Tucumán, Argentina.

Accepted 9 October, 2013

A novel esterase producer strain named *Bacillus* sp. A60 was isolated from a soil sample contaminated with hydrocarbons. It was found to belong to *Bacillus subtilis* species through morphological, biochemical and 16S rRNA gene sequence analyses. This strain which can tolerate 15% (w/v) NaCl and growth at 55°C, produced an interesting esterase activity in Luria-Bertani medium. Two different molecular weight esterase activities were detected in zymographic assays. Culture supernatant and whole cells showed specific hydrolytic activities of 2.67 ± 0.11 U/mg of protein and 7.07 ± 0.09 U/mg of dry weight, respectively. Concerning ethyl acetate production, conversions of 88.00 ± 0 and 55.58 ± 0.78% were obtained with culture supernatant entrapped in polyacrylamine gel and whole cells, respectively. In addition, the effect of different concentration of LB medium components on both growth and extracellular esterase hydrolytic activity was also discussed.

Key words: *Bacillus*, esterases, hydrolytic activity, synthetic activity, ethyl acetate.

INTRODUCTION

The broad metabolic diversity exhibited by the genus *Bacillus* coupled to the low pathogenicity of several species, make it a commercially important and an environmental friendly source of enzymes, vitamins and other products. In this connection, the screening of spore-forming bacteria for the production of useful metabolites continues to be an important aspect of biotechnology. Among the enzymes, esterases (E.C. 3.1.1) represent a diverse group of hydrolases catalyzing the cleavage and the formation of ester bonds. They have been extensively exploded in the synthesis of flavor esters, in the resolution of racemic mixtures, and in the degradation of natural material as well as industrial pollutants (Panda and Gowrishankar, 2005). However, the enzymatic production of ethyl acetate, an important environmental friendly solvent, was relatively little investigated (Alvarez-Macarie and Baratti, 2000; Bélafi-Bakó et al., 2003). Thus, the search of new microbial esterases suitable for this application is therefore, very attractive. As an acid inhibition during the esterification of acetic acid and ethanol by Novozyme 435® lipase was also reported by Bélafi-Bakó et al. (2003), the enzyme immobilization could improve its utilization at extreme pH, and also in different solvents, temperatures and high substrate concentrations (Hanefeld et al., 2009). Alternatively, the use of cell-bound enzymes (that is, naturally immobilized enzymes) is an interesting

*Corresponding author. E-mail: lymb@arnet.com.ar.

strategy because it eliminates complex procedures of purifications, so this kind of biocatalysts is cost effective since the biomass can be directly utilized in the reaction (Stergiou et al., 2013). In addition, the cell structure may act as natural matrix protecting the enzymes from the possible negative action of external agents. On the other hand, the selection of an appropriated fermentation technique as well as the optimization of culture conditions for the production of an industrially important target product must be done to ensure good yields and quality (Panda and Gowrishankar, 2005).

In our laboratory, the purification and characterization of esterases and lipases from both spore-forming bacteria and filamentous fungi were evaluated. In addition, the enzymatic synthesis of isoamy acetate and ethyl steareate were also explored (Baigorí et al., 1996; Torres et al., 2009; Romero et al., 2012). In this work, hydrolytic and synthetic activities of esterases produced by *Bacillus* sp A60 isolated from an oil contaminated soil were reported. Some aspects of the enzyme production were also analyzed.

MATERIALS AND METHODS

Microorganisms and fermentation conditions

Bacteria were isolated from different sources such as soils contaminated with hydrocarbons and soils from a river cost in the North West of Argentine. Soil suspensions were heated at 80°C for 15 min, plated on Luria-Bertani (LB) agar and incubated at 37°C. Liquid cultures were carried out in flasks of 125 ml containing 10 ml of LB medium on an orbital shaker at 37°C. The reference strain *Bacillus subtilis* 1A571 (Lindgren and Rutberg, 1974), a derivative of *B. subtilis* wild-type strain 168 (*trp* C2), was kindly provided by the Bacillus Genetic Stock Center, Columbus, Ohio.

Screening of esterase-producing spore-forming microorganisms

Separated colonies of isolated bacteria were grown 48 h on LB agar at 37°C. The enzyme activity was studied by pouring the plates with α-naphtil acetate and Fast Blue dissolved in 50 mM phosphate buffer pH 7 supplemented with 0.75% agar. A brown halo around the colonies was considered as a positive result for extracellular enzyme activity. The ratio between the diameter of each colony and the corresponding hydrolysis halo ($Ratio_{C/H}$) was evaluated as an indicator of the strains efficiency to produce an esterase activity; the values ranged from 0 to 1, and the highest level of enzymatic activity was the nearest to 0.

Identification of the spore-forming strain A60

Phenotypic characterization

Gram staining, motility assays, catalase test and starch hydrolysis were performed using standards protocols. Growth at different pH values, temperatures, NaCl concentrations, and motility were carried out as described by Garrity et al. (2005). The API 50 CHB system (bioMérieux) was used according to the manufacturer's instructions. Zymograms were performed in native- polyacrylamide gel electrophoresis (PAGE) using a 10% polyacrylamide gel (Davis, 1964).

Molecular characterization

Total DNA was extracted from cells harvested in the mid-exponential growth phase as described by Miller (1972). Polymerase chain reaction (PCR) amplification was performed in a 25 µl reaction mix containing 2.5 µl 10X STR reaction buffer (Promega), 20 ng total DNA, 0.5 µM of each primer and 1 U of Taq DNA polymerase (Promega). Primers 27 F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492 R (5´-GGTTACCTTGTTACGACTT-3´) were used to generate partial sequences of 16S rDNA. Samples were amplified as follows: 5 min at 94°C; 35 cycles of 1 min at 94°C, 2 min at 50°C and 2 min at 72°C, and at the end, 7 min at 72°C for final extension. PCR products were analyzed by electrophoresis in 2% (wt/vol) agarose gels. DNA sequencing was carried out by Macrogen Services. Sequences were compared and aligned with those from the GenBank database with BLAST software. The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrogram was constructed with the neighbor-joining method, and tree topology was evaluated by performing bootstrap analysis of 1000 data sets using MEGA 4 software (http://www.megasoftware.net/). Partial nucleotide sequence of the 16S rDNA gene of *Bacillus* sp. A60 was deposited in GenBank database under accession number EF513611.

Substrate specificity of esterase activity on LB agar plates

The selected strain *Bacillus* sp. A60 as well as the reference strain *B. subtilis* 1A571 were grown 48 h on LB agar at 37°C. The substrate specificity of the hydrolytic activity was also studied by pouring the plates with α-naphtil derivatives (α-acetate, α-propionate, α-caproate, α-laurate, α-miristate, α-palmitate and α-stearate) as described for screening assays.

Determination of hydrolytic activity

Hydrolytic activity was measured spectrophotometrically at 405 nm by using *p*-nitrophenyl acetate (*p*NPA) as substrate. All assays were performed at 37°C, and controls were included in which no enzyme or no substrate was added. One unit of enzyme activity (U) was defined as the amount of enzyme that released 1 µmol of *p*-nitro phenol per min. For extracellular hydrolytic activity, specific activity was expressed as U per mg of protein. Protein concentration was determined with Bradford (1976) reagent. For cell-bound hydrolytic activity determination, cell pellets were washed twice with 50 mM phosphate buffer pH 7, centrifuged and resuspended in the reaction mixture. The reaction was shaken at 150 rpm, and the absorbance of the supernatant was then measured. Specific activity was expressed as U per mg dry weight.

Determination of synthetic activity

Ester synthesis was performed with either entrapped culture supernatant in polyacrylamide gels or dried cells. In the first case, 4.25 ml of culture supernatant were added to a solution containing 0.75 ml of acrylamide-bis acrylamide (30:0.8), 20 µl of ammonium persulfate solution (0.1 g/ml) and 6 µl of TEMED solution. Polymerization was performed on a 1 mm width glass case. Pieces of gel (1 x 1 cm) were then cut and used for enzymatic synthesis. Ester synthesis was also evaluated in the presence of microbial biomass; in this experiment, the cells were first washed with n-hexane. Then, 1 mg of cells as well as a gel slice were resuspended

Table 1. Medium composition tested for esterase activity.

Medium	NaCl (g/l)	Yeast extract (g/l)	Tryptone (g/l)
1	0	5.0	10.0
2	5.0	5.0	10.0
3	12.5	5.0	10.0
4	10.0	5.0	0
5	10.0	5.0	5.0
6	10.0	5.0	12.5
7	10.0	0	10.0
8	10.0	2.5	10.0
9	10.0	7.5	10.0
10 (LB)[a]	10.0	5.0	10.0

[a]LB, Luria Bertani medium.

in 10 ml of n-hexane containing 20 mM of acetic acid and an excess of ethanol. Reactions were incubated at 37°C on an orbital shaker at 150 rpm. After a reaction time of 24 h, the residual acid content was determined by titration with a 0.1 N sodium hydroxide solution. The molar conversion was determined from the values obtained for the blank and the test samples. Qualitative analysis of ethyl acetate production was carried out by thin layer chromatography (TLC) using silica gel 60 as support and chloroform as developing solvent. Spots were visualized in iodine vapor. All assays were carried out at least in duplicate.

Effect of LB components on *Bacillus* sp. A60 growth and hydrolytic esterase production

Different concentrations of LB components were evaluated. The proportions of the components were varied once at a time as detailed in Table 1. All experiments were carried out in triplicate and average values of specific hydrolytic esterase activity as well as biomass were calculated.

Statistical analysis

Statistical analyses was performed using the Minitab (version 14; Minitab Inc) software for windows. Statistical significance values of the means were evaluated using a one-way analysis of variance. Subsequent comparisons were performed using Tukey's post-hoc test. Results were presented as the mean ± SD. Differences were accepted as significant when $P<0.05$.

RESULTS AND DISCUSSION

Isolation and selection of the indigenous spore-forming strain A60

A total of 44 spore-forming bacteria showing esterase activity in LB agar were isolated from natural environments. Thirty-four strains (78%) showed both, extracellular and biomass-bound esterase activity (Figure 1a,b), and 10 strains (22%) only displayed a biomass-bound esterase activity (Figure 1c). Interestingly, considering the ratio between the diameter of each colony and the corresponding hydrolysis halo, 17 strains were better producers than the reference strain *B. subtilis* 1A571 (Table 2).

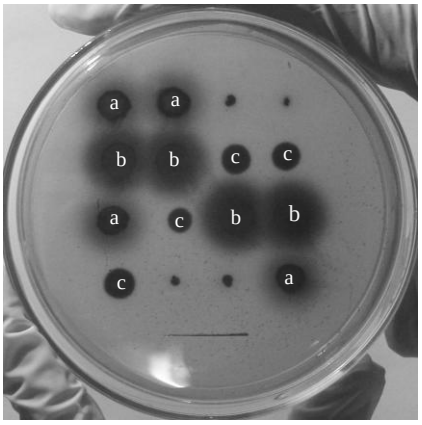


Figure 1. Hydrolytic esterase activity on LB agar plate. Esterase activity was detected using 1.3 mM of α-naphthyl acetate (C2) and 1 mM Fast Blue RR dissolved in 50 mM phosphate buffer pH 7. a, Bacillus subtilis 1A571; b, Bacillus sp. A60; c, Bacillus sp. M2. Scale: 2 cm.

Esterases from this species hydrolyze different substrates as antibiotic esters, and were utilized in chiral drug resolution, among other applications (Bornscheuer, 2002). In our study, the most promising strain named A60 showed the lowest colony diameter to halo diameter ratio ($Ratio_{C/H} = 0.33$), and was then selected for further studies.

Phenotypic and molecular characterization of *Bacillus* sp. A60

The spore-forming strain A60 stained Gram-positively and formed central oval spores. Growth was observed at pH 5.8, not at pH 10, and the strain was able to grow in LB agar supplemented with 7, 10 and 15% NaCl. Maximum growth temperature was 55°C. According to API 50CH system, carbohydrates fermented were: Glycerol, ribose, D-glucose, D-fructose, N-acetyl-glucosamine,

Table 2. Ratio between the diameter of each isolated colony and the corresponding hydrolysis halo produced by an esterase activity ($Ratio_{C/H}$).

Strain	$Ratio_{C/H}$	Strain	$Ratio_{C/H}$
A6	0.7	A34	1.00
A7	0.64	A35	1.00
A8	0.49	A36	0.46
A9	0.56	A37	0.40
A10	0.6	A38	0.40
A11	0.62	A39	0.58
A12	0.63	A41	0.35
A13	0.35	A43	0.40
A15	1.00	A45	0.64
A16	0.35	A46	0.70
A18	0.38	A49	0.40
A19	0.5	A52	0.40
A20	0.8	A59	0.57
A23	0.42	A60	0.33
A24	1.00	A61	1.00
A25	0.35	M1	1.00
A26	0.4	M2	1.00
A27	0.52	M5	1.00
A28	0.56	M15	0.44
A30	0.37	M19	0.38
A31	1.00	M27	0.40
A32	0.57	1A571	0.42
A33	1.00		

amygdaline, arbutine, esculine, salicine, cellobiose, maltose, saccharose, trehalose, starch, glycogene and gentiobiose. Phenotypic analyses and morphological characteristics strongly suggested A60 strain as *B. subtilis*.

Additionally, the partial sequencing of 16S rDNA gene confirmed the biochemical and morphological characterization. Basic Alignment Search Tool (BLAST) search and alignment analyses showed a similarity of 99% to *B. subtilis* subsp. *subtilis* str. 168. The phylogenetic tree is shown in Figure 2. It is important to mention the biotechnological importance of *B. subtilis* as an organism which lacks of toxicity (considered generally regarded as safe (GRAS) by FDA, USA). Moreover, it is recognized as a source of different metabolites potentially useful in food and detergent industry, pharmaceutical and health technologies and agriculture products (Harwood and Wipat, 1996; Schallmey et al., 2004).

Hydrolytic esterase activity from *Bacillus* sp. A60

The substrate specificity of the hydrolytic activities produced in LB agar by *Bacillus* sp. A60 as well as by the reference strain *B. subtilis* 1A571 was compared. Both esterase activities were able to hydrolyze acetate, propionate and caproate α-naphthyl derivatives. However, only the esterase from *Bacillus* sp A60 was capable to hydrolyze the laurate derivative. In addition, none of them were able to hydrolyze chromogenic substrates with longer carbon chains. These results strongly suggested that the enzyme activity produced by *Bacillus* sp. A60 corresponds to an esterase activity (Bornscheuer, 2002). Specificity of esterase from *Bacillus* sp. A60 as well as the esterase specificity reported from *Bacillus* sp. 4 (Ateslier and Metin, 2006) were different to that from the reference strain and other known esterases, which commonly showed no activity on substrates with chains longer than 10 carbons. However, it cannot be ruled out that other proteins or carbohydrates originating from the culture broth could contribute to a more robust catalytic system.

Zymograms of *Bacillus* sp. A60 supernatant showed two bands of activity with apparent molecular weights of 101.35 ± 0.31 and 50.10 ± 0.07 kDa, respectively (Figure 3). Higerd and Spizizen (1973) also reported two acetyl esterases from *B. subtilis* 168 extracts. By employing gel filtration chromatography, the estimated molecular weights for these esterases, named A and B, are 160,000 and 51,000, respectively. Finally, no significant difference was detected between the hydrolytic activities of the both whole cell biocatalysts tested. However, the supernatant activity obtained from *Bacillus* sp. A60 almost doubled that obtained from reference *B. subtilis* 1A571 (Table 3).

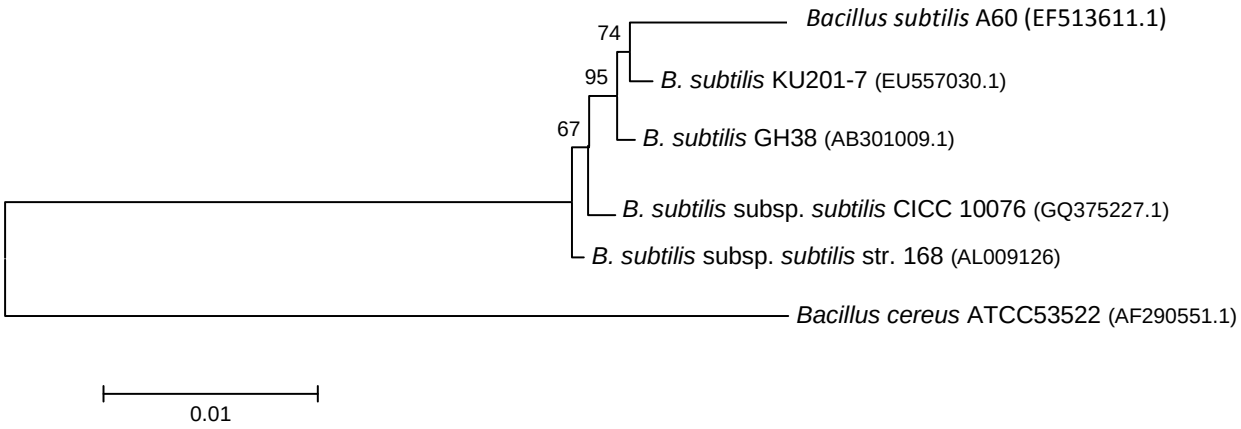


Figure 2. Neighbor-joining phylogenetic tree of partial 16S rDNA sequences. The method of Jukes and Cantor was used to calculate evolutionary distances, and tree topology was constructed using MEGA 4. Bootstrap values (*n* = 1000 replicates) were indicated at the nodes. Scale bar represents observed number of changes per nucleotide position. In parenthesis, accession numbers of the reference strains 16S rDNA sequences.

Table 3. Comparison of hydrolytic and synthetic activities from supernatant and whole cells between *Bacillus* sp. A60 and the reference strain *B. subtilis* 1A571.

Reaction	Biocatalyst	*Bacillus* sp. A60	*B. subtilis* 1A571
Hydrolysis	Supernatant	2.67 ± 0.11 (a)	1.62 ± 0.08 (b)
(U/mg)	Whole cell	7.07 ± 0.09 (a)	6.94 ± 0.75 (a)
Synthesis	Supernatant	88 ± 0 (a)	74.5 ± 0.35 (b)
(% conversion)	Whole cell	55.58 ± 0.78 (a)	66.47 ± 0 (b)

Values across lines followed by the same letters do not differ significantly ($p<0.05$).

Synthetic reactions catalyzed by culture supernatants and whole cells

One of the process for producing ethyl acetate is by esterification of ethanol with acetic acid. As shown in Table 3, enzymatic esterifications of ethanol with acetic acid in n-hexane using several biocatalysts preparations were investigated. The immobilized supernatant of *Bacillus* sp A60 had a better performance (88.0% of conversion) than that of *B. subtilis* 1A571 (74.50% of conversion) ($p<0.0001$). In contrast, whole cells of *B. subtilis* 1A571 showed a higher level of conversion (66.47%) than those of *Bacillus* sp. A60 (55.58%). In addition, the TLC profiles of synthetic reactions showed a new spot that could correspond to the ethyl acetate production (data not shown). It is interesting to note that although a high tolerance towards organic solvents has been described for *B. subtilis* 168 (Kataoka et al., 2011), the proposed biotransformation catalyzed by either immobilized supernatant or whole cells from *B. subtilis* 1A571 constitutes an important contribution to the knowledge of this reference strain.

Concerning the enzymatic synthesis of ethyl acetate, the conversions obtained in this work were similar to or higher than those reported in some chemical processes such as the ethanol dehydrogenation to ethyl acetate by using copper and copper chromite (Santacesaria et al., 2012) or Cu-Zn-Zr-Al-O (Inui et al., 2004) as catalysts.

Effect of medium components on *Bacillus* sp. A60 growth and hydrolytic esterase production

Esterase production requires complex nitrogen and carbon sources and its appropriate type and concentration differ from organism to organism (Kademi et al., 1999). To efficiently utilize *Bacillus* sp. A60 as a biocatalyst, high biomass density and high esterase activity would be necessary. In our assays, the concentrations of the components of LB medium were varied in order to determine any effect on either growth or hydrolytic esterase activity produced by *Bacillus* sp. A60. As shown in Figure 4, the nutrient media composition strongly affected both growth and enzyme activity under study. As expected, the LB medium represented the right proportions for growth. Concerning the extracellular esterase production, it was increased as the concentration of either tryptone or yeast extract increased. Others authors have obtained similar results, where tryptone and yeast extract act as enhancers for esterase and lipase production, without a growth

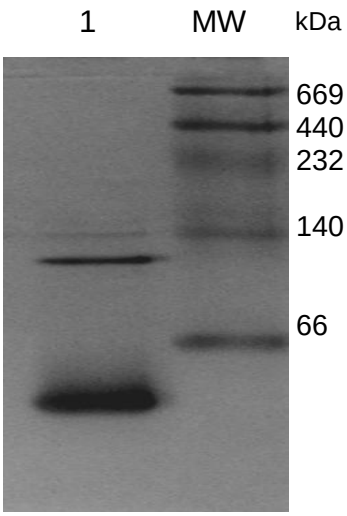


Figure 3. Native-PAGE using 10 % polyacrylamide gel revealed for esterase activity. Lane 1, *Bacillus* sp. A60 supernatant; MW, molecular weight maker.

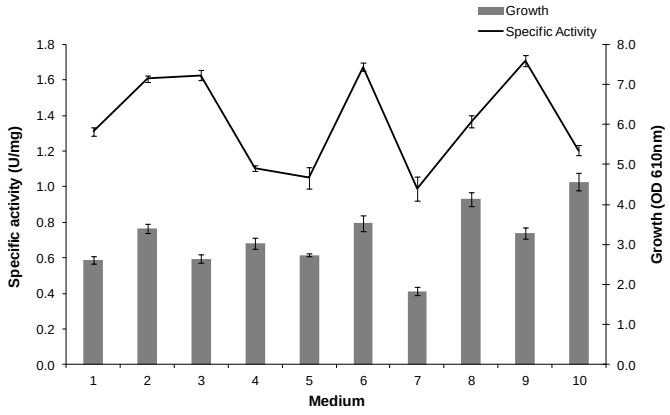


Figure 4. Effect of medium components on *Bacillus* sp. A60 growth and hydrolytic esterase production.

correlation (Kademi et al., 1999; Mimura and Nagata, 1998). Under our assay conditions, the effect of yeast extract was the most notorious; its absence meant slower growth and lower esterase hydrolytic activity. Adding yeast extract involved the recovery of both parameters, especially the esterase hydrolytic activity. On the other hand, according to its halotolerant condition, the presence of NaCl influenced positively on the production of the esterase activity. This is an interesting issue, since high concentration of NaCl could have an effect of thermal protection, as described for *Brevibacterium* sp. (Mimura and Nagata, 1998). For these reasons it is possible to improve the esterase activity of *Bacillus* sp.A60 by selecting appropriate concentration of the culture medium. These results combined with the previous ones prove the potential utility of esterases from *Bacillus* sp. A60 with a great application in food and chemical industries.

ACKNOWLEDGEMENT

This work was supported by grants PICT 2011-2158 (FONCyT), CIUNT 26/D409 (UNT) and PIP 297 (CONICET).

REFERENCES

Alvarez-Macarie E, Baratti J (2000). Short chain flavour ester synthesis by a new esterase from *Bacillus licheniformis*. J. Mol. Catal. B Enzym. 10: 377-383.

Ateslier ZBB, Metin K (2006). Production and partial characterization of a novel thermostable esterase from a thermophilic *Bacillus* sp. Enzyme Microb. Tech. 38:628-635.

Baigorí M, Castro GR, Siñeriz F (1996). Purification and characterization of an extracellular esterase from *Bacillus subtilis* MIR-16. Biotechnol. Appl. Biochem. 24: 7-11.

Bélafi-Bakó K, Kabiri Badr A, Nemestóthy N, Erenstein U, Gubicza L (2003). Kinetics of ethyl acetate formation by lipase in organic solvent and solvent-free system. Chem. Pap. 57: 278-281.

Bornscheuer UT (2002). Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS Microbiol. Rev. 26: 73-81.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Davis BJ (1964). Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404-247.

Garrity GM, Bell JA, Lilburn TG (2005). Taxonomic Outline of the Prokaryotes: Bergey´s Manual of Systematic Bacteriology. Doi No. http://dx.doi.org/10.1007/bergeysoutline. Published online by Springer-verlag, New York.

Hanefeld U, Gardossi L, Magner E (2009). Understanding enzyme immobilization. Chem. Soc. Rev. 38: 453-468.

Harwood CR, Wipat A (1996). Sequencing and functional analysis of the genome of *Bacillus subtilis* strain 168. FEBS Lett. 389: 84-87.

Higerd TB, Spizizen J (1973). Isolation of two acetyl esterases from extracts of *Bacillus subtilis*. J. Bacteriol. 114: 1184-1192.

Inui K, Kurabayashi T, Sato S, Ichikawa N (2004). Effective formation of ethyl acetate from ethanol over Cu-Zn-Zr-Al-O catalyst. J. Mol. Catal. A Chem. 216: 147-156.

Jukes TH, Cantor CR (1969). Evolution of protein molecules. In Munro HN, editor, *Mammalian Protein Metabolism*, pp. 21-132, Academic Press, New York.

Kademi A, Fakhreddine L, Ait-Abdelkader N, Baratti JC (1999). Effect of culture conditions on growth and esterase production by the moderate thermophile *Bacillus circulans* MAS2. J. Industrial Microbiol. Biotechnol. 23: 188-193.

Kataoka N, Tajima T, Kato J, Rachadech W, Vangnai AS (2011). Development of butanol-tolerant Bacillus subtilis strain GRSW2-B1 as a potential bioproduction host. AMB Express 1:10.

Lindgren V, Rutberg L (1974). Glycerol metabolism in Bacillus subtilis: gene-enzyme relationships. J. Bacteriol. 119: 431-442.

Miller JH (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mimura H, Nagata S (1998). Effect of hyper-salt stress on the heat resistance of a halotolerant *Brevibacterium* sp. JCM6894. J. Ferment. Bioeng. 85: 185–189.

Panda T, Gowrishankar BS (2005). Production and applications of esterases. Appl. Microbiol. Biotechnol. 67: 160-169.

Romero CM, Pera LM, Loto F, Vallejos C, Castro G, Baigori MD (2012). Purification of an organic solvent-tolerant lipase from *Aspergillus niger* MYA 135 and its application in ester synthesis. Biocatal. Agric. Biotechnol. 1: 25-31.

Santacesaria E, Carotenuto G, Tesser R, Di Serio M (2012). Ethanol dehydrogenation to ethyl acetate by using copper and copper chromite catalysts. Chem. Eng. J. 179: 209-220.

Schallmey M, Singh A, Ward O (2004). Developments in the use of Bacillus species for industrial production. Can. J. Microbiol. 50: 1-17.
Stergiou P, Foukis A, Filippou M, Koukouritaki M, Parapouli M, Theodorou L, Hatziloukas E, Afendra A, Pandey A, Papamichael E M (2013). Advances in lipase-catalyzed esterification reactions. Biotechnol. Adv. http://dx.doi.org/10.1016/j.biotechadv.2013.08.006.
Torres S, Baigorí MD, Swathy SL, Pandey A, Castro GR (2009). Enzymatic synthesis of banana flavour (isoamyl acetate) by *Bacillus licheniformis* S-86 esterase. Food Res. Int. 42:454-460.

academicJournals

Vol. 12(47), pp. 6544-6567, 20 November, 2013
DOI: 10.5897/AJB11.1139
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Biological degradation of oil sludge: A review of the current state of development

Ubani, O.[1]*, Atagana, H. I.[2] and Thantsha, M. S.[3]

[1]Department of Environmental Sciences, University of South Africa, Pretoria, South Africa.
[2]Institute for Science and Technology Education, University of South Africa, Pretoria, South Africa.
[3]Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

Accepted 10 October, 2013

Oil sludge is a thick viscous mixture of sediments, water, oil and hydrocarbons, encountered during crude oil refining, cleaning of oil storage vessels and waste treatment. Polycyclic aromatic hydrocarbons (PAHs), which are components of crude oil sludge, constitute serious environmental concerns, as many of them are cytotoxic, mutagenic and potentially carcinogenic. Improper management and disposal of oil sludge causes environmental pollution. The adverse effects of oil sludge on soil ecology and fertility have been of growing interest among environmental scientist and an important consideration in the development of efficient technologies for remediation of contaminated land, with a view to making such land available for further use. Oil sludge can be treated by several methods such as physical, chemical and biological processes. The biological processes are mostly cost effective and environmentally friendly, as they are easy to design and implement, as such they are more acceptable to the public. Compost, the product of biological breakdown of organic matter is a rich source of hydrocarbon-degrading microorganisms such as bacteria and fungi. These microorganisms can degrade the oil sludge to less toxic compounds such as carbon dioxide, water and salts. Compost bioremediation, the application of composting in remediation of contaminated environment, is beginning to gain popularity among remediation scientists. The success or failure of compost bioremediation depends on a number of factors such as nutrients, pH, moisture, aeration and temperature within the compost pile. The bioavailability and biodegradability of the substrate to the degrading microorganisms also contributes to the success of the bioremediation process. This is a review on the biological remediation technologies employed in the treatment oil sludge. It further assesses the feasibility of using compost technology for the treatment of oil sludge, as a better, faster and more cost effective option.

Key words: Biodegradation, bioremediation, composting, oil sludge, polycyclic aromatic hydrocarbons (PAHs).

INTRODUCTION

Oil sludge is a thick, viscous mixture of sediments, water, oil and high hydrocarbon concentration, encountered during crude oil refining, cleaning of oil storage vessels and refinery-wastewater treatment. The chemical composition of oil sludge is complex and depends on the source. Oil sludge is mainly composed of alkanes, aromatics, asphaltenes and resin (Diallo et al., 2000). It has high content of aromatic hydrocarbons in the range

*Corresponding author. E-mail: onyedika.ubani@gmail.com. Tel: +27 78 419 2899. Fax: 086 2245 824.

Abbreviations: PAHs, Polycyclic aromatic hydrocarbons; **VOCs,** volatile organic carbons; **SVOCs,** semivolatile organic carbons; **CNS,** central nervous system; **PCBs,** polychlorinated biphenyls; **TPHs,** total petroleum hydrocarbons.

of 1 - 40 carbon atoms (US EPA, 1997).The two major sources of oil sludge are oil storage tanks and refinery-wastewater treatment plants (Shie et al., 2004; Wang et al., 2010). Oil sludge found in crude oil storage tanks is typically made up of sulphides, phenols, heavy metals, aliphatic and polycyclic aromatic hydrocarbons (PAHs) of 4, 5, 6 and more rings, in over 10-20 fold concentration (Li et al., 1995). More than 90% of oil sludge material is composed of paraffin, asphaltenes and aromatic hydrocarbon mixtures. Paraffins are saturated hydrocarbon (alkanes) that have the general formula $C_nH_{2n}+_2$ and can either be straight chains (n-paraffins) or branched chains (isoparaffins). Asphaltenes are polycyclic aromatic clusters, substituted with varying alkyl side chain.

Aromatics hydrocarbons are unsaturated ring type (complex polycyclic of three or more fused aromatic rings) compounds, which reacts readily because they have carbon atoms that are deficient in hydrogen. All aromatics hydrocarbons have at least one benzene ring as part of their molecular structure. These components are highly recalcitrant under normal conditions. Such characteristics are attributed to their strong molecular bonds, high molecular weights, hydrophobicity and relative low solubility in water.

Oil sludge has been classified by the United States Environmental Protection Agency (US EPA) as a hazardous organic complex (US EPA, 1997; Liu et al., 2010). This contaminant enters the environment as a result of human activities, which includes deliberate dumping, improper treatments and management, storage, transportation and landfill disposal. This calls for concern because many of the oil sludge components have been found to be cytotoxic, mutagenic and potentially carcinogenic (Bojes and Pope, 2007).

The environmental impact of oil sludge contamination includes physical and chemical alteration of natural habitats, lethal and sub-lethal toxic effects on aquatics and terrestrial ecosystem. Oil sludge contains volatile organic carbons (VOCs) and semivolatile organic carbons (SVOCs) (for example, PAHs) which over the years have been reported as being genotoxic (Mishra et al., 2001; Bach et al., 2005; Bojes and Pope, 2007). They have cumulative effect on the central nervous system (CNS) leading to dizziness, tiredness loss of memory and headache, and the effect depends on duration of exposure.

In severe cases, PAH metabolism in human body produces epoxide compounds with mutagenic and carcinogenic properties that affects the skin, blood, immune system, liver, spleen, kidney, lungs, developing foetus, it also causes weight loss (TERA, 2008; API, 2008; Sidney, 2008; Bayoumi, 2009). However, environmental regulations in many parts of the world have stressed on the necessity to decrease emission of volatile organic compounds as well as PAHs, and have placed more restriction on land disposal of oil sludge (Mahmoud, 2004).

SOME IMPORTANT COMPOUNDS PRESENT IN OIL-REFINERY-SLUDGE

Some important PAHs of environmental concern present in oil sludge include Naphthalene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene and indenol(1,2,3-cd)pyrene. These PAHs which may be acute or chronic hazardous organic compounds are present in substantial quantities in oil sludge and are susceptible to microbial degradation (Gibson and Subramanian, 1984; Mueller et al., 1991; Field et al., 1992; Sutherland et al., 1995). Below are the catabolic pathways by aerobic bacteria for some of the PAHs.

Naphthalene

It is an aromatic hydrocarbon, with molecular formula $C_{10}H_8$ and the structure of two fused benzene rings. Biodegradation of naphthalene involves the microbial utilization of naphthalene as described by Gibson and Subramanian (1984) and documented in a catabolic pathway as shown in Figure 1 (Ri-He et al., 2008). The initial reaction in the bacterial oxidation of naphthalene involves the formation of dihydrodiol intermediates. Bacteria oxidised naphthalene to D-*trans*-1, 2-dihydroxy-1, 2-dihydronaphthalene (Gibson and Subramanian, 1984). Bacteria utilises a dioxygenase reaction to initiate the degradation of naphthalene, a reaction which is further catalysed by dehydrogenase to give 1, 2-dihydroxynaphthalene (Gibson and Subramanian, 1984; Sutherland et al., 1995).

Phenanthrene

Phenanthrene is a polycyclic aromatic hydrocarbon composed of three fused benzene rings. Many species of bacteria found in soil are capable of utilising phenanthrene as a growth substrate. The degradation of this compound by bacteria follows an oxidative pathway (Gibson and Subramanian, 1984; Sutherland et al., 1995). Bacteria can oxidise phenanthrene to cis-1, 2-dihydroxy-1,2-dihydrophenanthrene, which forms 1,2-dihydrophenanthrene when it undergoes enzymatic dehydrogenation. The compounds can be oxidised further to 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, o-phthalic acid, protocatechuic acid as shown in Figure 2 (Ri-He et al., 2008).

Pyrene

Pyrene is a PAH consisting of four fused benzene rings. It is the smallest peri-fused PAH (the rings are fused

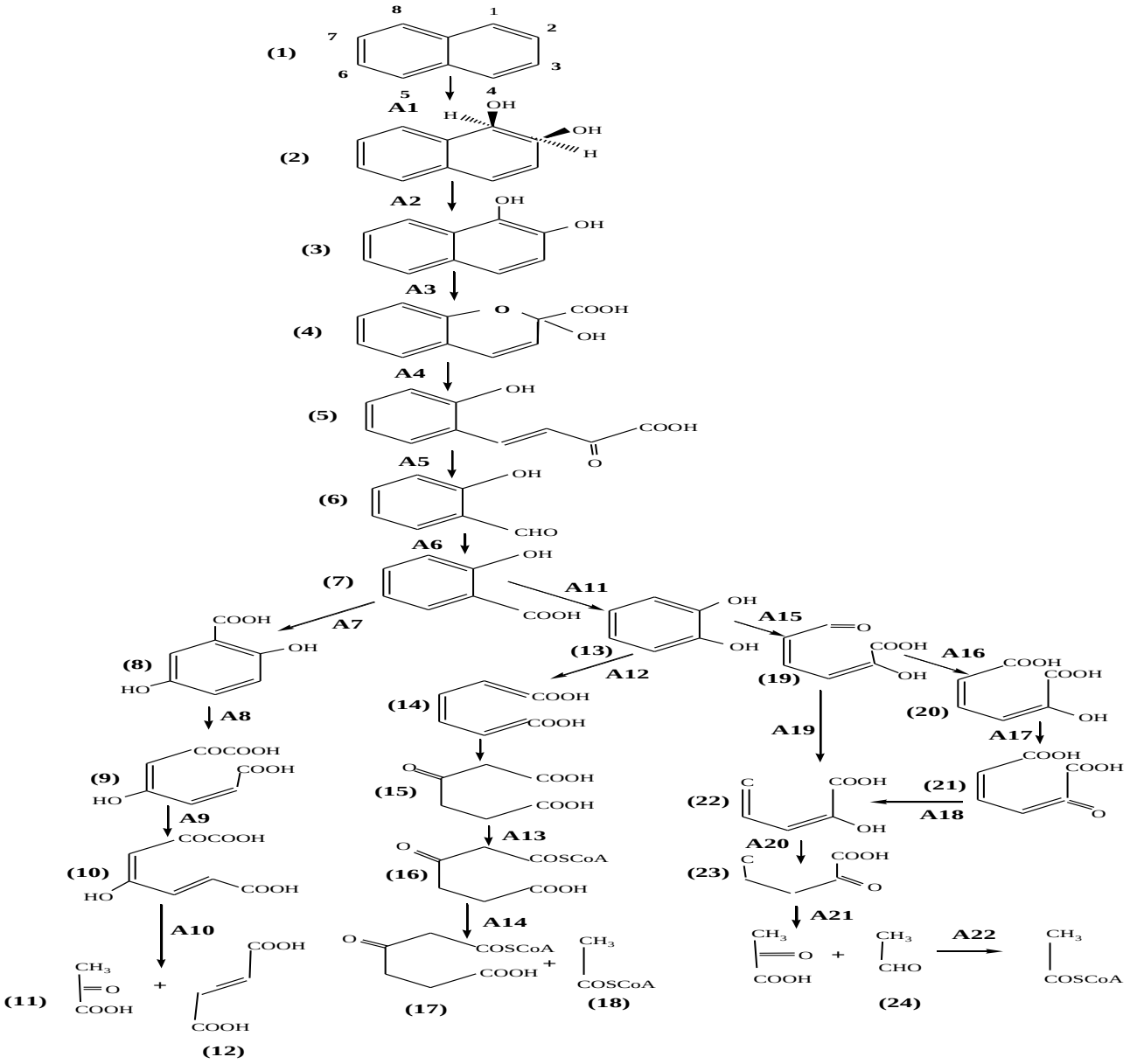


Figure 1. Proposed catabolic pathways of naphthalene by aerobic bacteria. The compounds are 1, Naphthalene; 2, cis-1,2-dihydroxy-1,2-dihydronaphthalene (cis-naphthalene dihydrodiol); 3, 1,2-dihydroxynaphthalene; 4, 2-hydroxy-2H-chromene-2-carboxylic acid; 5, trans-o-hydroxybenzylidenepyruvic acid; 6, salicylaldehyde; 7, salicylic acid; 8, gentisic acid; 9, maleylpyruvic acid; 10, fumarylpyruvic acid; 11, pyruvic acid; 12, fumaric acid; 13, catechol; 14, cis,cis-muconic acid; 15, β-ketoadipic acid; 16, β-ketoadipyl-CoA; 17, succiny-CoA; 18, acetyl-CoA; 19, 2-hydroxymuconic-semialdehyde; 20, 2- hydroxymuconic acid; 21, 4-oxalocrotonic acid; 22, 2-oxo-4-pentenoic acid; 23, 4-hydroxy-2-oxovaleric acid; 24, acetaldehyde. The enzymes involved in each reaction step are naphthalene dioxygenase (NahAaAbAcAd) (step A1), cis-naphthalene dihydrodiol dehydrogenase (NahB) (A2), 1,2-dihydronaphthalene dioxygenase (NahC) (A3), 2-hydroxy-2H-chromene-2-carboxylate isomerise (NahD) (A4), trans-o-hydroxybenzylidenepyruvic hydratase-aldolase (NahE) (A5), salicylaldehyde dehydrogenase (NahF) (A6), salicylate 5-hydroxylase (NagGHAaAb) (A7), gentisate 1,2-dioxygenase (Nahl) (A8), maleylpyruvate isomerise (NagL) (A9), fumarylpyruvate hydrolase (NagK) (A10), salicylylate hydroxylase (NahG) (A11), catechol1,2-dioxygenase (A12), β-ketoadipate:succinyl-CoA transferase (A13), β-ketoadipyl-CoA thiolase (A14), catechol 2,3-dioxygenase (NahH) (A15), hydroxymuconic-semialdehyde dehydrogenase (Nahl) (A16), 4-oxalocrotonate isermerase (NahJ) (A17), 4-oxalocrotonate decarboxylase (NahK) (A18), hydroxymuconic-semialdehyde hydrolase (NahN) (A19), 2-oxo-4-pentenoate hydratase (NahL) (A20), 2-oxo-4-hydroxypentennoate aldolase (NahM) (A21) and acetaldehyde hydrogenase (NahO) (22) (Ri-He et al., 2008).

through more than one face). Many microorganisms have shown the capability of utilising four ringed aromatic hydrocarbons such as pyrene (Heitkamp et al., 1988b; Meyer and Steinhart, 2001). Bacteria such as *Rhodococcus* sp. strain UW1 are capable of growing on pyrene as sole carbon source (Walter et al., 1991). This

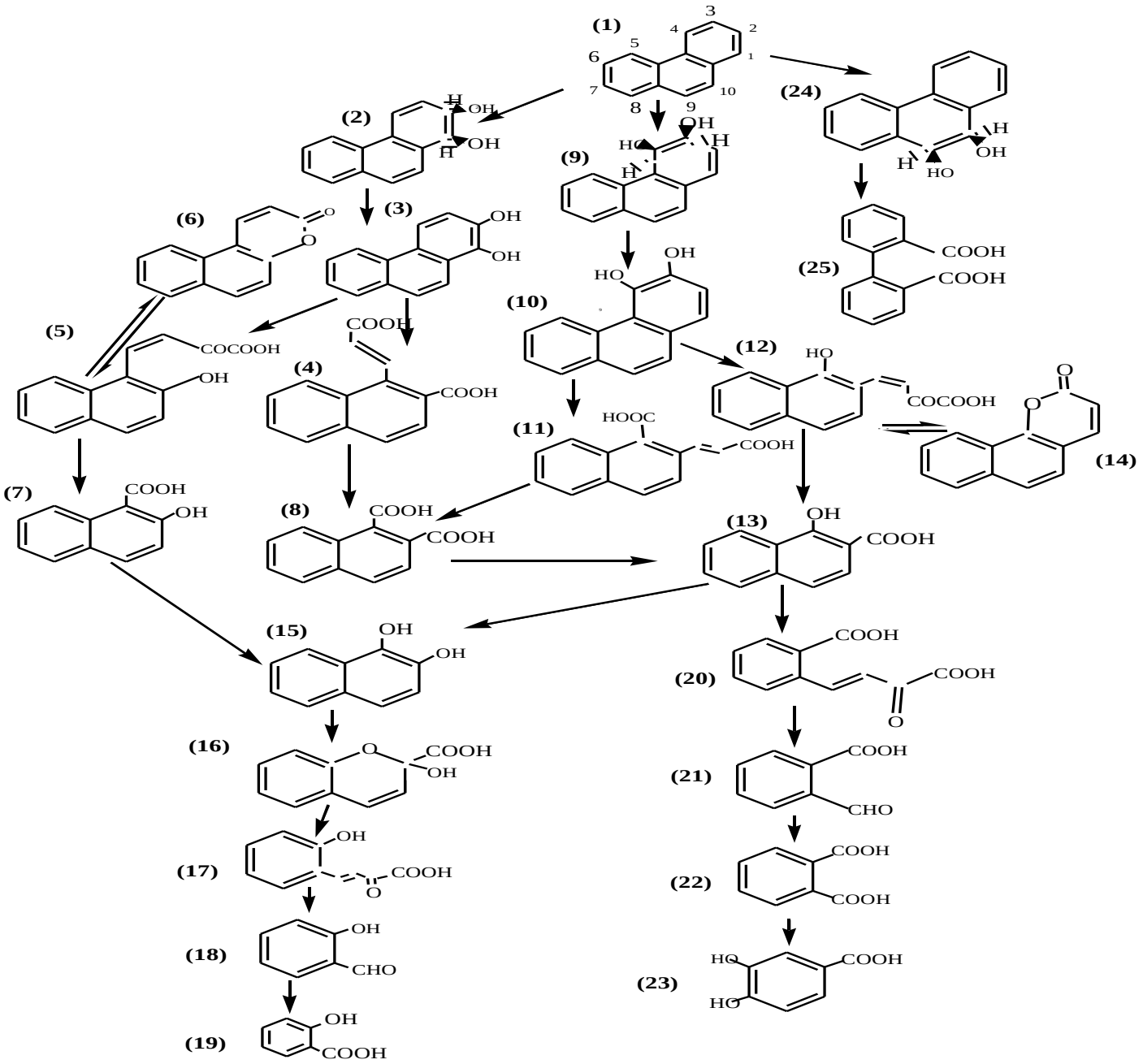


Figure 2. Proposed catabolic pathways of phenanthrene by aerobic bacteria. The compounds are 1, Phenanthrene; 2, cis -1,2-dihydroxy-1,2-dihydrophenanthrene; 3, 1,2-dihydroxyphenanthrene; 4, 2-[(E)-2-carboxyvinyl]-1-naphthoic acid; 5, trans-4-(2-hydroxynaph-1-yl)-2-oxobut-3-enoic acid; 6, 5,6-benzocoumarin; 7, 2-hydroxy-1-naphthoic acid; 8, naphthalene-1,2-dicarboxylic acid; 9, cis-3,4-dihydroxy-3,4-dihydrophenanthrene; 10, 3,4-dihydroxyphenanthrene; 11, 1-[(E)-2-carboxyvinyl]-2-naphthoic acid; 12, trans-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid; 13, 1-hydroxy-2-naphthoic acid; 14, 7,8-benzocoumarin; 15, 1,2-dihydroxynaphthalene; 16, 2-hydroxy-2H-chromene-2-carboxylic acid; 17; trans-o-hydroxybenzalpyruvic acid; 18, salicylaldehyde; 19, salyclic acid; 20, trans-2-carboxybenzalpyruvic acid; 21, 2-carboxybenzaldehyde; 22, o-phthalic acid; 23, protocatechuic acid; 24, cis-9,10-dihydroxy-1,2-dihydrophenanthrene; 25, 2,2′-diphenic acid (Ri-He et al., 2008).

organism was found to mineralize up to 72% of pyrene to CO_2 within two weeks. Three percent of the labelled carbon was found in the organic phase and 25% was present as water-soluble metabolites in the aqueous phase. Pyrene-4, 5-dihydrodiol was identified as the initial ring oxidation product and 4-phenanthroic acid as the major metabolite of the degradation of pyrene by a *Mycobacterium spp* (Heitkamp et al., 1988b). Also, a

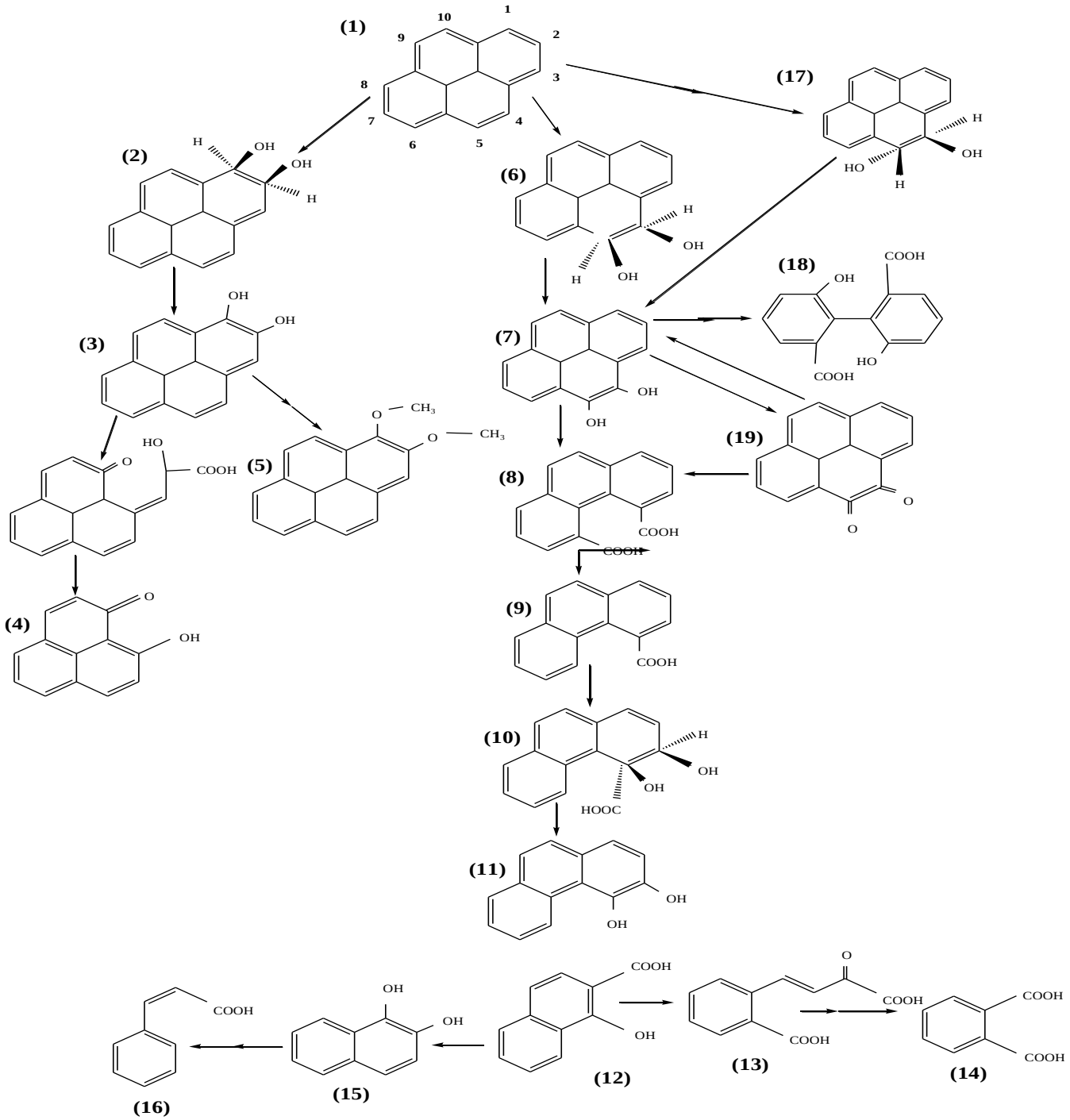


Figure 3. Proposed catabolic pathways of pyrene by aerobic bacteria. The compounds are 1, Pyrene; 2, cis-1,2-dihydroxy-1,2-dihydropyrene; 3, 1,2-dihydroxypyrene; 4, 4-hydroxyperinaphthenone; 5, 1,2-dimethoxypyrene; 6, cis-4,5-dihydroxy-4,5-dihydropyrene; 7, P2,4,5-dihydroxypyrene; 8, phenanthrene-4,5-dicarboxylate; 9, phenanthrene-4-dicarboxylate; 10, cis-3,4-dihydroxyphenanthrene-4-carboxylate; 11, 3,4-dihydroxyphenanthrene; 12, 1-hydroxy-2-naphthoate; 13, trans-2′-carboxybenzalpyruvate; 14, phthalate; 15, 1,2-dihydroxynaphtharene; 16, cinnamic acid; 17, trans-4,5-dihydroxy-4,5-dihydropyrene; 18, 6,6′-dihydroxy-2,2′-biphenyl dicarboxylic acid; 19, pyrene-4,5-dione (Ri-He et al., 2008).

proposed catabolic pathway of pyrene by aerobic bacteria has been suggested as shown in Figure 3 (Vila et al., 2001; Liang et al., 2006; Ri-He et al., 2008).

Fluorene

Fluorene is a polycyclic aromatic hydrocarbon and has

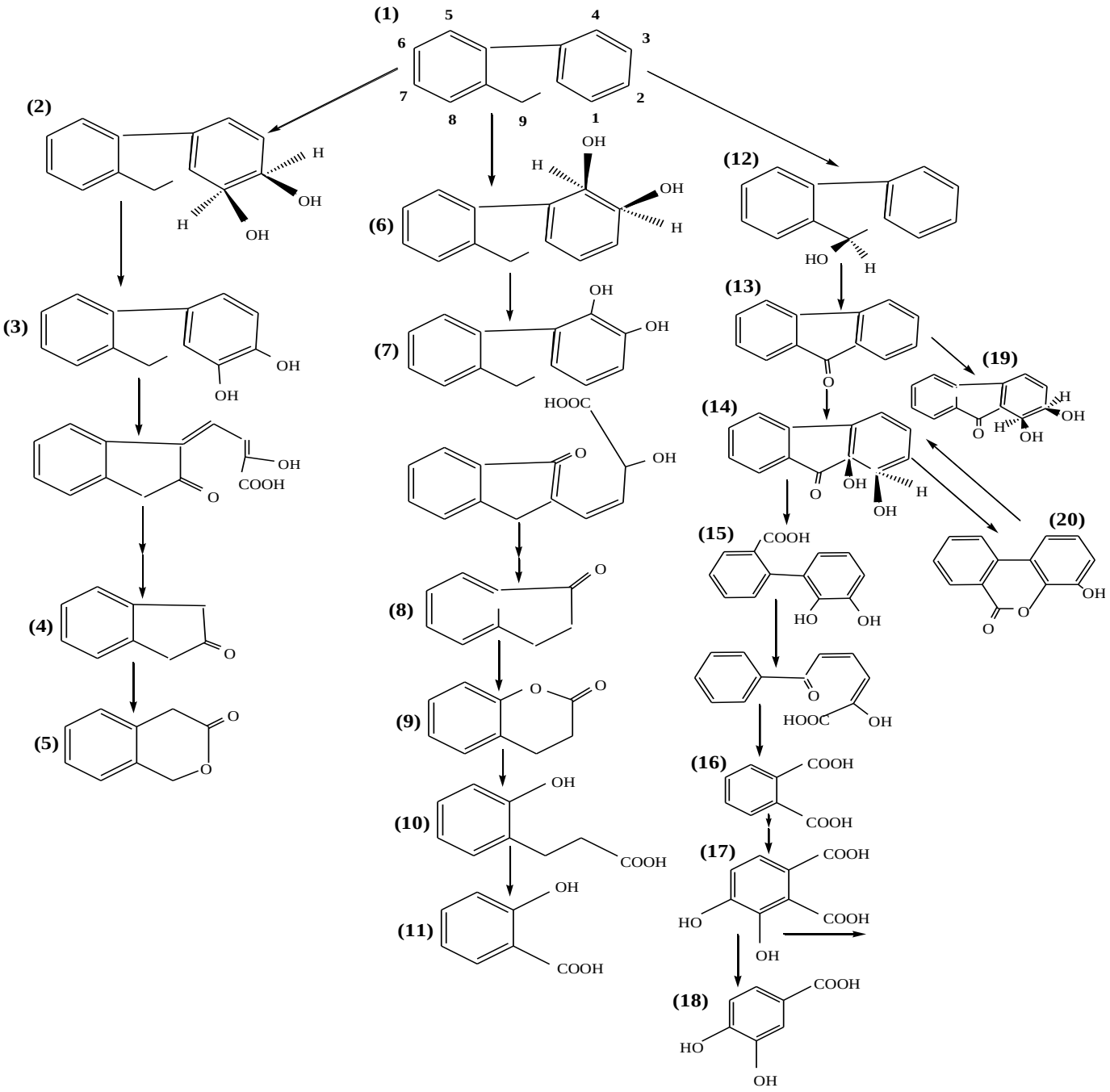


Figure 4. Proposed catabolic pathways of fluorene by aerobic bacteria. The compounds are 1, Fluorine; 2, cis-1,2-dihydroxy-1,2-dihydrofluorene; 3, 1,2-dihydroxy fluorine; 4, 2-indanone; 5, 3-isochromanone; 6, cis-3,4-dihydroxy-3,4-dihydrofluorene; 7, 3,4-dihydroxyfluorene; 8, 1-indanone; 9, 3,4-dihydrocoumarin; 10, 3-(2-hydroxyphenyl) propionic acid; 11, salicylic acid; 12, 9-fluorenol; 13, 9-fluorenone; 14, 1,1a-dihydroxy-1-hydro-9-fluorenone; 15, 2/-carboxy-2,3-dihydroxybiphenyl; 16, phthalic acid; 17, 4,5-dihydroxyphthalate; 18, protocatechuic acid; 19, 1,2-dihydro-1,2dihydroxy-9-fluorenone; 20, 8-hydrixy-3,4-benzocoumarin (Ri-He et al., 2008).

been found to be susceptible to microbial degradation to varying extents. (Gibson and Subramanian, 1984; Mueller et al., 1991; Field et al., 1992; Sutherland et al., 1995).

The initial attack on fluorene is catalysed by dioxygenase to yield 9-fluorenol and 1, 1a-dihydroxy-1-hydro-9-fluorenone. The catabolic pathway for fluorene degradation has been proposed as shown in Figure 4 (Kasuga et al., 2001; Wattiau et al., 2001; Habe et al., 2004; Ri-He et al., 2008).

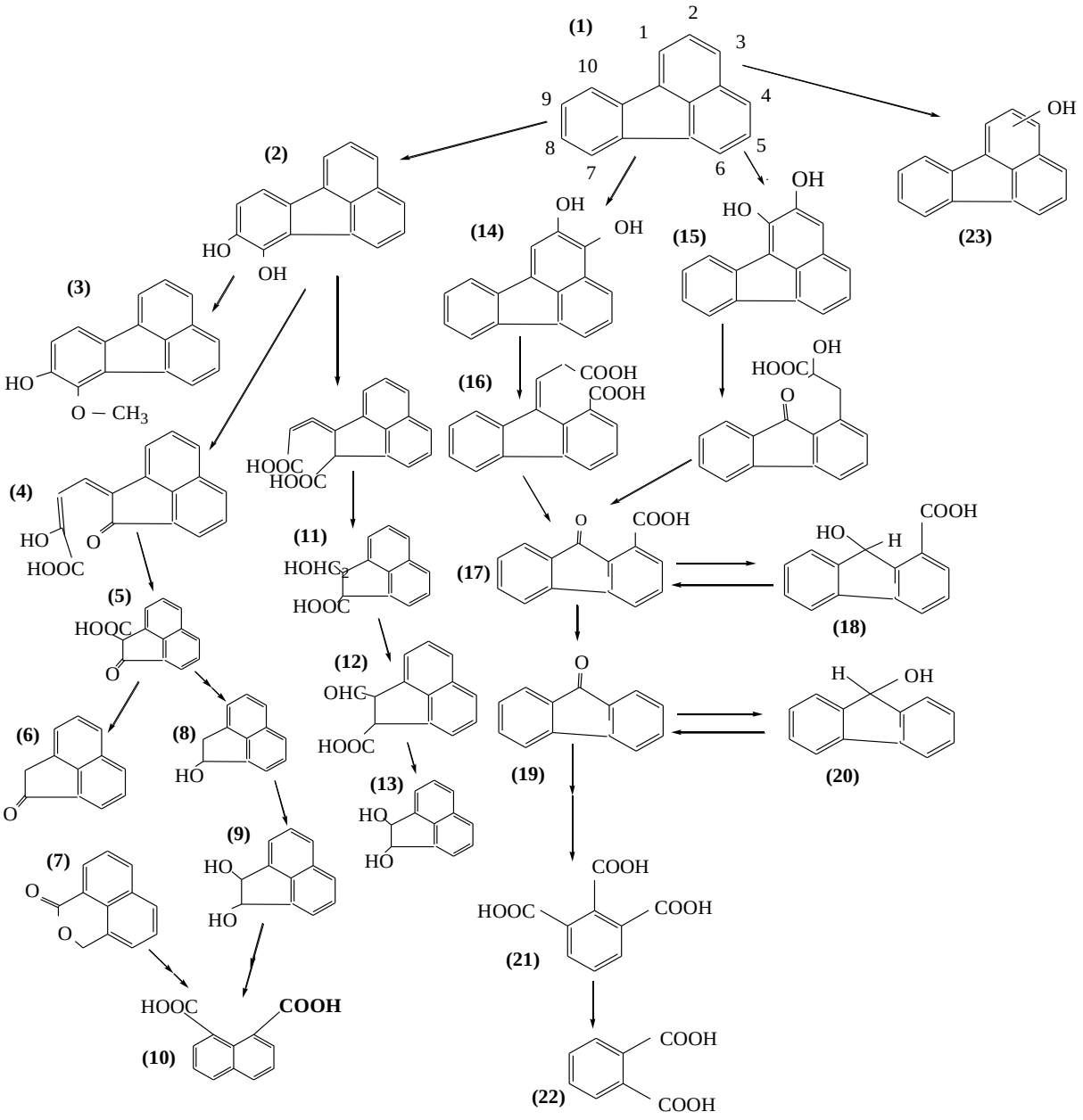


Figure 5. Proposed catabolic pathways of fluoranthene by aerobic bacteria. The compounds are 1, Fluoranthene; 2, 7,8-dihydroxy fluoranthene; 3, 7-methoxy-8-hydroxy-fluoranthene; 4, (2Z,4Z)-2-hydroxy-4-(2-oxoacenaphthylen-1(2H)-ylidene) but-2-enoic acid; 5, 1-acenaphthenone-2-carboxylic acid; 6, acenaphthylene-1(2H)- one; 7,1H,3H-benzo[de] isochromen-1-one; 8, acenaphthylen-1-ol; 9, acenaphthylen-1,2-diol; 10, naphthalene-1,8-dicarboxylic acid; 11, 2-(hydroxymethy)-acenaphthylene-1-carboxylic acid; 12, 2-formylacenaphthylene-1-carboxylic acid; 13, 1,2-dihydroacenaphthylene-1,2-diol; 14, 2,3-dihydroxy fluoranthene; 15, 1,2-dihydroxy fluoranthene; 16, (9E)-9-(carboxymethylene)-9H-fluorene-1-carboxylic acid; 17, 9-fluorenone-1-carboxylic acid; 18, 9-hydroxy-9H-fluorene-1-carboxylic acid; 19, 9-fluorenone; 20, 9-hydroxyfluorene; 21, 1,2,3-benzene-tricarboxylic acid; 22, monohydroxyfluoranthene; 23, phthalic acid (Ri-He et al., 2008).

Fluoranthene

This is a polycyclic aromatic hydrocarbon consisting of naphthalene and it is a four fused benzene ring. Many microorganisms showed the capability of utilising fluoranthene (Heitkamp et al., 1988a; Meyer and Steinhart, 2001). The catabolic pathway describing the biodegradation of fluoranthene by *M. vanbaalenii* PYR-1, initiated by mono-and deoxygenated reactions has been discovered recently, Figure 5 (Ri-He et al., 2008).

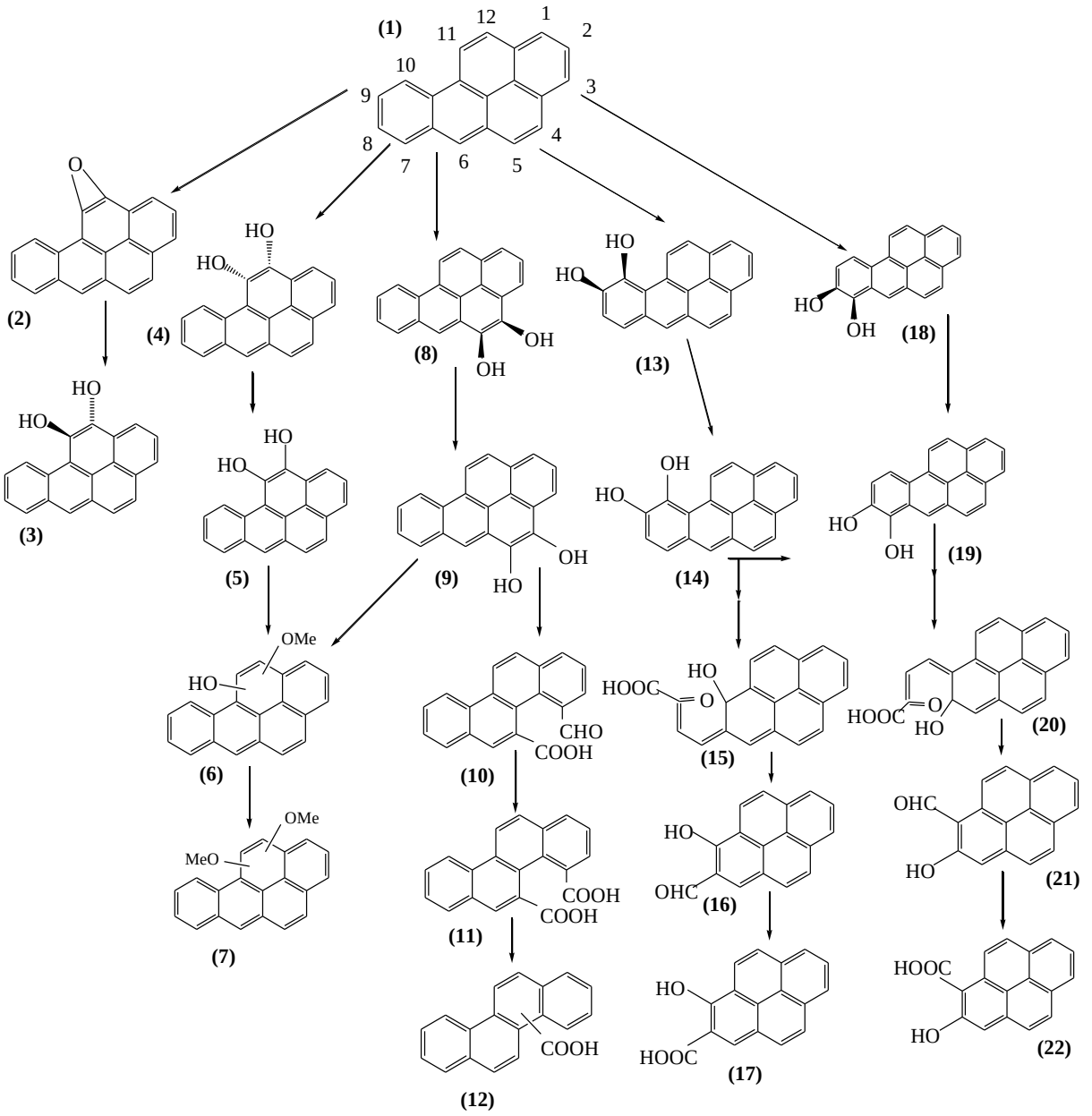


Figure 6. Proposed catabolic pathway of benzo[a]pyrene by aerobic bacteria. the compounds are 1, Benzo[a]pyrene; 2, benzo[a]pyrene-11,12-epoxide; 3, trans-benzo[a]pyrene-11,12-dihydrodiol; 4, cis-benzo[a]pyrene-11,12-dihydrodiol; 5, 11,12-dihydroxy-benzo[a]pyrene; 6, hydroxymethoxybenzo[a]pyrene; 7, dimethoxybenzo[a]pyrene; 8, cis-benzo[a]pyrene-4,5-dihydrodiol; 9, 4,.5-dihydroxy-benzo[a]pyrene; 10, 4-formylchrysene-5-carboxylic acid; 11, 4,5-chrysene-dicarboxylic acid; 12, chrysene-4(5)-carboxylic acid; 13, cis-benzo[a]pyrene-9,10-dihydrodiol; 14, 9,10-dihydroxy-benzo[a]pyrene; 15, cis-4-(8-hydroxypyrene-7-yl)-2oxobut-3-enoic acid; 16, pyrene-8-hydroxy-7-aldehyde; 17, pyrene-8-hydroxy-7-carboxylic acid; 18, cis-benzo[a]pyrene-7,8-dihydrodiol; 19, 7,8-dihydroxy-benzo[a]pyrene; 20, cis-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid; 21, pyrene-7-hydroxy-8-aldehyde; 22, pyrene-7-hydroxy-8-carboxylic acid (Ri-He et al., 2008).

Benzo[a]pyrene

This is a five ring polycyclic aromatic hydrocarbon ($C_{20}H_{12}$) whose metabolites are mutagenic and highly carcinogenic (Le Marchand et al., 2002). Benzo[a]pyrene can be oxidised by different microorganisms to various metabolites, which include: *trans*-7, 8-dihydroxy-7, 8-dihydrobenzo[a]pyrene, 3-hydroxybenzo[a]pyrene and 9-hydroxybenzo[a]pyrene, *trans*-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene, benzo[a]pyrene-1,6-quinone, benzo[a]pyrene-3,6-quinone Figure 6 (Gibson and Subramanian, 1984; Cerniglia et al., 1992; Ri-He et al.,

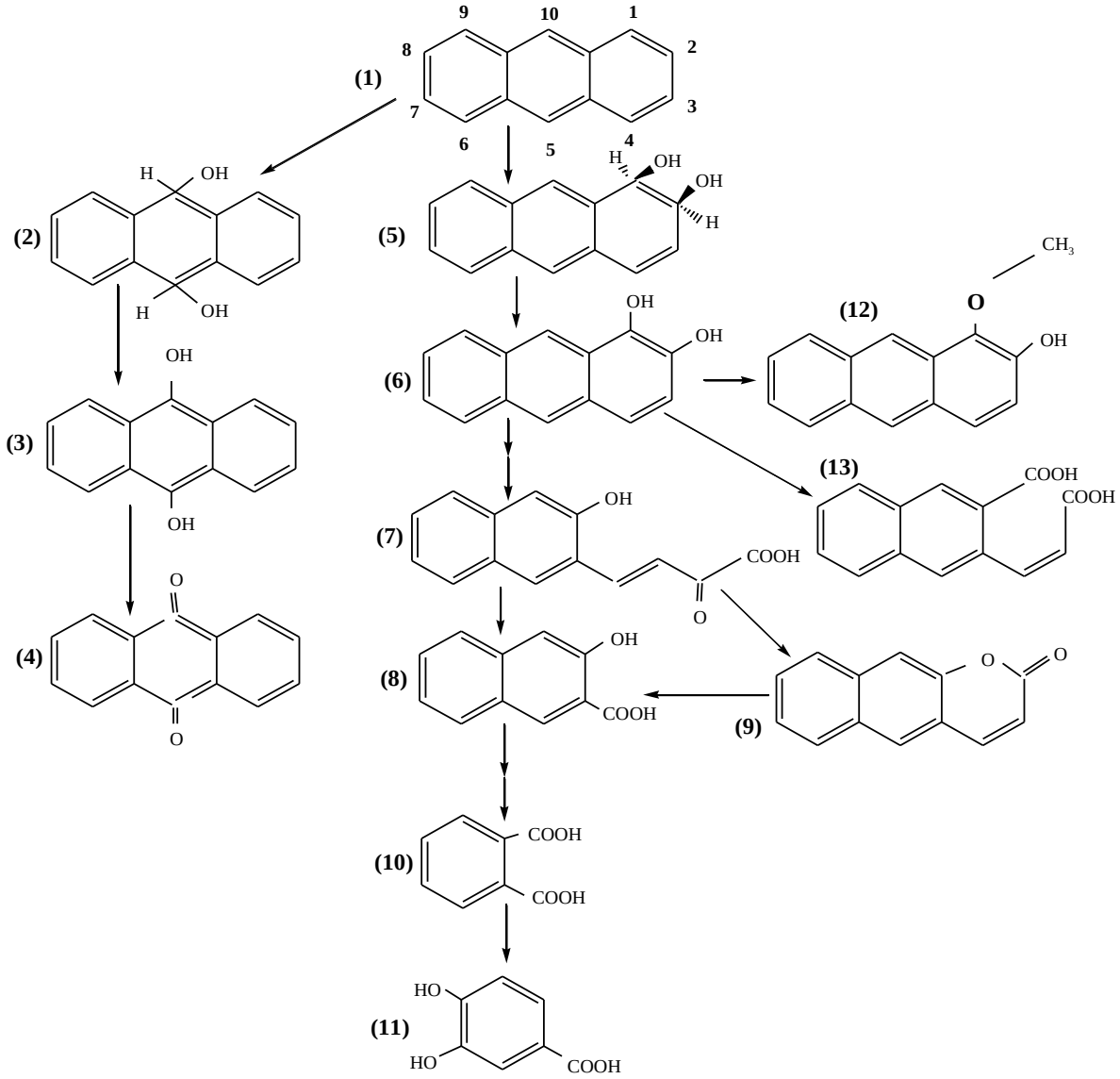


Figure 7. Proposed catabolic pathways of anthracene by aerobic bacteria. the compounds are 1, Anthracene; 2, anthracene-9,10-dihydrodiol; 3, 9,10-dihydroxyanthracene; 4, 9,10-anthraquinone; 5, cis-1,2-dihydroxy-1,2-dihydroanthracene; 6, 1,2-dihydroxyanthracene; 7, cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid; 8, 2-hydroxy-3-naphthoic acid; 9, 6,7-benzocoumarin; 10, o-phthalic acid; 11, protocatechuic acid; 12, 1-methoxy-2-hydroxyanthracene; 13, and 3-(-2carboxyvinyl)-napthalene-2-carboxylic acid (Ri-He et al., 2008).

2008).

Anthracene

This is a polycyclic aromatic hydrocarbon consisting of three fused benzene rings. It is also component of coal tar (Iglesias et al., 2010). The initial reactions in the bacterial degradation of anthracene involve the formation of *trans*-1, 2-dihydroxyanthracene prior to ring fission (Gibson and Subramanian, 1984).

Additional studies showed that *Pseudomonas putida* strain 199 and *Beijerinckia* sp. strain B-836 oxidised anthracene to (+)-*cis*-1, 2-dihydroxy-1, 2-dihydroxy-anthracene. Bacteria grown in a medium of naphthalene are shown to oxidise anthracene, 1, 2-dihydroxy-anthracene to 2-hydroxy-3-naphthaldehyde (Gibson and Subramanian, 1984; Sutherland et al., 1995). Also, the reactions in the degradation of anthracene are catalyzed by multicomponent dioxygenases to produce cis-1, 2-dihydrodiols.

The proposed catabolic pathway involves the ortho-cleavage of 1, 2-dihydroxyanthracene into 3-(2-carboxyvinyl) naphthalene-2-carboxylic acid for *Mycobacterium* sp. PYR-1 and *Rhodococcus sp.* Figure 7 (Dean-Ross et al., 2001; Moody et al., 2001).

Other PAHs

The other PAHs are classified as acute/chronic toxic hazardous organic compounds. They include compounds such as benzo[a]anthracene, benzo[k]fluoranthene with five fused benzene rings and indenol (1, 2, 3-cd) pyrene with six fused benzene rings. They are present in substantial quantities in oil sludge and can also be susceptible to microbial degradation (Gibson and Subramanian, 1984; Mueller et al., 1991; Field et al., 1992; Sutherland et al., 1995).

OIL SLUDGE IN THE ENVIRONMENT

It is generally recognised that land as a component of the environment deserves the same attention and protection as water and air (Okieimen and Okieimen, 2005). This recognition has perhaps risen because of increased incidents of land pollution, the scarcity of land, awareness and concern about long-term effects of land pollution on terrestrial and aquatic ecosystems. The adverse effects of oil sludge on soil ecology and fertility have been pivotal in the development of efficient technologies for the degradation of these contaminants in the environment (Okieimen and Okieimen, 2005). As oil sludge is dumped into the environment, lighter compounds volatilize and heavier ones remain. Most oil sludge components have high affinity for soil material and particulate matter. Overtime, they accumulate to the extent that they become difficult to eliminate because they reside in fine pores. Then, they become protected from attack by biota in the soil; hence they are not bio-available. Their fate and behaviour are controlled by factors such as soil type and their physico-chemical properties (Reid et al., 2000). Such properties include their concentration, structures of the components and their solubility, environmental conditions (temperatures, pH, moisture content and wind), and the available microorganisms (physiology and genetics). Their solubility is the key factor of their fate in the environment. The solubility of oil sludge components differs from one compound to another, some are infinitely soluble polar compounds, and others are of low solubility for example, the PAHs (Mahmoud, 2004). As the contact time with the environment increases, chemical and biological availability of the compounds decreases, a process termed “ageing”, which has attracted considerable attention in recent years.

Oil sludge can be biodegraded by microorganisms such as bacteria and fungi. A large number of bacteria species have the ability to degrade majority of natural hydrocarbon components from oil sludge especially low-molecular-weight contaminants (Ward et al., 2003). Microbial biodegradation is an effective and inexpensive approach to the degradation of petroleum hydrocarbons from oil sludge. This is possible as long as a large population of degrading microorganisms is present and the conditions encouraged the microbial growth and activities (Philips et al., 2000).

MICROBIAL BIODEGRADATION OF OIL SLUDGE

Oil sludge exhibits some biodegradable properties in the environments such as transformation, conversion or mineralisation, specific adhesion mechanisms and production of extracellular emulsifying agent by micro-organisms (Leahy and Colwell 1990; Research triangle institute, 1999; Laskova et al., 2007; Paulauskiene et al., 2009). To successfully exploit the microbial degradation of oil sludge, it is imperative to understand and master the mechanism needed in order to manipulate the microbial activities.

For oil sludge containing large quantities of hydrocarbons, microorganisms must be able to use hydrocarbons as substrates (Tabuchi et al., 1998). They must be able to synthesize enzymes that can catalyse the reaction in which these contaminants are degraded to simpler, lower molecular chains and less toxic compounds (CO_2 and H_2O), through obtaining the nutrients and energy necessary for their survival in the process (Johnson and Scow, 1999). The initial step in this mechanism is the catabolism of oil sludge by bacteria and fungi, which involves the oxidation of the substrate by oxygenases, in which molecular oxygen is required.

Aerobic conditions are necessary for this route of microbial oxidation of hydrocarbons to take place (Marin Millan, 2004). Microbial bioremediation of oil sludge is dependent on a number factor which includes: characteristics of the oil sludge, microbial population present and other physic-chemical factors such as temperature, pH and moisture. However, the characteristics and fate of oil sludge depends on its molecular size and topology or stoichiometry (Kanaly and Harayama, 2000). The removal of low molecular weight petroleum hydrocarbons (4-ring or less), is first done through evaporation. As the molecular sizes increases, biodegradation rates become slower.

Oil sludge, albeit very slow, is susceptible to degradation by naturally occurring microflora, but this process reduces nutrient and oxygen level in soil which in turn impedes other environmental processes such as transformation or mineralisation. In order to enhance the oil sludge biodegradation processes and make it economically realistic and fast, it is necessary that the bioavailability of hydrocarbons present in the oil sludge matrix be increased. This may be done by biostimulation, which is simply the addition of nutrients to stimulate the growth and degradative capabilities of the indigenous microorganisms present (Piskonen and Itävaara, 2004). Many microbial strains are capable of degrading only specific components of oil sludge. However, oil sludge is a complex mixture of different petroleum hydrocarbon (Mac Naughton et al., 1999). Single bacterial species

have limited capacities to degrade all the fractions of hydrocarbons presents (Loser et al., 1998). Hence, a mixture of different bacterial species that can degrade a broad range of the hydrocarbon constituents such as present in oil sludge would show more potential. Steps should be taken to ensure that the original indigenous bacterial communities be part of the regiment. Mishra et al. (2001) suggested that indigenous microorganisms isolated from a contaminated site will assist in overcoming this problem, as the microorganisms can degrade the components and have a higher tolerance to toxicity that may wipe off other introduced species.

Factors affecting the biodegradation mechanisms

There are many factors, including physical, chemical and biological that may ultimately determine the effectiveness of strategies for microbial bioremediation of oil sludge (van Hamme et al., 2003). These include: Biosurfactants, effect of pH, nutrients, salinity, oxygen, temperature and water activity/ moisture contents, according to Micky (2006) and discussed below.

Biosurfactants

Biosurfactants are important agents that enhance the effective uptake of petroleum hydrocarbons by bacteria and fungi (Leahy and Colwell, 1990; Cort and Bielefeldt 2000a; b; Shiohara et al., 2001). Bacteria are known to produce biosurfactants, which they use to form emulsions of oil substrates (Calvo et al., 2004; Bayoumi, 2009; Liu et al., 2011; Plaza et al., 2011). Most bacterial strains can efficiently produce biosurfactant on petroleum hydrocarbon medium and in soil. The biosurfactants they produce can emulsify petroleum hydrocarbon in oil sludge so that they can be bioavailable to bacteria for biodegradation in the system. They do this by increasing the surface area of the substrates therefore, increased their solubility (Ahimou et al., 2000; Ron and Rosenberg, 2001; Maier, 2003; Mukherjee and Das, 2005). Biosurfactant production by bacteria comes with the advantage of being natural, non-toxic, biodegradable and a cost effective approach that can help in solubilisation of oil sludge hydrocarbons during biodegradation (Sim et al., 1997; Calvo et al., 2004; Bayoumi, 2009; Liu et al., 2011; Plaza et al., 2011).

These biosurfactants secreted by bacteria are more effective than chemical surfactants in enhancing the solubility and biodegradation of petroleum hydrocarbons (Cybulski et al., 2003; Wong et al., 2004). The production of biosurfactant is proportional to the usage of hydrophobic PAHs substrates by the bacteria present in the system. Hydrocarbon catabolism in the environment can be enhanced by the production of biosurfactants and supplementary application of additives and bulking agents (Ward et al., 2003). Bulking agents such as compost will enhance metabolism of organic contaminants because they provide extra nutrients, additional carbon source and assist in retaining moisture contents of the pile (Namkoog et al., 2002).

Effect of pH

Most oil degrading heterotrophic bacteria and fungi perform at their optimum when pH is neutral. However, fungi are known to be tolerant of acidic conditions (Al-Daher et al., 1998). The mineralization of hydrocarbon components in the environment is generally optimal at pH 7 to 7.8, thus overall biodegradation process is enhanced (van Hamme et al., 2003). The metabolic pathways for degradation differ in both fungi and bacteria (Cerniglia et al., 1979).

According to report by Sutherland (1992), fungal decomposition of PAHs may produce mutagenic intermediates (Frick et al., 1999). In such instance, liming may be used to increase the pH from acidic to alkaline state so that bacterial growth may be favoured than fungal growth.

Nutrients

The growth of heterotrophic bacteria and fungi depends on a number of nutrient elements, an electron acceptor and organic compound that serves as the source of carbon and energy (Adriano et al., 1999; Boettcher et al., 2001). For aerobic microorganisms, the electron acceptor is oxygen. Some microorganisms can utilise some inorganic compounds such as nitrates, sulphates, carbon dioxide, ferric iron and some organic compounds, as electron acceptors for electrons released by the oxidation of the substrate carbon source. Some bacteria and fungi also require low concentrations of some amino acids, vitamins or other organic molecules as growth factors. The absence of any of these essential elements from the environment may prevent growth and metabolism of microorganisms (Atagana, 2003). Microorganisms that degrade oil sludge are dependent on fixed forms of nitrogen (NH_3, NO_3^-, NO_2^- and organic nitrogen) to meet their nitrogen requirements.

These forms of nitrogen are frequently limiting for microbial populations in soil, ground water and surface water (Atlas, 1991). Microbial synthesis of adenosine triphosphate (ATP), nucleic acids and cell membranes require phosphorus.

For nitrogen to be available to soil microorganisms it must be, in most cases, present in inorganic form such as ammonium or nitrate (Swindell et al., 1988; Tate, 1995) while phosphorus is available in the form of orthophosphate (Alexander, 1999). According to report by van Hamme et al. (2003), nitrogen and phosphorus contents have great effects on microbial degradation of oil sludge.

Salinity

Studies have shown that there are generally positive correlations between salinity and rates of mineralization of PAHs such as phenanthrene and naphthalene (Leahy and Colwell, 1990). However, it has been noted that hypersalinity will result in the decrease in microbial metabolic rates (Micky, 2006).

Oxygen

Aerobic biodegradation is the most effective pathway for bioremediation. This means that, the presence and concentration of oxygen is important in such process. Also, lack of aeration, in the system may be a rate-limiting parameter in the biodegradation and catabolism of hydrocarbons by bacteria and fungi (van Hamme et al., 2003).

The breakdown of oil sludge components may possibly involve the utilization of oxygenase, in which molecular oxygen is required. Great efficiency of natural microbial hydrocarbon degradation occurs mostly when oxygen is available (Ward et al., 2003). Although anaerobic degradation of PAHs by microorganisms has been shown to occur, the rates are somewhat negligible and limited to halogenated aromatics compounds such as the halobenzoates, chlorophenols and alkyl-substituted aromatic (Suflita et al., 1982; Boyd and Shelton, 1984; Angelidaki et al., 2000).

Temperature

Temperature is another important variable that has effect on oil sludge biodegradation. Microorganisms can grow at temperatures below 0 to above 100°C with good water supply (Atlas and Barther, 1987). Optimum temperature dictates the rate of oil sludge metabolism by micro-organisms and also the pattern of the microbial community. Temperature has direct effect on the physical nature and chemical composition of the PAHs constituents (Atlas, 1981). Increases in temperature have been reported to be proportional to the solubility of contaminants and induces higher metabolic activity in a compost system (Gibb et al., 2001). When temperatures are low, PAHs tend to be more viscous and their water solubility is greatly reduced (Leahy and Colwell, 1990). Low temperature also affects microbial growth, propagation and subsequently results in decrease in the rate of degradation (Gibb et al., 2001). Low temperature also results in a decrease in enzymatic activities, which is essential for degradation of the oil components. The optimum temperature for hydrocarbon degradation has reported to be in the range of 30 to 40°C. At temperatures above this range, enzymatic activities are inhibited as proteins denature at higher temperature (Leahy and Colwell, 1990).

Water activity/ moisture content

According to Vinas et al. (2005), the rates at which PAHs are degraded are also determined by moisture level. The reason is that water is needed for microbial growth and enzymatic/biochemical activities (Leahy and Colwell, 1990). Elemental uptake by microorganisms is by absorption and transportation of solubilised molecules across the cell membrane. The availability of target molecules to the microorganisms depends on the amount of water present in the treatment matrix.

Optimal activity occurs when the soil moisture and water content for aerobic bioremediation treatment matrix is usually between 50 and 80% of saturation (moisture holding capacity) (Kosaric, 2001). When the moisture content falls below 10% bioactivity becomes marginal (Kosaric, 2001). However, if the soil water holding capacity is high above the optimal ranges, biodegradation rates are usually small because of possible water-logging. The water-logging may promote anoxic conditions, thereby reducing aerobic bioremediation efficiency.

TREATMENT TECHNOLOGIES AND DISPOSAL OF OIL SLUDGE

Safe disposal and treatment of huge quantity of oil sludge generated during the processing of crude oil are some of the major challenges faced by oil refineries and petrochemical industries (Srinivasarao et al., 2011). In recent years, most refineries treat oil sludge using conventional methods which includes; physical treatment (storage, landfilling, combustion and incineration in a rotary kiln, lime stabilization, stabilization and solidification) (Wright and Noordhius, 1991; Karamalidis and Voudrias, 2001; Bhattacharyy and Shekdar, 2003; Radetski et al., 2006; Beech et al., 2009; Liu et al., 2010), chemical treatment (oxidative thermal treatment, treatment with fly-ash, pyrolysis treatment and solvent extraction) (Bonnier et al., 1980; Atlas, 1984; Taiwo and Otolorin, 2009) and biological treatment (landfarming, bio-reactor treatment and composting) (Pereira-Neta, 1987; Piotrowski, 1991; Lees, 1996; Singh et al., 2001; Hejazi et al., 2003; Mahmoud, 2004; De-qing et al., 2007; Srinivasarao et al., 2011; Udotong et al., 2011; Besalatpour et al., 2011). Most of the physical and chemical methods require expensive equipments and high energy to treat the oil sludge. Some of these methods convert oil sludge into lighter products and reduce the quantity before disposal. Some of the methods may generate by-products that may need to be treated using other methods before disposal to a landfill (Liu et al., 2010), making them more expensive. Exam-

ples of the conventional methods used are discussed below.

Incineration

Incineration is a technology commonly used in large refineries. The common types are rotary kiln and fluidized bed incinerators. In rotary kiln incinerator, the combustion temperature is from 980 to 1200°C and the residence time is about 30 min. While in fluidized bed incinerators, the combustion temperature is from 732 to 760°C, and the residence time may be in order of days. The incineration process requires sophisticated equipments and experienced operators to achieve adequate combustion of oil sludge. Usually the incineration of oil sludge using fluidized bed technique produces ash scrubber sludge, with low contents of heavy metals. These products are usually disposed of in a landfill (Liu et al., 2010). Incineration is an expensive technique and oil sludge contains high concentration of hazardous compounds including those that are resistant to incineration. Incineration is not only expensive but generates toxic residues such as ash, scrubber water, scrubber sludges, sulphur dioxide, nitrogen monoxide, carbon monoxide and some organic compounds (Srinivasarao et al., 2011). Some of these residues such as ash containing metals need to be treated before being disposed of. During the incineration process, waste feed rates, oxygen: air ratio, residence time, combustion temperature and gas emission are critical parameters that needs to be controlled (Mahmoud, 2004).

Treatment with fly-ash

This is the treatments of oil sludge with aqueous slurry of fly-ash and a small amount of polymers. This process mixes light sludge in a small tank equipped with a mixer before thickening. Sludge with high oil and solids contents are de-watered in a centrifuge before being treated with ash slurry in a screw mixer. The settled products from the thickener and mixer are transported in closed truck containers directly to a landfill, which must be well drained to minimize leaching. During the dry season the deposit quickly become hard enough to be used for roadbeds. Sludge treated in this way may be covered with a layer of soil and the deposit area can be used to grow grass and trees (Atlas, 1984; Mahmoud, 2004). This method may be expensive, since equipments, energy and operating persons are needed for this process.

Lime stabilization

Stabilization involves mixing a solid additive material to the oil sludge in order to produce a matrix within which the oil and metal are fixed and will not leach out. The use of lime for this purpose has being established in the literature, the addition of lime produces physical and chemical changes in the oil sludge which facilitates hydrocarbon adsorption and immobilization of metals as insoluble salts (Wright and Noordhius, 1991; Mahmoud, 2004).

The high pH provided by adding the lime is essential in this process, some additives can be added to produce hydrophobic matrix to prevent contaminants from becoming acidic due to rainfall percolation in the landfill (Mahmoud, 2004).

This technique may also generate residual products that may need to be treated by using other methods before disposal in a landfill which will make the techniques expensive.

Solvent extraction

In this method, the oil sludge is extracted with a solvent to remove oil and other organics, the solvent is recovered and recycled. Many refineries believe that recycling is the most desirable environmental option for handling oil sludge, due to the possibility of recovering valuable oil for reprocessing, reformulating and energy recovery (Bonnier et al., 1980; Taiwo and Otolorin, 2009). During recycling, the condensed solvent and water are continuously separated in a trap. The condensed liquid contains water and hydrocarbon.

The hydrocarbons in condensed liquid may amount to 73.24% of the sludge, and they are both volatile and non-volatile hydrocarbons. The solvent extraction technique has a tendency to greatly reduce sludge contaminants from 100 to 30% water and solid wastes. The method may possibly reduce the pollution effects of oil sludge on the environment with the recovery of recyclable hydrocarbons. If the optimum conditions are carefully selected, solvent extraction approach can significantly mitigate the non-compliance to standard limit of industrial discharge into the environ-ments and the permissible allowances for oil sludge.

Evaluation of the extent of sludge treatment before disposal can be done and can make significant impact on refinery and petrochemical industries. The advantage of solvent extraction techniques is that the recovery approach to oil sludge treatment explored can serve as a precursor to *in-situ* treatment and cleaning of oil storage facilities (Taiwo and Otolorin, 2009). It will also reduce economic losses and out of operation period, since there will be a reduction in time requirements for treatments, also the oil, water and mud can be effectively used and extraction solvents can be recycled. The limitation is the adaptation of selected solvent to the sludge treatment. Solvent extraction may not remove heavy metals such as arsenic, lead and selenium; these residues must be treated using other methods before disposal (Mahmoud, 2004; Taiwo and Otolorin, 2009).

Stabilization and solidification method

This technology is used to minimize potential environmental impact of oil sludge by enhancing the non-leachable properties of the treated oil sludge. The treatment uses advanced chemical oxidation (Fenton's reagents) followed by stabilization and solidification with lime-clay and Portland cement-lime to yield oil sludge degradation and immobilization. In this process, PAHs and BTEX compounds are reduced after stabilization and solidification process (Beech et al., 2009). The reduction of these compounds may be due to the dilution which occurred by the addition of clay and lime, and by immobilization promoted by the lime and cement (Radetski et al., 2006). The stabilization and solidification process is cheap compared to many other technologies for treating and disposing oil sludge. This technique reduces the mobility of hazardous substance and contaminants in the environments through physical and chemical means, and can be applied *ex-situ* and *in-situ* (Karamalidis and Voudrias, 2001). If the ecotoxicity potential of oil sludge is considered, the initial waste has high toxicity in PAHs and high concentration of phenolic compounds before treatment while after treatment the final products would be less toxic, and can be reused as concrete road bed blocks (Karamalidis and Voudrias, 2001). Despite the fact that the process enables the change of the initial dangerous waste to non-dangerous waste, the mass and volume ratio of residual product increases after the treatment (3 kg of waste yield 20 kg of commercial concrete block). In some cases, potential of oil sludge components leaching in long-term is possible. This calls for evaluation by a temporal series of leaching test to ensure environmental protection, in terms of public health and ecotoxicological perturbation of terrestrial and aquatic ecosystems (Karamalidis and Voudrias, 2001).

Oxidative thermal treatment

In this process, the oil sludge is not combusted but heated to remove organics and water from solids, the water is converted to steam to help strip off high boiling point semivolatile compounds, which can be condensed for recovery and disposal. The treatment is carried out using different concentration of oxygen at a constant heating temperature. This minimized waste and oil is recovered while producing a solid residue that meets environmental standards that are directly disposed into landfill. The flaw with this process is its high energy consumption and complex operation (Shie et al., 2004).

Pyrolysis treatment

It is a technique for recovering oil and organic liquid gas by breaking down large molecules into smaller ones. The treated sediments that met the standard land disposal restriction level are directly discharged. In pyrolysis treatment of oil sludge, the initial step produces CO_2, hydrocarbons (volatile organics), water, CO, char and tar. The next stage, char and tar are combusted to release heat which is needed for the endothermic pyrolysis reaction. The hydrocarbons consist mainly of low molecular weights paraffins and olefins (C_1-C_2). The advantage of this process is that about 70 - 84% of the oil could be separated from the solids. The disadvantage of pyrolysis is that a significant amount of vacuum residue is produced during the process. The energy required in pyrolysis of oil sludge is very high because it is close to energy required to distillate diesel from crude oil. Oxidative pyrolysis of oil sludge performed with insufficient oxygen produces alkyl and alkene compounds rather than being oxidized to produce CO_2, CO and H_2O. Therefore, oxygen is important in this technique to yield a better result (Liu et al., 2010).

After considering the limitations of physical and chemical processes in treating and disposing oil sludge, it becomes imperative to consider biological process, which have lauded as cost effective and environmentally friendly for treating contaminated environment. Biological treatment methods have numerous applications, which include the clean-up of ground water, soil, surface water and treatment of effluent from industrial process waste streams. Most biological techniques are developed as a result of simple emulation of nature and how nature does bioremediation (Okieimen and Okieimen 2005). Biological methods have been proposed as a possible remedy for oil sludge treatments. However, most biological methods are economically unsound, prone to prolonged treatment times and they are not permanent solutions (Ward et al., 2003). The observed time lag in biological treatment may be attributed to the stability of the compounds, their complex molecular structures and the ability of oil sludge components to adsorb onto sediments (Bach et al., 2005). Despite the complications, the biological methods are still considered as potential technologies for the treatment of oil sludge (Leung, 2004).

BIOREMEDIATION

Bioremediation is defined as the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous waste (Gibson and Sayler, 1992). Bacteria are generally used for bioremediation, but fungi, algae and plants could also be used. Bioremediation is not a new technology however; perspectives on the use of bioremedial technologies to treat contaminants vary. There are three classifications of bioremediation. The first defines biotransformation as the alteration of contaminant molecules into less or non-hazardous molecules; the second defines

biodegradation as the breakdown of organic substances into smaller organic or inorganic molecules while the third definition defines bioremediation as mineralization which is the complete biodegradation of organic materials into inorganic constituents such as CO_2 or H_2O (Leung, 2004). These three classifications of bioremediation can occur either *in situ* (at the site of contamination) or *ex situ* (contaminant taken out of the site of contamination and treated elsewhere) (Das and Mukherjee, 2007). There are advantages and disadvantages to both *in situ* and *ex situ* strategies. *Ex situ* strategies also known as 'pump and treat', removes the contaminants and places them in a contained environment. It involves excavation and transportation (relocation) from the natural or original contaminated site to elsewhere. This allows for easier monitoring and maintaining of conditions and progress, thus making the actual bioremediation process faster. However, the removal of the contaminant from the contaminated site is time consuming, costly and potentially dangerous.

By moving contaminants, the workers and the general public have increased exposure to the toxic material. In contrast, the *in situ* strategy does not require removal of the contaminant from the contaminated site. *In-situ* bioremediation method involves the treatment of contaminants at the natural or original contaminated sites without relocation. The *in-situ* methods include biostimulation and bioaugmentation and are usually cost effective because there is no need for excavation and transporttation, however, it is less controllable and time consuming. A major advantage of biological techniques is that the product is reusable.

Bioremediation is a promising strategy for the treatment of oil sludge. The technologies employed are nature-compatible, reliable, cheaper and easy to adopt compared to physical and chemical methods (Machin-Ramirez et al., 2008). The end products are usually harmless and include carbon dioxide, water and fatty acids. Bioremediation is often less disruptive and eliminates waste permanently. It reduces long term liability, and has greater public acceptance and regulatory encouragement. It can also be coupled with other physical or chemical methods (Idris and Ahmed, 2003). Bioremediation has its limitations; some chemicals are not amenable to bioremediation, for instance, heavy metals, radionuclides and some chlorinated compounds. In some cases, microbial metabolism of contaminants may produce toxic metabolites. Bioremediation therefore is a scientifically intensive procedure, which must be tailored to the site-specific conditions. There are different number of *ex-situ* and *in-situ* methods which include biostimulation, bioaugmentation, landfilling, landfarming, bioreactors, and composting.

Biostimulation

This involves the management of the natural environment to optimise the growth and activity of the natural microbial population (Crivelaro et al., 2010). The growth and activity of existing microorganisms are accelerated which may be inhibited under normal conditions in water and soil. According to Crivelaro et al. (2010), the low biodegradation efficiency observed in the treatments of oil sludge mixed with soil (landfarming, biopile and composting) are as a result of the imbalanced nutritional amendments. However, biostimulation is one of the possibilities to tackle this kind of problem since oil sludge has limited amounts of nitrogen as well as phosphates. This may be because in oil sludge, most of the nitrogen is not available since it is part of complex structures relatively inaccessible to the degrading microbial population (Crivelaro et al., 2010). In their study, Crivelaro et al. (2010), evaluated the potentials of biostimulation process using vinasse a byproduct from processed sugarcane which contains adequate nutrients such as nitrogen, phosphorus and potassium (Cortez and Brossard Perez, 1997; Carmen Baez-Smith, 2006), as the microbial stimulating nutrient agent for biodegradation of oil sludge.

The application of vinasse stimulated the activities and growth of microbial population greater in treatments with vinasse than the controls without vinasse. Furthermore, vinasse helped the microbial population to overcome the toxicity effects of oil sludge. Although, an increase in the soil microbial population was obtained with vinasse, it was not adequate to enhance the bioremediation efficiency of the oil sludge at the mineralisation level (Crivelaro et al., 2010). However, biostimulation process can enhance the activities and growth of microbial population capable of degrading oil sludge to mineralisation level. Therefore, biostimulation of indigenous degrading bacteria as a tool in bioremediation process should be encourage, because the process relies on the degrading bacteria that have already adapted to the site's conditions (Dzantor, 1999; Ausma et al., 2002; Singh and Lin, 2010). The constraints in this technique are time and limited knowledge of microbial process, since if compared with other technologies, bioremediation is a slow process. Also, favourable conditions such as temperature, pH, nutrients and additives such as surfactants must be optimised to stimulate the microbial growth and activities during bioremediation (Atlas and Bartha, 1972; Kim et al., 2004; Mahmoud, 2004).

Bioaugmentation

This technique refers to the introduction of specialized or genetically engineered microorganisms that target specific chemical compounds. These organisms have been developed to biodegrade most common organic contaminants ranging from polychlorinated biphenyls (PCBs), organic solvents and petroleum hydrocarbons (Mehrashi et al., 2003; Atlas and Philip, 2005). The identi-

fication of the key microorganisms that play a major role in pollutant degradation processes is relevant to the development of optimal *in-situ* bioremediation strategies (Abed et al., 2002; Watanabe, 2002). The use of such specialised formulations of microorganisms is often dictated whereby the indigenous bacteria cannot metabolize the contaminants concerned. It could be used if the contaminants are toxic to the naturally occurring bacteria. Introduction of specialized bacteria also may be used to increase the biological activity (Van Veen et al., 1997). The dynamic growth of a bacterial consortium on PAHs has been studied. The results showed varied individual ability of the bacterial strains to grow on PAHs. The growth was further improved by mixing the PAHs with non-ionic surfactant and optimising favourable conditions such as temperature and nutrients (fertilizer). The rates in reduction of petroleum hydrocarbon varied from 16.75 to 95% (Lazar et al., 1999; van Hamme et al., 2003; Mishra et al., 2001). This means that some bacterial strains have the ability to degrade PAHs present in oil sludge. The results have shown that the performance of the microbial cultures is dependent on several factors including the composition of the sludge that varies depending on the type of crude oil and the source of the sludge inside the refinery (Mahmoud, 2004).

However, very little information is available about the use of microbial cultures that can be used to treat oil sludge on pilot scale and full scale studies. The limitation to successful bioaugmentation has always been attributed to poor survival of the introduced strains. The use of readily degradable substrate has been found as a limitation, due to low concentration and non biodegradability of targeted pollutants (Alexander, 1994). Again, if the soil (or the media that contains the contaminant) is heterogenous, there will be uneven flow of liquid or gas containing the nutrients or microbes, so different areas will undergo different rates of remediation.

Del'Arco and de Franca (2001) observed that various efforts have been attempted to improve the success of bioaugmentation process. Strategies that were employed to improve bioaugmentation process for the effective degradation of petroleum hydrocarbons include the use of adapted strains or the field application vector (Lajoie et al., 1994). Bioaugmentation of oil sludge may be a slow process if compared to landfarming and composting. Hence, more research needs to be conducted to stimulate the growth of microorganisms on oil sludge and improve the performance of the bioaugmentation process.

Landfilling

Landfilling is a deliberate dumping of oil sludge into land (pit) without formal treatment. It has been the most common form of sludge disposal. This process has limitations as it requires a large land area and volatile organic compounds are emitted if the oil sludge is not treated before disposal. Most times, the locations of landfill sites for oil sludge disposal have been selected according to availability of land and convenience rather than consideration of the hydro geological features of the sites. This calls for more strict legislative restrictions on landfilling (Bhattacharyya and Shekdar, 2003).

Landfarming

Landfarming involves the controlled application of the oil sludge on the land surface. This method requires tilling of the topsoil (for easy mixture with oil sludge), addition of water and addition of desired nutrient such as organic fertilizers and manures. Tilling in this process is important as it aids aeration, proper mixture of sludge and nutrient, thereby making the sludge bio-available for microbial degradation. Proper landfarming practice has minimal impact on the environment (good site appearance, absence of odour, relatively low-cost compliance with sound industrial practices and government regulation, minimal residue disposal problems and compatibility of the method with the climate, location and type of sludge treated).

Landfarming gained popularity over incineration and landfilling following its advantages such as low energy consumption, low risk of pollution of the surface and groundwater due to the immobility of hydrocarbons and metals through the soil (Hejazi et al., 2003; Besalatpour et al., 2011). Landfarming technique only lost its popularity when the USA Environmental Protection Agency (USEPA), issued the land disposal restriction conservation and recovery act (RCRA), establishing treatment standards under the land disposal restriction program (USEPA, 1997). The restriction prohibited the land disposal of untreated oil sludge. This led to treating the oil sludge to meet EPA treatment standards and making sure that there was no migration of hazardous constituents from the injection zone (Hejazi et al*.,* 2003). However, landfarming was an acceptable disposal method as long as it is within EPA guideline that aims to minimize the possibility of wash out and groundwater contamination. Simplicity and cost-effectiveness are some of the major advantages of the technology (Hejazi et al., 2003). It is simple in that, typical equipments which are used for landfarming is used widely in the farming community and is therefore readily available.

Although, landfilling is reported as the most cost effective oil sludge treatment method, landfarming gained popularity among refineries following restrictions on landfilling oil sludge (Mahmoud, 2004). The challenges of landfarming include the release of hydrocarbon compounds (VOCs) during the application and degradation of oil sludge (greenhouse structure can help minimise emission), and its requirement of a large land area for treatment (just as in landfilling). There is also risk

of residues such as the branched n-alkanes not degrading. There are also health related concerns as the sludge poses serious carcinogenic risks to workers during the early sludge application period (Hejazi et al., 2003).

Bioreactors

This is the use of a bioreactor process as a fermentation technology to degrade oil sludge into non-hazardous effluents with very low level of hydrocarbon (Daubaras and Chakrabarty, 1992; Oolman et al., 1996; Singh et al., 2001; Soriano and Pereira, 2002). These methods uses a naturally selected and acclimated indigenous bacterial culture supplemented with a carefully designed blend of nutrients such as nitrogen, phosphate, essential minerals and a surfactant for degradation. The design and process operating conditions of the technique promoted the growth of highly active microbial population, which rapidly converted the oil sludge components to carbon dioxide and water (Soriano and Pereira, 2002).

It was further reported that the bacteria involved are known oil-degrading bacteria such as *Pseudomonas, Acinetobacter, Rhodococcus and Alcaligenes* (Singh et al., 2001). In Singh et al. (2001) study, more than 90% of the total petroleum hydrocarbons contained in the oil sludge were degraded. After a successful treatment, 80% of the processed materials were disposed of and the reactor were reloaded with another batch of oil sludge using the remaining 20% left in the reactor to serve as inoculums for the next run (Singh et al., 2001; Soriano and Pereira, 2002). The analysis of the total petroleum hydrocarbons obtained from the treatment process indicated that oil sludge was treatable to non-hazardous levels (Daubaras and Chakrabarty, 1992; Oolman et al., 1996; Singh et al., 2001; Soriano and Pereira, 2002).

However, the effects of the oxygen supply on the biodegradation of PAHs was more important as increase in PAHs degradation was observed in one of the experiment by Soriano and Pereira (2002), from 1.7 to 10.2% per day with high oxygen availability and the result obtained after 21 days was very promising (Field, 1991; Salameh and Kabrick, 1992; Hahn and Loehr, 1992; Huesemann et al., 1993). The aqueous low total petroleum hydrocarbons (TPHs) can be sent to the wastewater system, solid residues can be disposed of in a landfarm, to a non-hazardous landfill, dewatered and reused in other industrial purposes (Singh et al., 2001). This technique can be used in the process recovery of recyclable oil, biodegradation of oil sludge and disposal of treated oil sludge. It also eliminates the need to spray high concentration oil sludge on large areas of land. Bioreactor processes has high rates and extent of degradation than landfarming process due the minimization of mass-transfer limitation, high organic matter biodegradation and progressive reduction in the total operational time (Soriano and Pereira, 2002). This technique controls the environmental and nutritional factors such as pH, temperature, moisture, bioavailability of nutrients and oxygen promotes microbial growth and activity for the rapid degradation of oil sludge. The limitations faced by this technique are that it is an *ex-situ* process therefore, substantial cost can be incurred during excavation and transportation of oil sludge. The reactor mixer consumes energy and availability of well trained personnel is required for this method. It involves the risk of pollutant exposure and the unravelling limiting factors during bioremediation (Piotrowski, 1991; Lees, 1996). Despite their potentials, the use of bioreactors is limited and most studies have focused on synthetic residues (Field, 1991; Salameh and Kabrick, 1992; Hahn and Loehr, 1992; Huesemann et al., 1993). Also, oxygen limitation affects the contaminants consumption rates (Soriano and Pereira, 2002).

Composting

Despite decades of research, successful biological remediation of oil sludge in the environment remains a challenge. It is noticed that, there are physical, chemical and biological aspects of landfilling, landfarming and bioreactor treatments that can hamper the degradation processes of oil sludge, making them partially effective and sometimes prohibitively expensive. It is necessary to search for cheaper and environmentally friendly options that can enhance bioremediation of oil sludge. Such options should be able to take care of the limitations of the previous methods while improving oil sludge bioremediation. Therefore, composting process which involves the careful control and addition of nutrients, watering, tilling, addition of suitable microbial flora and bulking agents (wood-chips or hay) were considered an alternative option to improve the bioremediation of oil sludge (De-qing et al., 2007). The process leads to the production of carbon dioxide, water, minerals and stabilized organic matter (Pereira-Neta, 1987).

Composting is a controlled biological process of a mixture of substrates carried out by successive microbial populations combining both mesophilic and thermophilic activities. It is applied to solid and semi-solid organic waste such as nightsoil, sludge, animal manures, agricultural residues and municipal refuse, whose solid content are usually higher than five percent. The process can be classified into mechanical and non-mechanical processes (aerobic and anaerobic composting system); using technology as the key (the classification is divided into static pile or windrow, and mechanical or enclosed composting). Compost systems can be on three general bases: oxygen usage, technological approach and temperature. Oxygen usage is divided into aerobic and anaerobic. Aerobic composting involves the activity of aerobic microbes, and hence the provision of oxygen

during the composting process. Aerobic composting generally is characterized by high temperatures, the absence of foul odours, and is more rapid than anaerobic composting. The addition of oxygen promotes bacterial and fungal growth within the compost pile. The organisms that grow in aerobic compost piles produce less methane and sulphur-based gases than in anaerobic composting, resulting in less odour. This method requires much higher maintenance, regular turning and mixing to incorporate air into the material than in anaerobic composting. Moisture loss is more likely in aerobic composting and frequent watering of the material is required. Anaerobic composting is characterized by low temperatures, the production of odorous intermediate products, and generally proceeds at a slower rate than aerobic composting. In anaerobic composting, the material stacks in layers to form an environment completely free of air within the layers. Bacteria, fungi and a higher form of bacteria, such as *actinomycetes*, that thrive in this environment begin to grow to breaking down the material. Anaerobic composting requires little maintenance, as there is no need to turn the material within the compost pile. The bacteria, however, produce more methane and sulphur-based gases as by-products, which can produce a strong odour. The odour indicates the composting process is progressing.

Composting could be divided with respect to the modes of operations such as batch operation and continuous or semi-continuous operation. When temperature is the basis, composting can be divided into mesophilic composting (25 - 40°C) and thermophilic composting (50 - 65°C). The main advantage of composting is waste stabilization. The biological reactions occurring during composting will convert organic wastes into stable, mainly inorganic forms. These stable inorganic forms may cause little pollution effects if discharged onto land or into a water course. The degradation of organic matter in aerobic composting system depends on the presence of oxygen. Oxygen serves two functions in the metabolic reaction; the terminal electron acceptor in aerobic respiration and as a substrate required for the operation of the class of enzymes called oxygenase (Finstein et al., 1980). Briefly, essential factors are those features of the physical, chemical, and biological background that are necessary to the establishment and proliferation of the microorganisms specific to the desired process. Five essential factors that have become key design features in recent compost technology are suitable microbial populations, aeration (oxygen availability), temperature, moisture content, and carbon availability.

Compost bioremediation relies on the mixing of primary ingredients of compost with the contaminants and oil sludge is compostable which is enhanced when bulking agents are added to the treatment process (Milne et al., 1998). As the compost matures, the pollutants are degraded by the active microflora within the mixture. It is called tailored compost (designed compost), in the sense that, it is specially made to treat specific contaminants at specific sites (US EPA, 1997). In most cases, temperature, pH and nutrients are the important factors. An increase in temperature in the compost pile increases solubility of contaminants and induces higher metabolic activity of the compost (Gibb et al., 2001). Oil sludge degrading bacteria and fungi performance are affected by pH level; while on the other hand, nutrients like nitrogen and phosphorus have great effect on microbial degradation of oil sludge constituents (van Hamme et al., 2003).

Jose et al. (2006) attempted to ascertain the efficacy of composting technology in the reduction of hydrocarbon contents of oil sludge with large total hydrocarbon content (250-300g kg^{-1}) in semiarid conditions. They designed three composting systems with open air piles, which were turned periodically over a period of 3 months. This system proved to be inexpensive and reliable. Jose et al. (2006) also studied the effect of bulking agent (wood shavings) addition on the oil sludge biodegradation and inoculation of the composting pile with pig slurry (a liquid organic fertilizer which adds nutrients and microbial biomass to the pile). The most effective treatment was composting pile with the bulking agent. Initially, hydrocarbon content was reduced by 60% in 3 months. It seems that the bulking agent encourages the diffusion of oxygen inside the pile. It also facilitates microbial developments and raising the temperature quicker. The temperature increase in the composting process may be due to the differing capacity of microorganisms to degrade the hydrocarbons. Since oil sludge contains highly degradable materials, these microorganisms accept the hydrocarbons as substrates, which enhance their activities, leading to the higher increase in temperature (Bengtsson et al., 1998; Jose et al., 2006). The reduction of petroleum hydrocarbon achieved in the compost bioremediation was 85-90% over a period of 11 months. The composting pile without a bulking agent was reduced by 32% in 3 months. The introduction of the organic fertilizer did not significantly improve the hydrocarbon degradation because it only degraded 56% of the hydrocarbon content.

Oxygen content is known to be a key factor in composting. In pile containing bulking agent, the oxygen content measured was always high after turning (10-14%). However, in piles without a bulking agent, oxygen content remained at 2-9% (Zhou and Crawford, 1995). This result demonstrates the effectiveness of a bulking agent for fostering microbial activity during the composting process (Zhou and Crawford, 1995). The humidity of the pile maintained at 40-60% encouraged microbial activities and the biodegradation of the hydrocarbons. Low moisture level and low oxygen content explain the low temperature reached in piles without bulking agent. As time progressed, the moisture level of the piles decline and water had to be added. This proved to be difficult because the material may not

readily absorb water due to the high hydrophobic nature of oil sludge (Zhou and Crawford, 1995). This is one of the challenges which always arise in bioremediation process involving co-composting with oil sludge.

The initial degradation of the hydrocarbons in oil sludge may possibly be catalysed by mono and dioxygenase enzymes (Britton, 1984; Singer and Finnerty, 1984). The enzymes gradually oxidise the hydrocarbons to alcohol and aldehydes in the presences of oxygen, producing acids that finally follow a metabolic pathway to produce carbon dioxide (CO_2) and water (H_2O) (Britton, 1984; Singer and Finnerty, 1984). This is synthesized by aerobic microorganisms, which is clearly the benefit from the addition of the bulking agent. Therefore, the addition of suitable bulking agent improves aeration and the performance of the composting process of the oil sludge. When the total hydrocarbons present in the composting undergo a great degree of degradation, the process results in detoxification of the mass and the loss of their toxic substances.

The limiting step of composting process is maintaining a suitable level of humidity in the pile. Furthermore, the challenges of composting bioremediation are the nature of the oil sludge, the composting conditions, microbial communities and time. Lack of sufficient readily decomposable organic matter may give inadequate substrate to stimulate microorganisms in the decomposition of untreated disposed oil sludge (Cole et al., 2003; Fountoulakis et al., 2009). In many cases, during composting process, organic material degrades along with target organic compounds and this has been found to interfere with degradation results, therefore, complicating understanding of the treatment efficiency (Kriipsalu et al., 2007). Also during composting, if the degradation rate of target compound is slower than the biodegradable organics added as amendments; then the relative concentration of the contaminants may be affected by the reduction of the dry mass content, transformed into CO_2 and H_2O (Kriipsalu et al., 2007). Similar phenomenon has been described for heavy metals remaining in composting piles meanwhile the organic degrade (Parẻ et al., 1999; Kriipsalu et al., 2007). All these affect the mechanism of conversion in compost. Composting bioremediation tends to treat oil sludge in a cost-effective and environmental friendly way by utilizing effectively its biological, physical and chemical process. Many factors are considered in the design of an optimal oil sludge treatment process. These factors include time, nutrients, pH, moisture and microbial biodegraders; they are also considered as attributes of composting processes.

Amidst limitations that may hamper the composting processes, co-composting techniques for bioremediation of oil sludge have its advantages. It is economically sound, natural process that destroys organic contaminants and the residues obtained are no more harmful. The process eliminates the transfer of residue from one from one environmental medium to another. The biological reactions occurring during composting will convert organic wastes into stable, mainly inorganic forms. These stable inorganic forms may cause little pollution effects if discharged onto land or into a water course. As already stated, composting could be *ex situ* or *in situ* process depending on whether the oil sludge is taken out from its source or not. It is often less expensive and disruption is minimal. It eliminates waste permanently, eliminates long term liability, and has greater public acceptance, with regulatory encouragement, it can also be coupled with other physical or chemical methods. As far as the effectiveness of the by-products is concerned, the treated sludge is found enriched in organic matter along with sufficient amount of nutrients (nitrogen, phosphorus and potassium). This technique does not only reduce the PAHs concentrations, but tends to improve soil quality through the addition of organic matters. Also, if compared to landfill or landfarming and destructive treatment methods, such as incineration, the use of composted material and co-composting as bioremediation technique may possibly promote soil sustainability and re-use.

It is reported in the literature that animal manure co-composted with oil sludge can enhance the degradation of oil sludge. Also composting and the use of compost for the bioremediation of oil sludge have been reported to be cost-effective and environmental friendly. Hence, this technique has some physical, chemical and biological limitations. Such limitations can be addressed by applying the current findings as the way forward on biodegradation and bioavailability of oil sludge constituents to the degrading bacteria.

It also addresses the partitioning of oil sludge between environmental media, genetic transfer of the biodegradation knowledge to indigenous microbial communities and impact of oil sludge constituents on soil microbial diversity. These findings and knowledge on biodegradation and bioavailability of oil sludge add on the advantages that have been reported about composting techniques. Stimulated biological process and co-metabolism of recalcitrant (heavy molecular weight PAHs) will be an added advantage of the composting technique. Therefore, it is important to implement the technique in such a way that takes care of all the limitations.

Amidst limitations that may hamper the composting processes, co-composting techniques for bioremediation of oil sludge have its advantages. The technique has not only reduced the PAHs concentrations, but tends to improve soil quality through the addition of organic matters. However, if compared to landfill or landfarming and destructive treatment methods, such as incineration, the use of composted material and co-composting as bioremediation technique may possibly promote soil sustainability and re-use. The reports described in this study, have shown that co-composting of oil sludge with compost materials can promote degradation of oil sludge.

As the addition of mature/ripe compost or any other nutrients constituents to the composting processes can also enhance the biodegradation of oil sludge and can reduce the toxicity of oil sludge. In all, there must be precautions to bear in mind. The precautions must be applied where there is a comparison between a laboratory, pilot and field studies. This is because the chemical behaviour of the oil sludge constituents present may be different in each of the studies. The study may give false results different from the laboratory to that of the field studies, where the conditions are not the same. Such results may possibly lead to expectations that may not be reached at the end of the process. However, composting bioremediation tends to treat oil sludge in a cost-effective and environmental friendly way, by utilizing the removal efficiencies of its biological, physical and chemical process. It is possibly through conversion of the oil sludge to CO_2 and H_2O. However, this aim may not be thoroughly achieved due to the limitations of the technique or the design applied. In many cases, an important fraction of the oil sludge and their metabolites remain untouched by the treatment process. The amount of oil sludge residue remaining constitutes a major concern and source of debate in relation to risk assessment. Therefore, it is recommended that as composting techniques 'rely' on the biological process to remove or reduce the hydrocarbon content of oil sludge. There is a need to first gather and put into considerations all the information about the subsequent limiting factors during bioremediation (biological, chemical and physical limitation associated with composting), while looking for a way forward in the biotreatability studies. The limiting factors should help in the choice to design the process to optimise the treatment of oil sludge even after the removal of easily degradable constituents such as 2, 3 and 4 ringed PAHs.

These limiting factors (time, nutrients, pH, moisture level, biodegraders, toxic metabolites), during composting processes should be investigated, considered, adequately addressed and managed to optimise the biodegradation of both low and high molecular weight PAHs. Optimised biodegradation can be achieved by first conducting the laboratory and pilot studies before applying the technology to the full scale. Such strategy will reveal the information about the type and metabolic activities of the indigenous bacteria, the presence of possible inhibitors, biodegradability of oil sludge under optimal conditions, effects of nutrients and bioavailability of oil sludge in the co-compost process. The pilot study may possibly help to decide whether biostimulation, bioaugmentation or the combination of both is the relevant method for addressing the limiting factors that may occur during composting bioremediation of oil sludge. Most importantly, there is need to really understand microbial processes and environmental conditions conducive for seeding biodegradation information to the indigenous microbial communities.

CONCLUSION

In conclusion, it is evident that researchers and most refineries have tried to treat oil sludge using conventional methods which includes physical treatment (storage, landfilling, combustion and incineration in a rotary kiln, lime stabilization, stabilization and solidification) chemical treatment (oxidative thermal treatment, treatment with fly-ash, pyrolysis treatment and solvent extraction) and biological treatment (landfarming, bio-reactor treatment and composting).

However, safe disposal and treatment of huge quantity of oil sludge generated during the processing of crude oil have been the major challenges faced by oil refineries and petrochemical industries. This is because these methods may require expensive equipments and high energy to treat the oil sludge. Although, some of these methods convert oil sludge into lighter products and reduce the quantity before disposal but some of the methods may generate by-products that may need to be treated using other methods before disposal to a landfill, making their cost significantly higher. Nevertheless, this paper has reviewed the current position in the composting of oil sludge, the extent of development in methodology, the successes and the challenges encountered.

The pathways of aerobic transformation have been reported and it is established that microorganisms capable of degrading oil sludge could be found in the contaminated environments. This have been of growing interest to the potential use of microbes to degrade oil sludge and more recent work has established that it is possible to use microbial-based processes to remediate contaminated environments. It is clearly evident from the review that substantial progress has been made in the development and application of biological techniques in the degradation of oil and oil sludge in the environment. However, application of these technologies to the degradation of relatively complex organic substrates has continued to be a challenge. In all, bioremediation of oil sludge is feasible given the depth of our current knowledge. Although the inherent limitations of bioremediation of oil sludge are known, further research is required to test these limitations and to exploit the potential of the *in-situ* microbial communities to metabolise the oil sludge.

The findings will also help to deeply understand the microbial ecology and their activities in the degradation of oil sludge. It was necessary for further improvement of compost bioremediation process.

ACKNOWLEDGEMENT

The authors wish to acknowledge the financial support given by the South African National Research Foundation for this project.

REFERENCES

Abed MMR, Safi NMD, Koster J, deBeer D, El-Nahhal Y, Rullkotter J, Garcia-Pichel F (2002). Microbial diversity of heavily polluted microbial mat and its community changes following degradation of petroleum compounds. Appl. Environ. Microbiol. 68(4):1674-1683.

Adriano DC, Bollag JM, Frankenberger Jr WT, Sims RC (1999). Bioremediation of contaminated soil. Agronomy, No.37, American Society of Agronomy.

Ahimou F, Jacques P, Deleu M (2000). Surfactin and Iturin A effects on *Bacillus subtilis* surface hydrophobicity. Enzyme Microb. Technol. 27:749-754.

Al-Daher R, Al-Awadhi N, El-Nawawy A (1998). Bioremediation of damaged desert environment with the windrow soil pile system in Kuwait. Environ.Int. 24:175.

Alexander M (1994). Biodegradation and bioremediation. Academic Press Inc. California.233.

Alexander M (1999). Biodegradation and Bioremediation. Academics Press, San Diego.

Angelidaki I, Mogensen AS, Ahring BK (2000). Degradation of organic contaminants found in organic waste. Biodegradation 11:377.

API (2008). The Relationship between the Aromatic Ring Class Content and Selected Endpoints of Repeat-dose and Developmental Toxicity of High-boiling Petroleum Substances. PAC Analysis Task Group. American Petroleum Institute. Washington, DC.

Atagana HI (2003). Bioremediation of creosote- contaminated soil by microbial intervention. A PhD thesis submitted to the Department of Microbiology, University of Natal, Pietermaritzburg, South Africa.

Atlas RM (1981). Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol. Rev. 45:180.

Atlas RM (1991). Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol. Rev. 45:180.

Atlas RM, Bartha R (1972). Degradation and mineralization of petroleum in sea water: limitation by nitrogen and phosphorus. Biotechnol. Bioeng. 14(3):308-318.

Atlas RM, Bartha R (1987). Microbial ecology :Fundamentals and Application. Benjamin/Cummings Publishing company. MenloPark, California.

Atlas RM, Philip J (2005). Bioremediation: Applied microbial solutions for real-world environmental cleanup. ASM Press, Wasington, D.C. 1-292.

Ausma S, Edwards GC, Fitzgerald-Hubble CR, Halfpenny-Mitchell L, Gillespie TJ, Mortimer WP (2002). Volatile hydrocarbon emissions from a diesel fuel contaminated soil bioremediation facility. J. Air Waste Manage. Assoc. 52:769-780.

Bach QD, Kim SJ, Choi SC, Oh YS (2005). Enhancing the intrinsic bioremediation of PAH-contaminated anoxic estuarine sediments with biostimulating agents. J. Microbiol. 43:319.

Bayoumi RA (2009). Bacterial bioremediation of polycyclic aromatic hydrocarbons in heavy oil contaminated soil. J. Appl. Sci. Res. 5 (2):197-211.

Beech JF, Elder S, Weeks N (2009). Case-study: Use of circulating fluidized bed boiler by- product to solidify oil sludge. World of coal ash (WOCA) conference may 4-7, in Lexington, KY, USA.

Bengtsson A, Quednau M, Haska G, Nilzen P, Persson A (1998). Composting of oil sludges-degradation, stabilized residues, volatiles and microbial activity. Waste Manage. Res. 3:273-284.

Besalatpour A, Hajabbasi MA, Khoshgoftarmanesh AH, Dorostkar V (2011). Landfarming process effects on biochemical properties of petroleum-contaminated soils. Soil and sediment contamination. 20:234-248.

Bhattacharyya JK, Shekdar AV (2003). Treatments and disposal of refinery sludges: Indian scenario. Waste Manage Res. 21:249-261.

Boettcher G, Nyer EK, Palmer PL, Carman EP, Bedessem J, Kidd DF, Lenzo F, Rorech GJ, Crossman TL (2001). In-Situ bioremediation. In-situ Treatment Technology, Second Edition CRC Press.

Bojes HK, Pope PG (2007) Characterization of EPA's 16 priority pollutant polycyclic aromatic hydrocarbons (PAHs) in tank bottom solids and associated contaminated soils at oil exploration and production sites in Texas. Regul Toxicol Pharmacol. 47(3):288-295

Bonnier PE, Akoun GL, Cardon EC, Edwards ED, Hocknel W, Holtari U, Leygue G, Long DV, Moorse JH, Peterse JL, De Roocker A, Levi JD (1980). Sludge farming: A technique for the disposal of oil refinery waste, the oil companies' European association for environment, health and safety in refining and distribution (CONCAWE) report no.3/80

Boyd SA, Shelton DR (1984). Anaerobic biodegaradation chlorophenols in fresh and Acclimated sludge. Appl. Environ. Microbiol. 47:272.

Britton LN (1984). Microbial degradation of aliphatic hydrocarbons. In: Gibson D T (eds) Microbial degradation of organic compounds. Marcel Dekker, New York. 89-129.

Calvo C, Toledo FL, Gonzalez-lopez J (2004). Surfactant activity of a naphthalene degrading Bacillus pumilus strain isolated from oil sludge. J. Biotechnol. 109, 255-262.

Carmen Baez-Smith PE (2006). Anaerobic digestion of Vinasse for the production of methane in the suger-cane distillery, SPRI conference on sugar processing, smith-baez consulting inc, Loxahatchee, florida, USA.

Cerniglia CE, Herbert RL, Dodge RH, Szaniszlo PJ, Gibson DT (1979). Some approaches to studies on the degradation of aromatic hydrocarbons by fungi, In: Bourquin AL and Pritchard H (eds), Microbial degradation of pollutants in marine Environments. EPA Report No. EPA-600/9-79-012.EPA, Washington, DC.

Cerniglia CE, Sutherland JB, Crow SA (1992). Fungal metabolism of aromatic hydrocarbon. In: Winkelmann G (ed) Microbial degradation of natural products VCH press, Weinhein, 193-217.

Cole JR, Chai B, Marsh TL (2003). The ribosomal database project (RDP - II): Previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 31:442 - 443.

Cort T, Bielefeldt A (2000). Effects of surfactants and temperature on PCP biodegradation. J. Environ. Eng. 126:635-643.

Cort T, Bielefeldt A (2000). Mechanism of non-ionic surfactant inhibition of pentachlorophenol biodegradation. In proceeding of the 2000 conference on hazardous waste research, Denver, Colorado, USA. May 23-25, 2000.

Cortez, L.A.B., L.E. Brossard Perez. Experiences on vinasse disposal. Part III:Combustion of vinasse #6 fuel oil emulsions. Mar. 1997. São Paulo, Brazil. Br. J. Chem Eng. 14:1

Crivelaro SHR, Mariano AP, Furlan LT, Gonçalves RA, Seabra PN, Angelis DF (2010). Evaluation of the use of vinasse as a biostimulation agent for the biodegradation of oily sludge in soil. Int. J. Brazil Biol Technol. 53(5):1217-1224.

Cybulski Z, Dziurla E, Kaczorek E, Olszanowski A (2003). The influence of emulsifiers on hydrocarbon biodegradation by *Pseudomondacea* and *Bacillacea strains*. Spill Sci. Technol. Bull. 8:503-507.

Das K, Mukherjee AK (2007). Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Biores. Technol. 98:1339-1345.

Daubaras D, Chakrabarty AM (1992). The environment, microbes and bioremediation: Microbial activities modulated by the environment. Biodeg. 3:125-135.

Dean-Ross D, Moody JD, Freeman JP, Doerge DR, Cerniglia CE (2001). Metabolism of anthracene by a Rhodococcus species. FEMS Microbiol Lett. 204:205-211.

Del'Arco JP, de Franca FP (2001). Influence of oil contamination levels on hydrocarbon biodegradation in sandy sediments. Environ. Pollut. 112:515-519.

De-qing S, Jain Z, Zhao-long G, Jian D, Tian-Li W, Murygina V, Kalyuzhnyi S (2007). Bioremediation of oil sludge in shengli oilfield. Water Air Soil Pollut. 185:177 - 184.

Diallo M, Cagin T, Faulon JL, Goddard WA (2000). Thermodynamic properties of asphaltene: A predictive approach based on computer assisted structure elucidated and atomistic simulations. In: Asphaltene and asphalts II (eds TF Yen and GV Chilingarian), developments in petroleum science 40B, Elsevier Amsterdam. 103-127.

Diehl SV, Borazjani H (1998). Enhanced biodegradtion of organic wood-preservative contaminated wastewater by commercial surfactants. In RA Meyers (ed), Encyclopedia of environmental analysis and remediation. 3436-3451.

Dzantor EK (1999). Bioremediation of contaminated soil- what it is and how to do it. University of Agriculture and Natural Resources. 1007-1019.

Field SD (1991). Advanced biological treatment and separation of hazardous constituents from petrolchemicals sludges. J. Hazard. Mat. 28:101-113.
Field JA, De Jong E, Costa GF, de Bont JAJ (1992). Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. 58(7):2219-2226.
Finstein MS, Morris MI, Storm PF (1980). Microbial ecosystem responsible for anaerobic digestion and composting. J. Water. Pollut. Control Fed. 52:2675-2685.
Fountoulakis MS, Terzakis S, Georgaki E, Drakopoulou S, Sabathianakis I, Kouzoulakis M, Manios T (2009). Oil refinery sludge and green waste simulated windrow Composting. Biodegradation 20:177-189.
Frick CM, Farrel RE, Germida JJ (1999). Assessment of phytoremediation as an *in-situ* technique for cleaning oil-contaminated sites. Petroleum Technology Alliance of Canada (PTA) Calgary, AB.
Gibb A, Chu A, Wong RCK, Goodman RH (2001). Bioremediation kinetics of crude oil at 5^{o}C. J. Environ. Eng. 127(9):818.
Gibson DT, Sayler GS (1992). Scientific foundation for bioremediation:Current status and future needs: American Academy of Microbiology, Washington DC. 1-24.
Gibson DT, Subramanian V (1984). Microbial degradation of aromatic hydrocarbons. In Gibson D T (ed), Microbial degradation of organic compounds. Marcel Dekker, inc., New York. 182-252.
Habe H, Chung JS, Kato H (2004). Characterization of the upper pathway genes for fluorene metabolism in *Terrabacter* sp.strain DBF63. J. Bacteriol 186:5938-5944.
Hahn WJ, Loehr RC (1992). Biological treatment of petroleum oily sludge in proceedings of the Permian Basin oil and gas recovery conf. Texas, SPE-Soc. Of Pet. Engineers.
Heitkamp MA, Franklin W, Cerniglia CE (1988a) Microbial metabolism of polycyclic aromatic hydrocarbons: Isolation and characterization of a pyrene-degrading bacterium. Appl. Environ. Microbiol. 54:2549-2565.
Heitkamp MA, Freeman JP, Miller DW, Cerniglia CE (1988b). Pyrene degradation by a mycobacterium sp:identification of ring oxidation and ring fission products . *Appl. Environ. Microbiol* 54:2556-2565.
Hejazi RF, Husain T, Khan FI (2003). Landfarming operation of oil sludge in arid region-human health risk assessment. J. Hazard. Materi. B99:287-302.
Huesemann MH, Moore KO, Johnson RN (1993). The fate of BDAT polunuclear aromatics compounds during biotreatment of refinery API oil separator sludge Environ. Proj. 12:30-38.
Idris A, Ahmed M (2003). Treatment of polluted soil using bioremediation - A review. Faculty of Chemical and Environmental Engineering, University of Putra, Malaysia. 1-18.
Iglesias-Groth S, Manchado A, Rebolo R, Gonzalez Hernandez JI, Garcia-Hernandez DA, Lambert DL (2010). A search for interstellar anthracene toward the Perseus anomalous microwave emission region. Bibcode:2010arXiv1005.4388I.
Johnson CR, Scow KM (1999). Effect of nitrogen and phosphorus addition on phenanthrene biodegradation in four soils. *Biodegradation.* 10:43 - 50.
Jose AM, Jose LM, Teresa H, Carlos G (2006). Bioremediation by composting of heavy oil refinery sludge in semiarid conditions. *Biodegrad.* 17:251-261.
Kanaly RA, Harayama S (2000). Biodegradation of high-molecular weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol*. 182, 2059-2067.
Karamalidis AK, Voudrias EA (2001). Stabilization/Solidification of oil refinery sludge: immobilization of heavy metals. 7th conference of environmental Science and Technology Ermonpolis, Syros Island, Greece.
Kasuga K, Habe H, Chung JS, Yoshida T, Nojiri H, Yamane H, Omori T (2001). Isolation and characterization of the genes encoding a novel oxygenase component of angular dioxygenase from the gram-positive dibenzofuran-degrader *Terrabacter* sp.strain DBF63. Biochem Biophy Res Co. 283:195-204.
Kim S, Choi DH, Sim DS, Oh Y (2004). Evaluation of bioremediation effectiveness on crude oil-contaminated sand. Chemosphere 59(6):845-852.
Kosaric N (2001). Biosurfactant for soil bioremediation. Food, Technol. Biotechnol. 39 (4) 295-304
Kriipsalu M, Marques M, Nammari DR, Hogland W (2007). Biotreatment of oily sludge:The contribution of amendment material to the contents of target contaminants and the biodegradation dynamics. J. Hazard. Mat. 148 (3):616 - 622.
Lajoie CA, Layton AC, Sayler GS (1994). Cometabolic oxidation of polychlorinated biphenyl in soil with a surfactant-based field application vector. Appl. Environ. Microbiol. 60 (8):2826- 2833.
Laskova T, Zabukas V, Vaitiekunas P (2007). Influence of meterological conditions on volatile organic compound spread in the atmospheric boundary layer. J. Environ Eng. Landscape Manag. 15 (3):135-143.
Lazar I, Dobrota S, Voicu A (1999). Microbial degradation of waste hydrocarbons in oily sludge from some Romanian oil fields. *J.Petroleum Sci Eng.* 22:151-160.
Le Marchand L, Hankin JH, Pierce LM, Sinha R, Nerurkar PV, Franke AA, Wilkens LR, Kolonel LN, Donlon T, Seifried A, Custer LJ, Lum-Jones A, Chang W (2002).Well-done red meat, metabolic phenotypes and colorectal cancer in Hawaii. 2002 Sep Mutation Res. 30:506-507:205-14.
Leahy JG, Colwell RR (1990). Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 3:305.
Lees ZM (1996). Bioremediation of oil-contaminated soil:A South African case study. A PhD thesis submitted to the University of Natal, Pietermaritzburg, South Africa.
Leung M (2004). Bioremediation:Techniques for Deaning up Emess. J. Bioteach. 2:18- 22.
Li CT, Lee WJ, Mi HH, Su CC (1995). PAH emission from the incineration of waste oil sludge and PE plastic mixtures. *Sci. Total Environ.* **170**, 171-183.
Liang Y, Gardner DR, Miller CD, Chen D, Anderson AJ, Weimer BC, Sims RC (2006). Study of biochemical pathways and enzymes involved in pyrene degradation by *Mycobacterium* sp. strain KMS. *Appl Environ Microbiol* 72:7821-7828.
Liu T, Hou J, Zuo Y, Bi S, Jing J (2011). Isolation and characterization of a biosurfactant producing bacterium from Daqing oil-contaminated site. Afr. J. Microb. Res. 5(21):3509-3514.
Liu W, Luo Y, Teng Y, Li Z, Ma L Q (2010). Bioremediation of oily sludge-contaminated soil by stimulating indigenous microbes. *Environ Geochem Health*. 32:23-29.
Loser C, Seidel H, Zehnsdarf A, Stoltmeister U (1998). Microbial degradation of hydrocarbons in soil during aerobic/anaerobic changes and under purely aerobic condition. Appl. Microbiol. Biotechnol. 49:631.
Mac Naughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ, White DC (1999). Microbial population changes during bioremediation of an experimental oil spill. Appl. Environ. Microbiol. 65:3566.
Machin-Ramirez, CAI, Okoh DK, Morales M, Mayolo-Deloisa R, Quintero, Trejo-Hernaindez (2008). Slurry-phase biodegradation of weathered oily sludge waste. Chemosph. 70:737-744.
Mahmoud S (2004). Novel technology for sustainable petroleum oil sludge management:Bio-neutralization by indigenous fungal-bacterial co-cultures. A Masters Degree submitted to the Department of Building, Civil and Environmental Engineering at Concordia University, Montreal, Quebec, Canada.
Maier RM (2003). Biosurfactant:Evolution and diversity in bacteria. Adv. Appl. Microbiol. 52:101-121.
Marin Millån JA (2004). Bioremediaciòn, mediante tècnicas biolôgicas, de hidrocarburos contenidos en lodos de refinerîa. Experinecias en clima semiårido. Doctoral thesis, Murcia University.
Mehrashi MR, Haghighi B, Shariat M, Naseri S, Naddafi K (2003). Biodegradation of petroleum hydrocarbon in soil. Iran. *J. public Health.* 32 (3):28-32.
Meyer S, Steinhart H (2001). Fate of PAHs and hetero-PAHs during biodegradation in a model soil/compost-system:Formation of extractable metabolites. Water Air Soil Poll. 132:215-231.
Micky V (2006). Microbial bioremediation of polycyclic aromatic hydrocarbons (PAHs) in oily sludge wastes. Pp:1-12.
Milne BJ, Baheri HR, Hill GA (1998). Composting of heavy oil refinery sludge. Environ. Progr. 17:24 - 27.
Mishra S, Jyoti J, Kuhad RC, Lai B (2001). *In situ* bioremediation potential

of an oil sludge-degrading bacterial consortium. Curr. Microbiol. 43:328-335.
Moody JD, Freeman JP, Doerge DR, Cerniglia CE (2001). Degradation of phenanthrene and anthracene by cell suspensions of Mycobacterium sp.strain PYR-1. Appl Environ Microbiol 67:1476-1483.
Mueller JG, Lantz SE, Blattmann BO, Chapman PJ (1991). Bench-scale evaluation of alternative biological treatment processes for the remediation of pentachlorophenol- and creosote- contaminated material: solid-phase bioremediation. *Environ. Sci. Technol.* 25:1045-1055.
Mukherjee AK, Das K (2005). Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis strains* in a particular habitat. FEMS Microbiol. Ecol. 54:479-489.
Namkoong WE, Hwang JP, Choi J (2002). Bioremediation of diesel-contaminated soil with composting. Environ. Pollution. 119:23.
Okieimen CO, Okieimen FE (2005). Bioremediation of crude oil-polluted soil - Effect of poultry droppings and natural rubber processing sludge application on biodegradation of petroleum hydrocarbon. Environ Sci. 12(1):001 - 008.
Oolman T, Baker RR, Renfro NL, Marshall GE (1996). Refinery uses bioslurry process to treat RCRA wastes. Hydroc. Process. 71 - 76.
Parė T, Dinel H, Schnitzer M (1999). Extractability of trace metals during co-composting of biosolids and municipal solid wastes. Biol. Fert. Soils. 29:31- 37.
Paulauskiene T, Zabukas V, Vaitiekunas P (2009). Investigation of volatile organic compound (VOC) emission in oil terminal storage Tank Park. J. Environ Engnr. Landscape Manage. 17 (2):81-89.
Pereira-Neta JT (1987). On the treatment of municipal refuse and savage sludge using aerated static pile composting- A low cost technology approach. PhD. Dissertation. Leeds University. U.K.
Phillips TM, Liu D, Seech AG, Lee H, Trevors JT (2000). Monitoring bioremediation in creosote-contaminated soils using chemical analysis and toxicology tests. J. Ind. Microbiol. Biotechnol. 65:627.
Piotrowski MR (1991). Bioremediation of hydrocarbon contaminated surface water, groundwater and soils:The microbial ecology approach. In Kostecki, PT, Calabrese, EJ (Eds). Hydrocarbon contaminated soils and groundwater:Analysis fate Environmental and public health effects remediation. *Lewis publisher, Chelsea, Michigan. 1.*
Piskonen R, Itävaara M (2004). Evaluation of chemical pretreatment of contaminated soil for improved PAH bioremediation. Appl. Microbiol. Biotechnol. 65:627.
Plaza GA, Pacwa-Plociniczak M, Piotrowska-Seget Z, Cameotra SS (2011). Environmental applications of biosurfactants:Recent advances. Int. J. Mol. Sci. 12:633-654.
Radetski CM, Mater L, Sperb RM, Madureira LAS, Rosin AP, Correa AXR (2006). Proposal of a sequential treatment methodology for the safe reuse of oil sludge-contaminated soil. J. Hazard. Mat. *B* 136:967-971.
Reid BJ, Jones KC, Semple KT, Fermor TR (2000). Bioremediation potentials of PAHs in compost. In Leeson A, Alleman BC (eds), Bioremediation Technologies for polycyclic aromatics hydrocarbon compounds. Battelle, Columbus. 25-30.
Research triangle institute (1999). Toxicological profile for total petroleum hydrocarbons (TPH) prepared for US Department of health and human services.
Ri-He P, Ai-Sheng X, Yong X, Xiao-Yu F, Feng G, Wei Z, Yong-Sheng T, Quan-Hong Y (2008). Microbial biodegradation of polyaromatics hydrocarbons. Shanghai key laboratory of agricultural genetics and breeding, agro-biotechnology research institute, shanghai academy of agricultural sciences, shanghai china. Review. FEMS Microbiol Rev. 32:927-955.
Ron EZ, Rosenberg E (2001). Natural roles of biosurfactants. Environ. Microbiol. 3:229-236.
Salameh MF, Kabrick RM (1992). Treatment of petroleum oily sludge in liquid/solid contact reactors-results of batch testing. In:Proceedings University. of the 46th Ind. Waste Conf., West Lafayette, Indiana, Purdue.
Shie J, Lin J, Chang C, Wu C, Lee D. Chang C, Chen Y(2004). Oxidative thermal treatment of oil sludge at low heating rates. Energy Fuel 18:1272-1281.
Shiohara K, Diehl SV, Borazjani H (2001). Use of commercial surfactants for enhanced biodegradation of organic wood-preservative contaminated processwater. In proceedings of the 2001 Mississipi Water Resources Conference. Raymond, Mississipi, USA.
Sidney D (2008). Agency for toxic substances and diseases registry and national centre for environmental health.
Sim L, Ward OP (1997). Production characterization of a biosurfactant isolated from Pseudomonas aeruginosa UW-1. J. Ind. Microbiol. Biotechnol. 19:232.
Singer ME, Finnerty WR (1984). Microbial metabolism of straight-chain and branched alkanes. In: Atlas RM (Eds) Petroleum microbiology. Macmillan New York. 1-55
Singh C, Lin J (2010). Bioaugmentation efficiency of diesel degradation by *Bacillus pumilus* JL3 and Acinetobacter calcoaceticus LT1 in contaminated soil. Afr. J. Biotech. 9 (41):6881-6888.
Singh A, Mullin B, Ward O (2001). Reactor-based process for the biological treatment of petroleum wastes. Petrotech, petrozyme technologies inc.
Soriano AU, Pereira N Jr (2002). Oily sludge biotreatment. Biotech and Ecosys. Dept. Petro. Res. Centre, 9th Annul Int'l Petrol Brazil.
Srinivasarao-Naik B, Mishra IM, Bhattacharya SD (2011). Biodegradation of total petroleum hydrocarbons from oily sludge. *Bioremed. J.* 15 (3):140-147.
Suflita JM, Horowitz A, Shelton DR, Tiedje JM (1982). Dehalogenation:A novel pathway for the anaerobic biodegradation of haloaromatic compounds. Sci. 218:1115.
Sutherland JB (1992). Detoxification of polycyclic aromatic hydrocarbons by fungi. J. Ind. Microbiol. 9:53-62.
Sutherland JB, Rafti F, Khan AA, Cerniglia CE (1995). Mechanisms of polycyclic aromatic hydrocarbon degradation. In Microbial transformation and degradation of toxic organic chemicals (Eds) Young, LY, Cerniglia CE. Wiley Liss, New York. 269-306.
Swindell CM, Aelion CM, Pfaender FK (1988). Influence of minerals and organic nutrients anaerobic biodegradation and the adaptation response of surface microbial communities. Appl. Environ. Microbiol. 54:212-217.
Tabuchi K, Matu-ura K, Kawakani S, Shiratori T, Saitoh T (1998). Remediation and recycling of the soil contaminated with petroleum hydrocarbon. Metall. Rev. MMIJ. 1:14 - 25.
Taiwo EA, Otolorin JA (2009). Oil recovery from petroleum sludge by solvent extraction, petrol. Sci. and Technol. 27:8, 836-844.
Tate SM, Winegardner DL (1995). Soil Microbiology. John Wiley and Sons, Inc., New York. 398.
TERA (2008). "Peer Consultation on Relationship between PAC Profile and Toxicity of Petroleum Substances" (API Report), http://www.tera.org/peer/API/APIWelcome.htm, accessed 28 Oct 2009 and "Report of the Peer Consultation on Relationship between PAC Profile and Toxicity of Petroleum Substances Volume I" (TERA peer review) http://www.tera.org/peer/API/PAC MEETING REPORT Final.pdf, accessed 28 Oct 2009.
Udotong IR, Udotong IJ, Inam E, Kim K (2011). Bioconversion of crude oil production into soil conditioner Using sawdust as Organic amendment. *Geosyst. Engr.* 14 (2):51-58.US EPA method 3541.
USEPA (1997). EPA's contaminated sediment management strategy. Office of water and solid waste. USEPA, Washington D C.
van Hamme JD, Singh A, Ward OP (2003). Recent Advances in petroleum microbiology. Microbiol. Mol. Rev. 67:649.
Van Veen JA, Van Overbeek LS, Van Elsas JD (1997). Fate and activity of Microorganisms introduced into soil. Microbial. Mol. Biol. Rev. 61:121-135.
Vila J, Lopez Z, Sabate J, Minguillon C, Solanas AM, Grifoll M (2001). Identification of novel metabolites in the degradation of pyrene by *Mycobacterium* sp. strain AP1:actions of the isolate on two- and three- ring polycyclic aromatic hydrocarbons. Appl Environ Microbiol. 67:5497-5505.
Vinas M, Sabate J, Espuny MJ, Solanas AM (2005). Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. Appl. Environ. Microbiol. 71:7008.
Walter U, Beyer M, Klein J, Rehm HJ (1991). Degradation of pyrene by Rhodococcus sp. UW1. Appl. Microbiol. Biotechnol. 34:671-676.

Wang J, Yin J, Ge L, Shao J, Zheng J (2010). Characterization of oil sludge from two oil fields in China. Energy Fuel. 24:973-978.
Ward W, Singh A, van Hamme J (2003). Accelerated biodegradation of petroleum hydrocarbon waste. J. Ind. Microbiol, Biotechnol. 30:260.
Watanabe K (2002). Linking genetics, physiology and ecology:an interdisciplinary approach for advancing bioremediation. J. Biosci. Bioeng. 94 (6):557-562.
Wattiau P, Bastiaens L, van Herwijnen R, Daal L, Parsons JR, Renard ME, Springael D, Cornelis GR (2001). Fluorene degradation by *Sphingomonas* sp. LB126 proceeds through protocatechuic acids:a genetic analysis. Res Microbiol. 152:861-872.
Wright RA, Noordhius (1991). The treatment and disposal of oil solids, proceedings of the first international conference on health, safety and environment. In oil and gas exploration and production part. 2:521-526.
Wong JWC, Fang M, Zhao Z, Xing B (2004). Effects of surfactants on solubilisation and degradation of phenenthrene under thermophilic conditions. J. Environ. Qual. 33:2015-2025.
Zhou E, Crawford RL (1995). Effect of oxygen, nitrogen and temperature on gasoline biodegradation in soil. Biodegrad. 6:127-140.

academicJournals

Vol. 12(47), pp. 6605-6610, 20 November, 2013
DOI: 10.5897/AJB2013.13069
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Estimation of heterosis for yield and quality components in chilli (*Capsicum annuum* L.)

Alok Chaudhary[1], Rajesh Kumar[2] and SS Solankey[3*]

[1]Department of Horticulture, Udai Pratap Autonmous College, Varanasi, U.P, India.
[2]Division of Crop Improvement, Indian Institute of Vegetable Research, Varanasi, U.P. – 221 305, India.
[3]Department of Horticulture (Vegetable and Floriculture), Bihar Agricultural University, Sabour (Bhagalpur), Bihar – 813 210, India.

Accepted 7 November, 2013

Ten genotypes including five lines (Kashi Anmol, Pant C-1, Japani Longi, Kashi Sinduri and Pusa Jwala) and five testers (R-Line, VR-339, AKC-89/38, DC-16 and Punjab Lal) of chilli were crossed to derive 25 F_1 hybrids. The 35 genotypes (10 parents and 25 F_1 hybrids) were evaluated for yield and quality (capsaicin and oleoresin) traits. Highly significant correlation was observed between fruit yield per plant and average fruit weight (g). The direct and indirect effect on yield revealed that the maximum direct effect was exhibited by average fruit weight followed by number of fruits per plant via yield per plant. The crosses Pusa Jwala × VR-339, Pusa Jwala × DC-16 and Pant C-1 × VR-339 exhibited higher level of heterobeltiosis for most of the traits. The higher specific combing ability (SCA) for yield was obtained in crosses Kashi Sinduri × Punjab Lal followed by Pant C-1 × VR-339 and Pusa Jwala × VR-339. Among the hybrids, Pusa Jwala × VR-339 had higher yield as well as capsaicin content, moreover, Kashi Sinduri × AKC-89/38 exhibited highest oleoresin content. These best hybrids (Pusa Jwala × VR-339, Kashi Sinduri × Punjab Lal and Pant C-1 × VR-339) might be utilized for further chilli improvement programme.

Key words: Chilli, heterosis, correlation, combining ability, capsaicin, oleoresin.

INTRODUCTION

The genus *Capsicum* consists of a diverse range of plants and fruits, and varies enormously with respect to morphology, yield and nutrition related parameters. Chillies are grown as annual crop, although it can also be grown as perennial shrub in suitable climatic conditions. Among the five cultivated species, *Capsicum annuum* L. is most widely cultivated for its pungent (hot pepper) and non pungent (sweet pepper) fruits throughout the world (Bosland and Votava, 2000). Chilli comprises wide spectrum of chemicals including steam-volatile oil, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fiber and mineral elements (Bosland and Votava, 2000). The presence of capsaicinoids is specific to the genus *Caps*icum, which varies widely among varieties, seasons, places of origin, etc (Prasath et al., 2007). The chilli fruits are consumed at different ripening stage (green, red or partial red-ripe). India ranks first in dry chilli production in the world whereas, for the green chillies, the production is low (Anonymous, 2012). Heterosis breeding has been advantageous for increased chilli production. For effective transfer of desirable genes controlling both quantitative and qualitative traits in the resultant progenies,

*Corresponding author. E-mail: shashank.hort@gmail.com. Tel: +91-9934387357.

Abbreviations: GCA, General combing ability; **SCA,** specific combing ability; **PCV,** phenotypic coefficient of variation; **BP,** mean of better parent; **MP,** mean of mid parent value; **ECV,** environmental coefficients of variation; **h^2b,** heritability in broad sense.

Table 1. Genotypes of chilli employed in the present investigation.

Genotype	Specific traits	Source
Kashi Anmol	High yielding spreading	IIVR, Varanasi, Uttar Pradesh
Pant C-1	Pungent	GBPA&T, Pantnagar, Uttarakhand
Japani Longi	Erect bunch fruited	IIVR, Varanasi, Uttar Pradesh
Kashi Sinduri	Long paprika	IIVR, Varanasi, Uttar Pradesh
Pusa Jwala	Parrot green pungent	IARI, New Delhi
R-LINE	Restorer dark green pungent	AVRDC, Taiwan
VR-339	High yielding long dark fruited	IIVR, Varanasi, Uttar Pradesh
AKC89/38	Round fruited pungent dark colour	PDKV, Akola, Maharashtra
DC-16	Good general combiner	IIVR, Varanasi, Uttar Pradesh
Punjab Lal	High capsaicin and oleoresin content	PAU, Ludhiana, Punjab

it is necessary to exploit the better combining breeding materials (Kearsey and Farquhar, 1998). Since yield is a complex trait, governed by a large number of component traits, it is imperative to know the interrelationship between yield and its component traits to arrive at an optimal selection index for improvement of yield. Several hybrids have been developed in hot chillies; however, the hybrid development programme should be continuous so as to make the seeds available to the growers at affordable cost. This investigation was planned to identify good combiners and heterotic cross combinations for yield as well as quality and its component traits in chilli.

MATERIALS AND METHODS

This investigation was carried out to identify good combiners and heterotic cross combinations for yield and quality traits in chilli. Ten genotypes including five lines (Kashi Anmol, Pant C-1, Japani Longi, Kashi Sinduri and Pusa Jwala) and five testers (R-Line, VR-339, AKC-89/38, DC-16 and Punjab Lal) (Table 1) were crossed to obtain 25 F_1 hybrids. These 35 genotypes (10 parents and 25 resulting F_1 hybrids) were evaluated in a randomized complete block design (RCBD) with three replications at Indian Institute of Vegetable Research, Varanasi during 2007 - 2008. The maximum temperature during that period ranged from 18.9 to 27.2°C and maximum relative humidity ranged from 63 to 92% while the minimum relative humidity ranged from 30 to 83%. The soil type of experimental field was alluvial with average fertility level pH range of 6.6 - 7.4. Recommended package and practices were applied to raise good crops (Bose and Som, 1986).

The observations were recorded on nine horticultural traits, viz. number of fruits per plant, fruit length (cm), fruit diameter (cm), average fruit weight (mean of 10 fruits weight) (g), plant height (cm), yield per plant (g), capsaicin content (%) and oleoresin content (%).

Capsaicin content in chilli powder was estimated by the method of Thimmaiah (1999) and oleoresin content was estimated by the procedure as suggested by Mathew et al. (1971) as per the following formula:

$$\text{Oleoresin content (\%)} = \frac{\text{Dried weight of the residue}}{\text{Fresh weight of the sample}} \times 100$$

The heterosis of F_1s over the better parent (BPH) and mid parent (MPH) was calculated by using the formula:

$$\text{Percent heterosis over better parent (BPH)} = \frac{F_1 - BP}{BP} \times 100$$

$$\text{Percent heterosis over mid parent (MPH)} = \frac{F_1 - MP}{MP} \times 100$$

Where, F_1 = Mean value of F_1s, BP = mean of better parent and MP = mean of mid parent value.

Analysis of combining ability was carried out following Kempthorne (1957). The genetic parameters such as phenotypic, genotypic and environmental coefficients of variation (GCV, PCV and ECV), heritability in broad sense (h^2b), genetic advance and correlation coefficients for each character were estimated as suggested by Tsegaye et al. (2007).

RESULTS AND DISCUSSION

The analysis of variance (ANOVA) indicated significant differences between parents for all seven traits, namely, number of fruits per plant, fruit length, fruit width, average fruit weight, fruit yield per plant, plant height and yield per plant (Table 2). The mean sum of squares of parental genotypes for all the characters was highly significant, which indicates that there was substantial variation in characters among genotypes.

The ANOVA for general and specific combining ability for all seven traits were found significant (Table 2). The ratio of general combing ability (GCA) and specific combing ability (SCA) variance revealed preponderance of non-additive genetic variances for all traits except yield per plant (g) that is governed by additive gene action.

The magnitude of phenotypic coefficient of variation (PCV) values for all the traits were higher than the corresponding (GCV) values (Table 3) indicating that these characters may be under influence of the environmental effect to some extent. The highest difference between GCV and PCV was observed for the fruit width (GCV = 12.18, PCV

Table 2. ANOVA for line × tester analysis and its combining ability for seven quantitative traits in chilli.

Source of variance	D.F.	Fruits per plant	Fruit length (cm)	Fruit width (mm)	10 Fruit weight (g)	Fruit weight per plant (g)	Plant height (cm)	Yield per plant (g)
Replication	2	27.84	0.0694	0.0244	4.193	261.75	3.12	44.91
parents	9	1589.62**	11.936**	0.1611**	667.50**	9502.79**	274.54**	10755.50**
Females	4	2023.83**	10.81**	0.0725**	1046.38**	944.82	205.98**	10053.17**
Males	4	1313.95**	7.91**	0.02041**	41.43**	6809.10**	294.41**	12651.05**
Female × Male	1	955.41**	32.48**	0.3434**	164.26**	20509.44**	469.33**	5982.60**
Hybrids	24	915.79**	8.37**	0.07611**	249.83**	9462.16**	103.24**	12990.90**
Parents *vs.* hybrids	1	7630.10**	12.46**	0.1239**	107.28**	79321.41**	236.19**	103327.50**
Error (L × T)	68	71.79	0.1223	0.0123	4.88	1073.06	8.032	682.64
Line (Female)	4	1666.25**	5.72**	0.0384	835.84**	8348.37**	109.46**	17187.39**
Tester (Male)	4	1482.25**	28.49**	0.1982**	156.39**	11604.94**	349.03**	11886.38**
Line × Tester	16	586.56**	4.005**	0.550**	126.69**	9204.92**	40.24**	12217.92**
GCA variance (σ^2g)	-	65.85	0.87	-0.03	24.63	51.45	12.60	154.60
SCA variance (σ^2s)	-	-298.56	-8.16	0.12	-9.90	-800.01	-102.93	110.51
σ^2g/σ^2s	-	-0.22	-0.11	-0.25	-2.49	-0.06	-0.12	1.40
Error	48	66.96	0.083	0.01595	4.785	587.82	6.36	713.63

*, ** Significant at 5 and 1% level, respectively.

Table 3. Mean, range, genotypic and phenotypic coefficient of variation heritability and genetic advance in hybrid of chilli.

Character	Grand mean	Range	Coefficient of variation		Heritability (h^2b)	G.A in percent of mean
			G.C.V	P.C.V		
Fruits per plant	66.47	43.73 - 111.20	25.30	28.41	0.81	46.87
Fruit length (cm)	5.70	2.31 - 9.56	29.15	29.59	0.97	59.12
Fruit width (mm)	1.16	0.86 - 1.40	12.18	16.33	0.56	18.96
Average fruit weight (g)	26.71	87.67 - 322.67	33.84	34.81	0.95	67.76
Plant height (cm)	50.84	40.20 - 61.40	11.18	12.33	0.84	21.04
Yield per plant (g)	174.95	65.40 - 308.22	36.56	39.62	0.85	69.51

= 16.33), which means the environment has maximum influence on fruit width (Table 3).

The high genetic advance coupled with high heritability of average fruit weight (GA = 67.76%, h^2b = 95%), yield per plant (GA = 69.51%, h^2b = 0.85%), fruit weight per plant (GA = 60.13%, h^2b = 0.83%) and fruit length (GA = 59.12%, h^2b = 97%) suggested appreciable level of improvement for these characters subjected to selection. High estimates of heritability for above characters suggested that selection based on phenotypic performance would be effective as propounded by Johnson et al. (1955). Low heritability is noted for an important character, that is, number of fruits per plant (h^2b = 31.16%).

Therefore, it is obvious that selection for number of fruits per plant alone may not be effective in the early generation when the individual plants are selected on the basis of phenotypic performance. High heritability coupled with high genetic advance has been reported for yield and fruit weight per plant in chillies (Munshi and Behra, 2000; Sreelathakumary and Rajamony, 2004; Singh and Yadav, 2008).

Highly significant genotypic correlation was observed for average fruit weight (0.752), with significant correlation with fruits per plant (0.496) (Table 6). It suggests that increase in these characters would result in increase in yield. Average fruit weight was also significantly correlated with fruit length (0.472) and negatively correlated with fruits per plant (-0.183). Negative genotypic correlation was found between fruit length and number of fruits per plant (-0.118), fruit width and fruits per plant (-0.150), fruit and fruit length (-0.367); and average fruit weight and fruits per plant (-0.183), which indicate that increase in fruits per plant would result in decrease in fruit weight. The direct and indirect effect (Table 5) on yield revealed that the maximum direct effect was exhibited by average fruit weight (P = 732, G = 0.704) followed by fruits per plant (0.589) via yield per plant. Similar results have been reported in chillies by Palsudesai et al. (2006), Hosamani and Shivkumar (2008) and Ganeshreddy et al. (2008), where they observed significant correlation of various

Table 4. Best three heterotic F_1s and GCA and SCA effects for six characters in chilli.

Character	Hybrids (F_1)	BP (%)	MP (%)	Parent	GCA	Hybrids (F_1)	SCA
Fruits per plant	Pusa Jwala × DC-16	161.55	163.71	DC-16	16.73	Pant C-1 × VR-339	24.19
	Pusa Jwala × VR-339	127.63	160.19	PantC-1	13.87	Pant C-1 × DC-16	14.12
	Japanese Longi × DC-16	96.43	110.57	Pusa Jwala	5.71	Kashi Sinduri × R Line	15.14
Fruit length (cm)	Pant C-1 × Punjab Lal	52.38	71.63	R Line	2.15	Japanese Longi × AKC-89/38	2.24
	Pant C-1 × DC-16	51.68	59.42	Kashi Sinduri	0.80	Pant C-1 × Punjab Lal	1.34
	Pant C-1 × R Line	37.99	74.91	Pusa Jwala	0.52	PusaJwala × VR-339	1.04
Fruit width (mm)	Japanese Longi × DC-16	20.69	34.62	AKC-89/38	0.14	JapaneseLongi × DC-16	0.26
	Japanese Long I × Punjab Lal	12.68	21.48	Kashi Sinduri	0.08	Japanese Longi × VR-339	0.14
	Kashi Sinduri × R Line	7.57	9.04	VR-339	0.08	KA-2 × AKC-89/38	0.12
Average fruit weight (g)	Pant C-1 × Punjab Lal	123.33	123.33	Kashi Sinduri	12.95	Kashi Sinduri × PunjabLal	11.60
	Pant C-1 × DC16	90.00	93.22	VR-339	2.92	KA-2 × AKC-89/38	8.10
	Pusa Jwala × R Line	22.34	49.35	R Line	4.00	Pusa Jwala × VR-339	7.68
Plant height (cm)	Kashi Sinduri × Punjab Lal	17.15	20.63	R Line	4.96	PusaJwala × R Line	5.97
	Pusa Jwala × R Line	12.73	18.88	PantC-1	3.37	KA-2 × DC-16	3.72
	KA-2 × Punjab Lal	15.68	23.68	VR-339	2.69	JapaneseLongi × VR-339	3.16
Yield per plant (g)	Pusa Jwala × VR-339	220.53	264.47	Kashi Sinduri	37.11	Kashi Sinduri × PunjabLal	99.77
	Pusa Jwala × DC-16	205.53	312.85	VR-339	27.06	Pant C-1 × VR-339	92.59
	Pant C-1 × VR339	239.00	247.77	RLine	21.26	Pusa Jwala × VR-339	70.80

yield attributing traits with fruit yield.

Considering yield and number of fruits per plant, out of 25 F_1's, 3 cross combinations *viz.,* Pusa Jwala × DC-16 (161.55%) exhibited highest heterobeltiosis followed by Pusa Jwala × VR-339 (127.63%) and Japani Longi × DC-16 (96.43%) (Table 4). The corresponding values for these crosses for mid parent heterosis was 163.71, 160.19 and 110.57%, respectively. Regarding fruit length, Pant C-1 × Punjab Lal, Pant C-1 × DC-16 and Pant C-1 × R line showed 52.38, 51.68 and 37.99% heterosis over better parent, respectively. For these crosses, heterosis over mid parent for fruit length was observed to be 71.63, 59.42 and 74.91, respectively. For yield per plant (g) the crosses Pusa Jwala × VR-339 (220.53%), Pusa Jwala × DC-16 (205.53%) and Pant C-1 × VR-339 (239%) may be exploited for hybrid vigour in chilli (Tembhurne and Rao, 2012; Sharma et al., 2013). The high heterotic response as observed in most of the crosses further supported the predominant role of non-additive component in the inheritance of the character studied.

In the present study, none of the lines or testers exhibited significant GCA effects for all the characters (Table 6). The lines identified as promising combiner for different characters were Pant C-1 for number of fruits per plant and plant height, Pusa Jwala for number of fruits per plant and fruit length and Kashi Sinduri for fruit length, fruit width, and average fruit weight per plant and yield per plant. Among the testers, VR-339 was found to be good combiner for fruit width, average fruit weight per plant, plant height and yield per plant, R-Line for fruit length, average fruit weight, plant height and yield per plant, DC-16 for number of fruits per plant, and AKC-89/38 for fruit width. In terms of better general combiner, Pant C-1, DC-16 were found to be good general combiners for number of fruits per plant while Kashi Sinduri, VR-339 and R-Line were found to be good general combiners for yield per plant. It is therefore suggested that the above mentioned genotypes may be exploited in hybrid breeding program for increasing fruit weight and number of fruits in chilli. The crosses exhibiting best SCA effects for fruits per plant were Pant C-1 × VR-339, Pant C-1 × DC-16 and Kashi Sinduri × R-Line; for fruit weight per plant, Pusa Jwala × VR-339, Kashi Sinduri × R-Line and Pant C-1 × VR-339 (Table 4).

In general, the crosses which were heterotic as well as good specific combiner for fruits per plant, fruit weight per plant (g) and yield per plant were Pant C-1 × VR-339, Pant C-1 × DC-16, Pusa Jwala × VR-339, Kashi Sinduri × R-Line and Kashi Sinduri × Punjab Lal. Pant C-1 × VR-339, Pant C-1 × DC-16 and Kashi Sinduri × R-Line involving both the high general combiners for fruits per plant, fruit yield perplant and could; therefore, be due to additive and additive × additive type of gene interaction which are fixable in nature.

High general combining ability of the parents therefore seems to be reliable criterion for the prediction of specific combining ability. For number of fruits per plant, KA-2 × DC-16 and Kashi Sinduri × Punjab Lal involved low × high general combiner, while KA-2 × AKC-89/38 and Kashi Sinduri × R-Line involved low × low general combiners. Heterosis in the cross involving low × high general combiner might be due to dominant × additive type of interaction which is partially fixable and the crosses involving both the poor combining parents and showing high SCA

Table 5. Phenotypic and genotypic correlation coefficient for six characters in chilli.

Character		Fruit length (cm)	Fruit width (mm)	Average fruit weight (g)	Plant height (g)	Yield per plant (g)
Fruits/ plant	P	-0.091	-0.065	-0.152	0.222	0.535**
	G	-0.118	-0.150	-0.183	0.155	0.496*
Fruit length (cm)	P		-0.259	0.459*	0.289	0.332
	G		-0.367	0.472**	0.323	0.351
Fruit width (mm)	P			0.243	0.072	0.180
	G			0.325	0.094	0.211
Average fruit weight (g)	P				0.219	0.730**
	G				0.233	0.752**
Plant height (cm)	P					0.304
	G					0.278

P, Phenotypic; G, genotypic.

Table 6. Phenotypic and genotypic path analysis of six characters in chilli.

Character		Fruit length (cm)	Fruit width (mm)	Average fruit weight (g)	Plant height (g)	Yield per plant (g)	Fruit length (cm)
Fruits/ plant	P	0.589	0.001	-0.001	-0.112	-0.005	0.535
	G	0.496	0.010	0.008	-0.129	0.003	0.496
Fruit length (cm)	P	-0.053	-0.007	-0.003	0.336	-0.006	0.332
	G	-0.058	-0.087	0.019	0.332	0.006	0.351
Fruit width (mm)	P	-0.038	0.002	0.011	0.178	-0.001	0.180
	G	-0.074	0.032	-0.051	0.229	0.002	0.211
Average fruit weight (g)	P	-0.090	-0.003	0.003	0.732	-0.005	0.730
	G	-0.091	-0.041	-0.017	0.704	0.004	0.752
Plant height (cm)	P	0.131	-0.002	0.001	0.160	-0.021	0.304
	G	0.007	-0.028	-0.005	0.164	0.019	0.278

P, Phenotypic; G, genotypic.

Table 7. Fruit quality traits of parental lines of chilli.

Parent	Fruit colour	Oleoresin (%)	Capsaicin (%)
Kashi Anmol (KA-2)	Green	8.8	0.9
Pant C-1	Light Green	9.73	1.07
Japani Longi	Green	8.2	0.93
Kashi Sinduri	Green	12.13	0.3
Pusa Jwala	Yellow	9.4	0.88
R-Line	Dark Green	8.13	0.71
VR-339	Light Green	11.7	1.21
AKC 89/38	Green	9.86	1.25
DC-16	Dark Purple	12.06	0.93
Punjab Lal	Light Green	10.1	1.11

may be due to intra and inter-allelic interaction. Overall, the crosses Kashi Sinduri × Punjab Lal, Pant C-1 × VR-339 and Pusa Jwala × VR-339 showing high SCA for yield also exhibited high or average SCA effects for yield component traits. These crosses exhibited significant SCA effects indicating the presence of non-additive gene

Table 8. Fruit quality traits of promising chilli hybrids (F_1's).

Hybrid	Fruit colour	Oleoresin (%)	Capsaicin (%)
KA-2 × R Line	Green	12.26	0.71
KA-2 × VR339	Green	10.53	1.15
KA-2 × AKC-89/38	Green	8.93	1.06
KA-2 × Punjab Lal	Green	10.53	0.95
Pant C1 × R line	Green	10.86	1.09
PantC1 × VR 339	Light Green	8.73	1.19
Pant C1 × AKC-89/38	Green	9.60	1.13
Pant C1 × DC 16	Dark Purple	9.26	0.97
Japani Longi × VR-339	Green	10.26	0.76
Japani Longi × AKC-89/38	Green	11.06	1.15
Japani Longi × DC 16	Dark Purple	11.20	0.97
Kashi Sinduri × R line	Green	12.33	0.65
Kashi Sinduri × VR-339	Green	10.46	0.64
Kashi Sinduri × AKC-89/38	Green	11.80	0.69
Kashi Sinduri × DC 16	Dark Purple	10.46	0.70
Kashi Sinduri × Punjab Lal	Green	9.46	1.02
Pusa Jwala × VR 339	Light Green	9.86	1.72
Pusa Jwala × Punjab Lal	Light Green	10.05	1.36

action. Similar results were reported by Prasath and Ponnuswami (2008a).

For quality components, the parental lines AKC-89/38 (1.25%) and VR-339 (1.21%) recorded highest capsaicin content (%) while, Kashi Sinduri (12.13%) and DC- 16 (12.06%) recorded highest oleoresin content (Table 7). Among the hybrids (Table 8), Pusa Jwala × VR-339 (1.72%) followed by Pusa Jwala × Punjab Lal (1.36%) recorded highest capsaicin content moreover, Kashi Sinduri × R Line (12.33%) followed by KA-2 × R Line (12.26%) exhibited highest oleoresin content (%) as reported by Ben-Chaim et al. (2006), Prasath et al. (2007) and Prasath and Ponnuswami (2008b).

In the present study based on *per se* performance, heterosis and SCA effects, the hybrids Pusa Jwala × VR-339, Kashi Sinduri × Punjab Lal and Pant C-1 × VR-339 were found superior for yield and quality traits. The three short listed hybrids may be utilized for further chilli breeding programme.

REFERENCES

Anonymous (2012). F.A.O. Statistical Databases. Food and Agriculture Organization of the United Nations, Statistics Division, Rome, Italy.

Ben-Chaim A, Borovsky Y, Falise M, Mazourek M, Kang Byoung-Cheorl, Paran I, Jahn M (2006). QTL analysis for capsaicinoid content in Capsicum. Theor. Appl. Genet. 113:1481-1490.

Bose TK, Som MG (1986). Vegetable crops in India. In: Chilli and Capsicum. Naya Prokash. Calcutta,India. pp. 343-366.

Bosland PW, Votava EJ (2000). Peppers: Vegetable and Spice Capsicum. CABI Publishing, Wallingford, UK.

Ganeshreddy M, Kumar H, Salimath P (2008). Correlation and path analysis in chilli. Kar. J. Agril. Sci. 21:259-261.

Hosamani RM, Shivkumar (2008). Correlation and path analysis in chilli. Ind. J. Hort. 65: 349-352.

Johnson HW, Robinson HP, Comstoc RE (1955). Estimation of genetic and environmental variability in soybeans. Agron. J. 47:314-318.

Kearsey MJ, Farquhar AGL (1998). QTL analysis; where are we now? Heredity. 80(2):137-142.

Kempthorne O (1957). An introduction to genetical statistics. Jhon weley and sons (Eds.), New York, pp.458-470.

Mathew AG, Lewis YS, Jagdishan R, Nambuderi ES, Krishanamurthy N (1971). Oleoresin capsicum. Flavour Industry. 2:23.

Munshi AD, Behera TK (2000). Genetic variability, heritability and genetic advance for some traits in chillies (*Capsicum annuum* L.). Veg. Sci. 27:39-41.

Prasath D, Ponnuswami V, Muralidharan V (2007). Evaluation of Chilli (*Capsicum* spp.) germplasm for extractable colour and pungency. Indian J. Genet. and Plant Breed. 67(1):97-98.

Prasath D, Ponnuswami V (2008a). Heterosis and combining ability for morphological, yield and quality characters in paprika type chilli hybrids. Ind. J. Hort. 65(4): 441-445.

Prasath D, Ponnuswami V (2008b). Breeding for colour and pungency in *Capsicum annuum* L. – A review. Veg. Sci. 35 (1):1-9.

Sharma VK, Punetha S, Sharma BB (2013). Heterosis studies for earliness, fruit yield and yield attributing traits in bell pepper. Afr. J. Agric. Res. 8(29):4088-4098.

Singh VP, Yadav SK (2008). Genetic variability, heritability and genetic advance in chilli (*Capsicum annuum* L.). Int. J. Plant Sci. 3(2):498-501.

Sreelathakumary I, Rajamony L (2004). Variability, heritability and genetic advance in chilli (*Capsicum annuum* L.). J. Trop. Agri. 42 (1-2): 35-37.

Tembhurne BV, Rao SK (2012). Heterosis and Combining Ability in CMS Based Hybrid Chilli (*Capsicum annuum* L.). J. Agril. Sci. 4(10):89-96.

Thimmaiah SK (1999). Standard Methods of Biochemical Analysis. Kalyani Publishers. pp.301-302.

Tsegaye E, Dechassa N, Sastry D (2007). Genetic variability for yield and other agronomic traits in sweet potato. J. Agron. 6(1): 94-99.

academicJournals

Vol. 12(47), pp. 6568-6573, 20 November, 2013
DOI: 10.5897/AJB2013.12797
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular characterization of ten mango cultivars using simple sequences repeat (SSR) markers

M. Kumar[1]*, V. Ponnuswami[1], P. Nagarajan[2], P. Jeyakumar[3] and N. Senthil[2]

[1]Horticultural College and Research Institute, Periyakulam, Tamil Nadu, 625604, India.
[2]Centre for Plant Molecular Biology, TNAU, Tamil Nadu, India.
[3]Department of Plant Physiology, TNAU, Tamil Nadu, India.

Accepted 7 November, 2013

Simple sequence repeats (SSRs) which is an efficient genetic markers for comparative genome mapping can be helpful in the classification of genotypes, germplasm resource utilization and breeding programmes. Therefore, the present study was conducted to show genetic variation and investigate inter-relationship between ten mango genotypes. Twenty (20) SSR markers were tested with 10 genotypes: Kalepad, Neelum, Swarnarekha, Alphonso Rumani, Sendura, Banganapalli, Himayuddin, Mulgoa and Bangalora. The genomic DNA was extracted from the leaf samples using cetyltrimethyl ammonium bromide (CTAB) method. Polymerase chain reaction (PCR) amplification of the DNA isolated from 10 mango genotypes with 20 SSR primers produced a total of 240 amplified products, of which 184 were polymorphic and 56 monomorphic. The sizes of the alleles detected ranged from 120 to 369 bp. SSR markers were highly polymorphic with an average of 2.70 alleles per primers. SSRs gave moderate values of polymorphic information content (PIC) range of 0.320 to 0.774. The amplified products varied between 2 (LMMA 1, 5, 7, 12, 16, MiSHRS-1 and MiSHRS-37) to 3 and 4 (LMMA 4, 6, 9, 10, 11, 13, 14, 15 MiSHRS-4, 48, 18, 39 and LMMA 8) bands per primer. We obtained moderate degree of genetic diversity, with Jaccard's similarity co-efficient values ranging from 0.075 between cluster I and II to 0.285 between clusters II and III. The dendrogram generated from the unweighted pair group arithmetic average (UPGMA) cluster analysis broadly placed 10 mango cultivars into three major clusters at co-efficient similarity of 0.65. The cluster size varied from 1 to 6 and cluster III was the largest cluster comprising of six cultivars followed by cluster II possessing three and cluster I possessing one variety. Cluster I had the highest diverse cultivars namely, Kalepad, Neelum and Swarnarekha. Cluster II included cultivar of Alphonso. Cluster III contain the cultivars viz., Rumani, Sendura, Bangnapalli, Himayuddin, Mulgoa and Bangalora. Unique fingerprints were identified in the cultivars. The unique fingerprints size ranged from LMMA-8 (257-270 bp), LMMA-11 (232- 245 bp) to MiSHRS 39 (340-369 bp). The tendency of clustering among mango cultivars revealed that they have strong affinity towards further breeding programme.

Key words: Cultivars, genetic diversity, mango, simple sequence repeats (SSR).

NTRODUCTION

Mango (*Mangifera indica* L.) belonging to the family Anacardiaceae occupies paramount place among the fruit crops grown in India and christened as the "King of fruits" owing to its delicious flavor and taste; there are 1000 varieties found in the country (Singh, 1996). However, there is a lot of confusion in nomenclature of

*Corresponding author. Email: kumshorts@gmail.com.

the mango cultivars, which is attributed to the lack of systematic approach in nomenclature. Characterization of available cultivars is a prerequisite for their conservation as well as utilization in the further breeding programmes. Genetic analysis including assessment of genetic diversity, relatedness between or within species, population and individuals as well as genotype characterization, are central tasks for many disciplines of biological sciences. Conventionally, genetic analysis was dependent on morphological and/or biochemical markers. During the past few decades, classical strategies of genetic analysis have been increasingly complemented by molecular techniques. The most fundamental of these molecular techniques are DNA markers which portray genome sequence composition, thus, enabling the detection of differences in the genetic information carried by the different individuals. Therefore, these markers are powerful tools in genotype identification or fingerprinting and the estimation of relatedness between genotypes. Consequently, they provide the means to utilize our existing germplasm resources to understand fundamental plant processes and mechanisms. Furthermore, marker-mediated genetic analysis elucidates the genetic basis of agronomic characters and leads to their direct manipulation by plant breeders.

Microsatellites consist of highly variable tandem repeats of very short motifs (1-6 bp) (Litt and Lutty, 1989). Based on microsatellites, two types of DNA markers could be generated, that is, simple sequence repeats (SSRs) and inters simple sequence repeats (ISSRs). In SSRs, the polymorphism is detected by PCR amplification using primers complementary to unique flanking sequences. SSRs are becoming the markers of choice in genetic studies because they are transferable, multiallelic codominant markers, easily reproducible, randomly and widely distributed along the genome (Rafalski et al., 1995).

Genetic characterization serves the twin purpose of the identification of genotypes and estimation of their genetic relatedness (Ravishankar et al., 2000). Traditionally, the genetic variation in mango was estimated using morphological markers and isozymes. These techniques however do not provide an accurate estimation of variation and could lead to misidentification or duplication of genotypes. The incorrect labeling of cultivars and ambiguous identification of individual is a limitation that impedes progress in mango improvement programmes. Precise characterization of genetic variation at the molecular level is possible using DNA based markers. Different molecular marker, such as randomly amplified polymorphic DNA (RAPDs) (Bajpai et al., 2008), amplified fragments length polymorphism (AFLP) (Eiadthong et al., 2000), inter-simple sequence repeats (Pandit et al., 2007) and simple sequence repeats (Duval et al., 2005; Schnell et al., 2006; Viruel et al., 2005) have been employed for genetic diversity assessment in mango cultivars. SSR have gained considerable importance in genetic studies owing to their desirable attributes such as hyper-variability, multiallelic nature, co-dominant inheritance and reproducibility. Assessment of the genetic structure of closely related cultivars is also possible with SSRs. Based on informative and robustness; the use of SSRs has been preferred to determine the genetic relationships among the mango cultivars. Keeping in view these advantages, we analyzed the closely related mango cultivars with micro satellite markers.

MATERIALS AND METHODS

A total of 10 mango genotypes used in this study were collected from the mango orchard of State Horticultural Farm, Kanyakumari, Tamil Nadu, India.

DNA isolation

Total genomic DNA was isolated from fully expanded leaves using the hexadecyltrimethylammonium-bromide (CTAB) method (Murray and Thompson, 1980) with few modifications. Briefly, 2 g of leaves were ground in liquid nitrogen to a fine powder. The powder was added to 6 mL of extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (wv-1) CTAB, 2–mercaptoethanol 2% and 1% (wv-1) PVP) and incubated at 65°C for 30 min. The DNA was extracted with chloroform – octanol (24: 1). The DNA was washed with 70% ethanol and dissolved in 100 – 400 µL of TE (10mM Tris–HCl pH 8.0, 1 mM EDTA and 0.2 mg mL^{-1} RNase). The DNA concentration was determined spectrophotometrically at 260 nm. Stock DNA samples were stored at -20°C and diluted to 20 ng uL^{-1} when in use.

PCR procedure

The PCR reactions were performed on Perkin Elmer 9,600 thermocycler (USA). Each PCR reaction consisted of 2 µl of 10 x reaction buffers, 0.5 µl of 10 mM dNTPs, 2 µl of each forward and reverse primer, 0.3 µl of Tag DNA polymerase, 2 µl of DNA and 13.2 µl of sterile water in a final reaction volume of 20 µl. The PCR reaction profile was DNA denaturation at 95°C for 5 min followed by 40 cycle of 94°C for 1 min; primer annealing at 51°C for 1 min, 72°C for 1 min, and finally 72°C for a final extension of 5 min. Amplification products were separated by electrophoresis (8.3 V cm^{-1}) in 1.5% agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination.

Data analysis

Only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying species and cultivars were named with a primer number followed by the approximate size of the amplified fragment in base pairs. Polymorphism was calculated based on the presence or absence of bands. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using “Simqual” a subprogram of the NTSYS-PC program (numerical taxonomy and multivariate analysis system program) (Rohlf, 2000). The genetic associations between accessions were evaluated by calculating the Jaccard’s similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by the primers (Jaccard, 1908). The dendrogram was constructed by using a distance matrix using the unweighed pair group method with arithmetic average

Table 1. The sequence and details of the primer pairs.

Primer name	Primer details (5'-3')
LMMA1	f:atggagactagaatgtacagag
	r:attaaatctcgtccacaagt
LMMA 4	F:AGATTTAAAGCTCAAGAAAAA
	R:AAAGACTAATGTGTTTCCTTC
LMMA 5	F:AGAATAAGCTGATACTCACAC
	R:TAACAAATATCTAATTGACAGG
LMMA 6	F:ATATCTCAGGCTTCGAATGA
	R:TATTAATTTTCACAGACTATGTTCA
LMMA 7	F:ATTTAACTCTTCAACTTTCAAC
	R:AGATTTAGTTTTGATTATGGAG
LMMA 8	F:CATGGAGTTGTGATACCTAC
	R:CAGAGTTAGCCATATAGAGTG
LMMA 9	F:TTGCAACTGATAACAAATATAG
	R:TTCACATGACAGATATACACTT
LMMA 10	F:TTCTTTAGACTAAGAGCACATT
	R:AGTTACAGATCTTCTCCAATT
LMMA 11	F:ATTATTTACCCTACAGAGTGC
	R:GTATTATCGGTAATGTCTTCAT
LMMA 12	F:AAAGATAGCATTTAATTAAGGA
	R:GTAAGTATCGCTGTTTGTTATT
LMMA 13	F:CACAGCTCAATAAACTCTATG
	R:CATTATCCCTAATCTAATCATC
LMMA 14	F:ATTATCCCTATAATGCCCTAT
	R:CTCGGTTAACCTTTGACTAC
LMMA 15	F:AACTACTGTGGCTGACATAT
	R:CTGATTAACATAATGACCATCT
LMMA 16	F:ATAGATTCATATCTTCTTGCAT
	R:TATAAATTATCATCTTCACTGC
MiSHRS-1	F: TAACAGCTTTGCTTGCCTCC
	R: TCCGCCGATAAACATCAGAC
MiSHRS-4	F: CCACGAATATCAACTGCTGCC
	R: TCTGACACTGCTCTTCCACC
MiSHRS-37	F: CTCGCATTTCTCGCAGTC
	R: TCCCTCCATTTAACCCTCC
MiSHRS-48	F: TTTACCAAGCTAGGGTCA
	R: CACTCTTAAACTATTCAACCA
MiSHRS-18*	F: AAACGAGGAAACAGAGCAC
	R: CAAGTACCTGCTGCAACTAG
MiSHRS-39†	F: GAACGAGAAATCGGGAAC
	R: GCAGCCATTGAATACAGAG

(UPGMA) sub-program of NTSYS-PC.

RESULTS AND DISCUSSION

Twenty (20) SSR primers were used for generating banding profile (Table 1). Out of which 17 primers gave consistent and discrete bands. Three typical SSR profiles are shown in Figure 1. The details with respect to band statistics are shown in Table 2. The 20 SSR primers produced a total of 240 amplified products, of which 184 were polymorphic and 56 monomorphic. The number of alleles detected varied form 2 (LMMA 1, 5, 7, 12, 16, MiSHRS-1 and MiSHRS-37) to 3 and 4 (LMMA 4, 6, 9, 10, 11,13,14,15 MiSHRS-4, 48, 18, 39 and LMMA 8). The average number of alleles per primer pair was 2.70. The allele size ranged from 120 (MiSHRS-4 and MiSHRS-37) to 369 bp (MiSHRS-39). Earlier, Shareefa (2008) and Nayak (2010) reported similar values of SSR polymorphism (71 to 81.8), number of alleles and allele size in mango cultivars. In the present study, most of the SSR primers detected multiple loci, which can be attributed to the allopolyploid nature of mango (Mukherjee, 1950).

The characteristics of PCR products namely, the polymorphism information content (PIC) is presented in Table 2. In the present experiment, SSR markers gave low PIC values ranging from 0.320 (MiSHRS-37) and 0.683 (MiSHRS-4). The average PIC value for MiSHRS primer series was 0.552 whereas it was 0.518 in LMMA primer series in our study which is similar to the findings of Shareefa (2008) and Nayak (2010). PIC values of these markers were also low to moderate in Florida mango cultivars (Schnell et al., 2006). 20 SSR primers in the ten mango cultivars detected a total of 240 scorable bands with an average of 2.70 bands/SSR, ranging from 2 to 4 bands/SSR. This is lower than those reported by Viruel et al. (2004) in their work with 16 primer pairs among 28 mango genotypes, probably due to the lower number of analyzed samples as well as due to the less diverse genotypes analyzed.

The analysis of molecular data showed high level of genetic similarity within the analyzed cultivars, while different levels of genetic diversity were detected among ten mango genotypes determined based on the Jaccard's pair wise similarity co-efficient. We obtained moderate degree of genetic diversity, with Jaccard's similarity co-efficient values ranging from 0.075 between cluster I and II to 0.285 between clusters II and III. The dendrogram generated from the unweighted pair group arithmetic average (UPGMA) cluster analysis broadly placed 10 mango cultivars into three major cluster at co-efficient of 0.65 (Figure 2). The cluster size varied from 1 to 6 and cluster III was the largest cluster comprising of six cultivars followed by cluster II possessing 3 and cluster I possessing one variety. Cluster I has the most diverse cultivars namely, Kalepad, Neelum and Swarnarekha. Cluster II included cultivar of Alphonso. Cluster III has the cultivars viz., Rumani, Sendura, Bangnapalli, Himayuddin, Mulgoa and Bangalora.

The Jaccard's similarity values (65%) clearly depicted rich genetic variability in the cultivars studied. Our findings are supported by the earlier studies on genetic diversity analysis in mango using different marker system (RAPD) (Bajpai et al., 2008; Singh, 2005). The rich genetic variation found in cultivar progeny could be attributed to the cross pollinated nature of mango crop,

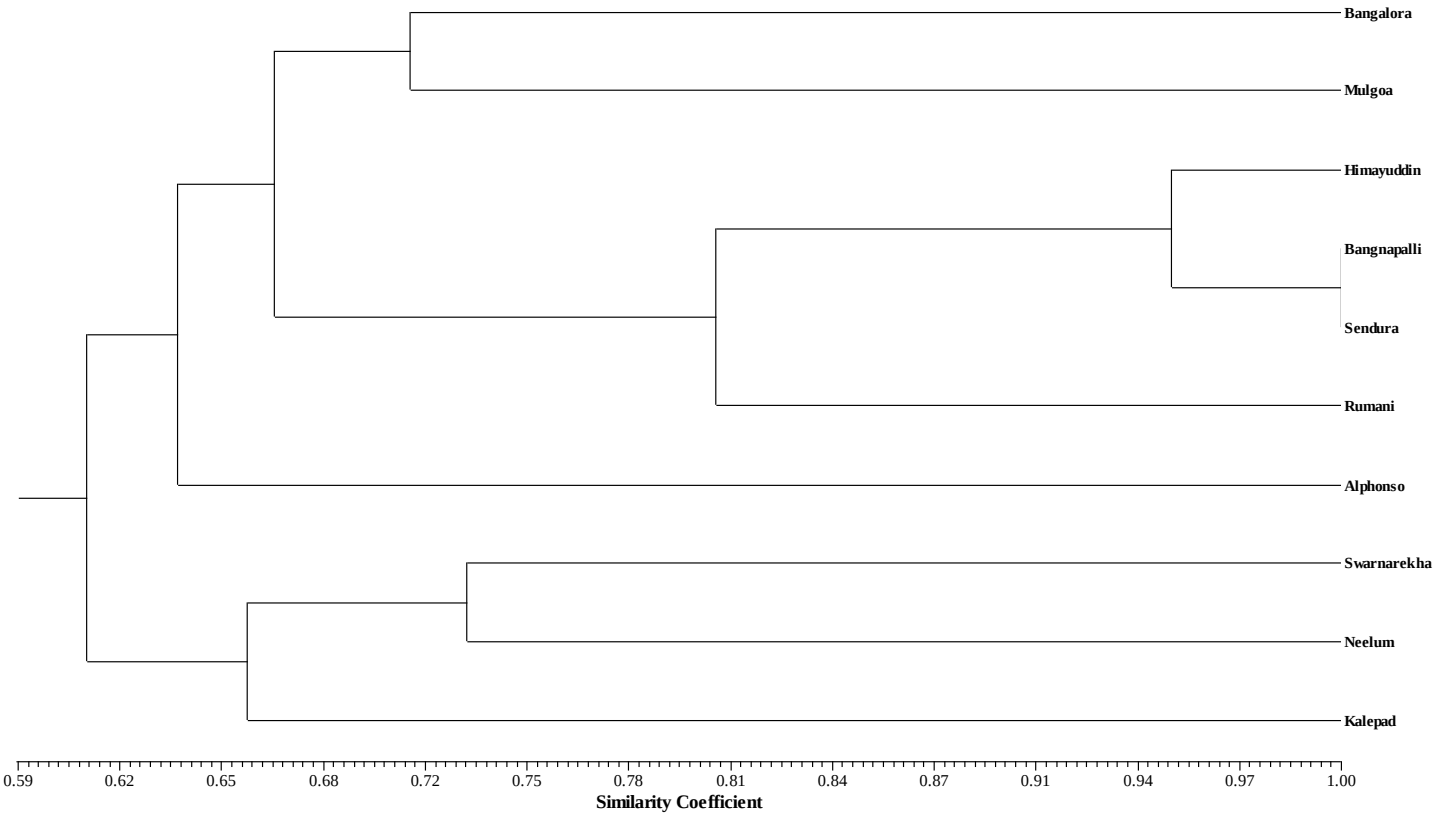
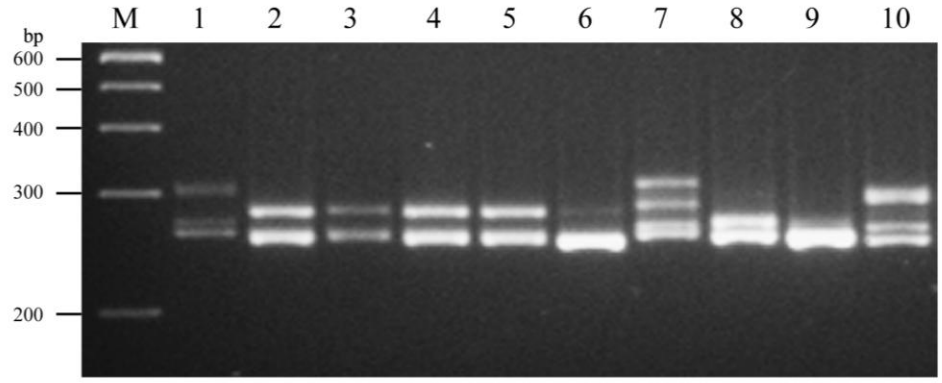


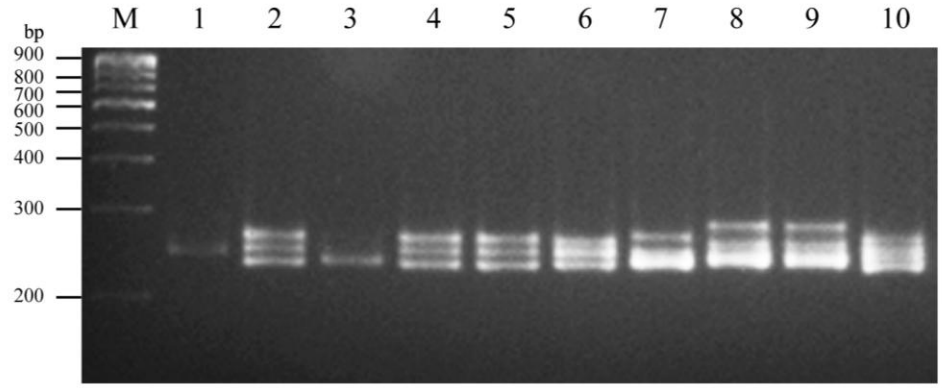
Figure 1. Phenogram of ten mango cultivars based on SSR data. The phylogenetic tree obtained from unweighted pair-group (UPGMA) cluster method of Nei's genetic distances option in the NTSYS-PC1.2 program.

Table 2. Allele variation and PIC values for SSR marker identification in mango genotypes.

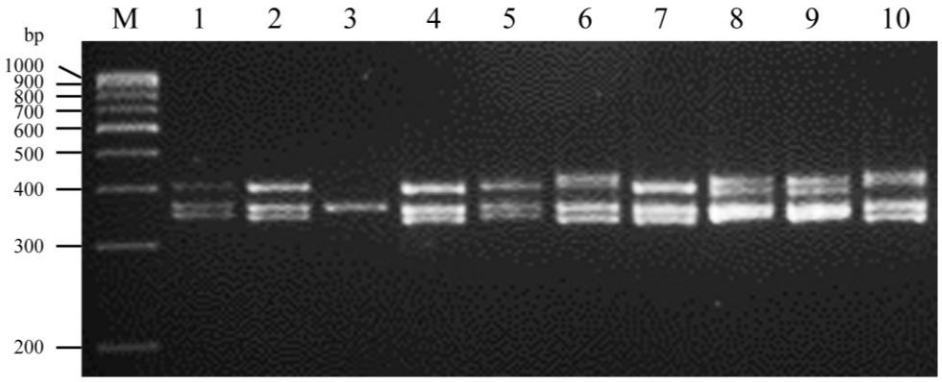
Locus	Number of alleles	Product size (bp)	PIC values
LMMA1	2	200-210	0.560
LMMA 4	3	235-240	0.520
LMMA 5	2	280-285	0.395
LMMA 6	3	220-235	0.382
LMMA 7	2	198-215	0.690
LMMA 8	4	257-270	0.774
LMMA 9	3	170-180	0.380
LMMA 10	3	145-172	0.642
LMMA 11	3	232-245	0.720
LMMA 12	2	195-205	0.430
LMMA 13	3	180-200	0.620
LMMA 14	3	165-170	0.320
LMMA 15	3	195-225	0.364
LMMA 16	2	220-245	0.465
MiSHRS-1	2	200-210	0.524
MiSHRS-4	3	120-130	0.683
MiSHRS-37	2	120-150	0.320
MiSHRS-48	3	205-225	0.520
MiSHRS-18*	3	210-240	0.615
MiSHRS-39†	3	340-369	0.650



A. SSR profile of mango cultivars generated by the primer LMMA8



B SSR profile of mango cultivars generated by the primer LMMA11



C. SSR profile of mango cultivars generated by the primer MiSHRS39

M - 100 bp ladder; 1 - Bangalora; 2 - Himayuddin; 3 - Rumani; 4 - Bangnapalli; 5 - Sendura; 6 - Alphonso; 7 - Swarnarekha; 8 - Neelum; 9 - Kalepad; 10 - Mulgoa

Figure 2. Simple sequence repeats (SSR) profiles of ten different mango cultivars.

high degree of heterozygosity and high discriminatory power of the SSR markers. The diverse genetic backgrounds of cultivars seem to have contributed to rich genetic variation observed in mango cultivars. In Kalepad and Neelum south Indian, especially Tamil Nadu, flowering cultivar of mango has regular bearing throughout

Table 3. Distribution of genotypes to different clusters based on UPGMA methods.

Cluster	Number of genotype	Name of genotype
Cluster I	3	Kalepad, Neelum and Swarnarekha
Cluster II	1	Alphonso
Cluster III	6	Rumani, Sendura, Banganapalli, Himayuddin, Mulgoa and Bangalora

the year. Swarnarekha cultivar prominently in Andra Pradesh has coloured fruit and is an early bearing cultivar. Alphonso has alternate bearing habit, which might be due to environmental, genetic and physiological factors. Other cultivars viz., Rumani, Sendura, Banganapalli, Himayuddin, Mulgoa and Bangalora, are popular varieties of South India. Thus, these diverse cultivars could have resulted in high genetic variability among the cultivars.

As with other vegetative propagated clonal crops, the differences among mango cultivars can result from epigenic modifications in response to the environment (Kaeppler et al., 2000). Somatic and bud mutations also play a minor role in clonal polymorphism in woody plants. Naik (1948) observed significant variation among the trees of some clones in mango with respect to fruit cha-racteristics and tree performance. It could be expected that most of the somatic mutations that occur during plant growth would have no effect on phenotype, although they could be identified at the molecular level.

DNA fingerprinting can be employed for individual identification of cultivars or rootstock for different horticultural purpose, such as breeder's right, identification of pollen parents and determination of genetic relatedness (Lavi et al., 1993). The potential of SSR markers in fingerprinting is well established in mango (Viruel et al., 2005; Shareefa, 2008). Unique fingerprints are genotype and marker specific alleles that may serve as indicators of a particular region of the genome specific to a parti-cular trait of horticultural importance. The genotypes carrying the unique alleles may prove useful for intro-ducing diversity in the future mango breeding programmes. The application of DNA fingerprinting technology has the potential of significantly improving mango breeding pro-jects in terms of cost, time and efficiency by enabling eventual use of marker-assisted selection (MAS) and reduction in the nature of backcross generations needed for gene introgression. Our study revealed that SSR markers are useful not only for varietal identification, but also in future mango breeding progrommes to maximize genetic variability among the mango cultivars.

ACKNOWLEDGMENTS

The authors thank the Department of Fruit Crops, Horticultural College and Research Institute, Periyakulam, Tamil Nadu Agricultural University, Coimbatore for providing the plant materials and for the financial support.

REFERENCES

Bajpai A, Srivastava N, Rajan S, Chandra R (2008). Genetic diversity and discrimination of mango accessions using RAPD and ISSR markers. Indian. J. Hort. 65:377-82

Duval MF, Bunel J, Sitbon C, Risterucci AM (2005). Development of microsatellite markers for mango (*Mangifera indica* L.*).* Mol. Eco. Notes. 5:824-26.

Eiadthong W, Yonemori K, Kanzaki S, Sugiura A, Utsunomiya N, Subhadrabandhu S (2000). Amplified fragment length polymorphism analysis for studying genetic relationship among *Mangifera* species in Thailand. J. Am. Soc. Hort. Sci. 125:160-64

Jaccard P (1908). Nouvelles recherches sur la distribution florale. Bul. Soc. Vaudoise Sci. Nat. 44:223-270.

Kaeppler SM, Kaeppler HF, Rhee Y (2000). Epigenetic aspects of somaclonal variation in plants. Plant Mol. Biol. 43:179-88

Lavi U, Cregan PC, Schaap T, Hillel J (1993). Application of DNA markers for identification and breeding of perennial fruit trees. Plant Breed. Rev. 7:195-226.

Litt M, Lutty JA (1989). A hypervariable microsatellite revealed by in vitro amplification of nucleotide repeat within the cardiac muscle actin gene. Amer. J. Hum. Genet. 44:397

Lodhi MA, Ye GN, Weeden NF, Reisch BI (1994). A simple and efficient method for DNA extraction from grapevine cultivars and *vitis* species. Plant Mol. Biol. Rep. 12(1):6-13.

Mukherjee SK (1950). Mango: its allopolyploid nature. Nature. 166:196-97

Naik KC (1948). Improvement of the mango (*Mangifera indica* L.) by selection and hybridization. Indian J. Agric. Sci. 18:35-41

Nayak D (2010). Characterization of mango hybrids for fruit quality traits. Ph.D. Thesis submitted to PG School, IARI, New Delhi.

Pandit SS, Mitra S, Giri AP, Pujari KH, Patil BP, Jambhale ND, Gupta VS (2007). Genetic diversity analysis of mango cultivars using inter simple sequence repeat markers. Curr. Sci. 93:1135-41

Rafalski JA, Morgante M, Vogel JM, Powell W, Tingey SV (1995). Generating and using DNA markers in plants. In: Birren B, and Lai E, (eds). Non-mammalian Genomic Analysis: a practical guide. Academic Press, London New York. pp. 75-134.

Ravishankar KV, Anand L, Dinesh MR (2000). Assessment of genetic relatedness among mango cultivars of India using RAPD markers. J. Hort. Sci. Biotech. 75:198-201

Rohlf FJ (2000). NTSYS-PC Numerical Taxonomy and Multivariate Analysis System Ver. 1.60. Exeter Publ. Ltd., Setauket, New York.

Schnell RJ, Brown JS, Olano CT, Meerow AW, Campell RJ, Kuhn DN (2006). Mango genetic diversity analysis and pedigree inferences for Florida cultivars using microsatellite markers. J. Am. Soc. Hort. Sci. 131:214-24

Singh RN (1996). Mango. Indian Council of Agricultural Research, New Delhi

Singh S (2005). Analysis of clonal variation in some mango cultivars using anchored Inter Simple Sequence Repeat (ISSR) markers. M.Sc. thesis, P.G. School, Indian Agricultural Research Institute, New Delhi.

Shareefa M (2008). DNA fingerprinting of mango (*Mangifera indica* L.) genotypes using molecular markers. Ph.D. thesis submitted to P.G. School, IARI, New Delhi.

Viruel MA, Escribano P, Barbieri M, Ferri M, Hormaza JI (2005). Fingerprinting, embryo type and geographic differentiation in mango (*Mangifera indica* L.) Anacardiaceae with microsatellites. Mol. Breed. 15:383-93.

academicJournals

Vol. 12(47), pp. 6583-6590, 20 November, 2013
DOI: 10.5897/AJB12.531
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Tracking the expression of photosensitive genic male sterility genes in rice

Njiruh Paul Nthakanio[1]* and Xue Qingzhong[2]

[1]The Kenya Polytechnic University College, Department of Biochemistry and Biotechnology P.O. Box 52428-00200, Nairobi, Kenya.
[2]Zhejiang University, Huajia Chi Campus, Agronomy Department, 310029, Kaixuan Road, Hangzhou-China.

Accepted 31 July, 2013

Photoperiod sensitive genic male sterile rice lines contain genes that induce complete sterility in high temperature and long day light length period, and revert to fertility in optimum low temperature and short day light length period. These lines are good candidates for hybrid rice seed production. The main challenge limiting their use in production of hybrid rice seeds is that of determining the exact time of their growth period when sterility gene(s) is expressed. The objective of this study was to determine the time in the rice growth period when the sterility gene(s) are expressed. Rice line ZAU11S106, a photoperiod sensitive genic male sterile line was used to test the hypothesis: it is possible to estimate time within ±2 days when photosensitive genic male sterility (PGMS) gene is expressed. Sowing was done in 9 rows in Hangzhou, China in the month of May and matured in August when day light length was over 14 h and day temperatures were over 30°C. At 57 days, old plants in row 1 were given short day length treatment and after every four days, the next row was included in the treatment. This was done until plants in row 1 flowered when the treatment was stopped. Plants given short day length treatment at 73 and 77 days old realized 6 and 0% seed set, respectively. At 73 and 77 days old, plants were at dyad and tetrad stages of pollen development, respectively. The conclusion was that, the sterility gene was expressed between the dyad and tetrad stage of pollen development.

Key words: Photoperiod, pollen abortion, PGMS rice.

INTRODUCTION

Heterosis or hybrid vigour realized in F_1 has been used to increase yield in many out-crossing crops like maize (*Zea may*). In particular, hybrid seed technology has been practiced in maize since 1939s and it accounts for over 30% increase in yield (Duvick, 1992, 1997). Two inbred genetically fixed varieties of a particular crop are crossed to obtain hybrids seeds. Plants from such seeds are special in that they express what is called *"heterosis"* or hybrid vigor. The basic principle is that, if two parents are crossed, which are genetically distant from each other, the offspring will be *"superior",* particularly in terms of yield. Hybrid seed technology is practiced in many other crops including wheat, sunflower, cotton and rice (Igor and Hari, 1969; Burke and Arnold, 2001; Xie and Hardy, 2009). Search for high yielding rice (*Oryza sativa* L.) line has gone through a number of breeding metamorphic stages. The first major breakthrough was the incur-poration of a semi-dwarfing gene (*sd-1*) in Chinese variety Dee-geo- woo-gen (Khush, 1994, 1995) into the ordinary rice plant around 1955 providing a plant

*Corresponding author. E-mail: nnpanj@yahoo.com.

Abbreviations: PGMS, Photosensitive genic male sterile line; **SDLLT,** Short day light length treatment; **LDLL,** Long day light length; **HYVs,** High yielding varieties; **IRRI,** International Rice Research Institute; **CMS,** Cytoplasmic male sterile; **I/KI,** Potassium iodide.

architecture that can accommodate use of more nitrogenous fertilizer hence, higher yield (Hu, 1993). Later came the high yielding varieties (HYVs) such that the International Rice Research Institute (IRRI) produced IR8 (Hossain et al., 1999). This was nicknamed *"miracle rice"* because of its improved yield. IR8 varieties churn out 10 metric tones/hectare in research stations, and for a long time this remained the yield ceiling. To break this barrier, two routes were adopted *"Super Rice"* or IRRI 15-tonner, which was achieved by a radical restructuring of the rice plant architecture, and the other route is by hybridizing rice. Super hybrid rice (Asia-Pulse, 1999; Yuan, 1997) has so far demonstrated the capacity to break the yield ceiling established by IR8 and other high yielding varieties (Heiling, 1999; Yuan, 1997). Production of hybrid rice started in 1970s with the discovery of cytoplasmic male sterile, *"wild rice with abortive pollen",* or WA rice line (*Oryza sativa f. spontanea*) (Virmani, 1996). This is what is called the "three line system" because it involves a sterile, a maintainer and a restorer line. Yield increase due to crossing breeding in rice exploits hybrid vigour or *heterosis* just like in maize. For rice, *heterosis* has been reported to increase rice yield by between 20 to 30% above the current dwarf lines (Kush et al., 1994; Virmani et al., 1996; Virmani et al., 2003). The yield gains led China to start commercial production of hybrid rice in 1976 and lines that can yield up to 17 tons per hectare have been developed (Yuan, 1997; Kuyek, 2000). Most available estimates suggest that China's hybrid rice yields average 15 to 20% more than the high-yielding inbred varieties (Yuan, 1994; Xie and Hardy, 2009).

To produce hybrid seeds, a sterile female parent and fertile male parent (pollen donor) are needed. This is achieved by male emasculation of the pollen recipient or the female parent which makes it labour and skill-intensive and increases cost of production especially if it is done manually (Virmani and Kumar, 1997). Despite this, hybridization remains the major method of increasing the rice yield. According to Yuan Longping, China has reached the yield plateau for hybrid rice (IRRI, 1998) using the three line system, however another major boost is expected from adopted "super hybrid" rice program (Xie and Hardy, 2009). The Green Revolution, led by IRRI's high-yielding varieties (HYVs), led to dominance of a few lines such that by the mid-1980s just two HYVs occupied 98% of the entire rice growing area of the Philippines (Kuyek, 2000) leading to genetic erosion and reduced biodiversity. The problem has been increased by use of cytoplasmic male sterile (cms) lines in hybrid seed production that has lead to increase of cytoplasmic uniformity leaving the hybrids vulnerable to disease and other environmental catastrophe (Levings, 1990).

Photosensitive genic male sterile (PGMS) rice is expected to reduce the problem of genetic degradation because it can be used with many restorer lines (both indica and japonica rice lines) to produce hybrid seeds, unlike in CMS where they are limited due to maternal (female parent) and paternal (male parent) incompatibility, which lead to F_1 sterility (Oka, 1974; Lin et al., 1992). Wide compatibility is realized because PGMS (female) lines with diversified germplasm background can be produced unlike in cms whereby wild abortive (WA) is the major maternal line used (Virmani, 1996). Since the cost of production of hybrid rice seed using PGMS is expected to be lower then, PGMS lines are suitable candidates in Hybrid rice seed production technology.

Discovery of PGMS rice line in 1970s (Shi, 1981, 1985) ushered in the use of two-line system as a major method of producing hybrid seeds (Mao and Deng, 1993). PGMS rice lines are completely sterile in long day light length (LDLL) and revert to fertility in short day (Shi, 1985; Shi and Deng, 1986). They do not require a maintainer line like in the case of cytoplasmic male sterile plants since they maintain themselves. In LDLL (above 14 h) and in temperature of above 26°C it is completely sterile and reverts to fertility in optimal day length and temperature (Yuan et al., 1993; Ku et al., 2001).

To effectively use PGMS rice lines in hybrid rice seed production, a good breeding program needs to be developed. This will include evaluation and monitoring of PGMS genes to determine the time when it is switched on and off. Under sterility inducing conditions, the fertility gene is off and the pollen is completely sterile and seed set rate is zero (Xu et al., 1995, Ku et al., 2001, Njiruh and Xue, 2011). When PGMS are grown in short day, low temperature plants revert to fertility; a time when they are used to propagate themselves for the next generation. The PGMS character is genetically controlled and can be inherited from one generation to another (Shi, 1985; Virmani, 1994). The trait is controlled by genes *pms1* and *pms2* in chromosomes 7 and 3, respectively (Zhang et al., 1994) and *pms3* on chromosomes 12 (Mei et al., 1999).

This has enabled breeding for new lines with the PGMS trait and among them include W6154s, W7415s, NS5047S, 31111s, WD1S, 8801S, 6334s, N5047S (Virmani, 1994; Xue et al., 1999). The objective of this research was to track PGMS gene and determine when it is expressed in rice growth cycle as this will enable development of a rice breeding program that ensures optimum sterility inducing condition, prevail at the time of gene expression. In this report, the PGMS gene has been tracked to identify the critical four days within rice growth cycle in which switching off and on of the gene is determined.

MATERIALS AND METHODS

Plant materials used were Sterile (PGMS) line ZAU11S106 and a fertile line ZAU11F121 (control- ck). ZAU11S106 is a PGMS developed from japonica line N5047S protoplasts (Xue et al., 1999). Sowing was done on May 14th, 2003 at the Zhejiang University–Huajiachi Campus experimental fields at Hangzhou in China, 30°15N. Plants were grown under natural conditions and sowing

was programmed so that the plants headed in summer during the LDLL and high temperature. In this research, LDLL refer to day length of over 14 h day time including morning and evening twilight while short day refer to 11 h daylight including the morning and the evening twilight. High temperature refer to >33°C and >26°C during day and night respectively and low temperature refer to 26 and 20°C during day and night, respectively.

Sowing and Short day length treatment of PGMS

Rice lines ZAU11S106 and control ZAU11F121 were sown on May 14th in nine rows each with six plants and allowed to grow up to 57th or the stage just before the primordial stage. After 57 days, the first row was covered with an opaque black cloth at 4.00 pm and uncovered at 9.00 pm Hangzhou-China time, so that it experienced only 11 h of normal daytime light. This is what is referred to as short day light length treatment (SDLLT) throughout this research. Time when first row was given SDLLT was referred to as day zero (0) and after four days, the first and the second row were put under SDLLT. After every four days, a new row was included in the SDLLT. This was done as described in Table 1 until plants in the first row flowered when SDLLT was stopped.

Relation between PGMS gene expression and Pollen development

Before plants in each row were given SDLLT, a panicle was collected and fixed in Canoys solution II for pollen analysis. Panicles were collected on days 57, 61, 65, 69 and 73 after sowing for rows 1, 2, 3, 4, and 5, respectively. At 73 days old and after, pollen had completely matured and therefore, pollen were collected from 77 days old plants or row 6 represents rows 7 and 8. A whole panicle from each sample was directly scanned using Uniscan 2100k scanner (Tsinghua, China). The fixed spikelets were stored at 4°C till use. This was followed by washing the glumes in 95% ethanol to wash away any residues of Canvoy's solution that could create artifacts. Anthers were extracted from the glume using forceps or a dissecting needle for very young glumes and placed on a microscope glass slide with a drop of 1% potassium iodide (I/KI) solution after which, they were macerated using the forceps to release the pollen cells. Anther-husks were removed from the glass slide leaving the microspores only after which a cover slide was placed on a glass slide, observed, and photographs taken under x40 of light microscope (Olympus 35AD2, Japan). Photos were scanned using Uniscan 2100k scanner (Tsinghua, China) and processed using Adobe Photo Element version 2.

Determination of plant fertility

At post ripening stage, three tillers with full grown panicles were picked from each hill in each row for seed set evaluation. Glumes with filled up grains were counted and fertility was calculated as a percentage of total number of grains per spike to total number of glumes per spike x 100.

RESULTS

Effects of short day length treatment on ZAU11S106 rice panicle fertility

ZAU11S106 plants in rows 1, 2, 3, 4 and 5 were given SDLLT for 28, 24, 20, 16 and 12 days respectively (Table 1). These plants recorded seed set of 16, 18, 8, 20 and 6%, respectively. The plants given SDLLT on 57, 61, 65, 69 and 73 days old flowered at 81, 81, 90, 96 and 101 days old after sowing respectively. In ZAU11S106 rows 6, 7 and 8 (Ck) were given SDLLT for 8, 4 and 0 days, or at 77, 80 and 80(Ck) days old all recorded 0% seed set and panicle appeared seedless (Figure 2b). These plants flowered after 101,102 and 105 days of age in this respect. The boundary on which seeds set was recorded and complete sterility was 77 days old or at 8 days of SDLLT or in row 6. ZAU11F121 plants in all row recorded over 39% seed set except row 2, which recorded 15% (Table 1).

A sample of panicles from ZAU11S106 plants under SDLLT in lines 1 to 5 and that from 6 and 7 lines are shown in Figure 2a and b, respectively. Glumes of panicle shown in Figure 2b were completely seedless while those from panicle shown in 2a recorded an average of 45% seed set.

Linkage of PGMS gene expression to Pollen developmental stages

Samples of panicles picked from rows 1 and 2 (corresponds 57 and 61 days old plants) did not have observable glumes. When these samples were stained with 1% I/K, no pollen grains were noticed (Figure 3a and b). However, samples obtained from rows 3 to 5 had distinctively grown glumes and after staining with 1% I/K, pollen were observed. These are the plants that received SDLLT on 65, 69 and 73 days old (Figure 3c to e).

Between days 57 and 61 after sowing panicles had no differentiated glumes and no pollen was observed for both ZAU11S106 (Figure 3f and g) and ZAU11F121 (Figure 3k and I). On 65th day after sowing pollen mother cell had formed for both ZAU11S106 and ZAU11F121 (Figure 3h and m) respectively. Dyads were observed in both ZAU11S106 (Figure 3i) and ZAU11F121 (Figure 3n) on day 69th after sowing; while on day 73 after sowing tetrad were observed for both ZAU11S106 and ZAU11F121 (Figure 3j and o, respectively).

Mature panicle from rows 5, 6, 8 (sterile control) and ZAU11F121 are shown in Figure 4a to d. Some pollen grains of plants in row 5 stained blue-black like the pollen from ZAU11F121 (Figure 4e and h). Plants in row six which was given SDLLT at 77 days old had their pollen all staining yellow same to those in rows 8 (ZAU11S106 control line). Pollen and panicle for row 7 were similar to those from row 6 and were not included.

DISCUSSION

Effects of Short length treatment on ZAU11S106 fertility

PGMS rice line ZAU11S106 given SDLLT in row 1 to 5

Table 1. Parameters used to track Critical Point of PGMS gene expression. Table a shows data collected from ZAU11S106 while b show data collected from ZAU11F121

Day length (h)	Plant Row	Date of initiation of SDLLT (Days after sowing)	Heading date	Total days of SDLT	Days from sowing to Heading	Seed set %
11	1	11-July (57)	4-Aug	28	81	16
11	2	15-July(61)	4-Aug	24	81	18
11	3	19-July(65)	11-Aug	20	90	8
11	4	23-July(69)	17-Aug	16	96	20
11	5	27-July(73)	22-Aug	12	101	6
11	6	30-August(77)	23-Aug	8	102	0
11	7	3- August(81)	26-Aug	4	105	0
14	8	CK (81)	26-Aug	0	105	0
11	1	11-July (57)	4-Aug	28	81	39
11	2	15-July(61)	4-Aug	24	81	15
11	3	19-July(65)	11-Aug	20	88	41
11	4	23-July(69)	11-Aug	16	88	53
11	5	27-July(73)	17-Aug	12	94	64
11	6	30-August(77)	17-Aug	8	94	67
11	7	3- August(81)	17-Aug	4	94	51
14	8	CK (81)	11-Aug	0	91	54

In both Tables a and b, column 1 shows number of hours of daylight the plants were exposed to SDLLT while “plants row” in column 2 refer to order of sowing in the concrete trough. Column three indicate the dates on which SDLLT was initiated and figures in brackets indicate the age of plant at the time of SDLLT. Heading dates in column 4 refer to date when all plants in each row flowered. Column 5 and 6 shows total number of short day length treatment and days from sowing to maturity respectively. Plant fertility (%) or seed set (%) shown in column 7 indicate percentage seed of all plants in each row treatment.

recorded a seed set rate of between 6 and 20% with an average of 14%. These were plants given SDLLT on days 57, 61, 65, 69 and 73 after sowing. For rows 6 and 7 seed set rate of 0% was recorded same as ZAU11S106 control line. There was a drastic reduction of 20% seed set recorded in row 4 to 0% seed set recorded in rows 6 and 7. Plants in row 5 received 12 days of SDLLT and recorded 6% seed set while plants in row 6 received 8 days of SDLLT and recorded seed set of 0%. At initiation of SDLLT, plants in row 5 were 73 days old; this was followed with 12 days of the SDLLT treatment after which the plants were left to grow under LDLL growth condition for 16 days when they flowered. The total time in days taken to completion of flowering was 73 days at initiation of SDLLT +12 days of SDLLT +16 days under LDLL growth conditions or 103 days.

Despite the 16 days under LDLL growth conditions before flowering, plants in this row still recorded 6% seed set (Table 1) and some pollen stained blue-black with 1% I/K same as non PGMS control plants of ZAU11F121 (Figure 4e and h). This implies that fertility gene must have been expressed within the 12 days of SDLLT and before the plants were left to grow under LDLL growth conditions. Plants in row 6 received SDLLT for 8 days starting at 77 days old and stopped when they were 85days old. After 8 days of SDLLT these plants were left to grow under LDLL growth conditions and flowered 17 days later, recorded a seed set of 0% (Table 1a) and mature pollen stained yellow with 1% same ZAU11S106 control plants in row 8 (Figure 4f and g) . Difference in SDLLT was 4 days (12 to 8 days) and within this period sterility inducing gene had already been expressed thus a seed set of 0% in row 6. Therefore the critical period when sterility genes were expressed lie between days 73 and 77 after sowing. At 77 days of age, exposure of plants to SDLLT for only 8 days resulted to complete sterility just like plants in the control row 8. At 73 days old, pollen corresponds to tetrad stage of meiosis (Figure 3); at this stage when the PGMS were exposed to SDLLT for only four days above 73 they were irreversibly fertile and LDLL growth conditions could not reverse it. Similarly, plants exposed to LDLL growth conditions for four days above 73 days old became completely sterile and a follow up with SDLLT could not induce any seed set. Growth of ZAU11S106 under LDLL growth conditions or under SDLLT before 73 days of age did not determine if the plant was to be completely sterile or fertile. This is why plants given SDLLT at 73 days old displayed spikelet fertility just like the ones given SDLLT at 57days old.

Panicle development in Figures 3a to e corresponds to growth in row 1 to 5 or to 28 to 12 days of SDLLT or 57 to 73 days of plants’ age at commencement of SDLLT. Pollen from plants in these growth stages had normal meiotic pollen development for ZAU11S106 (Figure 3f to j) same as ZAU11S121 (Figure 3k to o). Pollen from ZAU11S106 plants given SDLLT at 73 days old stained

Figure 1. Sowing pattern of ZAU11S106 and ZAU11F121 for SDLLT. PGMS rice ZAU11S106 was sown in concrete troughs in rows each with 6 plant and allowed to grow for 57 days after which SDLLT was started. Plants were covered from 4.00 to 9.00pm when complete darkness set in so as to be exposed to only 11 h of daylight. Figure a shows plants at initiation of SDLLT and Figure b shows plants at cessation of SDLLT. In Figure 1 a only four rows appear (others were not captured) but Figure b show the total size of trough and number of rows. Plants given SDLLT flowered earlier than those which did not (Figure b).

Figure 2. Effects of SDLT and LDLT on PGMS rice ZAU11S106 panicle fertility. Panicle in Figure 2a was a sample of plants in row 1 to 5 shown in Table 1. These plants which recorded some seed set received SDLLT for 12 to 28 days or when plants were between 57 and 73 days old. Panicle shown in Figure 2b with seedless glumes was obtained in ZAU11S106 sown in row 6 as recorded in Table 1. Plants in this row received SDLLT for only 8 days when they were 77 days old.

yellow or colourless with 1% I/KI indicating that they were of abortive type (Figure 4b). Therefore, SDLLT after 73 days old could not induce fertility and pollen cells were of abortive type. When plants were given SDLLT between 69 and 73 days old for 12 days they were fertile and allowing them to grow under LDLL growth conditions for 16 days could not prevent some pollen to stain blue-black with 1% I/KI solution indicating that they are of fertile type. Transformation of pollen from sterile to fertile and vise versa was found to occur between days 73 and 77 old after sowing. This is the critical period of pollen transformation. Once sterility or fertility reaction take place during the critical period, transformation is irreversible despite the conditions prevailing later. According to Yuan et al. (1993), at critical period, SDLLT make PGMS rice irreversibly fertile while LDLL treatment makes them irreversibly sterile.

Microscopic observation of pollen development from pollen mother cell to tetrad stage from both ZAU11S106 and ZAU11F121 (control) plants with or without SDLLT displayed same meiotic features up to tetrad. ZAU11S106 plants given SDLLT at tetrads stage (row 5) of pollen cell development recoded 6% seed set but those given SDLLT after tetrad (row6) had 0% seed set (Table 1). Apparently, the decision whether glumes will be fertile or sterile is taken between days 73 and 77 (4 days difference). Therefore, ZAU11S106 needed for production of self-breed seed for its own maintenance need to be given SDLLT before 77days old and those needed to be completely sterile for hybrid seedproduction need be given LDLL growth conditions between 73 and 77 days after sowing.

In photosensitivity male sterility (PGMS) rice, LDLL and high temperature induce up to 100% pollen sterility while in short day length growth conditions pollen recover their vitality and become fertile (Xue et al., 1999). Sterility is controlled by three major genes; *pms1, pms2* and *pms3* that have been mapped on chromosomes 7, 3 and 12, respectively (Zhang et al., 1994; Mei et al., 1999).

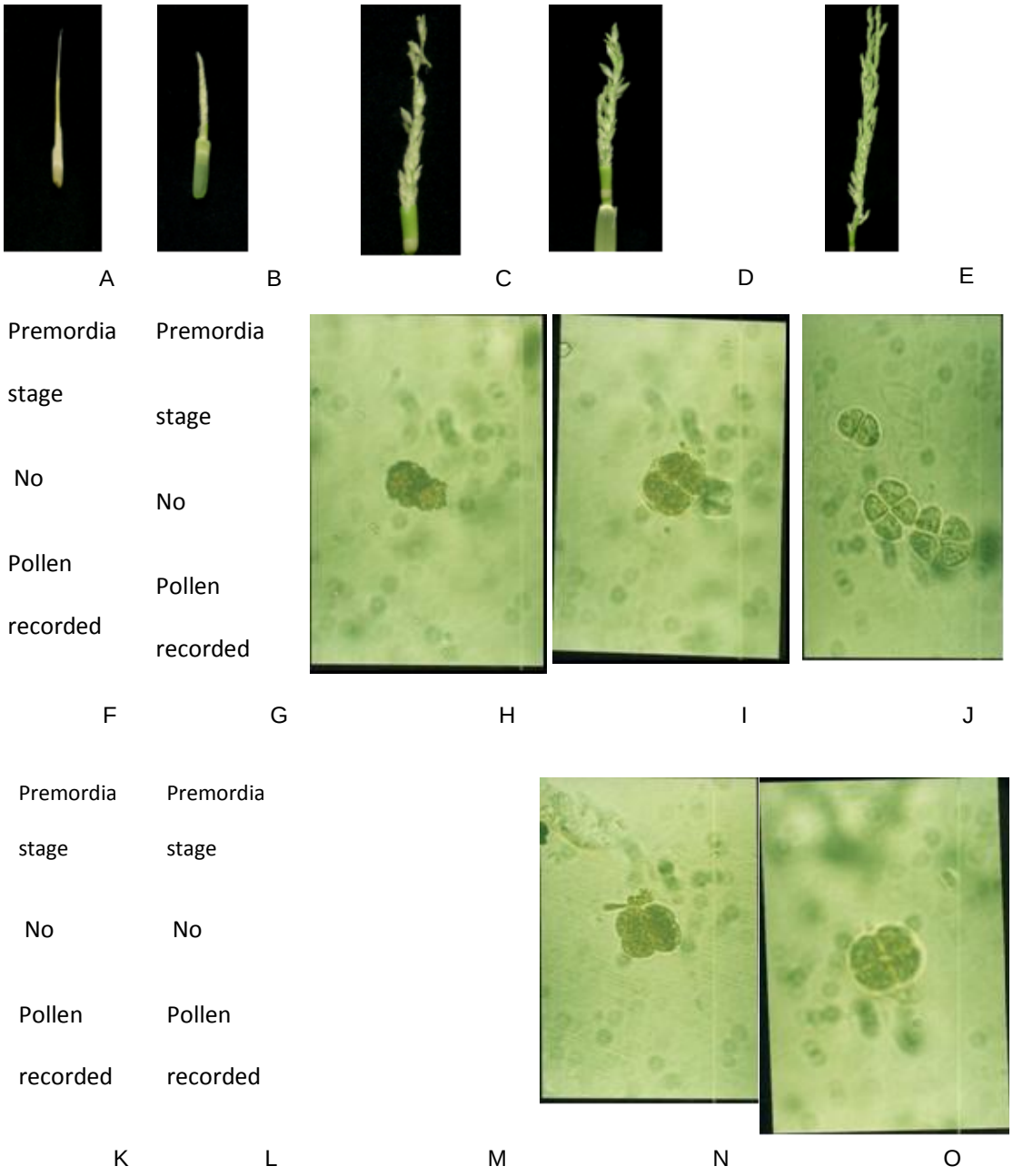


Figure 3. ZAU11S106/ZAU11F121 rice panicles and pollen cells at various stages of development. a to e shows samples of panicles obtained from ZAU11S106 in rows 1 to 5 taken before SDLLT initiation. At this stage panicles from ZAU11S106 and ZAU11S121 did not display any observable difference among them (therefore the samples represent both lines). Meiotic pollen from ZAU11F106 are presented in f to j while those from ZAU11F121are presented in k to o. Samples were taken up to completion (tetrad stage) of meiosis.

According to Yuan et al. (1993) the critical time determining sterility or fertility in PGMS rice is the time from primary premordia through secondary premordia differentiation to differentiation of stamen and pistillate. All pollen (100%) from ZAU11S106 rice given SDLLT at 77 days stained yellow with 1% I/KI and 0% seed set was realized (Figure 4f and g). At 73 days old, a time when pollen development was at tetrad stage of meiosis, SDLLT for four days gave 6% seed set. This is an indication that PGMS gene(s) were expressed between days surrounding tetrad and cytokinesis stages of pollen development or between 73 to 77 days old after sowing.

When breeding for hybrid rice seeds female parent need to be 100% sterile to prevent contamination of hybrid seeds with self-breed seeds. Precise determination of the most critical stage of sterility expressing genes

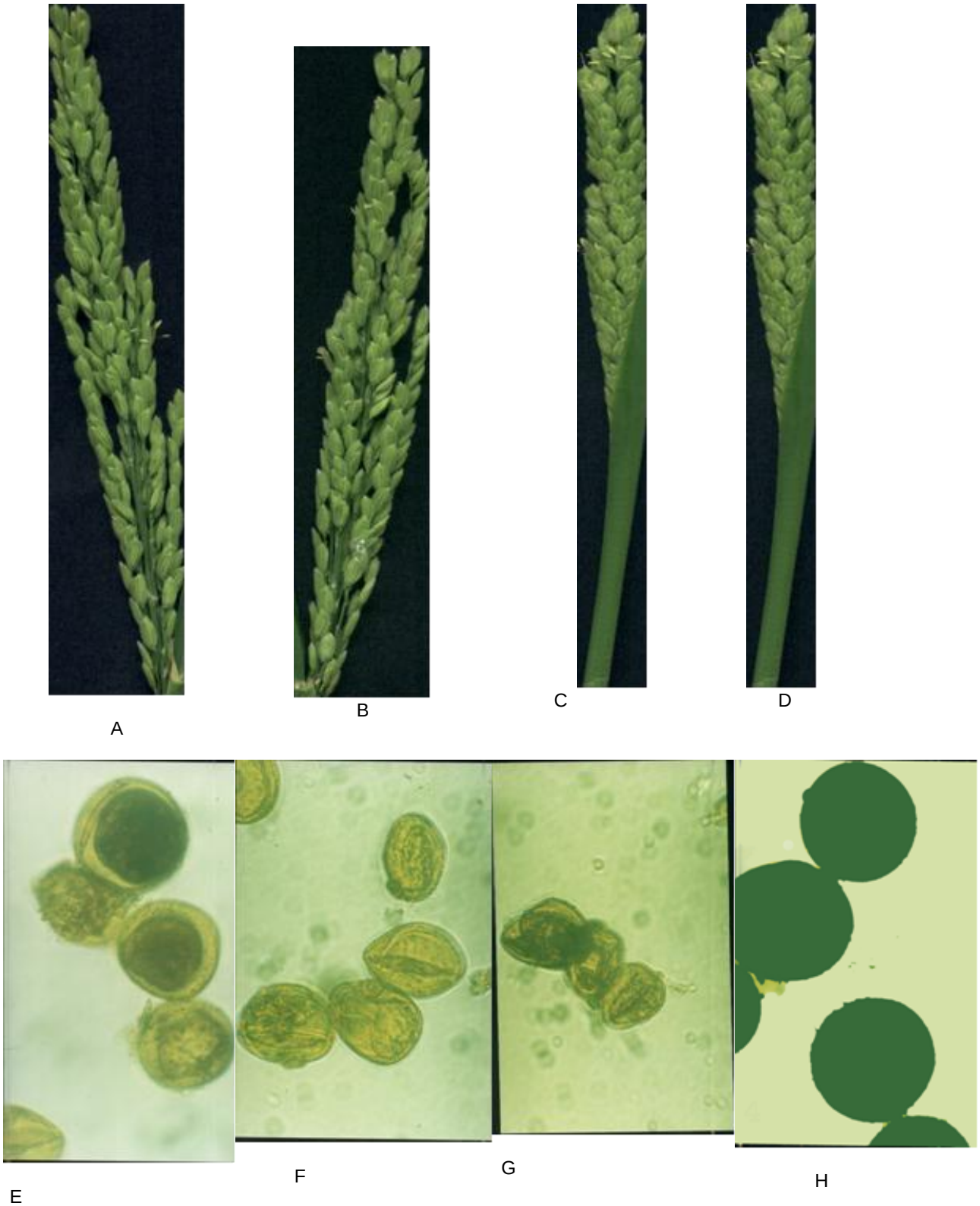


Figure 4. Manifestation of ZAU11S106 and ZAU11F121panilce and pollen cells after critical point of fertility/sterility determination. Figures a, b, c and d shows mature PGMS panicle collected from rows 5,6, 8(sterile control) and Fertile control (ZAU11F121). Pollen extracted from glumes were stained in 1% K/I. Figure e show pollen collected from mature panicle from row5 which shows effect of SDLLT at critical point of fertility determination and some pollen have stains of blue-black, resembling pollen from ZAU11F121 rice panicles in Figure h which stain blue black with 1% K/I. Figures f and g indicate pollen from row and row 8 (control), respectively.

will enable synchronization of sowing, so plants enter primordial stage of flowering in LDLL growth conditions. PGMS require sterility inducing conditions only at the critical sterility determining stage, once this is realized pollen become irreversibly sterile (Njiruh and Xue, 2011). Once expressed, the PGMS gene leads to deformed tapetum and exine (Kaul, 1988; Xu et al., 1995; Njiruh and Xue, 2011). Tapetum is the innermost wall of microsporogium that provide enzyme, hormones and food to the growing pollen mother cells (PMC). Biochemical substances interfere with the normal functioning of the tapetum by cutting off the pollen nourishment system and starve them to death since under sterility inducing conditions anther locules of PGMS plants are occupied by deformed pollen (Ku et al., 2001); such biochemical reaction may have taken place under SDLL growth conditions.

Conclusion

ZAU11S106 was completely sterile when given LDLL growth conditions at dyad and tetrad stage of pollen development. This corresponds to period 73 to 77 days old. Therefore, fertility or sterility was determined within these 4 days.

ACKNOWLEDGEMENT

This research was funded by IFS and Zhejiang University.

REFERENCES

Asia Pulse (1999). China research centre breeding super hybrid rice. Asia Pulse. 14 September 1999.

Burke JM, Arnold L (2001). Genetics and the fitness of Hybrids. Annu. Rev. Genet. 2001. 35:31–52.

Duvick DN (1992). Genetic contributions to advances in yield of U.5. maize. Maydica 37:69-79.

Duvick DN (1997). Heterosis: Feeding People and protecting natural resources. Department of Agronomy, Iowa State University, Ames, IA 50011, U.S.A. Book of Abstracts. The Genetics and Exploitation of Heterosis in Crops. An International Symposium. 17-22 August 1997 Mexico City, Mexico.

Heiling GK (1999). Can China feed itself? A System for Evaluation of Policy Options, International Institute for Applied Systems Analysis, Laxenburg,1999.http://www.iiasa.ac.at/Research/LUC/ChinaFood/argu/impact/ imp_81.htm.

Hossain M, Bennett J, Datta S, Leung H, Khush GS (1999). Biotechnology Research in Rice for Asia: Priorities, Focus and Directions Paper presented at the Conference “Agricultural Biotechnology in Developing Countries: Towards Optimizing the Benefits for the Poor” organized by ZEF and ISAAA in collaboration with AgrEvo and DSE in Bonn, 15-16 November 1999.

Hu CH (1993). Historical Review of Semidwarf Rice and Breeding of a New Plant Type for Sustainable Agriculture. Taichung District Agric. Improvement station. 38:45-63.

Igor VS, Hari KS (1969). High efficiency, heterosis, and homeostasis in mitochondria of wheat. Institute of Life Science, Texas A&A University, College Station. 302-309.

IRRI (1998). Hybrid rice breeding in China, Advances in Hybrid Rice Technology. IRRI. 1998. p. 29.

Kaul MLH (1988). Male sterility in higher plants. Springer Verlag Berlin.

Khush GS (1995). Modern Varieties - Their Real Contribution to Food Supplies and Equity. Geo J. 35(3):275-284.

Khush GS (1994). Increasing the genetic yield potential of rice: prospects and approaches. International Rice commission Newsletter. 43:1-7.

Ku SJ, Cho KH, Cho YJ, Baek WK, Kim S, Suh HS, Chung YY (2001) . Cytological Observation of Two Environmental Genic Male-Sterile Lines of Rice. Mol. Cells 12 (3):403-406.

Kuyek D (2000). Hybrid Rice in Asia: An Unfolding Threat. Current trends in agricultural R&D. Biothai (Thailand), GRAIN, KMP (Philippines), MASIPAG (Philippines), PAN Indonesia, Philippine Greens and UBINIG (Bangladesh), Drs. Romeo Quijano (UP Manila, College of Medicine, Philippines) and Oscar B. Zamora (UP Los Baños, College of Agriculture, Philippines). p. 1-20.

Levings CS III (1990). The Texas Cytoplasm of Maize: Cytoplasmic Male Sterility and Disease Susceptibility. Science 250:492-447.

Lin SY, Ikehashi H, Yanagihara S, Kawashima K (1992). Segregation distortion via male gametes in hybrids between *Indica* and *Japonica* or wide compatibility varieties of rice *(Oryza sativa* L.). Theor. Appl. Genet. 84:812-818.

Mao CX, Deng XL (1993). Two-Line hybrid in China. International Rice Research notes (IRRN). 18:3. Manila, Philippines.

Mei MH, Dai XK, Xu CG, Zhang QF(1999). Mapping and Genetic analysis of the genes for photoperiod-Senstive genic male sterile in rice using the original mutant Nongken58S. Crop Sci. 39:1711-1715.

Njiruh PN, Xue QZ (2011). Programmed cell death-like behavior in photoperiodsensitive genic male sterile (PGMS) rice. Afr. J. Biotechnol. 10(16):3027-3034.

Oka HI (1974). Analysis of genes controlling F_1 sterility in rice by the use of isogenic lines. Genetices 77:521-534.

Shi MS (1981). Preliminary research report on breeding and utilization of the natural two-uses line in late *japonica* rice. Sci. Agri. Hubei. 7:1-3.

Shi MS (1985). The discovery and study of the photosensitive recessive male-sterile (*Oryza sativa L. subsp. japonica*). Scientia Agriculturae Sinica 19:44-48.

Shi MS, Deng JY (1986). The Discovery, Determination and Utilization of the Hubei Photosensitive Genic Male-sterile Rice (Oryza sativa subsp. *japonica*). Acta Genetica Sinica 13(2):107-112.

Virmani SS (1994). Heterosis and Hybrid Rice Breeding. Monographs on Theoretical and Applied Genetics. Springer-Verlag. Berlin Heidelberg, Germany. 22:7(1)

Virmani SS (1996). Hybrid Rice, Adv. Agron. 57. Academic Press Inc. pp. 377-462.

Virmani SS, Kumar I (1997). Hybrid rice seed in Asia-Pacific. Asian Seed Planting Mater. APSA, Bangkok. 4(5):11.

Virmani SS, Sun ZX, Mao TM, Jauhar Ali A, Mao CX (2003). Two-Line Hybrid Rice Breeding Manual. IRRI. Manila, Philippines. pp. 4-85.

Xie F, Hardy B (2009). Accelerating Hybrid rice development. IRRI. Manila, Philippines. pp. 3-8.

Xu H, Knox RB, Tayor PE, Sing MB (1995). Bcp1, a gene required for male fertility in Arabidopsis. Proc. Natl. Acad. Sci. USA 92:2106-2110.

Xue QZ, Etoh K Li H, Zhang NY, Yan JQ, McCouch, S, Earl ED (1999). Production and testing of plants regenerated from proplasts of photoperiod sensitive genic male sterile rice (*Oryza sativa L*.). Euphytica 205:167-172.

Yuan LP (1994). Increasing yield potential in rice by exploitation of heterosis. In: Virmani SS, editor. Hybrid rice technology: new developments and future prospects. IRRI. p 1-6. Manila, Philippines.

Yuan LP (1997). Breeding of super hybrid rice for super high yield production. *Hybrid Rice* (in Chinese). 1:1.

Yuan SC, Zhang ZG, He HH, Zen HL, Lu KY, Lian JH, Wang BX (1993). Review and interpretation of two photoperiod-reactions in photoperiod-sensitive genic male-sterile rice. Crop Sci. 33(4):651-660.

Zhang QF, Zhen BZ, Dai XK, Mei MH, SaghaMaroof MA, Li ZB (1994). Using bulked extremes and recessive class to map genes for Photoperiod-sensitive genic male sterility in rice. Proc. Natl. Acad. Sci. USA 91:8675- 86789.

academicJournals

Vol. 12(47), pp. 6599-6604, 20 November, 2013
DOI: 10.5897/AJB2013.12151
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Growth, phytochemicals and antifungal activity of *Bryophyllum pinnatum* L. subjected to water deficit stress

C. E. Umebese* and F. D. Falana

Department of Botany, Faculty of Science, University of Lagos, P. M. B. 1029, UNILAG Post Office, Akoka – Yaba, Lagos, Nigeria.

Accepted 7 November, 2013

The impact of water stress on the growth, concentration of phytochemicals and antifungal potency of *Bryophyllum pinnatum* L. was investigated. Three weeks old seedlings were subjected to 3, 7 and 10 days water deficit regimes and a control (watered daily). Plant height, number of leaves, whole plant dry weight, net assimilation rate, leaf area ratio and relative growth rate were reduced by 6 to 50% as intervals of water deficit increased. Alkaloids, tannins, saponins and flavonoids were present in all plants and all but alkaloids increased with increase in intervals of water deficit. Alkaloid content decreased by 1.3 to 10.5% while the other phytochemicals increased by 12 to 206% in response to water deficit stress. Ethanolic extracts of plants from the four batches showed varying inhibition zones against *Candida albicans*. The inhibition zones decreased with increasing water deficit intervals corresponding with the decrease in alkaloid content. This research has revealed that water stress increases the tannin, saponin and flavonoid contents of *Bryophyllum* but decreases the alkaloid content.

Key words: *Bryophyllum*, water deficit, phytochemicals, inhibition zone.

INTRODUCTION

Bryophyllum pinnatum L., a perennial herb belonging to the Crassulaceae family and commonly known as resurrection plant, miracle leaf, air plant and life plant in English, is found growing widely in tropical Africa, tropical India, China, Australia and South America as a weed (Okwu, 2003). The phytochemical screening of *B. pinnatum* revealed the presence of alkaloids, flavonoids, phenolic compounds, tannins and saponins. The proximate profile shows that the plant is rich in magnesium, calcium, potassium, phosphorus, sodium, iron and zinc. It also contains vitamins: ascorbic acid, riboflavin, thiamine and niacin (Majaz et al., 2011). Three new components: bryophyllol, bryophollone and bryophollenone have also been isolated from the fresh leaves of *B. pinnatum*. *B. pinnatum* possesses significant antimicrobial, anti-inflammatory, analgesic, antihypertensive, wound healing, hepatoprotective and show neuropharmacological and antidiabetic activities (Kamboj and Saluja, 2009). The presence of phenolic compounds indicate that the plant possess anti-microbial activity. The plant is effective in the treatment of typhoid fever, bacterial and fungal infections particularly by *Staphylococcus aureus*, *Escherichia*

*Corresponding author. E-mail: cumebese@gmail.com.

coli, *Bacillus subtilis*, *S. typitic*, *Candida albicans* and *Aspergillus fumigatus* (Akinpelu, 2000). *B*. *pinnatum* has high saponin content which justifies the use of the extracts to stop bleeding and in treating wounds. Saponin has the property of precipitating and coagulating red blood cells. It has tannins that give astringent properties and strong analgesic potency of its alkaloids (Siddarta and Chaudhuri, 2002). It has antihypertensive, antidiabetic activity and it alters the general behavioural pattern (Ojewole, 2002, 2005). Both qualitative and quantitative variations of secondary metabolites have been shown to occur in response to various types of stress (Watermann and Mole, 1989). Water stress has been shown to increase the concentration of secondary metabolites in plant tissues (Mundree et al., 2002). It has also been shown to reduce leaf artemisin content in *Artemisia annua* (Charles et al., 1993). Thus, environmental factors including water deficit can increase or decrease phytochemicals in plants.

Since some of these compounds may have negative impact on health when taken in high doses or may affect the quality of products, it is important to investigate the extent to which environmental factors alter phytochemicals and subsequent bioactivity of plants. In this study, the impact of water deficit stress on the growth, phytochemicals and subsequent antifungal activity of *B*. *pinnatum* was investigated.

MATERIALS AND METHODS

Plant material

B. *pinnatum* L. plants were collected from the Botanic Garden of the University of Lagos and identified in the Herbarium of the Department of Botany. *C*. *albicans* was isolated and identified in the Pharmaceutical Microbiology Unit, Faculty of Pharmacy, University of Lagos. Pure culture of the fungus was obtained and later used for inoculation.

Planting procedure

Fresh leaves were placed in bowls containing water for faster germination of bulbils. After three weeks, 100 plants were transplanted into planting bags containing 2.5 kg of loamy soil. The young plants were separated into four groups of 25 plants and one batch each was subjected to daily watering (control), 3 days water deficit (WD), 7 days WD and 10 days WD regimes and each time 200 ml water was used for watering.

Growth parameters

Growth parameters (plant height, number of leaves, whole plant dry weight, leaf area, leaf area ratio, net assimilation ratio and relative growth rate) were measured in 10 and 14 weeks old plants using methods outlined by Eze (1965) and Noggle and Fritz (1976).

Preparation of plant extract

Plant samples were oven dried at 40°C, powdered and soaked in 300 ml of ethanol for 72 h. The crude ethanol extract was filtered and the filtrate was evaporated into dryness over a water bath (at 45°C) and weighed.

Phytochemical screening

Phytochemical screening was carried out on the extract and on the dried powdered specimens using standard procedures as described by Sofowora (1993), Trease and Evans (1989) and Harborne (1973). The phytochemicals tested were alkaloids, tannins, flavonoids and saponins.

Quantitative determination of the total amount of alkaloids

Alkaloid concentration was determined by the procedure outlined by Harborne (1973). Tannins were determined by the method of Van-Burden and Robinson (1981), flavonoids by Bohm and Kocipai-Abyazan (1994) and saponins by Obadoni and Ochuko (2001).

Determination of the total amount of alkaloids

Five grams (5 g) of the sample was mixed with 220 ml 10% acetic acid in ethanol, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed (Harborne, 1973).

Determination of the total amount of tannins

500 mg of the sample was mixed with 50 ml of distilled water and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then, 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min (Van-Burden and Robinson, 1981).

Determination of the total amount of flavonoids

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The solution was filtered through whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight (Bohm and Kocipai-Abyazan, 1994).

Determination of the total amount of saponins

20 g of each powdered sample were mixed with 100 ml 20% aqueous ethanol and placed in a hot water bath at 55°C for 4 h with continuous stirring. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added to the aqueous

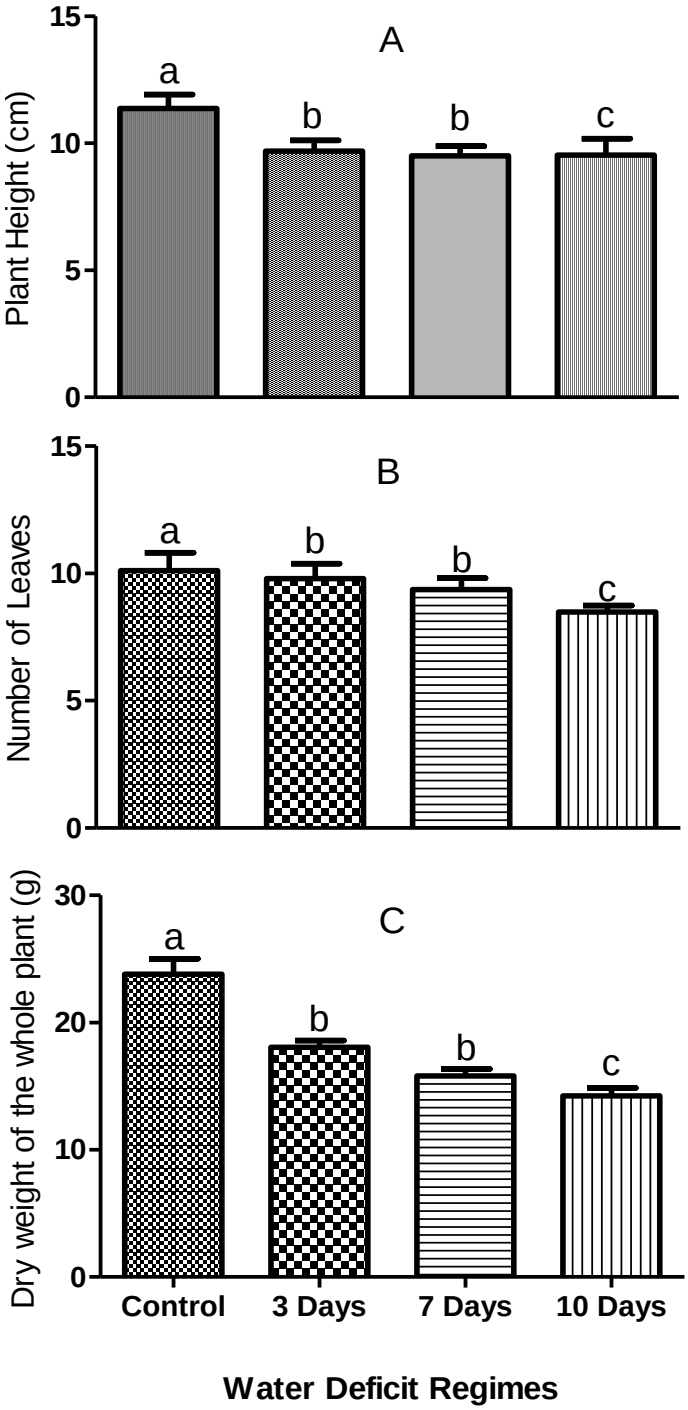


Figure 1. Mean height (A), number of leaves (B) and whole plant dry weight (C) of *Bryophyllum* plants subjected to different water deficit regimes (bars with similar letters for each parameter are not significantly different at p< 0.05 using Duncan's multiple range test.

extract. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight (Obadoni and Ochuko, 2001).

Innoculation and incubation

25 ml Saboraund dextrose agar (SDA) was measured aseptically and 1 ml of the calibrated organism was mixed with the medium and poured into a sterile Petri dish and was mixed and allowed to solidity. When the plates set, wells were bored using a cork borer of size 10 mm. Then, 0.2 ml of each extract and standard were introduced into the wells with the use of 1 ml sterile syringe. It was covered and kept in the incubator for 48 h at room temperature while the presence of measurable zone of inhibition was observed. Means of three replicates were recorded with the standard errors. Test of significance between treatments was done using analysis of variance (ANOVA) and Duncan's Multiple Range Test.

RESULTS AND DISUSSION

B. pinnatum L. subjected to 3, 7 and 10 days water deficit had marked reduction in growth. Plant height, number of leaves, whole plant dry weight, leaf area ratio, net assimilation rate and relative growth rate were reduced by 6 to 50% with greater reductions as intervals of water deficit increased (Figures 1 and 2). This corroborates an earlier study by Umebese et al. (2009) that water stress treatments lead to reduction in water potential in amaranth and tomato plants resulting in marked decreases in growth. The reduction in growth during water deficit stress has been attributed to the formation of reactive oxygen species (ROS). ROS which include oxygen ions, free radicals and peroxides, form as a natural by-product of the normal metabolism of oxygen and have important role in cell signaling. However, during environmental stress such as drought, ROS levels increase dramatically resulting in oxidative damage to proteins, lipids and DNA (Devasagayam et al., 2004). The reduction in growth caused by water deficit stress may have resulted from oxidative damage. The phytochemical screening and quantitative estimation of the phytochemicals of *Bryophyllum* showed that alkaloids, tannins, flavonoids and saponins were present in both stressed and unstressed plants (Table 1). These four phytochemicals are among those reported by Majaz et al. (2011) to be present in *Bryophyllum*; others being phenolic compounds, bryophyllol, bryophollone, bryophollenone and bufadienolides.

According to Cowon (1999), flavonoids, carotene and tannins are among several plant products utilized as antimicrobial agents, along with quinines, coumarines and terpenoids. Egunjobi (1969) reported that medicinal plants with high concentration of tannins, flavonoids and saponins are of greater medicinal uses and can be used as livestock and poultry feed. The water deficit treatments had resulting impact on the concentrations of phytochemicals of *Bryophyllum* plants (Figure 3). As the water deficit intervals increased from 3 to 10 days, the concentra-

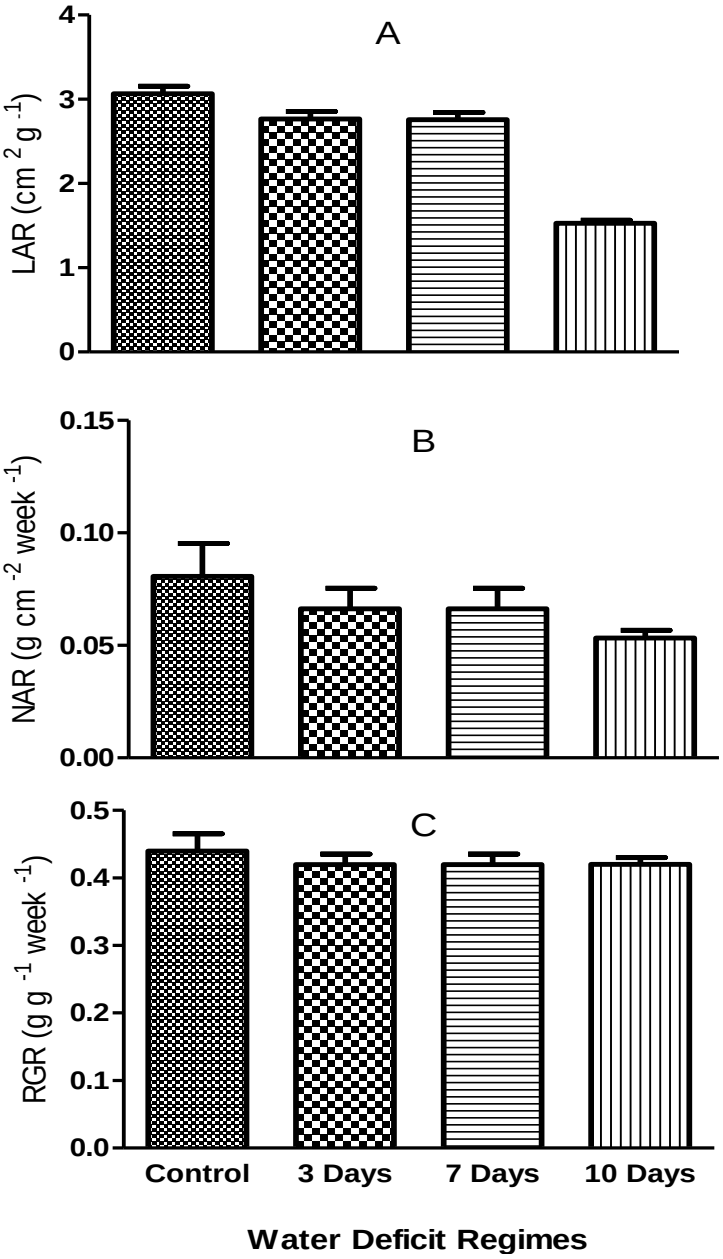


Figure 2. Leaf area ratio (A), net assimilation rate (B) and relative growth rate (C) of *Bryophyllum* plants subjected to different water deficit regimes.

Table 1. Qualitative analysis of the phytochemicals of *Bryophyllum* plants subjected to water deficit (WD).

Phytochemical	Control	3 days WD	7 days WD	10 days WD
Alkaloids	++	++	++	+
Tannins	+	++	+++	+++
Saponins	+	+	++	++
Flavonoids	+	+	+	++

+, Slightly present; ++, moderately present; +++, highly present.

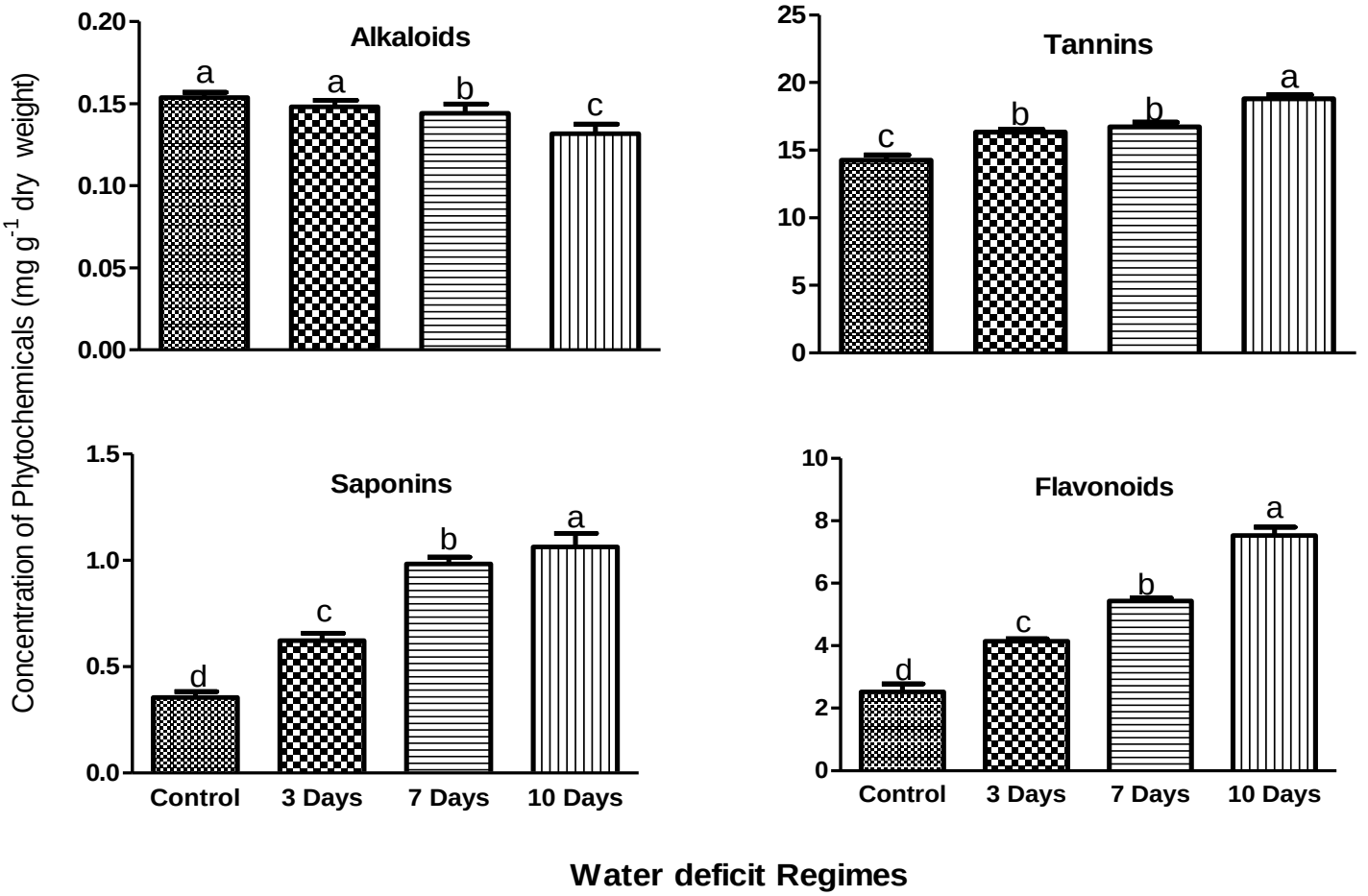


Figure 3. Concentrations of alkaloids, tannins, saponins and flavonoids of *Bryophyllum* plants subjected to different water deficit regimes (bars with similar letters for each phytochemical are not significantly different at p< 0.05 using Duncan's multiple range test.

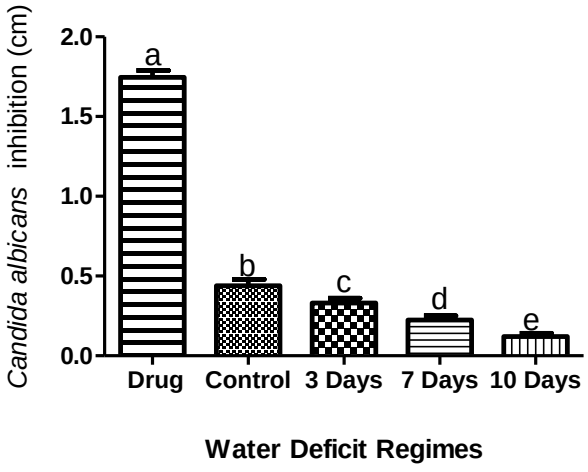


Figure 4. Mean inhibition lengths of *Candida albicans* by extracts of *Bryophyllum* plants subjected to different water deficit regimes (bars with similar letters for each phytochemical are not significantly different at p< 0.05 using Duncan's multiple range test.

trations of tannins increased by 12.9 to 29.5%, flavonoids increased by 22.1 to 45.1%. The increase in saponin content was remarkable; by 60 to 206%, respectively. The alkaloid content was reduced by water stress by 1.3 to 10.5%. Mundree et al. (2002) had earlier shown that water deficit increases the concentration of metabolites. Water stress leads to increases in cyanogenic glycosides, glucosinolates, terpenoids, alkaloids and tannins (David, 1998). However, the observed decrease in alkaloid concentration as a result of water deficit is supported by Belesky (1989) that water deficit regime decreases pyrrolizidine alkaloid yield in endophytes. It appears that not all plant secondary metabolites respond in similar ways to environmental stress. Many phytochemicals participate in the detoxification of active oxygen and are important for plant responses to biotic or environmental stress (Caldwell et al., 2005). Jain et al. (2008) and Tatsimo (2012) reported the antifungal property of *Bryophyllum* against the following fungi: *C*. *albicans*, *C. glabrata*, *C. tropicalis* and *C. pseodotropicalis.* The ethanolic extracts of the treated

plants showed varying lengths of inhibition zones of *C. albicans*; decreasing with increase in water deficit interval (Figure 4). This implies that the more the water deficit stress *Bryophyllum* is subjected to, the less is its potency against the growth of fungus.

The decrease in inhibition of *C. albicans* corresponded with the decrease in alkaloid content. Since concentrations of tannins, saponins and flavonoids increased with increasing water deficit intervals while alkaloids decreased corresponding with reduced inhibition of fungal growth, alkaloids may form the active antifungal property of *B. pinnatum*. Inhibition growth of *C. albicans* was remarkably high with the control antifungal drug (clotrimazole) compared with the plant extracts. This may imply that the concentration of extracts should be increased to obtain close antifungal activity.

Conclusion

Water deficit stress caused marked reduction in growth and increased the concentrations of tannins, flavonoids and saponins while the alkaloid content was reduced. The inhibition zones decreased with increasing water deficit intervals corresponding with the decrease in alkaloid content. Since the concentration of extracts used in this study showed low inhibition zones, higher concentrations of extracts of *Bryophyllum* may be required in the treatment of diseases caused by *Candida* spp.

REFERENCES

Akinpelu DA (2000). Antimicrobial Activity of *Bryophyllum pinnatum* Leaves. Fitoterapia 71(2):193-194.

Belesky CB (1989). Influence of Endophyte and water regime upon tall fescue accessions II pyrrolizidine and Ergopeptine alkaloids. Oxford J. 64(3):343-349.

Boham BA, Kocipal-Abyzan R (1994). Flavonoids and condensed tannins from leaves of *Hawaiian vaccinium vaticulatum and V. calycinium.* Pacific Sci. 48**:**458-463.

Caldwell CR, Britz SJ, Mirecki RM (2005). Effect of temperature, elevated carbon dioxide and drought during seed development on the isoflavone content of soybean (*Glycine max* L.) grown in controlled environments. J. Agric. Food Chem. 53:1125-1129.

Charles DJ, Simon JE, Shock CC, Feibert EB, Smith RM (1993). Effect of water stress and post-harvest handling on artemisinin content in leaves of *Artemisia annua* L. In: Janick J, Simon JE (eds.), New crops. Wiley, New York.

Cowan MM (1999). Plant Products as antimicrobial agents. Clinical Microbiol. Rev. 12:564-582.

David SS (1998). Plant Secondary Metabolism. Springer USA.

Devasagayam TPA, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD (2004). Free Radicals and Antioxidants in Human Health: Current Status and Future Prospects. J. Assoc. Phys. India 52: 796.

Egunjobi JK (1969). Some common weeds of West Africa. *Bull. Resource Division.* Ministry of Agriculture. National Resources western State, Ibadan, Nigeria.

Eze JMO (1965). Studies on Growth Regulation, Salt uptake and Translocation Ph.D. Thesis. University of Durham, England.

Harborne JB (1973). Phytochemical Methods, London. Chapman and Hall, Ltd.

Jain VC, Shah DP, Patel NM (2008). Microscopical and Preliminary Phytochemical Studies on Leaves of *Bryophyllum pinnatum*. J. Pharm. Res. 1(2):130-232.

Kamboj A, Saluja AK (2009). *Bryophyllum pinnatum* (Lam.) Kurz: Phytochemical and pharmacological profile: A review. Phcog. Rev. 3(6):364-374.

Majaz QA, Tatiya AU, Khurshid M, Nazim S, Siraj S (2011).The Miracle Plant (*Kalanchoe pinnata*): A Phytochemical and Pharmacological Review. Inter. J. Res. Ayur. Pharm. 2(5):1478-1482.

Mundree S, Baker B, Mowla, S, Govender K, Maredza S, Willingen A, Muyanga S, Farrant J, Thomson J (2002). Physiological and molecular insights into drought tolerance. Afr. J. Biotech. 1**:**28-33.

Noggle GR, Fritz GJ (1976). Introductory Plant Physiology. Prentice-Hall Publishers. New Jersey.

Obadoni BO, Ochuko PO (2001). Phytochemical studies and comparative efficacy of the crude extracts of some homeostatic plants in Edo and Delta states of Nigeria. Global J. Pure and Appl. Sci. 7 (3):455-459.

Okwu DE (2003). The potentials of *Ocimum gratissimum, Pengluria extensa* and *Tetrapleura tetraptera* as spice and flavouring agents. Nig. Agric. J. 34:143-148.

OjewoleJA(2002).Antihypertensivepropertiesof*Bryophyllumpinnatum* (Clam; Oken) leaf extracts. Am. J. Hypert. 15(4):143-148.

Ojewole JA (2005). Anti-nociceptive, Anti-inflammatory and Anti-diabetic Effects of *Bryophyllum pinnatum* (Crassulaceae) Leaf Aqueous Extract. J. Ethnopharm. 99(1):9-13.

Siddarta PAK, Chaudhuri N (2002). Studies on the anti-ulcer activity of *Bryophyllum pinnatum* leaf extract in experimental animals. J. Ethnopharm. 30:97-102.

Sofowora A (1993). Medicinal plants and Traditional medicine in Africa. Spectrum Books Ltd, Ibadan, Nigeria.

Tatsimo SJN, Tamokou JD, Havyarimana L, Csupor D, Forgo P, Judit H, Kuliate J, Tane P (2012). Antimicrobial and antioxidant activity of Kaempferol rhamnoside derivatives from *Bryophyllum pinnatum*. BMC Res. Notes 5:158-186.

Trease GE, Evans WC (1989). Pharmacognsy. 11th edition. Brailliar Tiridel Can. Macmillian Publishers.

Umebese CE, Olatimilehin TO, Ogunsusi TA (2009). Salicyclic Acid protects Nitrate reductase activity, growth and proline in Amaranth and Tomato Plants during water deficit. Am. J. Agric. Biol. Sci. 4(3):224-229.

Van-Burden TP, Robinson WC (1981). Formation of complexes between protein and Tannin acid. In: Medicinal plants and Traditional medicine in Africa. A Sofowora ed. Spectrum Books Ltd, Ibadan, Nigeria. p. 289.

Watermann PG, Mole S (1989). Extrinsic factors influencing production of secondary metabolites in plants. CRC press. USA.

academicJournals

Vol. 12(47), pp. 6611-6617, 20 November, 2013
DOI: 10.5897/AJB2013.12191
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Micropropagation of *Dioscorea alata* L. through nodal segments

Supriya Das[1*], Manabendra Dutta Choudhury[1] and Pranab Behari Mazumdar[2]

[1]Department of Life science and Bioinformatics. Tissue Culture Laboratory, Assam University, Silchar.
[2]Department of Biotechnology. Assam University, Silchar.

Accepted 27 August, 2013

Yams (*Dioscorea*) are well known for their medicinal use as well as nutritional values. *Dioscorea* plants are rapidly vanishing from nature due to their over-exploitation by human beings. In order to conserve *Dioscorea* plants, the present investigation was carried out with a view to regenerate plantlet of *Dioscorea alata* L. through *in vitro* culture using full strength Murashige and Skoog (MS) medium and indole-3-acetic acid (IAA) with and without sucrose. Nodal vine segments of *D. alata* were used as explants and nodal segments were cultured on MS (Murashige and Skoog's) medium supplemented with different concentrations of auxin (IAA) for axillary bud proliferation. Best shoot proliferation was observed in MS medium containing 1.5 mg/L kinetin + 2 mg/L IAA with highest rate of shoot multiplication (average of 9.90 shoots/explants). Micro shootlets were inoculated in half strength MS basal medium supplemented with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) of IAA and best rooting was observed in medium supplemented with 2.5 mg/L IAA with highest root length (8.14 cm). Regenerated plants were transplanted in hardening medium containing Brick bats + Charcoal + Dried moss + Leaf molds + Soil in 1: 1: 1:1:1 proportion. Maximum survival percentage was observed as 85 to 87% after one month of transfer in hardening medium. This work proposes an economic technique for the conservation of *D. alata.*

Key words: Yams, *Dioscorea alata* L., *in vitro culture,* growth regulators.

INTRODUCTION

Monocotyledonous *Dioscorea* is known as yam. The genus *Dioscorea* includes 600 species and is of considerable economic importance (Ayensu, 1972). Many wild *Dioscorea* species are a very important source of secondary metabolites used in pharmaceutical industry and medicine. A number of *Dioscorea* wild species are sources of compounds used in the synthesis of sex hormones and corticosteroids (Coursey, 1967) and cultivated species are the sources of food in some countries (Coursey, 1976). *Dioscorea alata* L. is an important tuber crop and is a staple food for millions of peoples in tropical and subtropical countries (Edison et al., 2006). Root and tuber crops are the most important food crops after cereals. Tuber crops find an important place in the dietary habits of small and marginal farmers especially in the food security of tribal population. India holds a rich genetic diversity of tuber crop especially yam (Hann, 1995).

Yams are valuable source of carbohydrates, fibers and low level of fats which make them a good dietary nutrient; they are also processed into various staple intermediate and end product forms (Jaleel et al., 2007). *D. alata* L. is a slender creeping vine reaching a length of several meters; it is a glabrous twinner, with a peculiar 4 winged stem. Its leaves are simple, opposite, ovate, cordate and

*Corresponding author. E-mail: Supriya1august@gmail.com. Tel: +91 9401238157.

acuminate. The flowers are unisexual and fruits are capsules. The plant has many aerial tubers that also aid in reproduction. Underground tubers are large and have many branches; the colour is dark brown and they are fleshy. It is edible and used as a staple food. Powdered tubers are used as a remedy for piles, gonorrhea and are applied externally to sores.

The tubers of some species of *Dioscorea* are important sources of diosgenin, a chemical used for the commercial synthesis of sex hormones and corticosteroids, which are widely used for anti-inflammatory, androgenic and contraceptive drugs (Satour et al., 2007). Many species of *Dioscorea* genus are economically important crops worldwide. For example, *D. alata, D. Cayenensis,* and *D. rotundata* are main crops in Caribbean Central and South America and West Africa (Tor et al., 1998). Out of six hundred species of *Dioscorea,* 14 are used as edible tubers. Tubers have a dual agricultural function. They supply nourishment as a source of food and also act as planting materials (Craufurd et al., 2006). *Dioscorea* species are vegetatively propagated by using tuber pieces. The production of tuber is hampered by several significant virus and fungal diseases (Saleil et al., 1990).

Induction and growth of micro tubers in *Dioscorea* has been found to be under the control of many factors. Among the environmental factors, photoperiod (Ng, 1988) is documented as important factor. *In vitro* propagation of some economically important *Dioscorea* sp. has been achieved using nodal cuttings (Chaturvedi, 1975), bulbils (Asokan et al., 1983), zygotic embryos (Viana and Mantell, 1989), meristem tips (Malaurie et al., 1995), immature leaves (Kohmura, 1995) and roots (Twyford and Mantell, 1996). Micro-propagation has been done in *D. abyssinica* (Martine and Cappadocia, 1991), *D. alata* (Mantell and Hugo, 1989), *D. batatas* (Koda and Kikuta, 1991), *D. Weightii* (Mahesh et al., 2010), *D. floribunda* (Sengupta et al., 1984). *D. alata* was also cultured by using liquid medium (Jova et al., 2011) and the effect of temporary immersion system on the growth of *D. alata* plantlet was also investigated (Yan et al., 2011).

Yam propagation by seeds using conventional methods is slow and not adequate for rapid multiplication. Tuber yield is drastically reduced by viral and nematode infections; through infected tubers it is transmitted to the next generation (Ng, 1992) and it also deteriorates the quality of the tuber (Mitchell and Ahmed, 1999). *In vitro* propagation may help to overcome constraints related to the availability of high quality of planting material (Wheatley et al., 2005; Vaillant et al., 2005). In the present investigation an attempt was made to cultivate *D. alata* L. *in vitro* using minimum number of growth regulators, so that the plant can be regenerated in mass for general use as well as commercial exploitation in minimum cost. Effect of indole acetic acid (IAA) on shoot and root initiation was also studied. It was noticed from review of literature, many workers propagated *D. alata* using growth regulators kinetin, benzyl aminopurine (BAP), naphthalene acetic acid (NAA), indole butyric acid (IBA) but effect of IAA was not investigated; so this work was done to explore this ground.

MATERIALS AND METHODS

Explant source

Field grown plants of *D. alata* L. propagated from a wild tuber were used as source of explants for *in vitro* culture.

Sterilization

The nodal segments were washed in tap water for some time and disinfected with 0.1%(w/v) mercuric chloride ($HgCl_2$) for 5 min followed by thorough rinsing in autoclaved distilled water for at least 7 to 8 times. The surface sterilized explants had length of 0.5 to 1cm containing single node.

Culture medium and condition

MS medium was selected for *in vitro* culture of explants; the pH of the medium was adjusted with 1N HCl and 1N NaOH solution between 5.7 to 5.8. After adjusting the pH, agar powder was mixed with the medium and boiled for some time to obtain clear solution. Thereafter, 40 ml of medium was taken in each 100 ml screw capped bottle autoclaved at 121°C at 15lbs/sq inch for 20 min.

The screw capped bottles containing the medium were then allowed to cool for 24 h in the culture laboratory. The surface sterilized explants were placed vertically on the MS media with and without growth regulators. Various concentrations of indole acetic acid (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) and kinetin (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) were used to observe its effect on axillary bud proliferation, shoot initiation and root initiation.

The cultures were maintained at 25 ± 1°C under 16 h photoperiod provided by white fluorescent tubes. After every two weeks, the culture materials were transferred into new medium for better growth of plants.

Acclimatization

The plants were found to be ready for transplanting in hardening medium after five months as they developed sufficient shoots and roots. Rooted plants were removed from the culture tube and roots were washed thoroughly to remove the medium. Then the plantlets were transferred to sterile small plastic cups containing vermiculite (a sterile inert medium for planting transferred plants) and kept inside the tissue culture room for acclimatization before exposing to the natural environment. During this period, the plants were sprayed with liquid MS medium without agar and sugar, thrice in a week. After one month. The plants were transferred to acclimatization media and medium comprised pre sterilized Brick bats + charcoal + dried moss + leaf molds + soil (1:1:1:1:1).

Statistical analysis

Ten explants were used per treatment on each multiplication and rooting medium. Experiments were repeated thrice and collected data were analyzed by using ANOVA; variation among means was compared by the Post- Hoc Multiple Comparison test at $P < 0.05$ level of significance.

RESULTS

The effect of various concentrations of IAA (0.5, 1.0, 1.5,

Table 1. Effect of various concentrations of IAA on axillary bud proliferation (Data scored after 40 days, 10 replicates for each treatment, repeated thrice).

Treatment	Hormonal supplements IAA (mg/L)	Percentage of explants response	Days to bud break
T0	0	46	35-37
T1	0.5	40	28-30
T2	1.0	53	15-17
T3	1.5	62	12-14
T4	2.0	68	7-9
T5	2.5	59	11-13

Values represent mean ± SE.

Table 2. Shoot formation in nodal explants of *Dioscorea alata* L. cultured on MS medium supplemented with various concentrations of Kinetin and IAA (10 replicates per treatment, data scored after three month, repeated thrice).

Treatment	Hormonal supplements (mg/L)		Mean no. of shoot /explant	Mean no. of shoot/explant ± SE	Mean shoot length (cm)	Mean shoot length ± SE
	IAA	KN				
T0	0	0	1.5	1.5±0.22	1.50	1.50±0.02
T1	2.0	0.5	1.9	1.9±0.23	6.62	6.62±0.12
T2	2.0	1.0	5.6	5.6±0.30	7.09	7.09±0.12
T3	2.0	1.5	7.7	7.7±0.29	9.90	9.90±0.11
T4	2.0	2.0	3.8	3.8±0.19	5.86	5.86±0.05
T5	2.0	2.5	3.1	3.1±0.23	4.90	4.90±0.05

Values represent mean ± SE.

2.0 and 2.5 mg/L) on axillary bud breaking of *D. alata* L. is listed in Table 1. Explants cultured on MS medium without IAA showed proliferation of axillary bud but required maximum time (35 to 37 days). Addition of low concentration (0.5 mg/L) of IAA was less effective in bud breaking and it took 28 to 30 days for proliferation, which was the second highest time period for bud proliferation. Nodal segments cultured on media with IAA (concentration 2.0 mg/L) proliferated within 7-9 days and percentage of explants' response (68) was also very satisfactory. Nodal segments cultured on MS medium with 1.5 mg/L IAA took a second minimum time period (12 to 14) for bud proliferation and it showed impressive response (62%) of explants also.

Bud proliferation was enhanced by the addition of IAA. After bud proliferation, for further growth, cultured plants were transferred to the media supplemented with kinetin and auxin (IAA). Response of different concentrations of kinetin with 2.0 mg/L concentration of IAA was recorded in terms of number of shoot and shoot length. 2.0 mg/L IAA when supplemented with MS media showed effective result in bud breaking. This concentration of IAA combined with different concentrations of kinetin was selected to study its effect on shoot proliferation in terms of shoot length and number of shoot per explants. Data obtained from the study are shown in Table 2.

MS medium with growth regulators produced better result in terms of percentage of explants' response, number of shoots/explants and average shoot length. Of the combination tested, MS+ kinetin (1.5 mg/L)+ IAA (2.0 mg/L) elicited optimal response, in which an average of 7.7± 0.29 shootlets with a mean shoot length of 9.90 ± 0.11cm per explant was recorded. The second highest shoot proliferation in terms of shoot number and shoot length was observed in the MS medium +Kinetin (1.0 mg/L)+ IAA (2.0 mg/L), in which 5.6±0.30 shootlets per explants with shoot length of 7.09 ± 0.12 cm was recorded. The well grown shoots were transferred to half strength MS medium containing IAA. The rooting responses of shoots in different concentrations of IAA were measured in terms of days required for root initiation, mean no. of roots/shoots and mean root length. Data are represented in Table 3. In different concentrations of IAA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L), the response of shoots was recorded.

IAA enhanced rooting and data were recorded. Shoots cultured on half strength MS media without any hormonal supplementation were unable to produce root; in some shoots, rooting was observed but in very negligible amount. Media supplemented with lower concentration (0.5 mg/L) of IAA produce few (mean 2.6±0.16) number of root with minimum root length (mean1.39 cm). Media treated with higher concentration of IAA (2.0, 2.5 and 3 mg/L) respond well .Half strength MS + 2.5 mg/L showed more impressive result where about 62% explants responded with average root length of 8.14 cm. In terms of response of explants, two concentrations of 2.5 and 3.0 mg/L showed

Table 3. Influence of different concentrations of IAA on rooting of *in vitro* generated shootlets of *Dioscorea alata* L. (Data scored after 3 month of inoculation, 10 replicates per treatment, repeated thrice).

Treatments	Concentration of hormone mg/L	Days to root initiation	% of explants response	Mean root number	Mean root number ± SE	Mean root length (cm)	Mean root length ± SE
T0	0	-	-	-	-	-	-
T1	0.5	60-62	46	2.6	2.6±0.16	1.39	1.39±0.02
T2	1.0	51-53	49	3.8	3.8±0.13	2.05	2.05±0.03
T3	1.5	43-45	58	4.9	4.9±0.17	3.73	3.73±0.07
T4	2.0	36-38	63	5.7	5.7±0.15	7.77	7.77±0.09
T5	2.5	22-24	62	6.6	6.6±0.16	8.14	8.14±0.06
T6	3.0	32-34	59	4.1	4.1±0.23	5.36	5.36±0.07

Values represent mean ± SE.

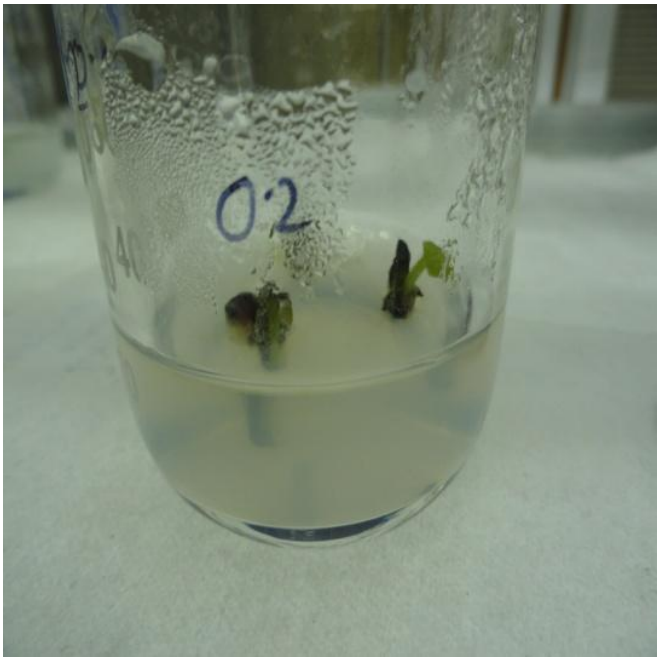


Figure 1. *Dioscorea alata* L. showing bud proliferation.

more or less similar result (62 and 59%, respectively). Shoots treated with 2.0 mg/L IAA showed the second highest response with root number of 5.7 and mean root length of 7.7 cm.

Plants were found to be ready for transplanting in hardening medium after five months. Rooted plants were removed from culture tube and washed thoroughly to remove adhering gel. They were then transplanted to sterile plastic cups containing vermiculite and kept inside the growth chamber. During this period, the plants were sprayed with liquid MS medium without agar and sugar. The plants were dipped in 2 mg/L Diethane for two minutes as precaution to resist fungal infection. After 1 month they were transplanted to earthen pots containing mixture of Brick bats + soil + Charcoal + Dried moss + Leaf mold (1:1:1:1:1).

The acclimatized plants grew normally without any morphological variation. 85 to 87% acclimatized plants survived.

DISCUSSION

Micro-propagation of various plant species including many medicinal plants have been described by many authors during the last two decades (Skirvin et al., 1990). In the present investigation micro-propagation of *D. alata* was done and for that nodal segment was used as explants. MS media supplemented with IAA and kinetin was used for root and shoot growth. In this work, we cultured *D. alata* in solid MS medium; similarly *in vitro* propagation of other yam species is tried by Martine and Cappadocia (1992) using solid medium.

Nodal segments of *D. alata* were cultured on MS media supplemented with auxin (IAA); within 7 to 9 days (Table 1) axillary bud proliferated (Figures 1 to 4) in culture media supplemented with IAA (2 mg/L). Results of many literatures indicate that addition of either IAA or NAA in culture medium improved shoot growth in a number of species and completely supported the effect of IAA on bud breaking in the present investigation. MS media was selected as culture media. Chen et al. (1995), while working with *Eucommia colomoides,* reported that MS medium is effective than WPM (Woody Plant Medium) and their report supports the findings of the present work where also MS medium was used as culture medium and satisfactory growth was observed using nodal segments as explants. For axillary bud proliferation nodal segments culture is recommended (Narula et al., 2007) for rapid clonal propagation when working with *D. bulbifera.*

In the present work, two growth regulators were added with full strength MS medium and results indicated that MS media supplemented with Kinetin (1.5 mg/L) and IAA (2.0 mg/L) induced maximum number of shoot per explant (mean no. 7.7) with highest shoot length of 9.90 cm (Table 2, Figures 5 and 6). Effects of different concen-concentration of Kinetin and indole acetic acid are shown in Table 2. It is observed that for shoot proliferation cyto-

Figure 2. Multiple shoot regeneration.

Figure 3. Showing Growth after 45 days.

Figure 4. Showing Growth after 75 days.

Figure 5. Showing Shoot number in regenerated plants.

cytokinin required optimal quantity in many genotypes but addition of low concentration of auxin with cytokinin triggered shoot proliferation (Sengupta et al., 1984). In this study, low concentration (1.5 mg/L) of kinetin with high concentration (2.0 mg/L) of IAA induced shoot proliferation; further increase of kinetin concentration after optimal concentration (1.5 mg/L) decreased shoot length. The result was supported by this work which shows that suppression of kinetin increases shoot length, node number and root length (Jha and Jha, 1998). In *Dioscorea composita* only auxin NAA, IAA and IBA at 1.25 and 2.5 mg/L account for promotive effects of *in vitro* shoot growth (Ondo et al., 2007).

Our experimental result is completely different from the cited work. The effects of auxins and cytokinins on shoot multiplication of various medicinal plants are reported by many workers (Alizadeh et al., 1998; Ahuja et al., 1982). Explants culture in 2.0 mg/L KN + 1.0 mg/L BAP + 0.5 mg/L NAA showed that highest rate of shoot multiplication is reported when working with *D. alata* (Borges et al., 2009) and rooting is more profuse in half strength MS basal media with 2.0 mg/l NAA. But in the present investigation, it was clearly observed that MS media supplemented with only auxin (IAA 2.0 mg/L) was effective in axillary bud proliferation and it initiated shoot formation within 7- 9 days. Kinetin was used to increase the shoot

Figure 6. Regenerated plants established in pots.

length. Explants cultured on media (MS + 2.0mg/L IAA + 1.5 mg/L Kn) showed highest shoot length within three month. Auxin (IAA) was very effective in bud breaking and shoots formation. Number of shoot induced per explant by the effect of IAA (2.0 mg/L) was also very satisfactory (Figure 5). Kinetin (1.5 mg/L) with IAA (2.0 mg/L) enhanced shoot multiplication and also increased number of node.

2 mg/L kinetin + 1.0 mg/L BAP+ 0.5 mg/L NAA + 100 mg/L ascorbic acid is supplemented with MS media for shoot proliferation of *D. oppositifolia* and results show 90% explants proliferate with highest rate of shoot multiplication (10.5 shoot/explant) (Behera et al., 2009). In our study, only one cytokinin (kinetin) was added with auxin (IAA) for shoot multiplication and IAA (2.0 mg/L) + Kinetin (1.5 mg/L) induced highest shoot length of 9.90 cm (Table 2) with 7.7 mean number of shoot per explant. Combination and interaction of BA and NAA plays an important role in *in vitro* propagation of nodal explants for multiple shoot induction (Shin et al., 2004). MS medium with 1.0 mg/L NAA and 0.5 to 1.0 mg/L BA is best concentration for induction of multiple shoot in *D. opposita*. The present study revealed that combination and interaction of Kinetin (1.5 mg/L) and IAA (2.0 mg/L) induced multiple shoot in MS media.

Half strength MS media supplemented with 2.5 mg/L IAA is most efficient for root initiation and it produces roots (average 6.67) with average length of 8.33 cm (Table 3). Addition of IAA, IBA or NAA to MS medium produces root (Barna and Wakhlu, 1988). Result of this work supports the observation of the present work. Shoot culture on full strength MS medium with auxin produces callus at the base of the shoots (Patra et al., 1998). The microshoots of various medicinal plants are rooted on only MS medium without growth regulators by many workers (Thomas and Maseena, 2006; Saxena et al., 1998). Observation of this work showed difference with the result of recent study because no rooting was observed in auxin free MS medium (Table 3). Rooting occurs in the presence and absence of NAA, IBA or IAA; it is also recorded that higher concentration of NAA, IBA or IAA (5.0 to 10 µM) induces root sooner than the lower concentration (0.1 to 1.0 µM/L) of IAA, NAA or IBA (Mao et al., 1995). The findings of this experiment are similar with the result of the present work. Nodal segments cultured on MS medium with IAA at concentration of 0.5 mg/L required 60 to 62 days to initiate rooting but IAA at concentration of 2.5 mg/L required only 22 to 24 days for root initiation. No rooting is observed in shoots planted on auxin free basal medium; lower concentration of auxin (NAA 0.5 mg/L) produced very few or no root (Behera et al., 2009). Result of this work show is similar with the present investigation because low concentration of IAA (0.5 mg/L) produced minimum number of root (average 2.56), with root length of 1.33 cm (Table 3). Work with *Saussurea lappa* indicated media containing 0.5 to 1.0µM NAA initiated rooting in 90% of culture shoots (Arora and Bhojwani, 1989).

The well rooted plants were transferred to sterilized plastic cups containing vermiculite for hardening and kept under controlled condition. Production of plantlets with profuse rooting in *in vitro* is essential for successful establishment of regenerated plants in soil (Ohyma, 1970). Later, plants were transferred to earthen pots containing mixture of Brick bats + soil + Charcoal + Dried moss + leaf mold (1: 1: 1: 1: 1) and survival rate was 85 and 87% after one month of hardening.

Here, an attempt was made to propagate *D. alata* using minimum number of growth regulators. In this Investigation, an efficient micropropagation technique (1.5 mg/L IAA + 2.0 mg/L KN for shooting and 2.5 mg /L IAA for rooting) was derived which may be useful for raising quality plants of *D. alata* for commercial purpose at lowest cost. This technique paves the way not only for *ex situ* conservation but also for the restoration of genetic stock of the species.

REFERENCES

Ahuja A, Verma M, Grewal S (1982). Clonal propagation of *Ocimum* species by tissue culture. Ind. J. Exp. Biol. 20:455-458.

Alizadeh S, Mantell SH, Viana AM (1998). *In vitro* shoot culture and microtuber induction in the steroidal yam *Dioscorea composite* Hemsl. Plant Cell Tissue Organ. Cult. 53:107-112.

Arora R, Bhojwani SS (1989). *In vitro* propagation and low temperature storage of *Saussurea lappa* C. B. Clarke: an endangered medicinal plant. Plant Cell Rep. 8:44-47.

Asokan MP, O'Hair SK, Litz (1983). *In vitro* plant development from bulbil explants of two Dioscorea species. Hortic. Sci. 18: 702-703.

Ayensu ES (1972). Anatomy of the monocotyledons VI Dioscoreales. Oxford Press, Oxford. p. 182.

Barna KS, Wakhlu AK (1988). Axillary shoot induction and Plant regeneration in *Plantago ovata* Forsak. Plant Cell Tissue Organ. Cult. 15: 167-169.

Behera KK, Sahoo S, Prusti A (2009).Regeneration of plantlet of water yam (*Dioscorea oppositifolia* L.) through *in vitro* culture of nodal segments. Not. Bot. Hort. Agrobot. Cluj. 37:94-102.

Borges GM, Alarcon SY, Malaurie B, Hernandez JY,Silva Pupo JJ (2009). *In vitr*o conservation of *D. alata.* Revista Peruana de Biologia 16:203-208.

Chaturvedi HC (1975). Propagation of *Dioscorea floribunda in vitro* culture single node segments. Curr. Sci. 44:839-841.

Chen LJ, Hu TW, Huang LC (1995). A protocol toward multiplication of the medicinal tree *Eucommia ulmoides* Oliver. In vitro Cell Dev. Biol. Plant 31:193-198.

Coursey DG (1976).Yams. *Dioscorea* spp. (Dioscoreaceae) In: Simmonds ED (ed) Evolution of crop plants.Longman. London, pp. 70-74.

Coursey DG(1967). *Yams* .Longman, Green and Co, London. pp. 230.

Craufurd PQ, Battey NH, Ile EI, Asedu R (2006). Phases of dormancy in yam tubers (*Dioscorea rotundata).* Ann. Bot. 97:497.

Edison S, Unnikrishnan M, Vimala B, Santha Pillai V, Sheela MN, Sreekumari MT, Abraham K(2006). Biodiversity of Tropical Tuber Crops in India, NBA Scientific Bulletin No. 7, National Biodiversity Authority. Chennai, India. p. 60.

Hann SK (1995).Yams. Dioscorea spp.(Dioscoreaceae), In Smartt J, Simmonds NW (eds) *Evolution of crop plants*, Longman Scientific and Technical, U.K. pp. 112.

Jaleel CA, Gopi R, Manivannan P, Kishorekumar A, Gomathinayagam M, Panneerselvam R(2007). Changes in biochemical constituents and induction of early sprouting by triadimefon treatment in white yam (*Dioscorea rotundata* Poir) tubers during storage. J. Zhejiang Univ. Sci. 8: 283-288.

Jha S, Jha TB (1998). Micropropagation of *Cephaelis ipecacuanha* Rich., Plant Cell Rep. 8: 437- 439.

Jova MC, Kosky RG, Cuellar EE (2011). Effect of liquid media culture systemson yam plant growth (Dioscorea alata L.'Pacala Duclos'). Biotechnol. Agron. Soc. Environ. 15:515-521.

Koda Y, Kikuta Y (1991). Possible involvement of jasmonic acid in tuberization of yam plants. Plant Cell Physiol. 32:629-633.

Kohmura H, Araki H, Imoto M (1995). Micropropagation of 'yamatoimo' Chinese yam (*Dioscorea opposite* Thun.) from immature leaves. Plant Cell Tissue Organ Cult. 40:271-276.

Mahesh R, Muthuchelian K, Maridass, Raju G (2010). *In vitro* propagation of wild yam, *Dioscorea wightii* through nodal cultures. Int. J. Biol. Technol. 1:111-113

Malaurie B, Pungu O, Trouslot MF (1995). Influence of meristem- tip size and location on morphological development *Dioscorea cayenensis* Lam., *Dioscorea rotundata Poir.* complex and *Dioscorea praehensilis* Benth. Plant Cell Tissue Organ. Cult. 42:215-218.

Mantell SH, Hugo SA (1989). Effects of photoperiod,mineral medium strength, inorganic ammonium, sucrose and cytokininon root, shoot and microtuber developmentin shoot cultures of *Dioscorea alata* L. and *D. bulbifera* L. Yams. Plant Cell Tissue Organ Cult. 16: 23-37.

Mao AH, Wetten A, Fay M, Caligari PDS (1995). *In vitro* propagation of *Clerodendrum colebrookianum* Walp, a potential natural antihypertension medicinal plant. Plant Cell Rep.14: 493-496.

Martine J, Cappadocia M (1991). *In vitro* tuberization in *Dioscorea alata* L. 'Brazo fuerte' and 'Florido'and D.abyssinica Hoch. Plant Cell Tissue Organ Cult. 26:147-152.

Martine J, Cappadocia M (1992). Effects of growth regulators on *in vitro* tuberization in *Dioscorea alata* L 'Brazo fuerte' and *D.abyssinica* Hoch. Plant Cell Rep.11: 34.

Mitchell SA, Ahmed MH (1999). Morphological changes of Dioscorea trifida L.cv. Short Neck Yampie and *Dioscorea cayenensis* Lam cv.round leaf yellow yam linked to the number and size of harvested tubers. J. Hort. Sci. Biotechnol. 74:531-539.

Narula A, Kumar S, Srivastava PS (2007). Genetic fidelity of *in vitro* regenerants, encapsulation of shoot tips and high diosgenin content in *Dioscorea bulbifera* L., a potential source of diosgenin. Biotechnol. Lett. 29:623-629.

Ng SYC (1988). *In vitro* tuberization in white yam (*Dioscorea rotundata* Poir.). Plant Cell Tissue Organ Cult. 14:121-128.

Ng SYC (1992). Micropropagation of white yam (Dioscorea rotundata Poir) In: Bajaj YPS (eds) Biotechnology in agriculture forestry, Springer, Berlin 19: 135-159.

Ohyma K (1970). Tissue culture in mulberry tree. Jpn. Agric. Res. Quart. 5:30-34.

Ondo Ovono P, Kevers C, Dommes J (2007). Axillary proliferation and tuberization of *Dioscorea cayenensis – D. rotundata* complex. Plant Cell Tissue Organ Cult. 91:107-114.

Patra A, Rai B, Rout GR, Das P (1998). Successful plant regeneration from callus cultures of *Centella asiatica* (Linn) Urban. Plant Growth Regl. 24:13-16.

Saleil V, Degras L, Jonard R (1990). Obtention de plantes indemmes de virus de la mosaique de l'igname americaine *Dioscorea trifida* L.. Agronomie 10:605.

Satour M, Mitaine-Offer AC, Lacaille-Dubois MA (2007). The *Dioscorea* genus : A review of bioactive steroid saponins. J. Nat. Med. 61:91-101.

Saxena C, Rout GR, Das P (1998). Micropropagation of *Psoralea corylifolia* ,Linn. J. Med. Aromat. Plant Sci. 20:15-18.

Sengupta J, Mitra GC, Sharma AK (1984) Organogenesis and tuberization in cultures of *Dioscorea floribunda.* Plant Cell Tissue Organ Cult. 3:325-331.

Shin JH, Kim SK, Kwon JB, Lee BH, Shon JK (2004). Factors affecting the production of *in vitro* plants from the nodal pieces of Chinese Yam (*Dioscorea opposita* Thunb). J. Plant Biotech. 6(2):97-102.

Skirvin RM, Chu MC, Young HJ (1990). Rose, In: Ammirato PV, Evans D R, Sharp WR, Bajaj YPS(eds) Handbook of plant cell cultures. MacMillan, New York. 5:716-743.

Thomas TD, Maseena EA (2006). Callus induction and plant regeneration in *Cardiospermum halicacabum* Linn. an important medicinal plant. Sci. Hortic. 108:332-336.

Tor M, Twyford CT, Funes I, Boccon-Gibod J, Ainsworth CC, Mantell SH (1998). Isolation and culture of protoplasts from immature leaves and cell suspension of *Dioscorea* yams: Tools for transient gene expression studies. Plant Cell Tissue Organ Cult. 53:113-125.

Twyford CT, Mantell SH (1996). Production of somatic embryos and plantlets from root cells of Greater Yam. Plant Cell Tissue Organ Cult. 46:17-26.

Vaillant V, Bade P, Constant C (2005). Photoperiod affects the growth and development of yam plantlets obtained by *in vitro* propagation. Biol. Plant. 49:355-359.

Viana AM, Mantell SH (1989). Callus induction and plant regeneration from excised zygotic embryos of the seed propagated yams *Dioscorea composita* Hemsl. and *D. cayenensis* Lam., Plant Cell Tissue Organ Cult. 16:113-122.

Wheatley AO, Ahmed MH, Asemota HN (2005). Development of salt adaptation in vitro greater yam (*Dioscorea alata*) plantlets. In vitro Cell. Dev. Biol. Plant. 39:346-353.

Yan H, Yang L, Li Yangrui (2011). Improved growth and quality of *Dioscorea fordii* Prain et Burk and *Dioscorea alata* plantlets using temporary immersion system. Afr. J. Biotechnol. 10: 19444-19448.

academicJournals

Vol. 12(47), pp. 6618-6624, 20 November, 2013
DOI: 10.5897/AJB2013.12416
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Bioconversion of ferulic acid to vanillin by combined action of *Aspergillus niger* K8 and *Phanerochaete crysosporium* ATCC 24725

Nazila Motedayen[1], Maznah B. T. Ismail[1,2]* and Forough Nazarpour[3]

[1]Laboratory of Molecular Biomedicine, Institute of Bioscience, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Department of Nutrition and Dietetic, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[3]Laboratory of Industrial Biotechnology, Institute of Bioscience, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

Accepted 29 October, 2013

Ability of 10 fungi strains for the degradation of ferulic acid and production of vanillic acid was examined. The findings suggested that all the fungi were able to degrade ferulic acid via different pathways producing variety of products. Vanillic acid was the main bioconversion product for all the fungi strains. *Aspergillus niger* K8 was chosen as a more suitable fungus for conversion of ferulic acid to vanillic acid, due to its highest potential to produce high concentration of vanilic acid (116 mg/l) compared to other fungi. Bioconversion proves was further carried out with *Phanerochaetechrysosporium* ATCC 24725 for production of vanillin from vanillic acid produced by *A. niger* K8. The vanillin production (44.8 mg/l) was significant from the economical aspect, due to the cheapness and available source of ferulic acid as a substrate and short time for bioconversion of ferulic acid to vanillic acid.

Key words: *Aspergillus niger* K8, biotransformation, ferulic acid, *Phanerochaetechrysosporium* ATCC 24725, vanillic acid, vanillin.

INTRODUCTION

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is a flavoring compound of high economic value. On the basis of its origin, it may be classified as natural or synthetic. Natural vanillin covers less than 1% of its total demand (Priefert et al., 2001) and is obtained from vanilla pods, whereas the synthetic vanillin is prepared chemically. Limited availability of vanilla beans has resulted in high prices of natural vanillin ($4000/kg), but still this yield is less than what is needed (Feron et al., 1996). To fulfill this demand, many biotechnological methods to produce natural vanillin from ferulic acid using fungi have been proposed. For example *Schizophyllum commune* degraded ferulic acid to 4-vinylguaiacol, then it was oxidized to vanillin and vanillic acid (Tsujiyama and Ueno, 2008). Biotransformation of ferulic acid using *Enterobacter* sp. Px6- has been studied by Li et al. (2008). During the bioconversion process, 4-vinylguaiacol and vanillin were produced; ferulic acid was decarboxylated to 4-vinyl guaiacol and converted to vanillin. Muheim and Lerch (1999) have screened two microorganisms (*Streptomyces setonii* and *Pseudomonas putida*) for testing their ability to accumulate vanillin from ferulic acid. They found that *P. putida* is the major producer of vanillic acid; whereas *S. setonii* accumulated vanillin. Falconnier et al. (1994)

*Corresponding author. E-mail: maznah@medic.upm.edu.my, myhome.e@gmail.com. Tel: +603-8947-2115. Fax: +603-8947-2116.

produced vanillin from ferulic acid by *Pycnoporus cinnabarinus*. Review of the related scientific literature indicates that natural ferulic acid exist indefinitely on the graminaceous plant cell walls as well as residues of agricultural wastes such as sugar beet pulp, cereal bran and rice bran oil (Harris and Hartley, 1980; Zheng et al., 2007) is the most appropriate precursor for production of vanillin because of its chemical resemblance to vanillin (Bonnin Estelle et al., 2002), abundance, being replenishable, capability to form fine substrate from agricultural feed supplies and low toxicity as compared to eugenol (Muheim and Lerch, 1999).

In this study, different fungi strains were screened, for their ability to bioconvert ferulic acid, extracted from waste residue of rice bran oil, to yield higher amount of vanillic acid. One of them is *Aspergillus niger* k8 which is a local isolated and is explored for the first time to biotransform ferulic acid to vanillic acid. Vanillic acid was used subsequently for vanillin production.

MATERIALS AND METHODS

Chemicals and reagents

Ferulic Acid (FA), vanillic acid, vanillin, vanilly alcohol, 4-vinyl guaiacol was purchased from Sigma Chemical Co. (St. Louis, USA). Waste residue of rice bran oil was provided by the Laboratory of Biomoloecular Science, Institute of Bioscience, University Putra Malaysia. Potato dextrose agar (PDA) and polystyrene resin HZ816 were purchased from Huachang polymer (Shanghai, China) and Merck (Germany), respectively. PDA medium was prepared according to the manufacturer's instructions. The used solvents were of high-performance liquid chromatography (HPLC) grade. All other chemicals were obtained from Fisher Scientific and Merck.

Microorganisms

Different fungi strains were used for the biotransformation of ferulic acid to vanillic acid. Original freeze-dried strains of the *A*. *niger* ATCC 200345, *Aspergillus terreus* ATCC 74135, *Aspergillus terreus* ATCC 10029, *Ceriporiopsis subvermispora* ATCC 90467, *Fusarium oxysporum* ATCC 11137, *Penicilliumdigitatum* ATCC 201167, *Phanerochaete chrysosporium* ATCC 24725 and *Trametes versicolor* ATCC 20869 were obtained from the American Type Culture Collection (ATCC). *Penicillium purpurogenum* PTCC 5212 was obtained from the Persian Type Culture Collection (PTCC). *Fusarium oxysporum* CBS 620.87 was obtained from Centraal bureau voor Schimmel cultures (CBS). *A*. *niger* K8 was isolated by the Laboratory of Industrial Biotechnology, Institute of Bioscience, University Putra Malaysia. The strains were kept at 4°C on potato agar slants.

Growth and bioconversion of ferulic acid to vanillic acid

The composition of basal medium used for the production of fungal cultures was according to the study of Zheng et al. (2007). Bioconversion of ferulic acid to vanillic acid was performed using, at least, one fungal culture. To carryout bioconversion, inoculation was performed with spores (10^6 spores/ml medium) or mycelia fragment into 100 ml basal medium of 250 ml flask at 30°C and agitated at 150 rpm for 48 h. The culture of fungus was filtered with filter paper whatman paper No. 1. Mycelium mass of the fungus was inoculated to flask containing 120 ml basal medium (yeast extract 4 g/l) at 30°C, 150 rpm (Zheng et al., 2007), until a biomass was obtained possessing the capacities needed for the bioconversion (biomass of at least 0.2 g/l). After 24 to 96 h, the cultures for biotransformation stage were ready. Ferulic acid solution (300 mg/l) was added to the cultures for bioconversion to vanillic acid. Three replicates were used for each time interval.

Growth and bioconversion of vanillic to vanillin

Mycelium of *P. chrysosporium* ATCC *24725* was collected after growth on basal medium for 48 h and inoculated into conical flask containing 120 ml medium containing (g/l): Cellobiose 2.5, beef extract 8, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 0.2, $CaCl_2$ 1.3 mg, $VB_2$2.5 mg, pH 5. Incubation was then carried out at 30°C for 48 h, followed by suspension of mycelium of *P*. *chrysosporium* ATCC 24725 in vanillic acid solution, which was produced by selected fungus. Bioconversion of vanillic acid to vanillin by *P*. *chrysosporium* ATCC 24725 was carried out at 35°C and 120 rpm (Zheng et al., 2007). Suitable amount of adsorbent resin HZ816 was applied to biotransformation system, 1 h, after adding fungi biomass to bioconversion of vanillic acid to vanillin. The molar yield of vanillic acid and vanillin formation to ferulic acid and vanillic acid consumed respectively was expressed as described by Baqueiro-Pe a et al. (2010).

Analytical procedures

Samples of bioconversion were withdrawn at different incubation times and applied to high performance liquid chromatography (HPLC) for analysis of bioconversion products. Each sample was centrifuged at 4000 rpm for 10 min, the supernatant was filtered through 0.2 µm Whatman nylon filter, acidified to pH = 2 and extracted with an equal volume of ethyl acetate. Ethyl acetate fractions were evaporated to dryness under reduced pressure. These were resuspended in 1 ml of methanol (50% v/v). The HPLC (Agilent 1200 series, Germany) analysis was done in order to detect any metabolites or byproducts. The C18 column (ZORBAX SB, 5 µm, 150 mm × 4.6 mm) was used and maintained at 22°C. Compounds were eluted with a gradient A 100:1 water: acetic acid solution and gradient B 95:5:1 methanol: aceonitrile: acetic acid mixture as used as: 0 to 2 min 5%, 2 to 10 min 5 to 25%, 10 to 20 min 25 to 40%, 20 to 30 min 40 to 50%, 30 to 40 min 50 to 100%, 40 to 45 min 100%. The flow rate was 1 ml/min. Sample detection was achieved with UV-Vis detector at 280 to 360 nm and injection volume was 20 µL. Quantitative data were obtained using external standards which were ferulic acid, vanillic acid, vanillin, vanilly alcohol and 4-vinyl guaiacol. Cell growth was measured by dry cell weight (DCW) measurement using filtration and drying method. In this method, filter paper was put at 70°C for 24 h, then weight and recorded. Ingredients in conical flask were filtered and remaining fungal mycelium was put on filter paper at 70°C for 24 h followed by weighting the filter paper with fungus. The difference between weights of the filter paper with and without fungus was taken as weight of dried mycelium.

The mean values of all the data obtained from the experiment were analyzed by using statistical analysis software (SAS) program (release 6.12, 1988.SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Screening of fungi for bioconversion of ferulic acid to vanillic acid

The objective here is to compare the production of vanillic

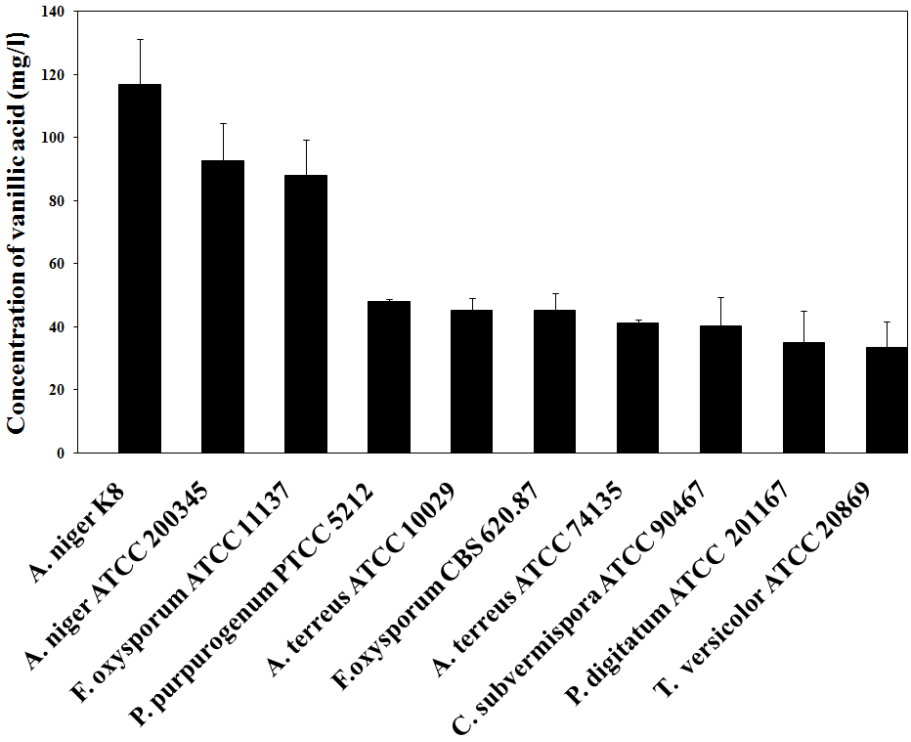


Figure 1. Vanillic acid concentration (mg/l) produced from ferulic acid through bioconversion process at 30°C with shaking speed of 150 rpm using 10 various strains fungi. Triplicate assay were carried out and the error bars represent standard deviations.

acid from ferulic acid using different fungi. The fungus, which yielded the highest concentration of vanillic acid, was used in fermentation process for the production of vanillin. The bioconversion results of vanillic acid produced by different fungi are depicted in Figure 1. The results suggest that the concentration of vanillic acid is different among fungi maybe due to different fungi having various pathways to degrade ferulic acid (Priefert et al., 2001). Ligninolytic enzyme activity also may be have effect on vaniilic acid formation whereas different fungi have various lignolytic enzyme for example *Trametes versicolor* have laccase, lignin peroxidase and manganese peroxidase and *Ceriporiopsis subvermispora* have laccase and manganese peroxidase (Johansson and Nyman, 1992; Lobos et al., 1994; Maciel et al., 2010) which play critical role on degradation of ferulic acid. The highest vanillic acid concentration (116.9 mg/l) was obtained using *A. niger* K8. The bioconversion time course of ferulic acid by *A. niger* K8 is shown in Figure 2; at the beginning of bioconversion process, the concentration of ferulic acid was 300 mg/l, while no vanillic acid could be detected. During bioconversion process, ferulic acid was converted to vanillic acid and concentration of vanillic acid reached 76.33 mg/l within 12 h. Meanwhile, ferulic acid concentration decreased to 92 mg/l, the formation of vanillic acid increased finally to 116.9 mg/l, within 36 h and subsequently decreased. In this case, ferulic acid conversion was found to be 69.16% with molar yield of 64.56%, which indicated it used most of ferulic acid to produce vanillic acid; however, in other fungi due to the production of unwanted byproduct, the final yield (vanillic acid) is lower compared to that of *A. niger* K8.

During the biotransformation of ferulic acid by *A. niger* K8, no coniferyl alcohol and vanillin was observed therefore from our result we do propose that reaction to be occurring via a propenoic chain degradation to vanillic acid as described for *A. niger* (Lesage-Meessen et al., 1996). A similar route for ferulic acid degradation was reported using *A*. *niger* C28B25 and 57% yield of vanillic acid was obtained by Baqueiro-Pea et al. (2010).

Effect of ferulic acid concentration on vanillic acid yield

Different concentrations of ferulic acid, that is, 100 to 1200 mg/l were used based on previous work (Lesage-Meessen et al., 1999). Initial ferulic acid concentration of 100 mg/l resulted in formation of 29.96 mg/l vanillic acid with molar yield of 33.3%. By using 300 mg/l ferulic acid as an initial substrate concentration of vanillic acid was found to be 116.9 mg/l with a molar yield of 64.56%. With increasing concentration of ferulci acid to 600 mg/l, molar yield of vanillic acid decreased to 40.6% (128.4 mg/l) was

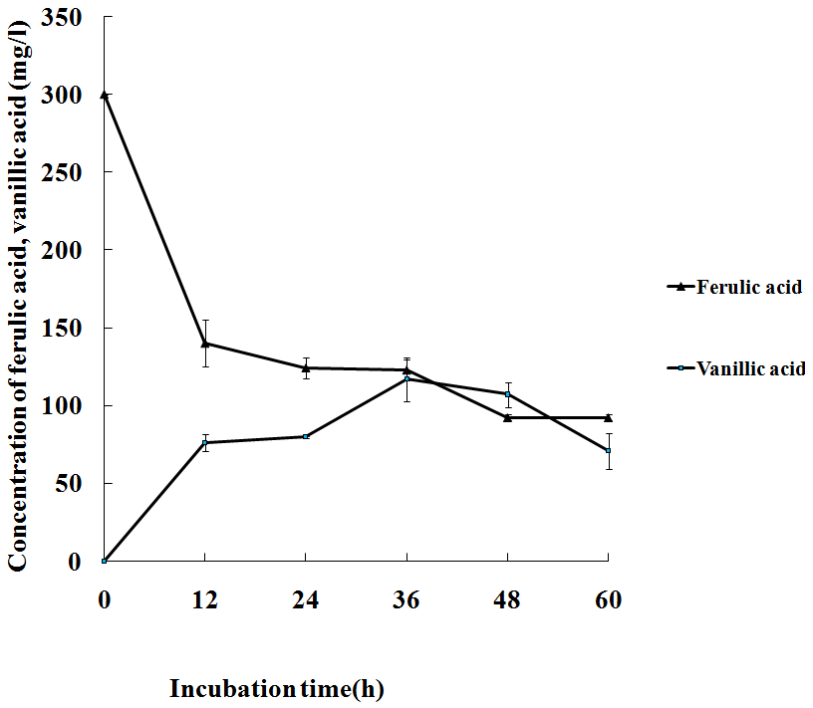


Figure 2. The bioconversion time course of ferulic to vanillic acids using *A. niger* K8 under condition of 30°C and 150 rpm. Triplicate assay were carried out and the error bars represent standard deviations.

observed. With the increase in concentration of ferulci acid, a proportionate decrease in yield of vanillic acid was observed. Higher ferulic acid concentration that is, 1200 mg/l resulted only in 11.34% yield of vanillic acid and 832.8 mg/l of ferulic acid remained unreacted (Figure 3). This maybe assumed due to substrate toxicity at high concentration, which indicated inhibitory of ferulic acid to mycelail growth and fungal metabolism (Gross-Falconnier, 1991). Conclusively, the best initial concentration of ferulic acid was decided to be 300 mg/l with 64.56% yield of vanillic acid. The toxicity of substrate at certain concentration was also reported by Allouche and Sayadi (2005). Ghosh et al. (2005) reported that increase in the yield of vanillic acid is not linearly correlated to increase in the concentration of ferulic acid.

Bioconversion process of vanillic acid to vanillin by *P. chrysosporium* ATCC 24725

Prior to bioconversion, the mycelium of *A. niger* K8 was filtered and *P. chrysosporium* ATCC 24725 was added to the filtrate medium. As shown in Figure 4, concentration of residual ferulic acid in filtrate medium decreased negligibly after 60 h. This result showed that *P. chrysosporium* ATCC *24725* had a slight degradation role on ferulic acid in our study therefore it preferentially used vanillic acid to produce vanillin. During the bioconversion process, maximum concentration of vanillin (44.8 mg/l) was produced by *P. chrysosporium* ATCC 24725 at 60 h of bioconversion time (Figure 4) with added HZ816 to this medium. The molar yield of vanillin was 42.6% and very low amount of vanilly alcohol was determined in bioconversion medium. According to the study of Falconnier et al. (1994), *P. chrysosporium* ATCC 24725 matabolized vanillic acid from two pathways; reduction pathway leading to the formation of vanillin and oxidative decarboxilated leading to formation of methoxyhydroquinone. In this study, the presence of cellobiose methoxyhydroquinone was not produced therefore *P. chrysosporium* ATCC 24725 metabolized vanillic acid from first step. Proposed pathways for the metabolism of ferulic acid to vanillin by *A. niger* K8 and *P. crysosporium* ATCC 24725 is shown in Figure 5. The bioconversion route of vanillic acid to vanillin by *P. chrysosporium* ATCC 24725 is in agreement with the study of Stentelaire et al. (1998), who showed the same metabolic pathway obtained using *P. chrysosporium* I-1471.

Lesage-Meessen et al. (1997) found cellobiose in culture medium was able to inhibit the production of methoxy-hydroquinone from vanillic acid. Bonnin et al. (1999) reported by adding cellobiose in culture medium of *Pycnoporus cinabarinus* vanillin production increased. In order to improve vanillin production and inhibited from transformation to vanilly alcohol (Lesage-Meessen et al., 2002), HZ816 resin was added to adsorb vanillin. Increase in the yield of vanillin in the fermentation liquor with adding resin has been reported elsewhere (Stentelaire et

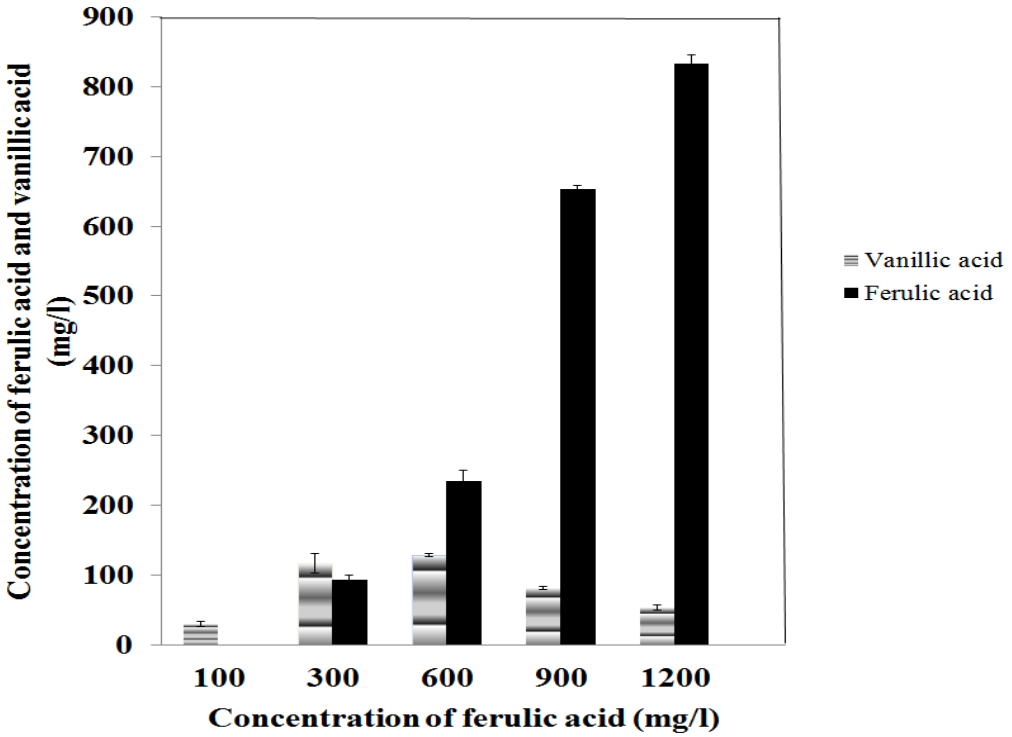


Figure 3. Effect of different concentarations of ferulic acid (100 to 1200 mg/l) on vanillic acid formation during the bioconversion process at 30°C and 150 rpm by *A. niger* K8. Triplicate assay were carried out and the error bars represent standard deviations.

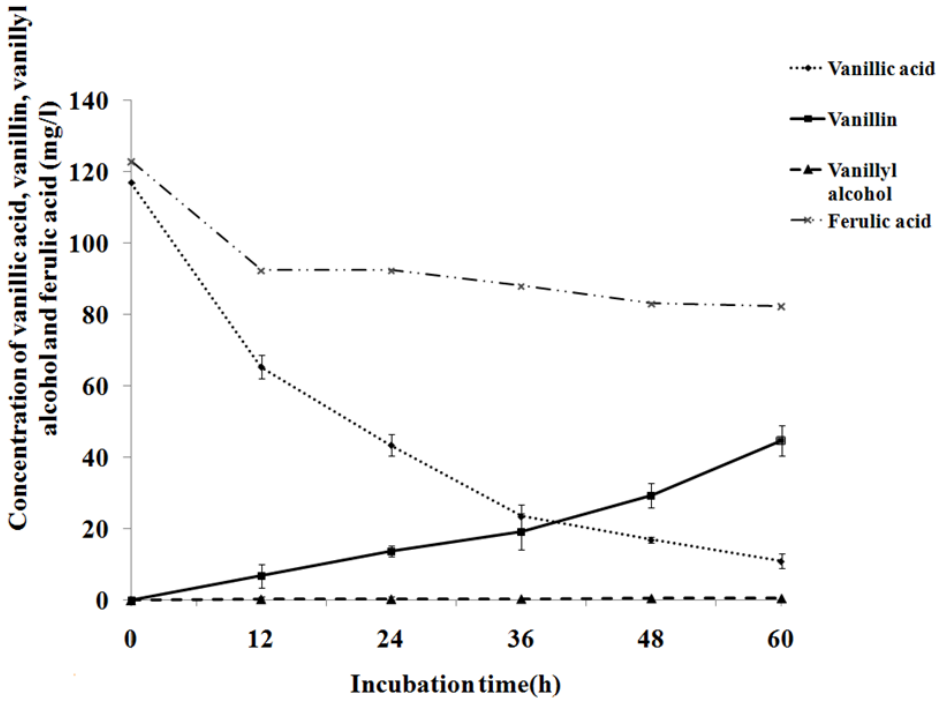
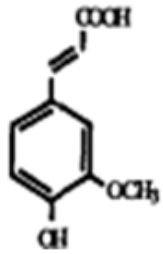


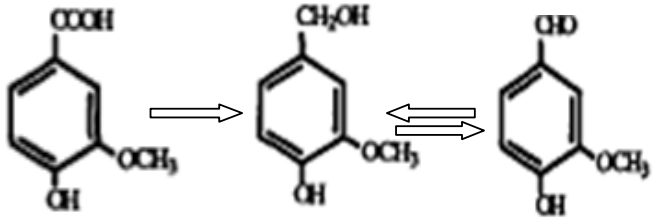
Figure 4. The bioconversion time course of vanillin production using *P. chrysosporium* ATCC 24725 from vanillic acid produced by *A. niger* K8 and residual ferulic acid. Experiment was performed at 35°C and 120 rpm with adding 5% HZ816 resin. Triplicate assay were carried out and the error bars represent standard deviations.

Pathway 1: Ferulic acid propenoic chain degradation



Ferulic acid

Pathway 2: Vanillic acid reduction



Vanillic acid Vanillin Vanillyl alcohol

Figure 5. Proposed pathways for the metabolism of ferulic acid to vanillin by *A. niger* K8 and *P. crysosporium* ATCC 24725.

al., 2000; Nilvebrant et al., 2001).

Optimization of adsorbent resin amount for vanillin production

This part of study is conducted to determine the significant amount of adsorbent resin for highest vanillin production. Different concentrations of HZ816 resin ranging from 0 to 6% were investigated. As shown in Table 1, no vanillyl alcohol was absorbed by resin. By adding resin up to 5%, the vanillin concentration got increased to 39 mg/l, while without resin, its concentration was low. However, subsequent increase in the amount of resin to 6% did not result in any improved sorption of vanillin showing that 5 and 6% of resin had almost same adsorption ability. So, 5% resin was chosen because of high ability to absorb vanillin and economy reason. The resin for adsorption of vanillin in the bioconversion of ferulic acid to vanillin was also applied by Hua et al. (2007), who reported that 8 and 10% (wet w/v) concentration of DM11 resin had same ability and upon increasing concentration of resin to 15%, its adsorption ability was decreased. It can be concluded that, the increasing demand for natural vanillin in the food industry highlights the significance of this study on the production of vanillin from ferulic acid by use of microorganisms. Screening of microorganisms was done using different fungal strains to select the best microorganism that may produce the highest concentration of vanillic acid. A local strain of *A. niger* K8 was chosen to cotablenvert ferulic acid to vanillic acid, where the yield of vanillic acid was 64.56%. Next, an experiment was conducted to identify the best concentration of substrate. The results showed 300 mg/l ferulic acid to be most suitable for the bioconversion process. *P. chrysosporium* ATCC 24725 converted vanilic acid broth to vanillin. Trace amounts of vanillin were obtained from the bioconversion process of vanillic acid to vanillin; therefore, application of resin was critical for the bioconversion of vanillic acid to vanillin by *P. chrysosporium* ATCC 24725. The amount of resin was optimized and 5% of resin HZ816 was found to be the best for bioconversion process. With the addition of resin, the molar yield of vanillin increased to 42.6% as compared to vanillin

Table 1. Effect of HZ816 resin on vanillin yield.

Percentage (%) HZ816 resin (w/v)	Residual in medium (mg/l)		Adsorbed onto HZ816 (mg/l)	
	Vanillin	Vanillyl alcohol	Vanillin	Vanillyl alcohol
2	18.9^{a}±1.2	0.81^{a}±0.02	25.8^{c} ± 2.1	-
3	11.5^{b}±1.2	0.71^{a}±0.01	29.3^{c}± 1.9	-
4	8.7^{c}±1.0	0.59^{b}±0.08	34.2^{b} ± 4.0	-
5	5.7^{d}±0.2	0.59^{b}±0.06	39.0^{a} ± 1.0	-
6	5.7^{d}±0.1	0.59^{b}±0.06	39.1^{a} ±0.9	-

production without resin. The present study obtained some findings regarding improvement in the biotechnological process for the production of vanillin, but there is still room for further investigation. Genetic modification of *A. niger* K8 and *P. chrysosporium* ATCC 24725 may also be employed in bioconversion process for increase vanillin production.

REFERENCES

Allouche N, Sayadi S (2005). Synthesis of hydroxytyrosol, 2-hydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid by differential conversion of tyrosol isomers using Serratia marcescens strain. J. Agric. Food Chem. 53:6525-6530.

Baqueiro-Pe A I, Rodríguez-Serrano G, González-Zamora E, Augur C, Loera O, Saucedo-Casta eda G (2010). Biotransformation of ferulic acid to 4-vinylguaiacol by a wild and a diploid strain of Aspergillus niger. Biores. Technol. 4721-4724.

Bonnin E, Saulnier L, Brunel M, Marot C, Lesage-Meessen L, Asther M, Thibault J.-F (2002). Release of ferulic acid from agroindustrial by-products by the cell wall-degrading enzymes produced by Aspergillus niger I-1472. Enzyme. Microbial. Technol. 31:1000-1005.

Carunchio F, Crescenzi C, Girelli A, Messina A, Tarola A (2001) Oxidation of ferulic acidby laccase: identification of the products and inhibitory effects of some dipeptides. J. Talanta. 55:189-200.

Civolani C, Barghini P, Roncceti A, Ruzzi M, Schiesser A (2001). Bioconversion of ferulic acid into vanillic acid by Means of a Vanillate-Negative Mutant of Pseudomonas fluorescens Strain BF13. J. Environ. Microbiol. 66:2311-2317.

Falconnier B, Lapierre C, Lesage-Meessen L, Yonnet G, Brunerie P, Colonna- Ceccaldi B, Corrieu G, Asther M (1994) Vanillin as a product of ferulic acid biotransformation by the white-rot fungus Pycnoporus cinnabarinus I-937: identification of metabolic pathways. J. Biotechnol. 37(2):123-132.

Feron G, Bonnarme P, Durand A (1996) Prospects for the microbial production of food flavours. Trends Food Sci Technol. 7: 285-293.

Ghosh S, Sachan A, Mitra A (2005). Degradation of ferulic acid by a white rot fungus Schizophyllum commune. World J. Microbiol Biotechnol. 21:385-388.

Gross-Falconnier B (1991) Production de molecules aromatisantes par des basidiomycetes: etudes d'une biosynthese et d'une bioconversion realisees par Pycnoporus cinnabarinus. These, Universite Paris-Sud, Centre d'Orsay (France): No. 1658.

Harris P J, Hartley R D (1980). Phenolic constituents of the cell walls of monocotyledons. Biochem. Systematics Ecol. 8:153-160.

Hua D, Ma C, Song L, Lin S, Zhang Z, Deng Z, Xu P (2007). Enhanced vanillin production from ferulic acid using adsorbent resin. Applied Appl. Microbiol. Microbiol. Biotechnol. 74:783-790.

Johansson L, Nyman P (1992) Characterization of a lignin peroxidase gene form the white root fungus Trametes versicolor, Biochemie 74:177-182.

Lesage-Meessen L, Delattre M, Haon M, Asther M (1999). Methods for bioconversion of ferulic acid to vanillic acid or vanillin and for the bioconversion of vanillic acid to vanillin using filamentous fungi, US Patent no 5866380.

Lesage-Meessen L, DelattrevM, Haon M, Thibault J. F, Ceccaldi B. C, Brunerie P, Asther M (1996). A two-step bioconversion process for vanillin production from ferulic acid combining Aspergillus niger and Pycnoporus cinnabarinus. J. Biotechnol. 50:107-113.

Lesage-Meessen L, Lomascolo A, Bonnin E, Thibault J. F, Buleon A, Roller M, Asther M, Record E, Ceccaldi B.C (2002). A biotechnological process involving filamentous fungi to produce natural crystalline vanillinfrom maize bran. Appl. Biochem. Biotechnol. 102:141-153.

Li Xu, Yang J, Li Xi, Gu W, Huang J, Zhang K (2008). The metabolism of ferulic acid via 4-vinylguaiacol to vanillin by Enterobacter sp. Px6-4 isolated from Vanilla root. Process Biochem. 43:1132-1137

Lobos S, Larrain J, Salas L, Cullen D, Vicuña R (1994). Isoenzymes of manganas dependent peroxidase and laccase produced by the lignin-degrading basidiomycete Ceriporiopsis pubvermispora. Microbiology 140:2691-2698.

Maciel l, Silva A, Ribeiro H (2010) Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota: A review. Elec. J. Biotechnol. ISSN: 0717-3458.

Muheim A, Lerch K (1999). Towards a high-yield bioconversion of ferulic acid to vanillin. Appl. Microbiol. Biotechnol. 51:456-461.

Nilvebrant N.O, Reimann A, Larsson S, Jönsson L.J (2001). Detoxification of lignocellulose hydrolysates with ion-exchange resins. Appl. Biochem. Biotechnol. 91:35-49.

Priefert H, Rabenhorst J, Steinbüchel A (2001). Biotechnological production of vanillin. Appl. Microbiol. Biotechnol. 56:296-314.

Stentelaire C, Lesage-Meessen L, Delattre M, Haon M, Sigoillot J. C, Ceccaldi B. C, Asther, M (1998). By-passing of unwanted vanillyl alcohol formation using selective adsorbents to improve vanillin production with Phanerochaete chrysosporium. World J. Microbiol. Biotechnol. 14:285-287.

Stentelaire C, Lesage-Meessen L, Oddou J, Bernard O, Bastin G, Ceccaldi B.C, Asther M (2000). Design of a fungal bioprocess for vanillin production from vanillic acid at scalable level by Pycnoporus cinnabarinus. J. Biosci. Bioengr. 89:223-230.

Tsujiyama S, Ueno M (2008). Formation of 4-vinyl guaiacol as an intermediate in bioconversion of ferulic acid by Schizophyllum commune. Biosci. Biotechnol. Biochem. 72:212-215.

Zheng L, Zheng P, Sun Z, Bai,Y, Wang J, Guo X (2007). Production of vanillin from waste residue of rice bran oil by Aspergillus niger and Pycnoporus cinnabarinus. Biores. Technol. 98:1115-1119.

academicJournals

Vol. 12(47), pp. 6632-6635, 20 November, 2013
DOI: 10.5897/AJB2013.13137
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Degradation of cyclohexane and cyclohexanone by *Bacillus lentus* strain LP32

Bolanle O. Opere, Oluwafemi S. Obayori* and Adebanji A. Raji

Department of Microbiology, Lagos State University, Ojo, Lagos, Nigeria.

Accepted 10 October, 2013

A Gram-positive bacterium, *Bacillus lentus* LP32, originally isolated on the basis of its ability to utilise pyrene as sole source of carbon was found to be able to grow luxuriantly on alicyclic compounds as sole substrates. It showed poor growth on anthracene, naphthalene, 1-naphthol and phenanthrene. Growth rate on cyclohexane was 1.32 d^{-1}, while doubling time was 0.76 d. The corresponding values for growth on cyclohexanone were 0.77 d^{-1} and 1.29 d, respectively. Within 10 days, the amount of cyclohexane in culture reduced from 317.62 to 102.55 mgl^{-1}, then to 23.04 mgl^{-1} on day 18. On cyclohexanone, substrate concentration decreased from 287.56 mgl^{-1} to 101.66 mgl^{-1} in 10 days before declining to 24.21 mgl^{-1} on day 18. The rate of degradation when growing on cyclohexane was 23.50 $mgl^{-1}d^{-1}$ in the first 10 days and 9.93 $mgl^{-1}d^{-1}$ between day 10 and day 18, with 67.71% degradation in 10 days and overall percentage degradation of 92.43%. On cyclohexanone, the corresponding values were 18.59 and 9.68 mg $l^{-1}d^{-1}$ as well as 64.65 and 91.58%, respectively. This organism is a potential candidate for bioremediation purpose.

Keywords: Degradation, cyclohexane, cyclohexanone, alicyclic compounds.

INTRODUCTION

Alicyclic compounds are major components of crude oil (Okoh, 2006). Their relative amount in the crude oil (20 to 70%) is dependent upon the nature and origin of the petroleum (Ilori, 1999; Maier, 2009). They usually find their way into soil and natural bodies of water, including aquifers, either accidentally or deliberately, during commercial operations such as oil drilling and oil transportation; or during industrial applications as solvents, though on a smaller scale (Ilori, 1999; Lee and Cho, 2008). They occur in nature as components of plant oils, paraffins, microbial lipids and pesticides (Maier, 2009).

Alicyclic compounds have been reported to be relatively persistent in the environment when compared to other components of the crude oil (Okoh, 2006). Indeed, they are reputed to be the saturated hydrocarbons most resistant to microbial attack (Sikkema et al., 1995; Ko and Lebault, 1999). Thus, their biological fate in the environment has been a major source of concern to environmentalists (Rio-Hernandez et al., 2003).

Reports on the use of axenic cultures of microorganisms to degrade alicyclic compounds are fewer in the literature compared with other fractions of petroleum (Mechichi et al., 2003; Amund et al., 2006). Their degradation is thought to occur primarily by commensalistic and cometabolic reactions under aerobic condition (Maier, 2009). The potential membrane toxicity of alicyclic compounds to microbial cells in little amounts and their low water solubility greatly account for the persistence in the environment (Sikkema et al., 1995; Amund et al., 2006).

It is equally noteworthy that most studies on the biodegradation of alicyclic compounds are based on

*Corresponding author. E-mail: femiobayori@yahoo.com. Tel: (234) 802-319-2652.

Abbreviations: API, Analytical profile index; **MSM,** mineral salts medium; **TVC,** total viable count; **GC,** gas chromatography.

cyclohexane (Tonge and Higgins, 1974; Donoghue et al., 1976; Stirling and Watkinson, 1977; Anderson et al., 1980; Trower et al., 1985; Brzostowicz et al., 2000). Axenic cultures including *Nocardia* sp., *Pseudomonas* sp., *Xanthobacter* sp. have been reported to grow on cyclohexane (Stirling and Watkinson, 1977; Anderson et al., 1980; Trower et al., 1985). Similarly, a microbial consortium of *Stenotrophomonas* sp. and *Rhodococcus* sp. was reported to be able to utilise this substrate (Lee and Cho, 2008). The *Rhodococcus* sp. was further shown to be able to utilise cyclohexane as sole source of carbon and energy, producing as part of the intermediates cyclohexanone and cyclohexanol, 2-cyclohexene-1-one and phenol (Yi et al., 2011).

On the other hand, reports on degradation of cyclohexanone have been scantier than envisaged. Brzostowicz et al. (2000) reported the ability of *Brevibacterium* sp. strain HCU to utilise cyclohexanone as sole source of carbon and energy. According to the authors, the monooxygenases required to degrade cyclohexanone are only induced in the presence of the hydrocarbon. Amund et al. (2006) reported the ability of strains of *Pseudomonas*, *Acinetobacter*, *Arthrobacter* and *Norcardia* to grow on cyclohexanone and cyclohexanol. This degradative ability was found to be chromosomally borne. In this paper, we report the degradation of alicyclic compounds, cyclohexane and cyclohexanone, by a *Bacillus* species originally isolated on the basis of its ability to grow with pyrene as sole source of carbon and energy.

MATERIALS AND METHODS

Microorganism and culture condition

The organism used in this study was *Bacillus lentus* strain LP32. The isolation and characterisation of the organism by continuous enrichment of run-off soil adjacent to an asphalt plant in Lagos, Nigeria on the basis of its ability to use pyrene as sole source of carbon and energy has been documented elsewhere (Obayori, 2008). The isolate was maintained in glycerol:nutrient broth (1:1) at -20°C. It was checked for purity by plating onto nutrient agar and observing the 18 h old culture under the microscope. The identity of the isolate was reaffirmed by analytical profile index (API) using the API ZONE and API V.20 test kit according to the manufacturer's specifications (Biomerieux Inc., Durham, NC, USA).

Colonies growing on Luria-Bertani agar with very low percentage of pyrene (0.005%) were harvested with sterile inoculating loop, pooled and transferred to screw-capped bottles containing 5 ml of physiological saline (0.9% NaCl). Enough culture was transferred to achieve an OD_{520} of approximately 1.5. The organism was grown in mineral salts medium (MSM) previously described by Kastner et al. (1994). The medium contained per litre Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g and $MgSO_4.7H_2O$, 0.20 g. The pH of the medium was adjusted to 7.2. Trace elements solution (1 ml/l) described by Bauchop and Elsden (1960) was sterilized separately and added aseptically to the medium. Unless otherwise stated, incubation was performed at room temperature (27 ± 2.0°C). The stock culture was aerobically grown routinely in MSM containing cyclohexane (SIGMA-ALDRICH Inc., USA) and cyclohexanone (SIGMA-ALDRICH Inc., USA) as the sole carbon sources in separate conical flasks.

Substrate specificity of isolate

The isolate was tested for its ability to grow on the following substrates: anthracene, 1-naphthol, pyrene, cinnamic acid, succinic acid, biphenyl and naphthalene. Substrate specificity was evaluated in MSM broth supplemented with 50 mg L^{-1} of respective solid hydrocarbons or 0.1% (*V*/*V*) in case of liquid substrates as a sole carbon and energy source. Degradation was monitored by cell increase with reference to uninoculated flasks, coupled with disappearance of the crystals in the case of pyrene and anthracene. Inocula were 24 h LB-grown cultures and inoculation was carried out to achieve initial optical density of 0.05 (OD_{520}). Incubation was performed at room temperature (27 ± 2.0°C).

Time course of growth of isolate on cyclohexane and cyclohexanone

Replicate flasks containing 50 ml of autoclaved MSM with 0.5 ml of filter-sterilised cyclohexane or cyclohexanone were prepared. Thus for each substrate, there were 10 triplicate sets, making a total of 30 flasks per substrate. The flasks were inoculated to achieve an initial concentration of the total viable count (TVC) about 2.0 x 10^7 cfu ml^{-1} and incubated at room temperature (27 ± 2.0°C) for a period of 18 days.

Flasks inoculated with heat-inactivated cells served as controls. Total viable counts were determined at 2 days interval by plating out appropriate dilutions of the cultures onto nutrient agar. Mean generation times and specific growth rates were calculated using nonlinear regression of growth curves for the period when growth rates were maximal (Prism version 5.0, Graphpad software, San Diego, CA, USA).

Chromatographic determination of residual alicyclic substrates

Residual concentration of the alicyclic compound in the culture medium was analysed by gas chromatography (GC) (Hewlett-Packard) fitted with flame ionization detector (FID). An aliquot (20 ml) of the sample was extracted with 5 ml hexane and concentrated to 1 ml. One microlitre of the concentrated sample was injected into the chromatograph column. The column was OV-101 and SE 30 with length of 60 m. The injector and detector temperatures were maintained at 200 and 300°C, respectively. The initial temperature of 70°C and a final temperature of 320°C were used for the analysis which was programmed to rise at 10°C per min. Nitrogen was used as the carrier with the gas flow rate of hydrogen at 45 ml per min, nitrogen at 22 ml per min and air flow rate of 450 ml per min. The GC runs were carried out on the sample at day 0, day 10 and day 18.

RESULTS AND DISCUSSION

Identification and characterisation of isolate

LP32 was a Gram-positive, catalase and oxidase positive, spore forming, motile rod, with β-haemolysis on blood agar. It was positive for starch hydrolysis, liquefied gelatine and reduced nitrate. It utilised citrate, lactose, sucrose, glucose and fructose. It was negative for urease, indole and hydrogen sulphide production, and equally failed to grow on sorbitol, ducitol, mannitol,

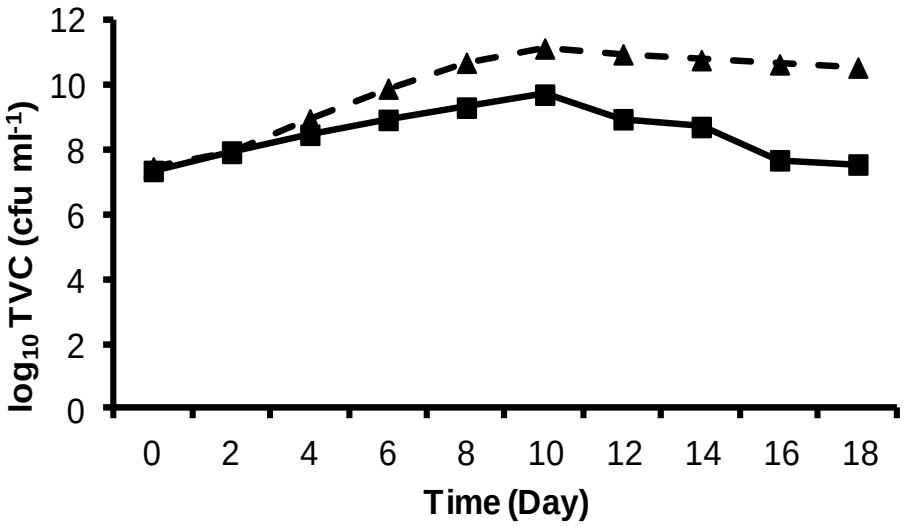


Figure 1. Time course of growth of *Bacillus lentus* strain LP32 on cyclohexane (▲) and cyclohexanone (■) in mineral salt medium (MSM) supplied with 0.1% percent of the respective substrates as sole source of carbon and energy. Incubation was carried out at room temperature (27 ± 2°C) with shaking at 150 rpm. Time point data are means of triplicate readings, error bars were eliminated for the purpose of clearity.

Table 1. Kinetics of degradation of alicyclic substrates by *Bacillus lentus* LP32.

Substrate	*K* (day $^{-1}$)	T (day)	DR_{10} (mg l^{-1} d^{-1})	DR_{18} (mg l^{-1} d^{-1})	PD_{10} (%)	PD_{18} (%)
Cyclohexane	1.32	0.76	23.50	9.93	67.71	92.43
Cyclohexanone	0.77	1.29	18.59	9.68	64.65	91.58

K, Growth rate; T, doubling time; DR_{10}, degradation rate in the first 10 days; DR_{18}, degradation rate between day 10 and 18; PD_{10}, percentage degraded in the first 10 days; PD_{18}, overall percentage degraded in 18 days. The substrates were supplied at a concentration of 0.1% (*V*/*V*) and separated on OV-101 column with length of 60 m.

arabinose and maltose. It was therefore putatively identified as *Bacillus lentus*. Substrate specificity showed that LP32 did not grow on biphenyl, and grew poorly on anthracene, naphthalene, 1-naphthol and phenanthrene. However, it showed heavy growth on cyclohexane and cyclohexanone.

Biodegradation of cyclohexane and cyclohexanone

The time courses of growth of *B. lentus* strain LP32 on cyclohexane and cyclohexanone are shown in Figure 1. On cyclohexane from an initial population density of 2.94 x 10^7 cfu ml^{-1} the organism concentration steadily increased to 3.27 x 10^{11} cfu ml^{-1} in ten days before dropping to 3.62 x 10^{10} cfu ml^{-1} on day 18. On the other hand, when grown on cyclohexanone population density increased from 2.3 x 10^7 to 5.28 x 10^9 cfu ml^{-1} on day 10 before declining to 3.59 x 10^7 cfu ml^{-1} on day 18. Growth rate on cyclohexane was 1.32 d^{-1} while doubling time was 0.76 d (Table 1) the corresponding values for growth on cyclohexanone were 0.77 d^{-1} and 1.29 d, respectively.

Kinetics of growth and degradation of alicyclic compounds (Table 1) showed that the rate of degradation when growing on cyclohexane was 23.50 $mgl^{-1}d^{-1}$ in the first ten days and 9.93 $mgl^{-1}d^{-1}$ between day 10 and 18, with 67.71% degradation in 10 days overall percentage degradation of 92.43%. When LP32 was grown on cyclohexanone the corresponding values were 18.59 and 9.68 mg $l^{-1}d^{-1}$ as well as 64.65 and 91.58%, respectively. The ability of *B. lentus* LP32 to degrade cyclohexane and cyclohexanone is of interest because *Bacillus* species had rarely been previously reported to play a major role in degradation of alicyclic compound. The only report on cyclohexane degradation by *Bacillus* till date was that of Lee et al. (2013). The authors isolated from loess four strains, *Bacillus cereus* strain VOC18, *Bacillus thuringensis* strain VOC 11, *B. thuringensis* strain VOC13 and *Bacillus megaterium* strain VOC 03 capable of utilizing or tolerating cyclohexane to varying extents.

However, strains of *Bacillus* have been previously and consistently reported as a hydrocarbon utilizers (Okerentugba and Ezeronye, 2003; Okoh, 2006; Nwaogu et al., 2008). Other organisms previously isolated from alicyclic compounds span genera such as *Pseudomonas, Norcardia, Brevibacterium, Acinetobacter, Xanthobacter, Alicycliphilus denitrificans and Arthrobacter* (Stirling and Watkinson, 1977; Anderson et al., 1980; Trower et al., 1985; Ilori, 1999; Brzostowicz et al., 2000; Mechichi et al., 2003; Amund et al., 2006).

The fact that LP32 which was originally isolated on pyrene was able to use alicyclic compounds and a variety of substrates as sole source of carbon is of interest. Partly because of differences in metabolic pathways, it is rare to find organisms which could degrade both aliphatic and aromatic compounds effectively. However, it has been suggested that long periods of exposure to mixture of hydrocarbon and preponderance of enabling intrinsic and extrinsic factors could lead to acquisition of such rare ability (Obayori et al., 2009). It is equally noteworthy that a degrader of alicyclics had been previously demonstrated to grow on pyrene, benzene and other diverse hydrocarbons and petroleum cuts (Lee and Cho, 2008). The steady increase in the population of organisms in both substrates between day 0 and 10 could be attributed to the utilisation of the sole carbon sources, cyclohexane and cyclohexanone (Okerentugba and Ezeronye, 2003; Amund et al., 2006). The observation of a downward trend in the total viable count of the organisms after day 10 could be attributed to nutrient exhaustion (especially the carbon source) or accumulation of toxic metabolites (Atlas, 1994). The main routes of degradation of cyclohexane and cyclohexanone metabolism involves the formation of 1-oxa-2-cycloheptane (*ε*-caprolactone) and 6-hydroxyhexanoate (6-hydroxycaproate) as intermediate products (Wagner et al*.,* 2002). These intermediate products are toxic to microorganisms that lack *ε*-caprolactonase and 6-hydroxyhexanoate dehydrogenase (Brzostowicz et al*.,* 2000). Thus, this may account for the downward trend of total viable count during the succeeding days.

The gas chromatography results further accentuated the result obtained from total viable counts. Over 67.71% of cyclohexane was metabolized by *B. lentus* within 10 days. This indicated that trend of degrading alicyclic compounds within the first ten days was better pronounced than the succeeding 11 days. In the same vein, 64.65% of cyclohexanone was degraded by *B. lentus* within the first ten days. The overall percentage degradation of cyclohexane of 92.2% is similar to the result obtained by Lee and Cho (2008) for *Rhodococcus* strain ECI which metabolised 9% hexane. The ability of strain LP32 to effectively degrade cyclohexane and cyclohexanone *in vitro* and its broad substrate susceptibility suggests that it could further investigation for application in production of value added bio-products from alicyclic compound and bioremediation of hydrocarbon polluted compartments.

REFERENCES

Amund OO, Ilori MO, Adebusoye SA, Musa KI (2006). Utilisation of alicyclic compounds by soil bacteria. Nat. Sci. 4:65-68

Anderson MS, Robert A, Hall A, Griffin M (1980). Microbial metabolism of alicyclic hydrocarbons: cyclohexane catabolism by a pure strain of *Pseudomonas* sp. J. Gen. Microbiol. 120:89-94.

Atlas RM (1994). Environmental Microbiology. In: Microorganisms in our World. 4th edition. Mosby Inc., St. Louis, USA. pp 758-762

Bauchop T, Elsden SR (1960). The growth of microorganisms in relation to their energy. J. Gen. Microbiol. 23:457-459.

Brzostowicz PC, Gibson KI, Thomas S, Blasko MS, Rouviere P (2000). Simultaneous identification of two cyclohexanone oxidation genes from an environmental *Brevibacterium* isolate using mRNA different display. J. Bacteriol. 182:4241-4248.

Donoghue NA, Griffin M, Norris dB, Trudgill PW (1976). The microbial metabolism of cyclohexane and related compounds. J. Gen. Microbiol. 120:43-56.

Ilori MO (1999). Utilisation of cyclohexanol by bacteria in tropical estuarine water. Folia Microbiol. 44:553-556.

Kastner M, Breuer-Jammali M, Mahro B (1994). Enumeration and characterisation of the soil microflora from hydrocarbon-contaminated soil sites able to mineralise polycyclic aromatic hydrocarbons. Appl. Microbiol. Biotechnol. 41:267-273.

Lee EH, Cho KS (2008). Characterization of cyclohexane and hexane degradation by *Rhodococcus* sp. EC1.Chemosphere 7:1738-1744.

Lee S, Oh H, Kim O (2013). Isolation and characterisation of volatile organic compounds-degrading *Bacillus* strains from Loess. J. Environ. Protect. 4:50-57

Maier RM (2009). Microorganisms and organic pollutants. In: Maier RM, Pepper IL, Gerba CP (eds) Environmental Microbiology, 2nd Edition. Academic Press, Burlington. pp. 387-420

Mechichi T, Stackebrandt E, Fuchs G (2003). *Alicycliphilus denitrificans* gen. nov., sp. nov., a cyclohexanol-degrading, nitrate-reducing *beta-proteobacterium.* Int. J. System. Evol. Microbiol. 53:147-152.

Nwaogu LA, Onyeze GOC and Nwabueze RN (2008). Degradation of diesel oil in polluted soil using *Bacillus subtilis*. Afr. J. Biotechnol. 7:1939-1943.

Obayori O (2008). Degradation of some petroleum hydrocarbon pollutants by microorganisms from contaminated tropical soils. PHD Thesis, University of Lagos, Lagos, Nigeria

Obayori OS, Adebusoye SA, Adewale AO, Oyetibo GO, Oluyemi OO, Amokun RA, Ilori MO (2009). Differential degradation of crude oil (Bonny Light) by four *Pseudomonas* strains. J. Environ. Sci. 21:243-248

Okerentugba PO, Ezeronye OU (2003) Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluents in Nigeria. Afr. J. Biotechnol. 2:288-292.

Okoh AI (2006). Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotechnol. Mol. Biol. Rev. 1:38-50.

Rios-Hernandez LA, Gieg LM, Suflita JM (2003). Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-contaminated aquifer. Appl. Environ. Microbiol. 69:434-443.

Sikkema J, de Bont JA, Poolma B (1995). Mechanism of membrane toxicity of hydrocarbons. Microbiol Rev 5a:201-222.

Stirling LA, Watkinson RJ, Higgins IJ (1977). Microbial metabolism of alicyclic hydrocarbons: isolation and properties of a cyclohexane-degrading bacterium. J. Gen. Microbiol. 99:119-125.

Tonge GM, Higgins IJ (1974). Microbial metabolism of alicyclic hydrocarbons: growth of *Nocardia petroleophilus* (NCIB 9438) on methylcyclohexane. J. Gen. Microbiol. 81:521-524.

Trower MK, Buckland M, Higgins M, Griffin M (1985). Isolation and characterization of a cyclohexane-metabolizing *Xanthobacter* sp. Appl. Environ. Microbiol. 49:1282-1289.

Yi T, Lee EH, Ahn YG, Hwang GS, Cho KS (2011). Novel biodegradation pathways of cyclohexane by *Rhodococcus* sp. EC1. J. Hazard. Mat. 191:393-396.

academicJournals

Vol. 12(47), pp. 6643-6649, 20 November, 2013
DOI: 10.5897/AJB2013.13241
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Phenolic components, antioxidant activity, and mineral analysis of *Capparis spinosa* L

Rezzan Aliyazicioglu[1]*, Ozan Emre Eyupoglu[2], Huseyin Sahin[2], Oktay Yildiz[3], Nimet Baltas[4]

[1]Department of Biochemistry, Faculty of Pharmacy, Karadeniz Technical University, 61080 Trabzon, Turkey.
[2]Department of Chemistry, Faculty of Science, Karadeniz Technical University, 61080 Trabzon, Turkey.
[3]Maçka Vocational School, Karadeniz Technical University, 61080 Trabzon, Turkey.
[4]Department of Chemistry, Faculty of Science, Recep Tayyip Erdoğan University, 53100 Rize, Turkey.

Accepted 10 October, 2013

In addition to being consumed as food, caper (*Capparis spinosa* L.) fruits are also used in folk medicine to treat inflammatory disorders, such as rheumatism. *C. spinosa* L. is rich in phenolic compounds, making it increasingly popular because of its components' potential benefits to human health. We analyzed a number of individual phenolic compounds and investigated *in vitro* biological activities of *C. spinosa* L. Sixteen phenolic constituents were identified using reverse phase-high performance liquid chromatography (RP-HPLC). Total phenolic compounds (TPCs), ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity were used as determinants of antioxidant capacity. *C. spinosa* L. exhibited strong antioxidant activity and contained high levels of antioxidant compounds. Gentisic, sinapic and benzoic acid were detected in *C. spinosa* L. No gallic acid, *proto*-catechuic acid, *proto*-catechuic aldehyde, chlorogenic acid, *p*-OH benzoic acid, vanillic acid, caffeic acid, syringic acid, vanillin, syringaldehyde, *p*-coumaric acid, ferulic acid or rosmarinic acid were identified. Iron and zinc were present at high levels in samples. *C. spinosa* L. appears to be a good source of antioxidants and minerals that might serve to protect health and combat several diseases.

Key words: Antioxidant, capers, *Capparis spinosa* L., phenolics.

INTRODUCTION

Reactive oxygen species (ROS) generation in excess of a biological system's antioxidant capacity results in oxidative stress (Zima et al., 2001). Free radical oxidative stress is involved in the pathogenesis of a range of human diseases. Cells and tissue generally possess antioxidant defense mechanisms to ensure the removal of ROS; while some are controlled endogenously (superoxide dismutase), others are supplied by diet and other means (ascorbic acid, α-tocopherol and β-carotene) (Haslam, 1996). In terms of cellular pro-oxidant states and lipid peroxidation, the consumption of simple plant components in regular diet, apart from supplying traditional nutrients, may provide benefits in the treatment, improvement or prevention of numerous chronic diseases, such as cancer, and cardiovascular and inflammatory damage including aging-related cellular degeneration (Steinmetz and Potter, 1991).

Capparis spinosa L. (Capparidaceae) is a particularly common aromatic in the Mediterranean area, and is also important in the commercial preparation of frozen food.

*Corresponding author. E-mail: rezzanaoglu@mynet.com. Tel: +905335113364.

Abbreviations: DW, Dry weight; **RP-HPLC,** reverse phase-high performance liquid chromatography; **TPC,** total phenolic compounds; **FRAP,** ferric-reducing/antioxidant power; **DPPH,** 2,2-Diphenyl-1-picrylhydrazyl; **GAE,** gallic acid equivalents; **TPTZ,** 2,4,6-tripyridyl-s-triazine; **BHT,** butylated hydroxytoluene; **ROS,** reactive oxygen species.

The aromatic part of the caper consists of the floral bud, collected immediately prior to blossoming. The plant is not generally cultivated, with wild buds being picked by seasonal workers. These are then stored in salt before packaging takes place. Earlier studies involving *C. spinosa* L. have identified alkaloids, lipids, flavonoids and glucosinolates (Brevard et al., 1992), naturally occurring products from the order Capparales, also known as flavor compounds, anticarcinogenic agents and biopesticides (Mikkelsen et al., 2000). Also, Bonina et al. (2002) had reported that methanolic extract of *C. spinosa* L. was shown to possess strong antioxidant/free radical scavenging effectiveness in different *in vitro* tests. Besides this information, *C. spinosa* L. is a source of phenolic compounds. According to the study of Argentieri et al. (2012), rutin was the dominant phenolic in it.

This study was designed to assess the phenolic composition, including phenolic acids and *in vitro* biological activities as antioxidant and mineral analysis of *C. spinosa* L., as well as to evaluate their nutritional and medicinal potentials.

MATERIALS AND METHODS

Chemicals and instrumentation

The phenolic standards (purity > 99.0%) gallic acid, protocathechuic acid, *p*hydroxybenzoic acid, vanillic acid, caffeic acid, chlorogenic acid, syringic acid, gentisic acid, vanilline, protocateculaldehyde, rosemarinic acid, sinapic acid, syringaldehyde, *p*-coumaric acid, ferulic acid and benzoic acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Methanol, acetic acid and acetonitrile were obtained from Merck (Darmstadt, Germany). Trolox (6-hydroxy–2,5,7,8-tetramethylchroman–2-carboxylic acid), 2,4,6-tripyridyl-s-triazine (TPTZ) and Folin-Ciocalteu's phenol reagent were obtained from Fluka Chemie GmbH (Buchs, Switzerland) and polytetrafluoroethylene membranes (porosity 0.45 µm) for extract filtration from Sartorius (Goettingen, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was supplied from Sigma-Aldrich (St. Louis, MO, USA).

Reverse phase-high performance liquid chromatography (RP-HPLC) (Agilent 1100, DAD 1200 Agilent Technologies, Waldbronn, Germany) and reverse phase waters spherisorb ODS2-C18 column (Water Corporation, Milford, USA) were used for analysis of phenolics. A Spectro UV-Vis Double PC–8 auto cell spectrophotometer (Labomed Inc., Los Angeles, CA, USA) was used in all absorbance measurements. Water solutions were prepared using deionized water purified in an Elgacan® C114 Ultra Pure Water System Deioniser (The Elga Group, Buckinghamshire, England). Ultrasonic bath (Transsonic Digital S, Elma®, Germany) was used during sample extraction procedure and rotary evaporator system (IKA® RV 05 Basic, Werke, USA) for evaporation. Epsilon 5 EDXRF Spectrometer (PANalytical Inc., Westborough, MA USA) was used for mineral analysis

Preparation of extracts

C. spinosa L. samples were selected from herb markets in Gaziantep, Turkey, in June 2013. Approximately 5-10 g of dry *C. spinosa* L. sample was extracted with 30 mL methanol in a flask attached to the condenser, in a sonicator device at 60°C over 3 h. Ten milliliters was separated from methanolic extract in order to determine antioxidant activities. The residual extracts of methanol were evaporated until dry and then concentrated in a rotary evaporator at 50°C. The crude extract was then dissolved in 10 mL distilled water, and liquid-liquid extractions were performed. The mixtures were extracted three times consecutively with 5 mL diethyl ether and 5 mL ethyl acetate. The organic moiety was picked up in the same flask and evaporated to dryness under reduced pressure in a rotary evaporator at 40°C. The residue was finally weighed and dissolved in methanol for high performance liquid chromatography (HPLC) analysis.

Reverse phase-high performance liquid chromatography (RP-HPLC) analysis of phenolic compounds

RP-HPLC analysis of phenolic compounds was conducted by using a reverse phase column (4.6 × 250 mm, 5 µm), on a gradient program with a two solvents system (A: 2% acetic acid in water; B: 0.5% acetic acid in acetonitrile: water [1:1]) at a constant solvent flow rate of 1.2 $mL.min^{-1}$. Injection volume was 20 µL. Signals were detected at 232, 246, 260, 272, 280, 290, 308, 328 nm by DAD and at 280 nm by UV detection. Column temperature was maintained at room temperature, 25°C.

Determination of antioxidant capacity

Total phenolic contents were analyzed with Folin-Ciocalteu's phenol reagent method, using gallic acid as the Standard (Singleton and Rossi, 1965; Akyuz et al., 2013). Briefly, 20 µL of various concentrations of gallic acid and 20 µL methanolic samples (1 $mg.mL^{-1}$), 400 µL of 0.5 Folin-Ciocalteu regents and 680 µL of distilled water were mixed and the mixture was vortexed. Following 3 min incubation, 400 µL of Na_2CO_3 (10%) solution was added, and after vortexing the mixture was incubated for 2 h. After the incubation period at room temperature, absorbances of the mixtures were measured at 760 nm. The concentration of total phenolic compounds was calculated as mg of gallic acid equivalents per 100 g of dry weight (DW) sample, by using a standard curve for gallic acid in the concentration range between 0.015 and 0.5 $mg.mL^{-1}$ ($r^2 = 0.99$).

Ferric reducing antioxidant power (FRAP) assay was used to determine antioxidant activity of the sample. The technique is based on the measurement of ferric reducing ability. FRAP assay was performed following the method described by Benzie and Strain (1996), with minor modifications. Working FRAP reagent was prepared as required by mixing 25 mL of 0.3 M acetate buffer at pH 3.6 with 2.5 mL of 10 mM TPTZ solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$. Subsequently, 100 µL of sample was mixed with 3 mL of freshly prepared FRAP reagent. The reaction mixture was then incubated at 37°C for 4 min. Absorbance was determined at 593 nm against blank prepared using distilled water and incubated for 1 h rather than 4 min. A calibration curve was employed, using an aqueous solution of ferrous sulphate Trolox concentrations in the range of 100–1000 µM, r^2=0.97. For purposes of comparison, Trolox® was also tested under the same conditions as a standard antioxidant compound. FRAP values were expressed as µmol Trolox equivalent of 100 g DW sample. Radical scavenging activity of *C. spinosa* L. extracts against DPPH radical was spectrophotometrically studied at 517 nm (Molyneux, 2004). The assay is based on the color change of the DPPH solution from purple to yellow as the radical is deactivated by the antioxidants. Briefly, various concentrations 0.75 mL of parts of *C. spinosa* L. methanolic extracts were mixed with 0.75 mL of a 0.1 mM of DPPH in methanol. Radical scavenging activity was measured by using butylated hydroxytoluene (BHT) as standards and the values are expressed as SC_{50} (mg sample per mL), the concentration of the samples that causes 50% scavenging of DPPH radical.

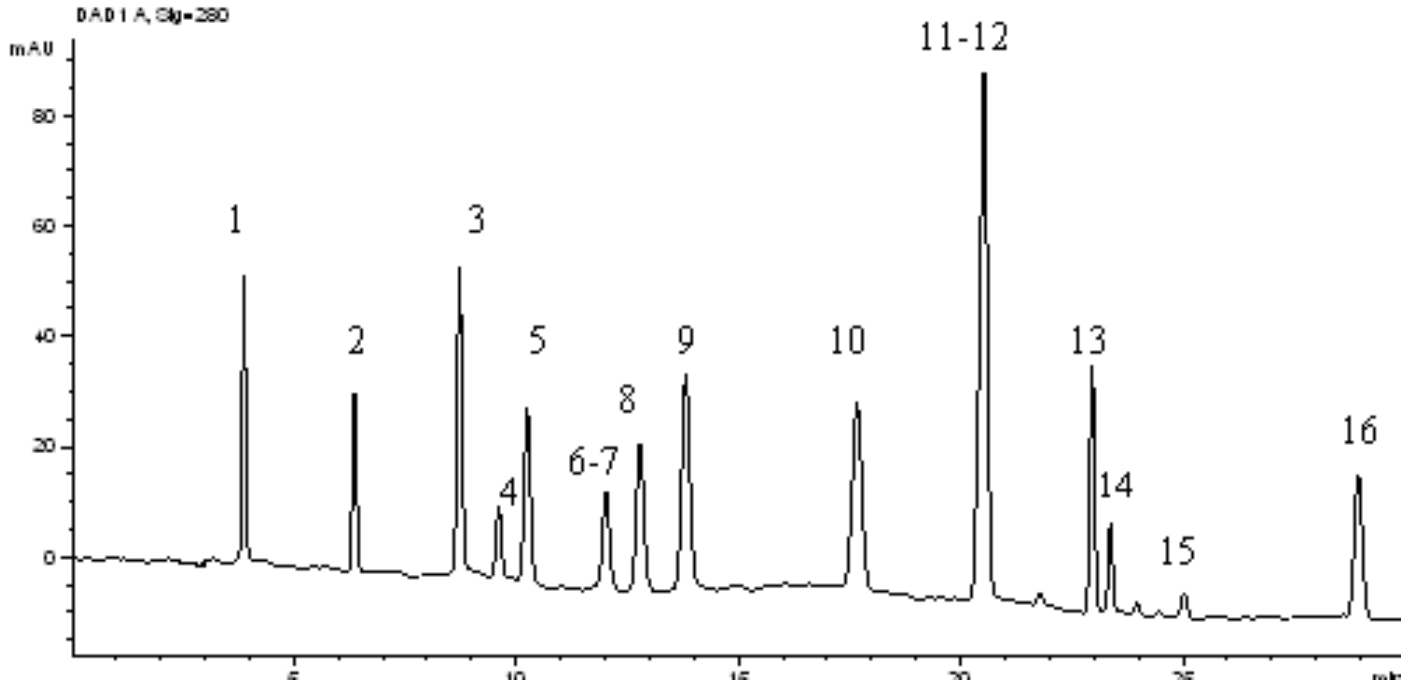


Figure 1. RP-HPLC chromatogram of phenolic standards at 280 nm. Peak identification: 1, Gallic acid; 2, *proto*-catechuic acid; 3, *proto*-catechuic aldehyde; 4, gentisic acid; 5, chlorogenic acid; 6, *p*-OH benzoic acid; 7, vanillic acid; 8, caffeic acid; 9, syringic acid; 10, vanillin; 11, syring aldehyde; 12, *p*-coumaric acid; 13, ferulic acid; 14, sinapic acid; 15, benzoic acid; 16, rosmarinic acid.

Sample preparation for mineral analysis

All the *C. spinosa* L. samples were dried in an incubator and then ground in a Spex mill. In order to minimize the effect of particle size, the resulting powder was sieved using a 400 mesh sieve and then stirred for 25 min to produce a well-mixed sample. The mixed samples were then pressed into 40 mm diameter pellets.

Mineral analysis

The measurement parameters were set up using the Epsilon 5 EDXRF system inbuilt software. Samples were irradiated by X-rays from a Gd tube under a vacuum equipped with a liquid nitrogen cooled PAN-32 Ge X-ray detector with a Be window thickness of 8 µm. Instrument power, current and high voltage were 600W, 6 mA and 100 kV, respectively. The system's software (Epsilon 5 software) automatically analyzed the sample spectrum and determined the net intensities of element peaks once measurement was completed. When elements overlap one another, accuracy is essential for trace element analysis. A set of secondary standards, available from PANalytical, was used for the calibration of this application. The resulting samples were again measured three times.

RESULTS AND DISCUSSION

Identification of phenolic compounds using RP-HPLC

Plants and fruits contain biologically active products that protect them against a range of physical and chemical hazards, including diseases, parasites and bacteria (Kolayli et al., 2003, 2010). Due to their phenolic constituents they may also possess biologically active properties. Any natural sample will contain numerous different phenolic compounds, making individual measurement difficult. This study was restricted to measuring only 16 phenolic substances using RP-HPLC. We also used RP-HPLC to analyze 16 phenolic compounds; gallic acid, *proto*-catechuic acid, *proto*-catechuic aldehyde, gentisic acid, chlorogenic acid, *p*-OH benzoic acid, vanillic acid, caffeic acid, syringic acid, vanillin, syring aldehyde, *p*-coumaric acid, ferulic acid, sinapic acid, benzoic acid, rosmarinic acid. The RP-HPLC chromatograms of the standard phenolic compounds are shown in Figure 1. When the individual phenolic compounds were compared with each standard, sinapic acid emerged as the main phenolic component in *C. spinosa* L (Table 1). Gentisic acid and benzoic acid were determined in very low concentrations, while no gallic, proto-catechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, rosmarinic, vanillin, syringaldehyde and *proto*-catechuic aldehyde were detected in *C. spinosa* L. (Figures 2 and 3). A previous study reported a methanolic extract of *C. spinosa* buds of 0.39% w/w of rutin as revealed by HPLC analysis (Germano et al., 2002).

Total antioxidant capacity

Total phenolic compounds (TPC)

TPC was determined in comparison with standard gallic acid and calculated at 37.01±0.03 mg GAE.100 g^{-1} DW of

Table 1. Retention times and peak areas of phenolic component peaks of *Capparis spinosa* L.

Peak number	Phenolic's Name	Retention time (RT)
1	Gentisic acid	9.65
2	Sinapic acid	23.57
3	Unknown	24.66
4	Benzoic acid	24.97
5	Unknown	26.56

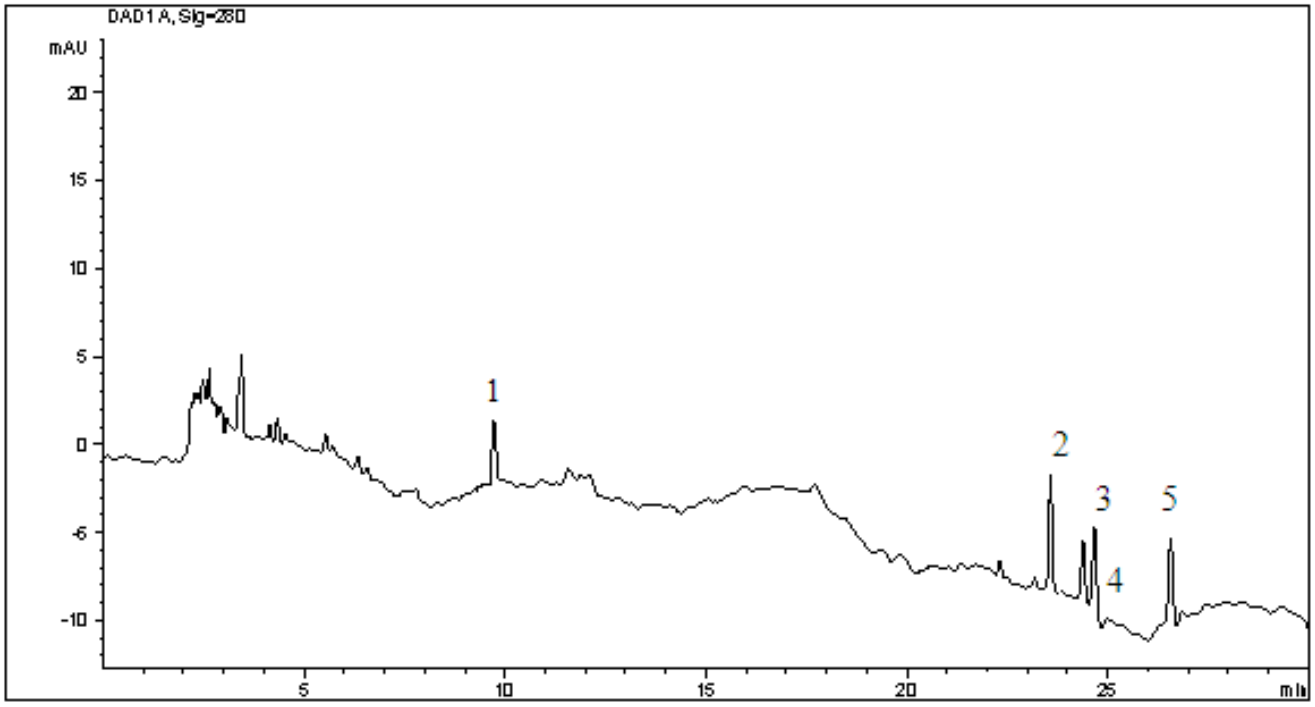


Figure 2. RP-HPLC chromatogram of *Capparis spinosa* L. (1 mg/mL) at 280 nm. Peak identification: 1, Gentisic acid; 2, sinapic acid; 3, unknown; 4, benzoic acid; 5, unknown.

C. spinosa L. using the Folin-Ciocalteu method (Table 2). Plants compounds are an important source of active natural products with a wide range of different structures and biological properties. Phenolic compounds are widely present in both edible and non-edible plants. Previous studies have reported that they exhibit multiple biological effects, including antioxidant activity (Yoshino and Murakami, 1998). Several studies have demonstrated a correlation between antioxidant activity and phenolic content (Nagai et al., 2003; Yang et al., 2002). The efficacy of natural antioxidants is closely associated with the chemical composition and structures of active extract components. It is therefore not possible to account for the antioxidant activity of an extract on the basis of its phenolic content, and characterization is also required (Heinonen et al., 1998). A high antioxidant activity may also be associated with compounds other than phenolics which are soluble in different solvents.

Ferric reducing antioxidant power (FRAP)

The FRAP test was used to measure the total antioxidant capacity of *C. spinosa* L. method is based on electron transfer and is regarded as accurate indicators of total antioxidant power, since total reducing power is defined as the sum of the reducing powers of the individual compounds contained in a particular sample (Tezcan et al., 2011). FRAP activity of sample is given in Table 2.

DPPH radical scavenging activity

The free radical scavenging activity of *C. spinosa* L. was determined using the DPPH test. When compared to reference antioxidants with the sample extract of *C. spinosa* L., it was found more efficient than BHT. This may be attributed to polyphenols in the samples varying

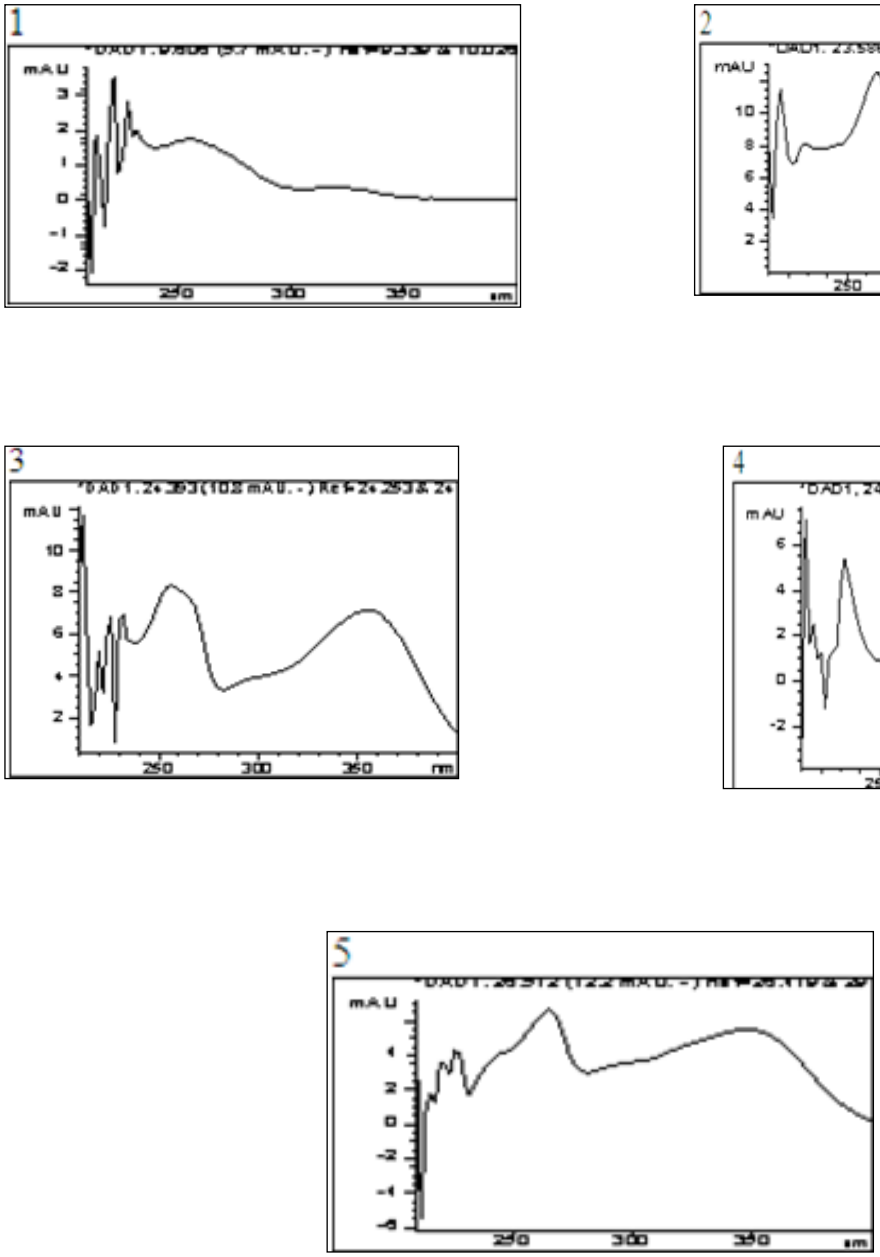


Figure 3. RP-HPLC-UV spectrums of phenolic component peaks of *Capparis spinosa* L. (1 mg/mL). Peak identification: 1, Gentisic acid; 2, sinapicacid; 3, unknown; 4, benzoic acid; 5, unknown (200–400 nm).

Table 2. Antioxidant activities of *Capparis spinosa* L.

Plant	TPC (mg GAE.100 g^{-1} DW)	FRAP (µmol Trolox.100 g^{-1} DW)	DPPH radical scavenging activity (SC_{50}: mg.mL^{-1})
Capparis spinosa L.	37.01±0.03	145.07 ± 0.04	0.32 ± 0.26

Values given are the mean and standard deviation of triplicate measurements. Standard antioxidants used were BHT (10 µg mL^{-1}, 0.01 ± 0.25).

significantly, in the same way as their scavenging capacities. The phenolic compounds may thus have acted as free radical scavengers on the basis of their hydrogen-donating ability (Molyneux, 2004). The sample extract possessed hydroxyl radical scavenging properties acting as donor for hydrogen atoms or electrons in the DPPH test.

Table 3. Elemental analysis values (X ± SD) of *Capparis spinosa* L. using EDXRF system.

Mineral	Sample
Al[a]	0.48 ± 0.05
P[a]	1.15 ± 0.01
S[a]	4.00 ± 0.06
K[a]	4.54 ± 0.03
Ca[a]	1.18 ± 0.01
Cl[b]	94.86 ± 25.51
Ti[b]	55.24 ± 2.30
Mn[b]	70.04 ± 1.00
Fe[b]	520.72 ± 4.05
Ni[b]	24.10 ± 0.05
Cu[b]	88.27 ± 0.45
Zn[b]	250.75 ± 0.80
Br[b]	11.92 ± 0.07
Rb[b]	79.03 ± 0.19
Sr[b]	40.20 ± 0.69
Y[b]	2.48 ± 0.38
Hf[b]	27.32 ± 0.87
Pb[b]	5.34 ± 0.13

a, % ; b, ppm. Values given are the mean and standard deviation of triplicate measurements.

This also increased iron autoxidation, while significantly suppressing the accessibility of iron to oxygen molecules by oxidizing ferrous ion to a ferric state, in turn inhibiting hydroxyl radical production (Yoshino and Murakami, 1998). These findings suggest that the antioxidant activeties of the sample extract of *C. spinosa* L. are associated with the high level of phenolic compounds.

The lyophilized and methanolic extract of *C. spinosa* L. exhibited a significant antioxidant effect (Germano et al., 2002). Extract concentrations of 100 and 1000 g.mL^{-1} significantly inhibit (p<0.01) lipid peroxidation by 71.50 and 90%, respectively. These antioxidant activities of methanolic extract are associated with the high phenolic compound levels (Tlili et al., 2010; Tlili et al., 2011). This protective effect may possibly be attributed to the richness of phenolic compounds, tocopherols and carotenoids. Many previous studies have suggested that these compounds possess very good antioxidative properties (Ihme et al., 1996; Burton et al., 1983; Mozaffarieh et al., 2003; Sommer and Davidson, 2002).

Mineral contents

The mineral contents of *C. spinosa* L. were shown to be excellent using the Epsilon 5 EDXRF system's inbuilt software (Table 3). Potassium, magnesium, manganese and sodium contents of *C. spinosa* L. buds as reported in Ozcan (2004) study were low compared to Ozcan and Akgul (1998) results. Some of our results concerning the mineral contents of condiments differ from those in the literature (Akgul, 1993). These differences may be attributed to growth conditions, genetic factors, geographical variations or the analytical procedures involved (Guil et al., 1998; Ozcan, 2004).

Calcium is the major component of bone and also assists in teeth development (Brody, 1994). Magnesium, iron and phosphorus levels are also high. These elements are highly important since they are required as cofactors by many enzymes (Akpanabiatu et al., 1998). There are also inorganic elements which may be involved in biological processes, but which have not been shown to be essential (Macrae et al., 1993a). Absence of toxic element contents such as mercury and arsenic is highly advantageous. Zinc is essential for growth in animals, human beings and plants. It is also crucial to crop nutriation since it is required in various enzymatic reactions, metabolic processes and oxidation-reduction reactions. It is present in the enzyme system as a co-factor and mental activator of many enzymes. Zinc has been reported as potentially limiting the growth of bacteria at higher levels (>13.60 mg.kg^{-1}) (Hafeez et al., 2013). In conclusion, *C. spinosa* L. possesses antioxidant activity and mineral content. The purpose of the study is to contribute to knowledge of the nutritional properties of *C. spinosa* L. knowledge of the mineral contents of condiments is also of great interest.

ACKNOWLEDGEMENTS

This study was supported by the KTU Research Fund (Project No: 2010.131.003.3). One of the authors (Huseyin SAHIN) would like to thank TUBITAK BIDEB for the financial support given to him.

REFERENCES

Akyuz E, Sahin H, Islamoglu F, Kolayli S, Sandra P (2013). Evaluation of phenolic compounds in *Tilia rubra subsp. caucasica* by HPLC-UV and HPLC-UV-MS/MS. Int. J. Food Prop. Doi: 10.1080/10942912.2011.631252.

Akgul A (1993). Spice science and technology. Turkish Association Food Technologists Pub l. No. 15, Ankara, Turkey.

Akpanabiatu MI, Bassey NB, Udosen EO, Eyong EU (1998). Evaluation of some minerals and toxicants in some Nigeriansoup meals. J. Food Compos. Anal. 11: 292–297.

Argentieri M, Macchia F, Papadia P, Fanizzi FP, Avato P (2012). Bioactive compounds from *Capparis spinosa subsp. Rupestris*. Ind. Crop. Prod. 36: 65–69.

Benzie IFF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. 239: 70–76.

Bonina F, Puglia C, Ventura D, Aquino R, Tortora S, Sacchi A, Saija A, Tomaino A, Pellegrino ML, De Capraris P (2002). *In vitro* antioxidant and *in vivo* photoprotective effects of a lyophilized extract of *Capparis spinosa L*. buds. J. Cosmet. Sci. 53: 321–335.

Brevard H, Brambilla M, Chaintreau A, Marion JP (1992). Occurrence of elemental sulphur in capers (*Capparis spinosa* L.) and first investigation of the flavour profile. Flavour Fragrance J. 7: 313–321.

Burton GW, Joyce A, Ingold KU (1983). Is vitamin E the only lipid-soluble, chain breaking antioxidant in human blood plasma and erythrocyte membrane? Arch. Biochem. Biophys. 221: 281–290.

Brody T (1994). Nutritional Biochem. San Diego, CA: Academic Press.
Germano MP, Pasquale R, D'angelo V, Catania S, Silvari V, Costa C (2002). Evaluation of extracts and isolated fraction from *Capparis spinosa* L. buds as an antioxidante source. J. Agric. Food Chem. 50: 1168–1171.
Guil JL, Martinez JJG, Isasa ME (1998). Mineral nutrient composition of edible wild plants. J. Food Compos. Anal. 11: 322–328.
Hafeez B, Khanif YM, Saleem M (2013). Role of zinc in plant nutrition- a review. Am. J. Exp. Agr. 3(2): 374–391.
Haslam E (1996). Natural polyphenols (vegetable tannins) as drugs: possible modes of action. J. Nat. Prod. 59: 205–215.
Heinonen M, Lehtonen PJ, Hopla A (1998). Antioxidant activity of berry and fruit wines and liquor. J. Agric. Food Chem. 48: 25–31.
Ihme N, Kiesewetter H, Jung F, Hoffmann KH, Birk A, Muller A, Grutzner KI (1996). Leg oedema protection from a buckwheat herb tea in patients with chronic venous insufficiency: a single-centre randomised, double blind, placebo-controlled clinical trial. Eur. J. Clin. Pharmacol. 50: 443–447.
Kolayli S, Küçük M, Duran C, Candan F, Dinçer B (2003). Chemical and antioxidant properties of *Laurocerasus officinalis* Roem. (cherry laurel) fruit grown in the black sea region. J. Agric. Food Chem. 51: 7489–7494.
Kolayli S, Kara M, Tezcan F, Erim FB, Sahin H, Ulusoy E, Aliyazıcıoğlu R (2010). Comparative study of chemical and biochemical properties of different melon cultivars: Standard, hybrid, and grafted melons. J. Agric. Food Chem. 58: 9764–9769.
Macrae R, Robinson RK, Sadler MJ (eds.) (1993). Encyclopaedia of food science, food technology and nutrition, vol. 5. SanDiego, CA: Academic Press INC.
Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA (2000). Cytochrome P450 CYP79B2 from Arabidopsis catalyzed conversion of triptophane to indole-3,3-acetaldoxime, a precursor of indole glucosinolates and indole-3 acetic acid. J. Biol. Chem. 275: 33712–33717.
Molyneux P (2004). The use of the stable free radical diphenyl picrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 26: 211-219.
Mozaffarieh M, Sacu S, Wedrich A (2003). The role of the carotenoids lutein and zeaxanthin in protecting against age related macular degeneration: a review based on controversial evidence. Nutr. J. 2: 20–28.
Nagai T, Reiji I, Hachiro I, Nobutaka S (2003). Preparation and antioxidant properties of water extract of propolis. Food Chem. 80: 29–33.
Ozcan M (2004). Mineral contents of some plants used as condiments in Turkey. Food Chem. 84: 437–440.
Ozcan M, Akgul A (1998). Influence of species, harvest date and size on composition of capers (*Capparis spp.)* flower buds. Nahrung, 42: 102–105.
Singleton VL, Rossi JL (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. Am. J. Enol. Viticult., 16: 144–158.
Sommer A, Davidson FR (2002). Assessment and control of vitamin A deficiency: The Annecy Accords. J. Nutr. 132: 2845S–2850S.
Steinmetz KA, Potter JD (1991).Vegetables, fruit, and cancer. II. Mechanisms. Cancer Causes Control 2: 427–442.
Tezcan F, Kolaylı S, Sahin H, Ulusoy E, Erim BF (2011). Evaluation of organic acid, saccharide composition and antioxidant properties of some authentic Turkish honeys. J. Food Nutr. Res. 50: 33–40.
Tlili N, Khaldi A, Triki S, Munné-Bosch S (2010). Phenolic compounds and vitamin antioxidants of caper (*Capparis spinosa*). Plant Foods Hum. Nutr. 65: 260–265.
Tlili N, Nasri, N, Khaldi A, Triki S, Munne-Bosch S (2011). Phenolic compounds, tocopherols, carotenoids and vitamin C of commercial caper. J. Food Biochem. 35: 472–483.
Yang JH, Lin HC, Mau JL (2002). Antioxidant properties of several commercial mushrooms. Food Chem. 77: 229–235.
Yoshino M, Murakami K (1998). Interection of iron with polyphenolic compounds: application to antioxidant characterization. Anal. Biochem. 257: 40–44.
Zima TS, Fialova L, Mestek O, Janebova M, Crkovska J, Malbohan I, Stıpek S, Mikulıkova L, Popov P (2001). Oxidative stress, metabolism f ethanol and alcohol-related diseases. J. Biomed. Sci. 8: 59–70.

academicJournals

Vol. 12(47), pp. 6650-6660, 20 November, 2013
DOI: 10.5897/AJB2013.13058
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Synthesis and characterization of cupric oxide (CuO) nanoparticles and their application for the removal of dyes

Ghulam Mustafa[1], Hajira Tahir[1]*, Muhammad Sultan[2] and Nasir Akhtar[1]

[1]Department of Chemistry, University of Karachi 75270, Pakistan.
[2]Federal Urdu University of Arts, Science and Technology Karachi- Pakistan.

Accepted 7 November, 2013

In the present work, cupric oxide (CuO) nanoparticles (NPs) were prepared by adopting aqueous precipitation method using copper sulphate 5-hydrate as a precursor and NaOH as a stabilizing agent. This gives a large scale production of CuO-NPs which are utilized for the removal of methylene blue (MB) dye. The CuO NPs were characterized for the studying of their structure and composition from XRD which reveals the single phase monoclinic structure. The surface morphology of these NPs was carried out by using scanning electron microscopy (SEM). Moreover, the effect of optimization parameters such as time, concentration and temperature was also examined. Spectrophotometric technique was used to evaluate the removal of MB in aqueous solution by NPs. The equilibrium adsorption of cationic dye (MB) was carried out at various temperatures ranging from 303 to 318 K ± 2K. The adsorption isotherm equations like Langmuir, Freundlich and Dubinin-Radushkevich were applied and the values of their respective constants were evaluated by adopting graphical method. Thermodynamic and kinetic studies were also performed to determine the feasibility of the process. The maximum MB removal was observed to be 88.93%. The pH of point zero charge (pH_{PZC}) of adsorbent was also estimated by pH drift method. The results indicate that aqueous precipitation method is a reliable and cheap method for the development of CuO-NPs which can be effectively used for the removal of dyes from effluents. This method is beneficial for the remediation of industrial waste.

Key words: CuO nanoparticles, XRD, SEM, methylene blue, adsorption, UV Spectrophotometer.

INTRODUCTION

There is a number of metal oxide in nature but some of the metal oxides are most useful in accordance with their applications of day to day life in science and technology. Physico-chemical properties of special relevance in Chemistry are mostly related to the industrial use of oxides as sensors, ceramics, adsorbents and catalysts. A nano-silica-Ag NPs composite material is proposed as a novel antifouling adsorbent for cost-effective and eco-friendly water purification (Das et al., 2013), novel magnetic Fe_3O_4@C nanoparticles have been synthesized and employed as high efficient adsorbent for removal of cationic dyes from polluted water (Zhang and Kong, 2011). The utilization of modified magnetite nanoparticles (Fe_3O_4 NPs) with a cationic surfactant (cetyl trimethyl ammonium bromide, CTAB) as an efficient adsorbent was successfully carried out to remove reactive black 5 (RBBA), reactive red 198 (RRR) and reactive blue 21 (RTB) dyes from aqueous solutions (Faraji et al., 2010).

The creation of environmental problems due to rapid development of technology causes a threat to our horizon. Dyes can be said to be colored, ionizing and aromatic organic compounds which shows an affinity

*Corresponding author. E-mail: hajirat@uok.edu.pk. Tel: +92-333-3621470. Fax: +92-21-6832216.

towards the substrate to which it is being applied. It is generally applied in a solution that is aqueous and required a mordant to improve the fastness of the dye on the material on which it is applied.

They have many different structural variations like acidic, basic, disperse, azo, anthra quinone based and metal complex dyes among others. The textile industry is the largest consumer of dye stuffs. During the coloration process, a large percentage of the synthetic dye does not bind and is lost to the waste stream (Weber and Adams, 1995). Approximately 10-15% dyes are released into the environment during dyeing process making the effluent highly colored and aesthetically unpleasant (Ratna and Padhi, 2012). Dyes have been applied in many industries such as textiles, printing, leather, pulp, food and plastics, etc. The effluent from these industries normally contains presence of these dyes. About 10,000 different commercial dyes and pigments exist and more than $7x10^5$ tons are produced per year worldwide (Wanchanthuek and Nunrung, 2011; Shah et al., 2010). Approximately 10-15% of these dyes are released after dying process Al-Degs et al. (2008) since many organic dyestuffs are harmful to human being and toxic to animals and microorganisms. The dye removal has been in considerable attention over the past decades before release to natural stream. Various dye removal process have been used such as coagulation, chemical oxidation, membrane separation, electrochemical process, biological treatment and adsorption techniques Durai and Rajasimman (2011). Adsorption was recognized to be an effective process for the removal of dyes from waste water effluents which are the easiest in separation after the process and highly effective in dye removal (Orthman et al., 2003). Different kinds of adsorbents have been developed for various applications such as activated carbon (Iqbal et al., 2007), active carbon from pyrolysis of bagasse (Lori et al., 2008), spent activated clay (Weng et al., 2007), sand (Rauf et al., 2008), soil Cheng et al. (2008), kaolin (Nandi et al., 2009), biomass from calotropis procera leaf (Ali et al., 2008), natural resin (Clinoptilolite) (Moazed , 2008), and Mg-Al-CO_3 layers (Gaini, 2009). CuO NPs Aparna et al. (2012) and carbon-encapsulated super magnetic colloidal NPs Wang et al. (2013). In the present work, CuO nanoparticles were prepared and utilized as nano adsorbents for the removal of cationic dye. The XRD and morphological study of CuO-NPs was observed by scanning electron microscopy (SEM).

MATERIALS AND METHODS

Synthesis of CuO nanoparticles

CuO nanoparticles were synthesized by aqueous precipitation method using 55 g of copper sulfate 5-hydrate (Merck) mixed with 25 g of hydroxyl ammonium chloride (Sigma Aldrich) in 125 ml distilled water. The mixture was allowed to cool in a cold water bath with swirling well and solution of 40 g of sodium hydroxide in 750 ml distilled water was added. The precipitate was allowed to settle down and the supernatant liquid was poured off according to a modified method of Kannaki et al. (2012). The oxide was transferred to 250 ml flask and the volume was made up. The content was washed by repeated decantation until the rinsing was chloride-free, and then suction filtration was applied and the residue was washed with 95% alcohol and ether. The residue was dried at 200 to 250°C in air oxidized conditions to obtain Cu_2O and then further the temperature was increaded to 300°C which leads to the formation of CuO. For the formation of CuO NPs from Cu_2O precipitant, the following reaction mechanism can be formulated as represented as:

$$2CuSO_4 + 2NH_2OH.HCl + 6NaOH \rightarrow Cu_2O + N_2 + 2NaCl + 2Na_2SO_4 + 7H_2O \quad (1)$$

$$Cu_2O \xrightarrow{\triangle} CuO \quad (2)$$

Characterization of nanoparticles

The XRD patterns of nano CuO were recorded on a (BRUKER AXS) diffractometer in the scanning range of 20-70° (2θ) using CuKα as radiation source having a wavelength of 1.54060 A° at the scanning rate of 15.50 s with the instrument temperature of 25°C. The morphology of prepared NPs before and after adsorption was examined by SEM technique. SEM images were taken by Joel Quick Auto Coater and by using ion sputtering device.

Treatment of methylene blue

The removal of methylene blue from aqueous solution was carried out by adsorption method using CuO-NPs.

Optimization of amount of adsorbent

CuO-NPs of known amounts 0.01 - 0.1 g was added in shaking flasks and placed in electric shaker for a desired time period. The amount of dye adsorbed (mg/g) increased with increase in time and then reached equilibrium. The contents of the flasks were filtered to separate the NPs. Concentration of methylene blue was quantified in the filtrate by UV spectrophotometer. The initial dye concentration provides the necessary driving force to overcome the resistances to the mass transfer of MB between the aqueous and solid phases (Rauf et al., 2007). A similar phenomenon was observed for the adsorption of Methylene Blue (MB) dye onto banana stalk waste by Hameed et al. (2007), pomelo (*C. grandis*) peel (Wang et al., 2007) and castor shell seed (Ni et al., 2007).

Optimization of shaking time

The shaking time also varied from 2 to 24 min by keeping the optimum amount of adsorbent and concentration of methylene blue. After the interval of 2 min each, flask was ejected and the content filtered. The filtrate was analyzed by UV spectrophotometer to study the adsorption of MB on NPs as a function of contact time in order to find out the equilibrium time for maximum adsorption. Sureshkumar et al. (2008) reported an equilibrium adsorption time of 135 min for the adsorption of Methylene Blue onto wheat shells and 150 min for the adsorption of methylene blue on fallen phoenix tree's leaves by Ofomaja et al. (2007)

Optimization of temperature

The shaking temperature also varied from 30 to 45°C by keeping

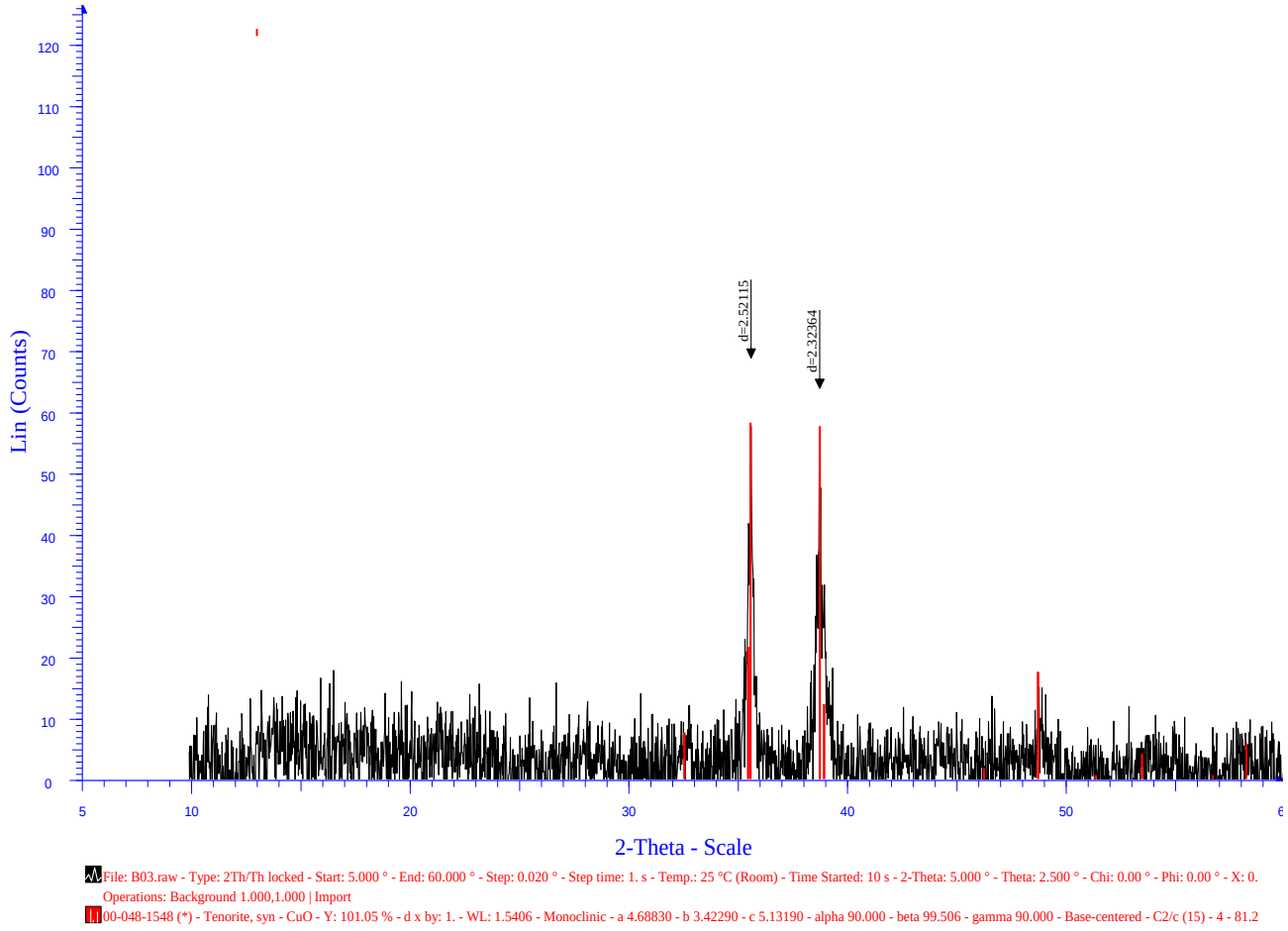


Figure 1. XRD pattern of CuO nanoparticles.

the optimum amount of adsorbent and time against known concentration of methylene blue ($1x10^{-5}$ to $1x10^{-4}$ M). The adsorption sets were run at 30, 35, 40 and 45°C for 60 min. The contents of the flasks were filtered to separate the adsorbent. The filtrate was analyzed by UV spectrophotometer. Alkan et al. (2008) and Abd El-Latif et al. (2009) reported that the changing of the temperature will change the equilibrium capacity of the adsorbent for a particular adsorbate.

pH at the point of zero charge (pHzpc)

The point of zero charge (pHzpc) is the pH at which the total number of positive and negative charges on its surface becomes zero (Jia et al., 2002). The pH at the point of zero charge (pHzpc) of CuO-NPs was measured by using the pH drift method. The pH of 0.005 mol/dm^3 NaCl solution was adjusted between 2-12 by adding either HCl or NaOH. The 0.03 g of adsorbent was added in 20 ml of the solution in Erlemmeyer flask and left at room temperature for 24 and 48 h. After the pH stabilized, the final pH was recorded. The graph of pHs was drawn and used to determine the points at which the initial and final pH values were equal (Vijayakumar et al., 2012).

RESULTS AND DISCUSSION

Powder X-ray diffraction study was carried out on the prepared CuO NPs. These NPs retained its monoclinic structure with lattice parameters: a = 4.688A°, b = 3.422A°, c = 5.131A°, β = 99.506 and V = 82.31 A°. The crystalline CuO NPs were confirmed by the powder X-ray diffraction study and diffraction peaks are indexed in Figure 1.

The crystal structure parameters obtained show that all the diffraction peaks can be indexed with lattice planes and compared to the International center for diffraction data (ICDD) Card No:41-0254. The d-spacing values of the peaks were well matched with the reported values in the card. The grain size for different FWHM (β) values was calculated using Debye - Scherrer's equation Lisa et al. (2007).

$$D = K\lambda / \beta \cos \theta \quad (3)$$

Where, K is a constant representing shape factor which is about 0.9, λ is the X-ray wavelength used which is 1.54060A°, while β is the full width half maximum (FWHM) of the diffraction angle. No peaks of impurities were found in XRD pattern. The peaks were broad due to the nano size effect. The average crystalline size of CuO

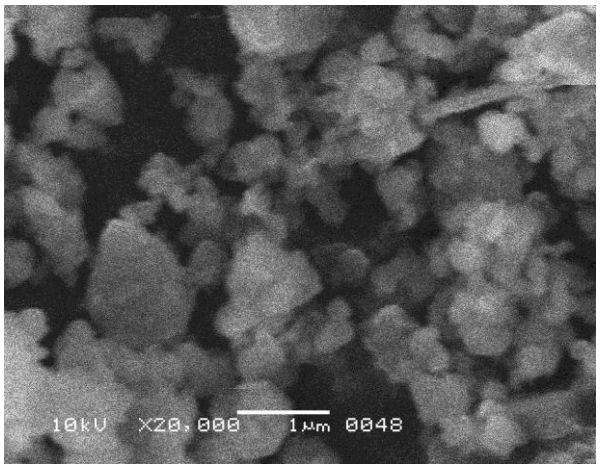


Figure 2. SEM of CuO-NPs before adsorption of methylene Blue.

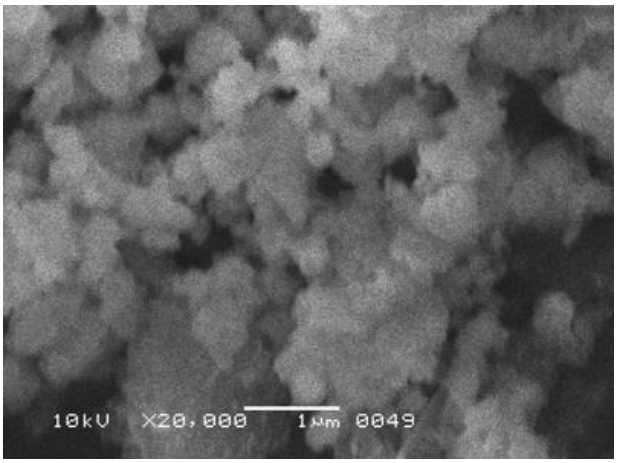


Figure 3. SEM of CuO-NPs after adsorption of methylene blue.

NPs was found to be 20 - 28 nm.

Surface morphological studies

The images of Scanning Electron Microscope of CuO-NPs before and after adsorption of MB dye are shown in Figures 2 and 3 at different magnification. SEM image of CuO before adsorption of MB dye indicates that the material is composed of irregularities in shape from 0.02 to 1 µm which provides a large surface area for the adsorption experiment. After the adsorption of MB dye, the structures become saturated with MB molecules.

Investigation of adsorption parameters

The present work represents the removal of methylene blue dye onto CuO-NPs by adsorption method. The effect of point of zero charge, thermodynamic and kinetic studies were run by batch adsorption processes under the optimized conditions of amount of adsorbent, contact time and temperatures.

Effect of amount of adsorbent dosage

The adsorption of methylene blue (MB) dye onto CuO-NPs was carried out by varying the amount of adsorbent from 0.02 - 0.2 g. The values of % removal of MB dye were increased with the increase in amount of CuO-NPs and 0.03 g of adsorbent showed the optimum adsorption as shown in Figure 4. This may be due to the increase in adsorbent surface area and viability of more adsorption sites. The amount of adsorbent at time t and K_D (mol/g) were calculated as:

$$K_D = (C_i - C_f)V / W \quad (4)$$

Where C_f (mol/dm^3) is the concentration of dye after removal, Ci (mol/dm^3) is the initial concentration of dye in the solution, V is the volume of the solution (ml) and w is the mass of the adsorbent (g).

The amount of equilibrium adsorption, x/m (mol/g), was calculated as:

$$x/m = (C_0 - C_e)\ V / W \quad (5)$$

Where, Co and Ce (mol/dm^3) are the initial and equilibrium concentrations of the dye in the solution.

The percentage removal was calculated as:

$$\% \text{ Removal} = C_o - C_e / C_o \times 100 \quad (6)$$

Effect of contact time

The rate of adsorption of dye decreased with the increase in contact time (02 to 24 min) and reached to an optimum value when the adsorption equilibrium was achieved. The rate of adsorption indicated that the removal of more than 86.28% of MB was observed in the first 2 min. The maximum adsorption capacity of MB on CuO-NPs was obtained at 06 min. The results show that the removal process was very fast and this is an important advantage of separation method as shown in Figure 5.

Effect of temperature

Adsorption isotherm is a graphical representation between the bulk activity of adsorbate and amount adsorbed at constant temperature. It characterizes the distribution of adsorbed solute between the adsorbate and solid phases at various equilibrium concentrations.

The effect of temperature on the removal tendency of dye was also studied at temperatures ranges from 303 to 318 K. The critical review of adsorption isotherm shows

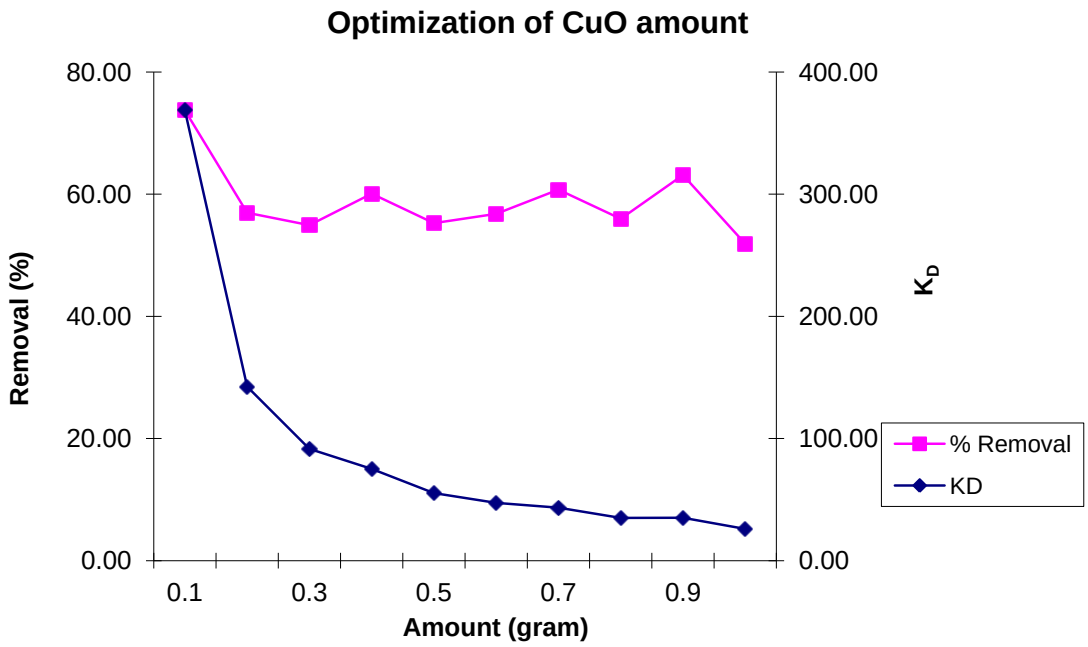


Figure 4. Optimization of amount of CuO for the removal of MB.

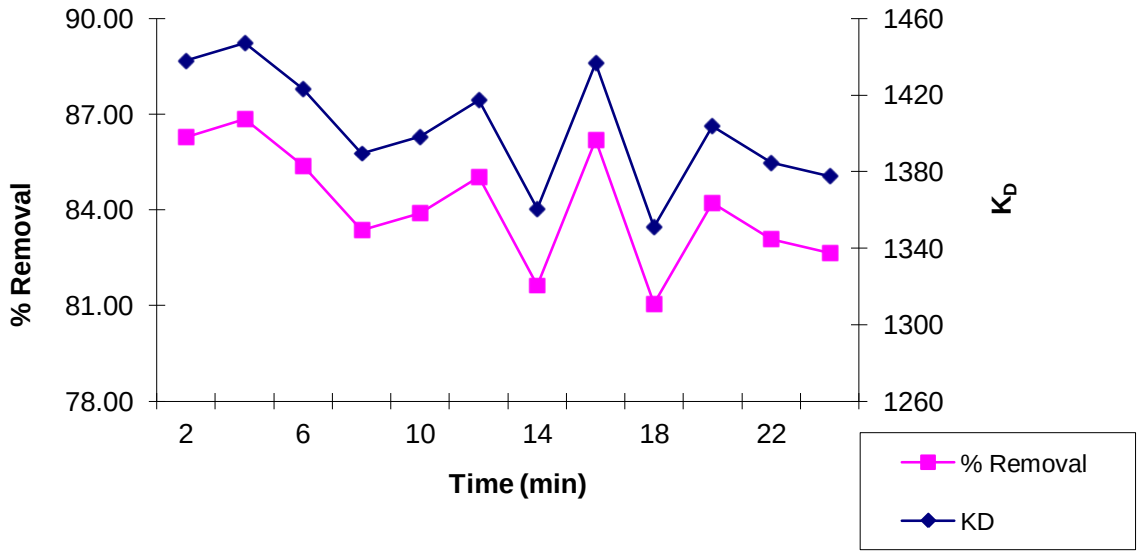


Figure 5. Optimization of time of CuO for the removal of MB.

an increase in the adsorption of dye with the rise in temperature. It shows that adsorption of dye on NPs is endothermic in nature.

Freundlich adsorption isotherm

Freundlich expressed an empirical equation for representing the isothermal variation of adsorption on the quantity of gas adsorbed by unit mass of solid adsorbent with concentration. It is expressed as:

$$\log X/m = \log K + 1/n \log C_e \quad (7)$$

Where, X/m is the amount adsorbed per unit mass of the adsorbent (mol/g), Ce is the equilibrium concentration (mol/dm^3) and the constant K relates to the degree of adsorption, while n provides the rough estimation of the

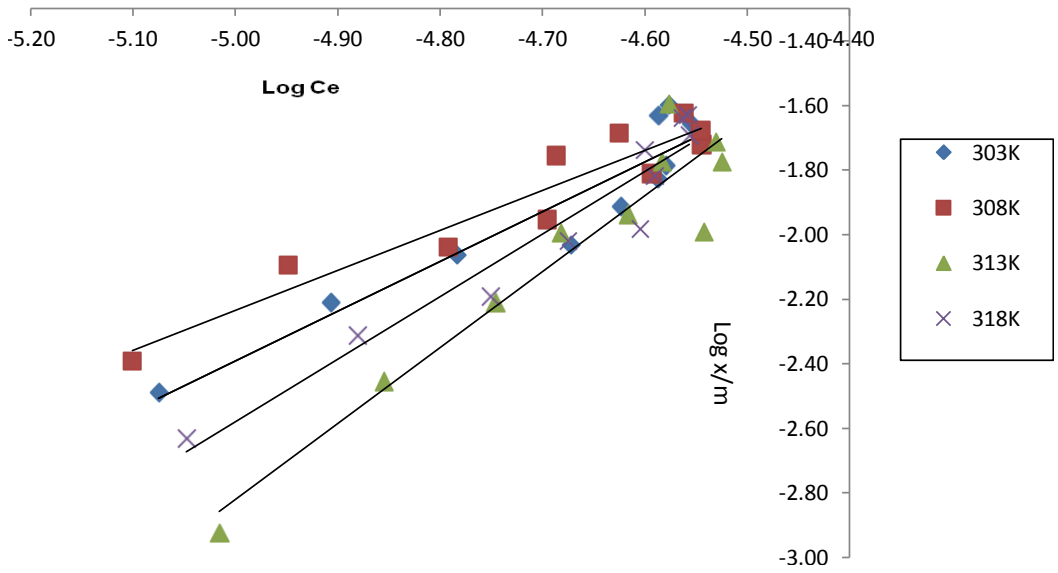


Figure 6. Freundlich Isotherm Plot of CuO NPs for the removal of MB dye.

Table 1. Freundlich parameters for methylene blue dye adsorption on CuO nanoparticles.

Adsorbent	Temperature (K)	K	N (10^{-1})
CuO-NPs	303	2.03E+05	3.46
	308	8.93E+03	1.73
	313	8.83E+08	22.5
	318	1.26E+07	8.63

intensity of the adsorption (Hema and Arivoli, 2008). Freundlich plots were obtained at various temperatures ranges from 303 - 318 K as shown in Figure 6 and the values of respective constant 'K' and 'n' are indicated in Table 1. The decrease in the values of K with the rise in temperature indicates that adsorption affinity of dye on CuO-NPs is less favorable at higher temperatures.

Langmuir adsorption isotherm

The Langmuir isotherm assumes the monolayer adsorption on a homogeneous surface with a finite number of adsorption sites. The Langmuir model assumes that the adsorptions occur at specific homogeneous sites on the adsorbent and is used successfully in many monolayer adsorption processes. The theory can be expressed by the following linear equation:

$$Ce/X/m = 1/kVm + Ce/Vm \quad (8)$$

Where, Ce is the equilibrium concentration (mol/dm^3), X/m is the amount adsorbed at equilibrium (mol/g) and V_m (mol/g) and k (dm^3/mol) are the Langmuir constants relates to monolayer capacity and adsorption coefficient respectively (Hameed and Daud, 2008). The linear plot of Ce/X/m vs Ce is shown in Figure 7 and the values of respective constants are shown in Table 2.

D-R adsorption isotherm

Dubinin Radushkevich (D-R) isotherm was also applied for the adsorption of methylene blue (MB) on to CuO-NPs. The D-R equation can be expressed as:

$$Ln\ X/m = Ln\ Xm - K\varepsilon^2 \quad (9)$$

Where, X_m is the monolayer capacity of adsorbent, K is a constant related to adsorption energy, while ε is the adsorption potential which can be obtained as:

$$\varepsilon = RT\ Ln\ (1+1/Ce) \quad (10)$$

Where, Ce is the equilibrium concentration of dye (mol/dm^3), R is a gas constant and T is the absolute temperature. The D-R plot of ln X/m versus ε^2 was

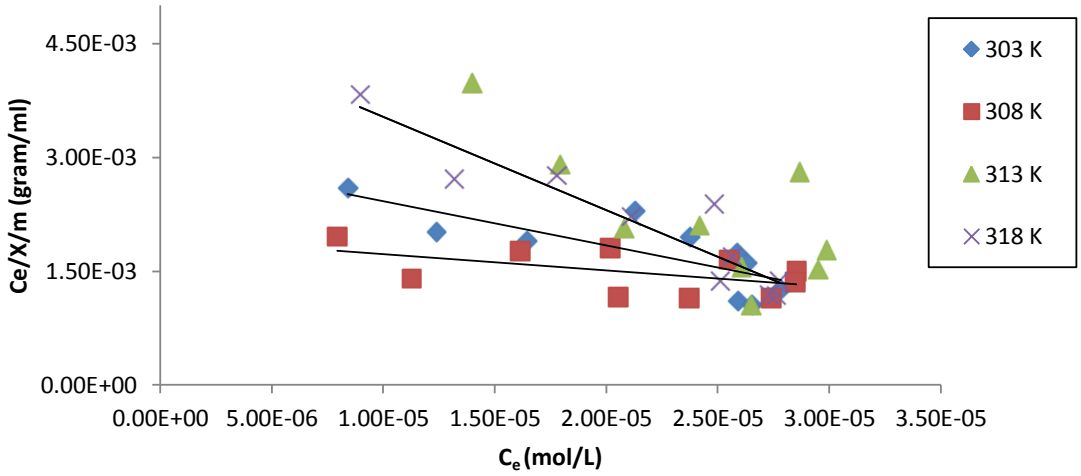


Figure 7. Langmuir Isotherm of CuO-NPs for the removal of MB Dye.

Table 2. Langmuir parameters for methylene blue dye adsorption on CuO nanoparticles.

Adsorbent	Temperature (K)	Xm	Es (KJ/mol) 10^{-3}
CuO-NPs	303	4.11E+01	7.07
	308	9.66E+00	7.45
	313	2.34E+03	5.00
	318	1.18E+01	10.0

obtained at various temperatures as shown in Figure 8 and in Table 3. The values of K and Xm were obtained from the slope intercept while the values of mean free energy of adsorption (Es) were estimated by using the value of K as expressed:

$$Es = (-2K)^{-1/2} \quad (11)$$

Investigation of point of zero charge (pH_{pzc})

For the investigation of point of zero charge CuO-NPs, the values of the initial and final pH were plotted at 24 and 48 h time intervals as shown in Figure 9. From the graph, the values of pH_{pzc} of CuO were determined from the points where the initial pH equals the final pH as shown in Table 4. The pH values implies that surface of CuO was basic in nature since the pH_{pzc} values were increased from acidic pH to neutral and then moved from basic to neutral and finally toward the basic behavior again.

Thermodynamic parameters

The thermodynamic parameters relates to the adsorption of dyes such as free energy change (ΔG°), enthalpy change (ΔH°) and entropy change (ΔS°). These parameters were calculated by using the following equations;

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (12)$$

$$\Delta G^\circ = -RTLnK \quad (13)$$

$$LnK = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (14)$$

The values of ΔH° and ΔS° were calculated from the slope and intercept of the linear variations of lnK_D with the reciprocal of temperature (1/T). The values are represented in Table 5. The negative values of ΔG^o indicate the spontaneous nature of adsorption process while the positive values of ΔH^o and E_a confirms the endothermic nature of the system (Aksu, 2002).

Investigation of adsorption kinetics

The time dependent behavior of adsorption was determined by varying the equilibrium time (2-24) min between adsorbate and adsorbent. The percentage removal of the MB dye against contact time plotted as shown in Figure 10 indicates that the equilibrium was established between MB dye and CuO NPs within 10 min. For the investigation of possible mechanism of adsorption, pseudo first order model was adopted by the

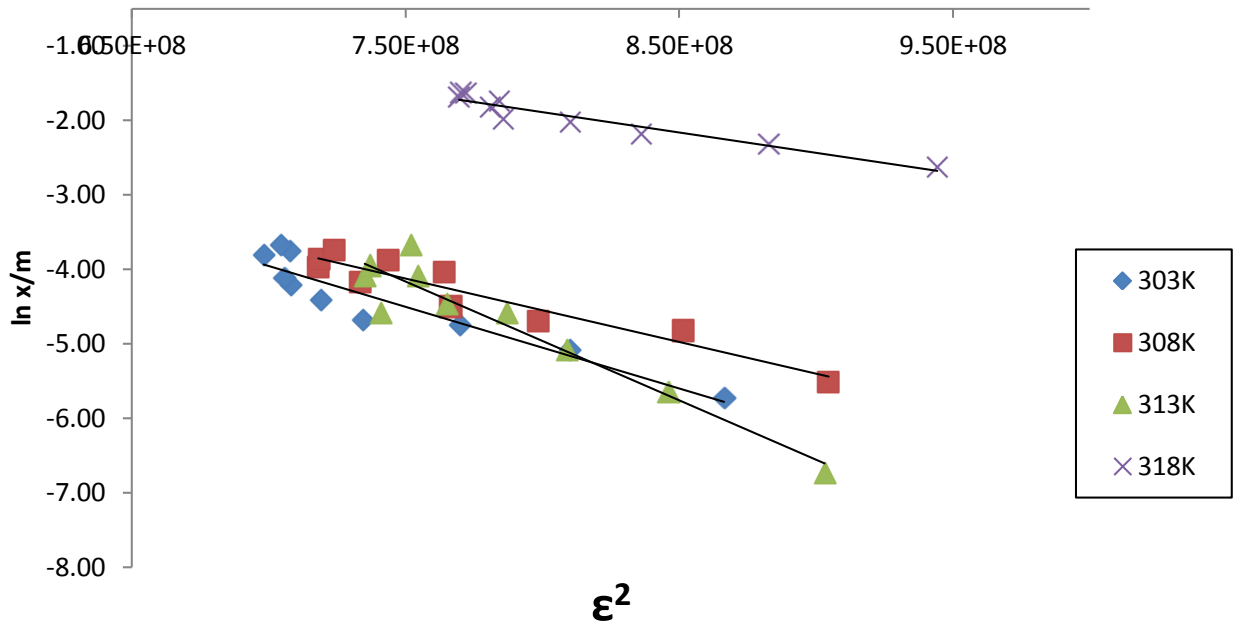


Figure 8. D-R Isotherm Plot of CuO-NPs for the removal of MB dye.

Table 3. D-R parameters for methylene blue dye adsorption on CuO nanoparticles.

Adsorbent	Temperature (K)	Es (J/mol) 10^{-3}	Xm (10)
CuO-NPs	303	7.07E+03	4.11E+01
	308	7.45E+03	9.66E+00
	313	5.00E+03	2.34E+03
	318	1.00E+04	1.18E+01

Table 4. Point of zero charge (pH_{pzc}) of CuO nanoparticles.

Adsorbent	Amount (g)	Initial pH	Final pH	
			After 24 h	After 48 h
CuO-NPs	0.03	2.20	5.55	5.62
	0.03	4.10	7.00	7.11
	0.03	6.04	7.25	7.31
	0.03	7.98	7.39	7.38
	0.03	10.00	7.88	7.96
	0.03	12.01	12.87	12.80

Lagergen (Lagergren, 1898) and pseudo second order models were adopted by the Ho and McKay (Ho and McKay, 1999). They are expressed as:

$$\log (q_e - q_t) = \log q_e - k_1 t/2.303 \quad (15)$$

$$t/q_t = ½ k_2 qe_2 + 1\ t/q_e \quad (16)$$

Where, q_e and q_t are the amount of the dye adsorbed on the adsorbent (mol/g) at equilibrium and time t, k_1 is the rate constant (min^{-1}) representing the pseudo first order kinetics and k_2 is the adsorption of pseudo second order rate constant (mol/g.min). The pseudo second-order adsorption kinetic plot is shown in Figure 10, which shows that it follows a pseudo-second order reaction.

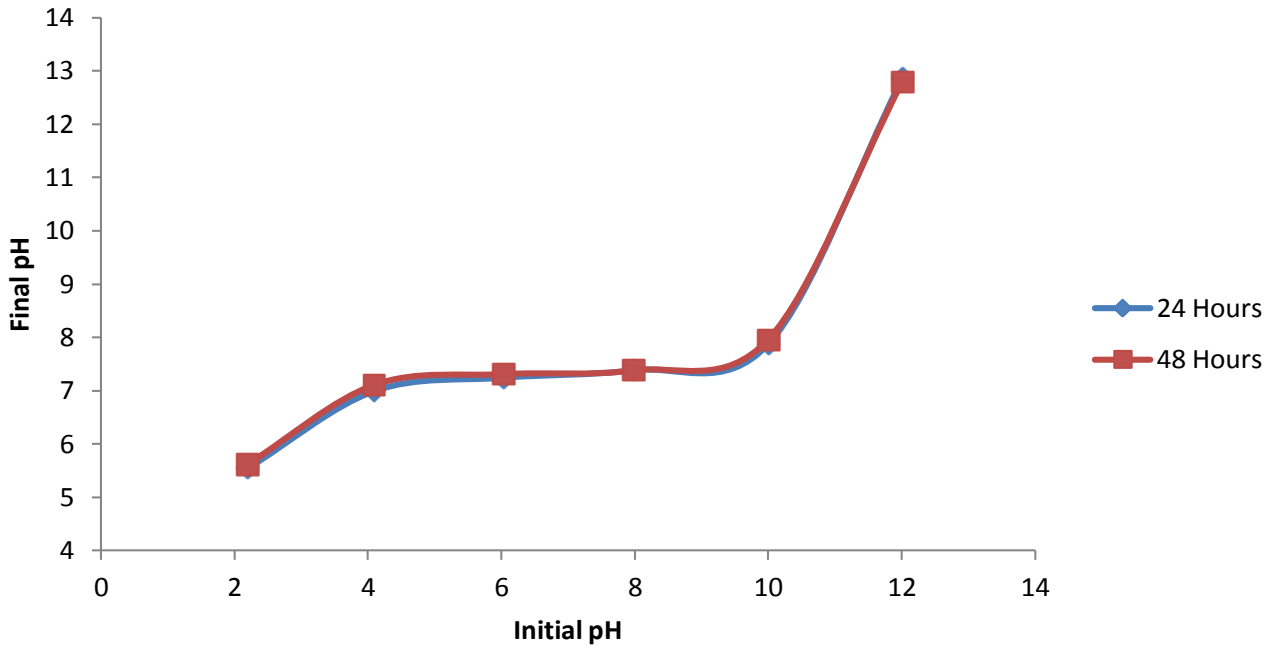


Figure 9. Effect of Point of Zero Charge on CuO-N

Table 5. Thermodynamic parameters for Methylene Blue dye adsorption on CuO nanoparticles.

Adsorbent	Conc. of dye (M) (10^5)	ΔH (KJ/mol) (10)	ΔS (J/mol)	ΔG (J/mol) x 10			
				303K	308K	313K	318K
CuO-NPs	1.00E-05	88.19	1.16	-263.29	-269.09	-274.89	-280.69
	2.00E-05	92.37	1.16	-259.11	-264.91	-270.71	-276.51
	3.00E-05	78.78	1.12	-260.58	-266.18	-271.78	-277.38
	4.00E-05	-0.60	0.86	-261.18	-265.48	-269.78	-274.08
	5.00E-05	65.91	1.07	-258.30	-263.65	-269.00	-274.35
	6.00E-05	21.66	0.93	-260.13	-264.78	-269.43	-274.08
	7.00E-05	-7.49	0.83	-258.98	-263.13	-267.28	-271.43
	8.00E-05	61.44	1.05	-256.71	-261.96	-267.21	-272.46
	9.00E-05	-3.89	0.83	-255.38	-259.53	-263.68	-267.83
	10.0E-05	-20.78	0.91	-296.51	-301.06	-305.61	-310.16

Conclusion

CuO-NPs were prepared successfully with monoclinic structure and confirmed by XRD analysis. The fabricated NPs are composed of irregular shapes ranges from 0.02 to 1 µm which provide a large surface area for the adsorption as determined by SEM results.

These NPs used as adsorbent for the removal of MB dye. Adsorption, desorption, point of zero charge pHpzc, thermodynamic and kinetics studies were proceeded to determine the validity of process. The adsorption experiments were run under the optimized conditions of amount of adsorbent, stay time, initial concentration at different temperatures. The adsorption models like: Freundlich, D-R and Langmuir adsorption isotherm models were applied to determine the adsorption equilibrium data. Thermodynamic parameters, free energy change (ΔG°), enthalpy change (ΔH°) and entropy change (ΔS°) show that the adsorption of MB dye onto CuO-NPs was spontaneous and endothermic process. The kinetic data showed that the adsorption process followed the pseudo second order kinetics.

The feasibility of adsorption / removal process was examined in the present study. It was estimated that inexpensive and cost effective materials can be prepared as adsorbents for the removal of dyes and metals. Undoubtedly low-cost adsorbents offer a lot of promising benefits for commercial purposes with respect to utili-

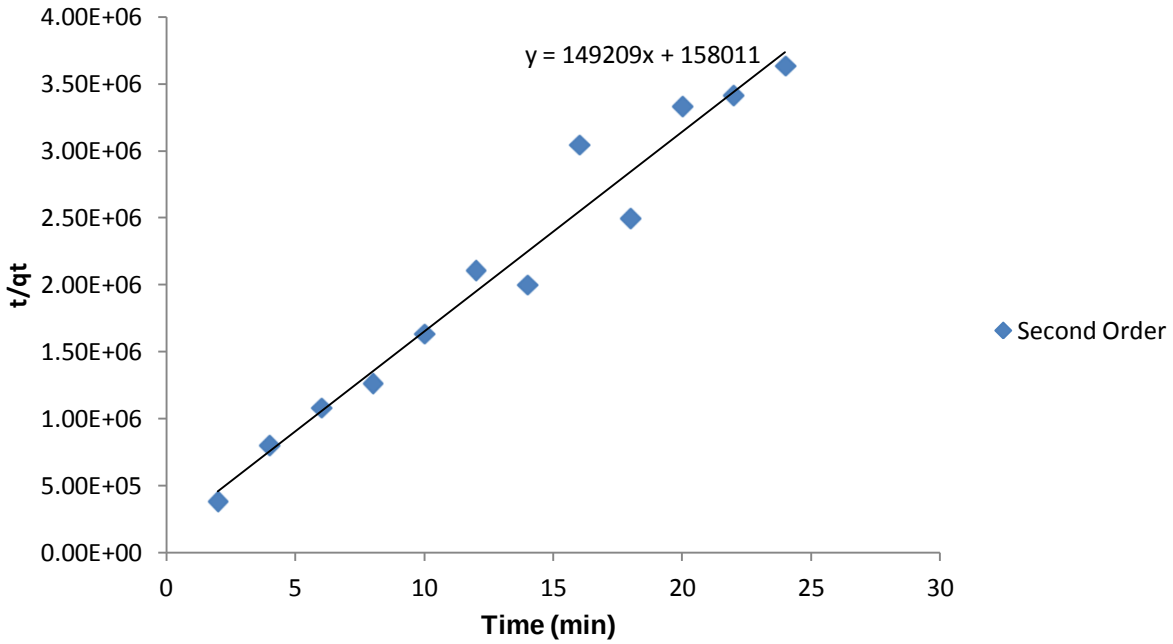


Figure 10. Pseudo second order adsorption kinetics of MB onto CuO-NPS.

zation and minimization of waste.

REFERENCES

Abd El-Latif MM, Ibrahim AM (2009). "Adsorption, kinetic and equilibrium studies on removal of basic dye from aqueous solutions using hydrolyzed Oak Sawdust," Desalination and Water Treatment. 6(1-3): 252-268.

Aksu Z (2002). Determination of the equilibrium, kinetic and thermodynamic parameters of the batch biosorption of nickel(II) ions onto *Chlorella vulgaris,*Pro.Biochem. vol 38, no.1, 89-99.

Al-Degs YS, El-Bargouthi MI, El-Sheikh AH, Walker GM (2008). Effect of solution pH, ionic strength and temperature on adsorption behavior of reactive dyes on activated carbon. Dyes Pigments. 77: 6-23.

Ali H and Muhammad SK (2008). Biosorption of crystal violet from water on leaf biomass of *Calotropis procera*. J. Environ. Sci. Technol. 1: 143-150.

Alkan M, Doğan M, Turhan Y, Demirbaş O, Turan P (2008). "Adsorption kinetics and mechanism of maxilon blue 5G dye on sepiolite from aqueous solutions," Chem. Engin. J. 139(2):213-223.

Aparna Y, Enkateswara Rao KV, Srinivasa SP (2012) synthesis and Characterization of CuO Nano Particles by Novel Sol-Gel Method, 2nd International Conference on Environment Science and Biotechnology, DOI: 10.7763/IPCBEE. 48: 30.

Cheng D L, Zhaoxia Y, Yinglong, Jianxiang J (2008). Adsorption behavior of p-chlorophenol on the reed wetland soils. J. Environ. Sci. Technol. 1:169-174.

Das SK, Khan MM, Parandhaman T, Laffir F, Guha A.K., Sekaran G, Mandal AB, (2013). Nano-silica fabricated with silver nanoparticles: antifouling adsorbent for efficient dye removal, effective water disinfection and biofouling control. Nanoscale. 5(12):5549-60.

Durai G Rajasimman M (2011). Biological treatment of tannery wastewater - A review. J. Environ. Sci. Technol. 4:1-17.

El-Gaini L, Lakraimi M, Sebbar E, Meghea A , Bakasse M (2009). Removal of indigo carmine dye from water to Mg-Al-CO_3 calcined layered double hydroxides. J. Hazard. Mater. 161:627-632.

Hameed BH, Din ATM, Ahmed AL (2007). "Adsorption of Methylene Blue onto bamboo-based activated carbon: kinetics and equilibrium studies", J. Hazard. Mat. 141:819-825.

Handan UCUN. (2011). Equilibrium, thermodynamic and kinetics of reactive black 5 biosorption on loquat (Eriobotrya japonica) Seed. Sci. Res. Essays, 6:4113-4124.

Iqbal, MJ, Ashiq MN (2007). Adsorption of dyes from aqueous solutions on activated charcoal. J. Hazard. Mater. B, 139:57-66.

Jia YF, Xiao B, Thomas KM (2002). Adsorption of metal ions on nitrogen surface functional groups in activated carbons. Langmuir. 18:470-478.

Kannaki K, Ramesh PS, Geetha D (2012). Hydrothermal synthesis of CuO Nanostructure and Their Characterizations, IJSER, 3 (9):1-4.

Lisa M, Daniel RL, Frost Huai YZ. (2007). Synthesis and characterization of clay-supported titania photo catalysts. J. Coll. and Interf. Sc. Vol. 316(1)72-79 .

Lori JA., Lawal AO, Ekanem EJ (2008). Adsorption characteristics of active carbons from Pyrolysis of Bagasse, sorghum and millet straws in ortho phosphoric acid. J. Environ. Sc. Technol. 1:124-134.

Moazed H (2008). Ammonium ion removal from wastewater by a natural resin. J. Environ. Sci. Technol. 1:11-18.

Nandi BK, Goswami A, Purkait MK (2009). Removal of cationic dyes from aqueous solutions by Kaolin: Kinetics and equilibrium studies . Appl. Clay Sci. 42:583-590.

Ni ZM, Xia SJ, Wang LG, Xing FF, Pan GX (2007). "Treatment of methyl orange by calcined layered double hydroxides in aqueous solution: Adsorption property and kinetic studies", J.Collo. Interf. Sci. 316:284-291.

Ofomaja AE, Ho YS (2007) "Equilibrium sorption of anionic dye from aqueous solution by palm kernel fibre as sorbent". Dyes Pigm. 74:60-66.

Orthman J, Zhu HY, Lu GQ (2003). Use of anionclay hydrotalcite to remove coloured oreganics from aqueous solutions . Sep. Purif. Technol. 31:53-59.

Ratna, Padhi BS (2012). Pollution due to synthetic dyes toxicity & carcinogenicity studies and remediation. IJES. 3:3.

Rauf MA, Bukallah SB, Hamour FA, Nasir AS (2008). Adsorption of dyes from aqueous solutions onto sand and their kinetics behavior. Chem. Eng. J.137:238-243.

Rauf MA, Shehadi I, Hassan WW (2007). "Studies on the removal of Neutral Red on sand from aqueous solution and its kinetic behavior", Dyes Pigm. 75:723-726.

Shah RA, Kumawat DM, Singh N, Wani K A (2010). water hyacinth (*Eichhornia Crassipes*) as a remediation tool for dye-effluent pollution, I.J.S.N.1(2):172-178.

Sureshkumar MV and Namasivayam C (2008) "Adsorption behavior of Direct Red 12B and Rhodamine B from water onto surfactant-modified coconut coir pith", Collo.Surf. A: Physico. Eng. Aspects, 317:277-283.

Vijayakumar G, Tamilarasan R, Dharmmendirakumar M (2012). Adsorption, Kinetic Equilibrium and Thermodynamics studies on the removal of baisc dye Rhodamine-B from aqueous solution by the use natural adsorbent perlite. J. Master. Environ. Sci. 3(1):157-170.

Weng CH and Pan YF (2007). Adsorption of a cationic dye (Methylene Blue) onto spent activated clay. J.H Hazard. Mater.144:355-362.

Wang S, Li H (2007). "Kinetic modelling and mechanism of dye adsorption on unburned carbon", Dyes Pigm. 72:308-314.

Wang H, Zhou X, Chen Q (2013). Removal of cationic dyes from aqueous solution using carbon-encapsulated superparamagnetic colloidal nanoparticles as adsorbent, Nano. 08: 1350006-9 .

Wanchanthuek R, Nunrung W (2011). The adsorption study of Methylene blue onto MgO from various preparation methods. J. Environ. Sci. Technol. vol. 4(5):534-542.

Weber EJ, Adams RL, (1995). Chemical and sediment mediated reduction of the azo dye Disperse Blue 79. Environ. Sci. Technol. 29:1163-1170

Zhang Z, Kong J (2011). Novel magnetic Fe_3O_4@C nanoparticles as adsorbents for removal of organic dyes from aqueous solution, J. Hazard. Mater, 193:325-329

academicJournals

Vol. 12(47), pp. 6661-6671, 20 November, 2013
DOI: 10.5897/AJB2013.12957
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Preliminary spectroscopic characterization of PEGylated mucin, a novel polymeric drug delivery system

Franklin Chimaobi Kenechukwu[1]*, Emmanuel Chinedum Ibezim[1], Anthony Amaechi Attama[1], Mumuni Audu Momoh[1], John Dike Nwabueze Ogbonna[1], Petra Obioma Nnamani[1], Salome Amarachi Chime[2] , Chukwuebuka Emmanuel Umeyor[3] and Emmanuel M. Uronnachi[3]

[1]Drug Delivery Research Unit, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria.
[2]Department of Pharmaceutical Technology and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria.
[3]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

Accepted 16 September, 2013

The objective of this study was to evaluate, spectrophotometrically, the compatibility of non-mucinated polyethylene glycol (PEG) 4000 and non-PEGylated mucin in a PEGylated mucin matrices for drug delivery application. Mucin was extracted from the giant African land snails (*Archachatina maginata*) using chilled acetone and characterized in terms of qualitative properties and solubility profile. Polymeric matrices composed of PEG 4000 and mucin in ratios of 2:0 (A), 1:1 (B), 2:1(C) and 3:1 (D) were prepared by co-precipitation using chilled acetone. The matrices were characterized with respect to compatibility using the Fourier transform infrared (FT-IR) spectroscopy. Results of the qualitative tests performed on the snail mucin showed that carbohydrates, proteins and trace amounts of fats were present; the extracted mucin was light-brownish in colour, with a pleasant meaty odour. Snail mucin, when dispersed in water yielded a slightly viscous dispersion, but is not soluble in ethanol, acetone, 0.1 M sodium hydroxide, ammonium hydroxide and sulphuric acid. The presence of different peaks in the FT-IR spectra of the PEGylated mucin matrices compared with the non-PEGylated mucin (2:0) matrix and non-mucinated PEG 4000 (0:2) matrix indicated the formation of new polymers, which could be employed in drug delivery. This study has shown that PEGylation of mucin gives rise to new polymeric system with principal FT-IR peaks quite different from those of non-PEGylated mucin and non-mucinated PEG, and this may be employed in the delivery of drugs.

Key words: PEGylation, drug delivery, mucin, Fourier transform infrared (FT-IR) spectroscopy, *Archachatina maginata*.

INTRODUCTION

Drug delivery can be of importance for both new chemical entities as well as established drugs. The basic goal of any drug delivery system is to achieve steady state of blood concentration or tissue level that is therapeutically effective and non-toxic for an extended period of time (Muller et al., 2000; Gillies and Frechet, 2004). Many biolo-

gically active molecules are limited in their therapeutic values by properties such as poor solubility, limited bioavailability, and rapid elimination (Muller et al., 2000). In addition, while the beneficial effects of many drugs arise from their interaction with specific tissues, their exposure to other cell types frequently leads to undesirable side effects and toxicity (Gillies and Frechet, 2004). In recent decades, there has been increased awareness of the need to develop drug delivery systems to improve the properties of therapeutic compounds, increase their effectiveness, and reduce their harmful side effects. Oral administration of therapeutic agents represents by far the easiest, safest and most convenient route of drug delivery, especially in the case of chronic therapies (Kreuter, 1991). Unfortunately, the oral delivery route is beset with problems such as gastrointestinal (GI) destruction of labile molecules and low levels of macromolecular absorption (Francis et al., 2004). The development of oral forms of many drugs however remains a challenge either on account of their stability or their absorption from the GIT thus leading to sub-therapeutic bioavailability (Kreuter, 1991). To reduce the impact of digestive enzymes and ensure the absorption of bioactive agents in an unaltered form, molecules may be incorporated into polymeric matrices, which could shield the encapsulated drug from the external harsh conditions, and may favour uptake by intestinal cells (Delie and Blanco-Prieto, 2005). Many polymers have been used in the formulation of polymeric matrices (Ravi-Kumar, 2000; Pamujula et al., 2004; Lagarce et al., 2005; Bernkop-Schnurch et al., 2004; Attama and Nwabunze, 2007). The engineering of new polymer biomaterials with special physicochemical properties appears to be an answer to the afore-mentioned problems. To obtain new polymer biomaterials with better physicochemical qualities that will meet the carrier needs of challenging drug molecules, blending of polymers with desirable properties becomes imperative. Thus, mucin and PEG could be blended to improve the physico-chemical properties of the individual polymers.

Mucins are high molecular weight (0.2 – 10 million Dalton) glycoproteins containing both highly glycosylated domains and naked domains. The glycosylated domains are enriched in serine and threonine residues which serve as anchoring points for oligosaccharide chains. These O-linked oligosaccharide side chains are complex both in terms of composition and length. The naked domains are typically found in the N-terminal and C- terminal parts of the protein and are enriched in cysteine residues (Carlstedt et al., 1983). The cysteine residues can form intermolecular bonds, and in the native state mucins are often found as oligomers composed of several end-to-end linked mucin subunits (Perez and Proust, 1987). A common feature of mucins, apart from a high molecular weight and a high carbohydrate content (68 - 81%), is the abundance of negatively charged groups. The negative charges arise mainly from sialic acid residues and in some cases from sulphated sugars. These acidic groups account for the low isoelectric point (2-3) of mucins (Durrer et al., 1995). The basic amino acids of the protein component are serine and threonine. The glycosylated regions of mucins interact favourably with water and force the molecule to an extended random coil conformation, and the high molecular weight enables individual mucin molecules to overlap and entangle at relatively low concentrations (Lee et al., 2005). These features are ideal with respect to the formation of hydrogels. Studies have shown that reconstucted mucous gels from mucins have similar rheological properties as native mucous gels at physiological concentrations (Raynal et al., 2003). The ability of mucin to form the structural backbone of the mucous gel is one of its most important functions (Raynal et al., 2003).

There is an on-going effort aimed at improving the potential use of mucin in controlled delivery of certain drugs. One of the strategies that have been successfully employed is PEGylation (Momoh et al., 2010; Momoh et al., 2011; Momoh et al., 2010). Previous studies on PEGylation (the process of attaching PEG to any polymer, or the molecular attachment to polyethylene glycols with different molecular weights to active drug molecules or surface treatment of drug-bearing particles with PEGs) have shown that the interaction between PEG and mucin is either molecular interaction in a solvent which resulted in the formation of new bond or attachment of the PEG to a functional group in the protein molecule to form a copolymer (Schnurrer and Lehr, 1996). This technology is one of the most promising and extensively studied strategies for improving the pharmacokinetics of drugs (Abuchowski et al., 1977). Clinically proven PEGylation technology can improve the performance and dosing convenience of peptides, proteins, some water soluble drugs, antibodies, oligonucleotides and many small molecules by optimizing pharmacokinetics, increasing bioavailability and decreasing immunogenicity and dosing frequency. PEGylation has been shown to increase therapeutic efficacy by enabling increased drug concentration, improved biodistribution, and longer dwelling time at the site of action (Kodera et al., 1998). This would result in the achievement of therapeutic drug concentrations with less frequent dosing which would be a significant benefit to patients who are taking injected drugs

*Corresponding author. E-mail: chimafrankduff@yahoo.com; frankline.kenechukwu@unn.edu.ng.
Tel: +234 8038362638. Fax: +234-42-771709.

Abbreviations: APIs, Active pharmaceutical ingredients; **FT-IR,** Fourier transform infrared; **DSC,** differential scanning calorimetry; **GI,** gastrointestinal.

and long time medication for example in diabetes management. Therefore, among the different polymer-based drug delivery systems, PEGylated polymeric bioadhesive microparticles represent a promising delivery vehicle especially intended for short-acting active pharmaceutical ingredients (APIs) employed in chronic therapies in order to improve their therapeutic effects.

Recently, PEGyalted mucin matrices were characterized with respect to thermal properties. The results of the thermal properties of PEGylated mucin which was measured by differential scanning calorimetry (DSC) indicated that an interaction occurred between PEG and mucin (Momoh et al., 2010). This serves as a basis for further characterization of the interaction between PEG and mucin in PEGylated mucin matrices for drug delivery application. Fourier transform infrared (FT-IR) spectroscopic analysis can be used to determine the functional groups of the molecules (Sahoo et al., 2012; Sahoo et al., 2012; Sahoo et al., 2011). Therefore, to obtain more information in detail about chemical interaction between PEG and mucin, the FT-IR analysis was carried out. In this study, admixtures of PEG 4000 and mucin obtained from African land snail (*Archachatina marginata*) were prepared by combining the two at different ratios. Some physicochemical properties as well as compatibility of the PEGylated mucin matrices were assessed. The objective of this study was to evaluate, spectroscopically, the compatibility of non-mucinated polyethylene glycol (PEG) 4000 and non-PEGylated mucin in a PEGylated mucin matrices for drug delivery application.

MATERIALS AND METHODS

The following materials were purchased from their local suppliers and used without further purification: polyethylene glycol-4000 (Ph. Eur. Carl Roth GmbH + Co.KG Karlsruhhe Germany), citric acid, sodium hydroxide (Merck, Germany), methyl red, ethanol, nitric acid, silver nitrate, tetraoxosulphate (vi) acid, sodium chloride, acetone, concentrated hydrochloric acid (BDH, England). The giant African land snails (*A. marginata*) used were procured from a local market in Nsukka, Enugu State. All other reagents were of analytical grade and were used as such.

Extraction of snail mucin (slime)

The method of Adikwu and Nnamani (2005) was employed for the extraction with modifications. The giant African land snails (*A. maginata*) were washed with clean water and the shells were knocked open at the apex and a spirally coiled rod was inserted to remove the fleshy body. The mucus layer (slime mucus) was gently scraped off from the fleshy parts, pooled together in a container and precipitated with chilled acetone. These precipitates were then dried with a flush of cold air (-4°C) to obtain greyish-brown flakes of the snail mucin, which were powdered and used for the study.

Physicochemical properties of snail mucin

Some physicochemical properties of the snail mucin were also assessed using standard procedures (Adikwu and Ikejiuba, 2005).

Test for proteins (amines; oxidation test)

Biuret test

Two drops of water and 1 ml of dilute sodium hydroxide were added to 2 % dispersion of mucin in water. Two drops of 1 % copper sulphate solution were added with the solution shaken thoroughly after each drop and observed. A purple or pink colour shows the presence of proteins.

Xanthoproteic reaction

Two drops of concentrated nitric acid were carefully added to 2% dispersion of mucin in water. A white precipitate was formed, which turned yellow on heating. The contents of the test tube were cooled, three drops of dilute sodium hydroxide solution added and the precipitate observed. A yellow colour which changes to orange indicates the presence of proteins.

Test for fats and oils

A drop of the acetone extract of the mucin was placed on a filter paper. The solvent was allowed to evaporate and the filter paper observed carefully for any transluscence.

Test for sugars

Three drops of freshly prepared Fehling's solutions I and II were added to 1% w/v aqueous dispersion of snail mucin which was then heated in a boiling water bath for 5 min and observed.

Test for carbohydrates

Iodine test

Two drops of 1% iodine solution were added to 1 ml of 1% w/v of mucin and then observed for blue-black colouration.

Molisch's test

Two drops of ɑ-naphthol solution was added to 2 ml of the snail mucin dispersion and the two mixed thoroughly. Then 1 ml of concentrated sulphuric acid was gently poured down the side of the tube and observed.

Tollen's reagent test

Tollen's reagent prepared as 1 ml of 5% silver nitrate solution was treated with a few drops of 5 % sodium hydroxide solution. A volume of aqueous ammonia just enough to redissolve the precipitate was added to 3 drops of the snail mucin dispersion and the mixture warmed in a boiling water bath for few minutes. The colour of the precipitate formed was observed.

Solubility profile of snail mucin

The solubility of snail mucin in several solvents was determined by dispersing 100 mg of the snail mucin in definite volumes of each solvent- acetone, ethanol, water, sodium hydroxide, hydrochloric acid and ammonium hydroxide respectively at different temperatures (25, 30, 40°C).

Table 1. Physicochemical properties of snail mucin.

Test	Observation	Inference
Carbohydrate	+++	Present
Protein	++	Present
Fats	+	Present

+, Present in trace amount; ++, moderately present; +++, copiously present.

Table 2. Solubility profile of snail mucin.

Temperature (°C)	Acetone	Ethanol	0.1 M NaOH	0.1 M H_2SO_4	0.1 M NH_4OH	H_2O
25	-	-	-	-	-	-
30	-	-	-	-	-	+
40	-	-	-	-	-	++

-, Not soluble; +, less soluble; ++, more soluble.

Preparation of PEGylated-mucin matrices

Polymer hybrids (matrices) were generated from mucin and poly(ethylene glycol) (PEG M_w 4000) by controlled coacervation in aqueous medium (Momoh et al., 2010; Momoh et al., 2011; Momoh et al., 2010). The matrices were prepared in the following molecular ratios (M:P): 1:1, 2:1, 3:1, 2:0, 0:2. Briefly, known quantities of mucin and PEG 4000 were weighed into separate 100 ml beakers and 20 ml of distilled water was added into the samples and allowed to stand for 72 h for interaction of the solvent and the polymers. The contents of the two beakers were then mixed and allowed to stand for another 72 h for molecular interaction. The hybrids were precipitated with chilled acetone, dried, pulverized and kept in an airtight container until used.

Spectroscopic characterization of the matrices

FTIR spectroscopy study was conducted using a Shimadzu FTIR 8300 Spectrophotometer (Shimadzu, Tokyo, Japan) and the spectrum was recorded in the wavelength region of 4000 to 400 cm^{-1} with threshold of 1.303, sensivity of 50 and resolution of 2 cm^{-1} range. The procedure consisted of dispersing a 5 mg sample in KBr and compressing into discs by applying a pressure of 5 tons for 5 min in a hydraulic press. The pellet was placed in the light path and the spectrum obtained (Sahoo et al., 2011, 2012, 2012; Builders et al., 2008).

RESULTS AND DISCUSSION

Physicochemical properties of snail mucin

Results of the physicochemical tests performed on the snail mucin (Table 1) showed that carbohydrates, proteins and trace amounts of fats were present. This is consistent with previous reports (Carlstedt et al., 1983; Perez and Proust, 1987; Durrrer et al., 1995; Lee et al., 2005; Raynal et al., 2003). In both wet and dry states, the extracted mucin was light-brownish in colour, with a pleasant meaty odour. Snail mucin when dispersed in water yielded a slightly viscous dispersion (Table 2). The snail mucin is not soluble in ethanol, acetone, 0.1 M sodium hydroxide, ammonium hydroxide and sulphuric acid (Table 2), in agreement with previous reported studies ((Momoh et al., 2010, 2010, 2011; Adikwu and Nnamani, 2005; Adikwu and Ikejiuba, 2005).

Fourier transform infrared (FT-IR) spectroscopy

The IR spectrum of a given compound is always unique and characteristic. Thus, IR spectroscopy is a quick and relatively cheap technique for identifying compounds (Sahoo et al., 2012; Sahoo et al., 2012; Sahoo et al., 2011; Builders et al., 2008). FT-IR spectra of the matrices (2:0, 0:2, 1:1, 2:1 and 3:1) are shown in Figures 1 – 5 . FT-IR spectra of non-PEGylated mucin (2:0) matrix, non-mucinated PEG (0:2) matrix and PEGylated mucin (1:1, 2:1 and 3:1) matrices were carried out as finger prints to identify PEGylated mucin relative to non-PEGylated mucin and non-mucinated PEG matrices. FT-IR spectrum of non-PEGylated mucin matrix (that is, mucin alone 2:0) (Figure 1) shows that principal peaks were observed at wave numbers of 1074.39, 1240.27, 1528.64, 1641.48, 2946.36, 3275.24 and 3421.83 cm^{-1} corresponding to C-N vibrations, -C-O stretching, N-H bending vibration for secondary amines, C=C stretching of α,β–unsaturated ring, C-H stretching, O-H stretching and N-H amide bending, respectively.

FT-IR spectrum of non-mucinated PEG matrix (that is, PEG 4000 alone 0:2) (Figure 2) shows characteristic peaks at 840.99, 950.94, 1114.89, 1273.06, 1355.04, 1466.91, 2882.71 and 3429.55 cm^{-1} corresponding to aromatic C-H bending (2 adjacent free H's), aromatic C-H bending (1 FT-IR spectra of PEGylated mucin matrices (that is, 1:1, 2:1 and 3:1) (Figures 3 - 5) were different from those of non-PEGylated mucin matrix (2:0) and non-mucinated PEG matrix (0:2). Spectrum of 1:1 matrix (Figure 3)

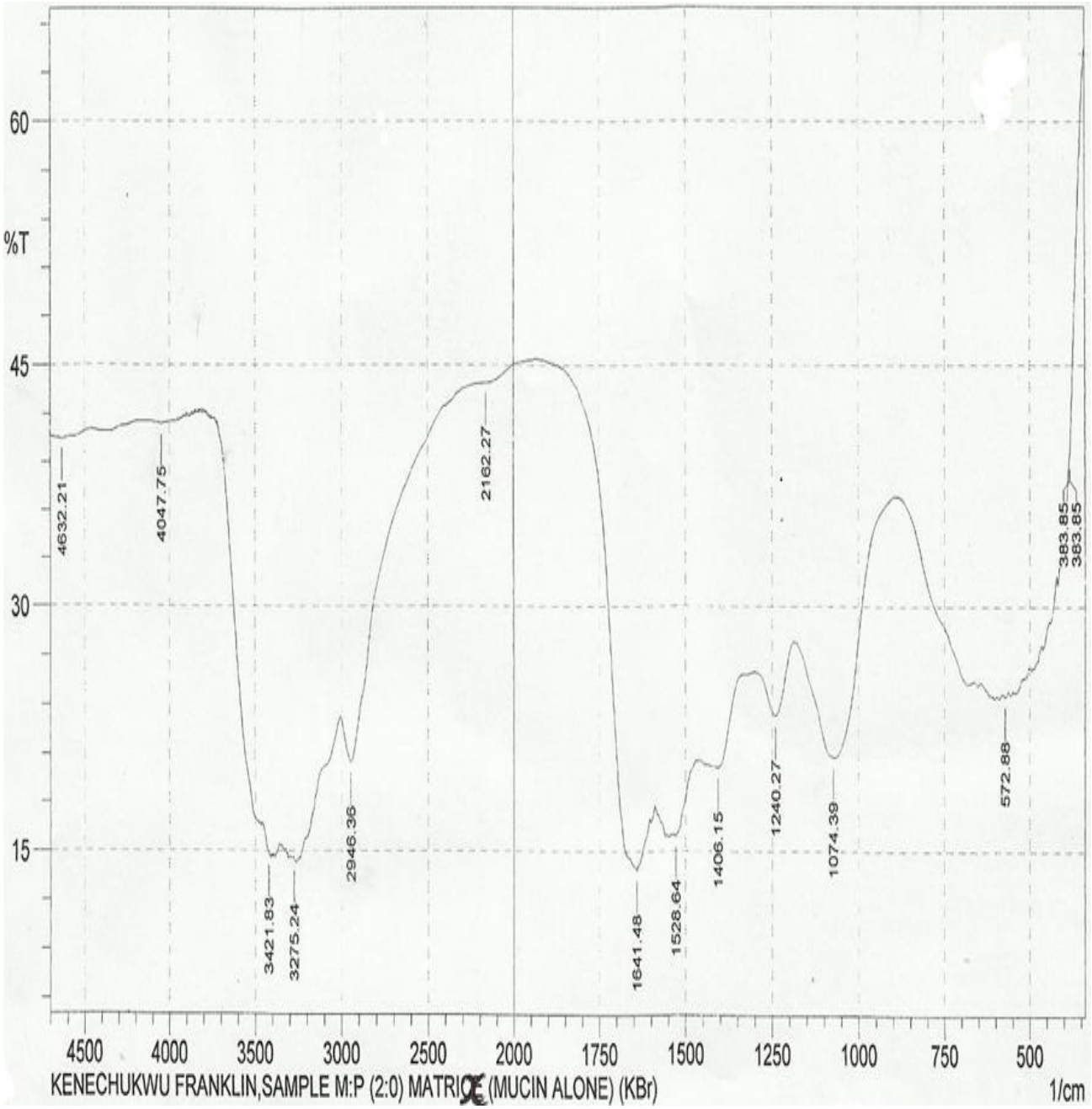


Figure 1. FT-IR spectrum of polymer matrix (2:0) (mucin).

shows strong peaks at 838.10, 956.72, 1116.82, 1264.38, 1362.75, 1536.35, 1665.59, 2885.60 and 3298.38 cm^{-1} corresponding to aromatic C-H deformation (2 adjacent free H's), C-N vibrations, C-O stretching (strong), C-O stretching, C-H deformation (CH_3), N-H bending vibration for secondary amines, C=C stretching of α,β–unsaturated ring, O=C-H stretching and N-H amide bending, respectively; in 2:1 matrix, the characteristic peaks were found at 625.92, 1113.93, 1251.84, 1404.22, 1679.09, 2888.50 and 3444.02 cm (Figure 4) due to aromatic C-H deformation, C-O stretching, C-O stretching, C-H deformation (CH_3), C=C stretching of α,β–unsaturated ring, C-H stretching and N-H amide bending, respectively; while the FT-IR spectrum of 3:1 matrix (Figure 5) shows that principal peaks were observed at wave numbers of 595.06, 1079.21, 1244.13, 1450.52, 1597.11, 2889.46

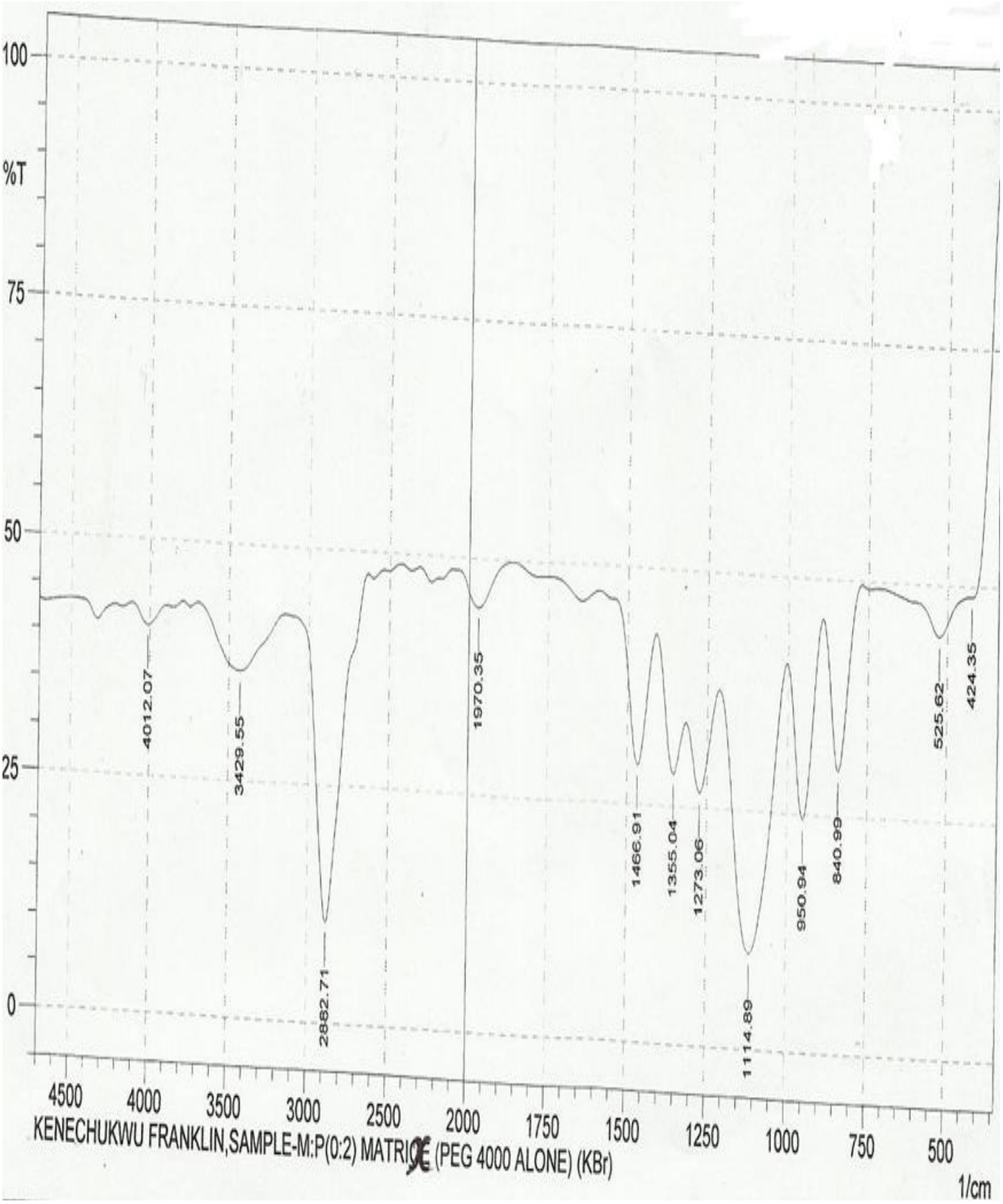


Figure 2. FT-IR spectrum of polymer matrix (0:2) (PEG-4000).

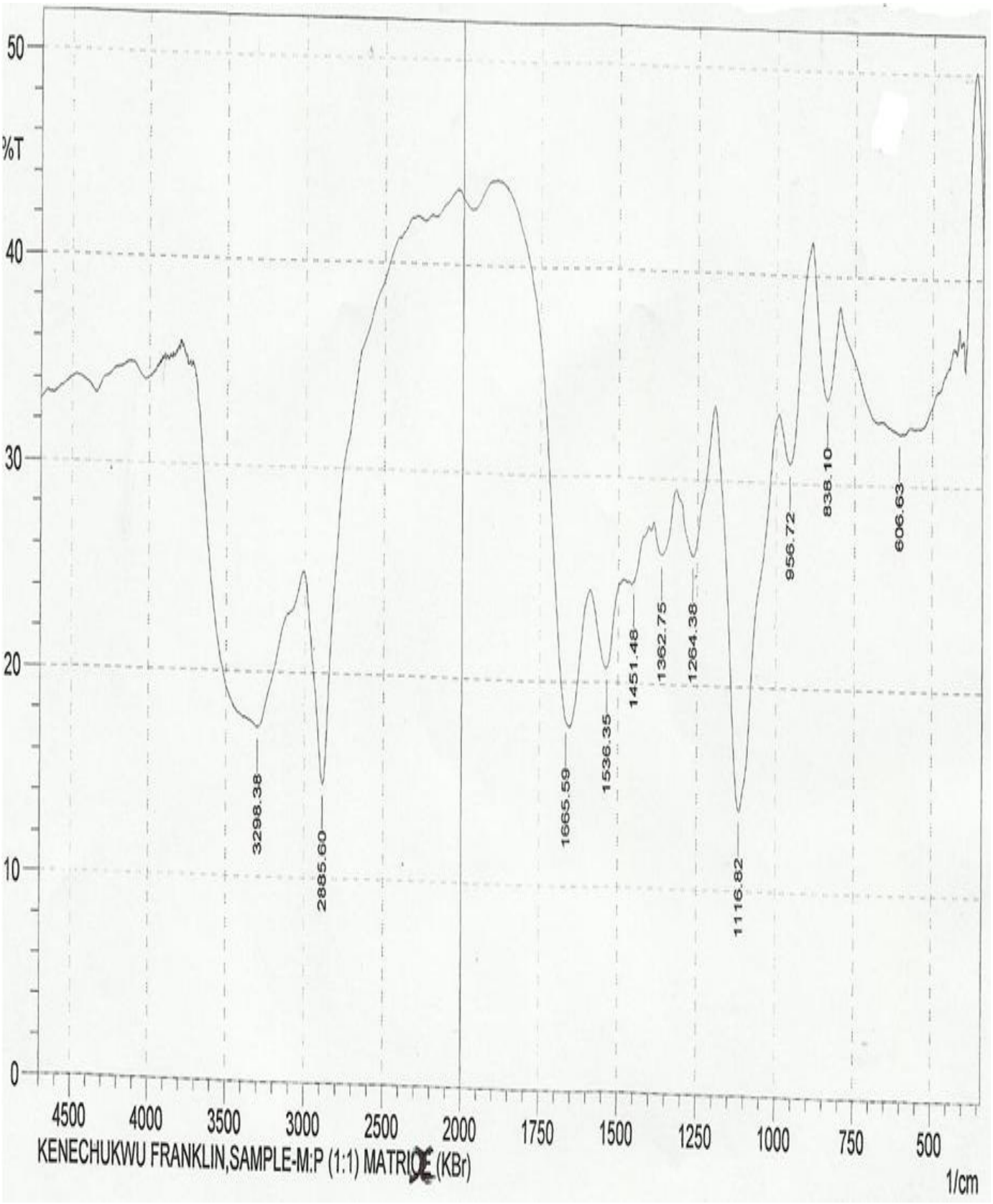


Figure 3. FT-IR spectrum of polymer matrix (1:1).

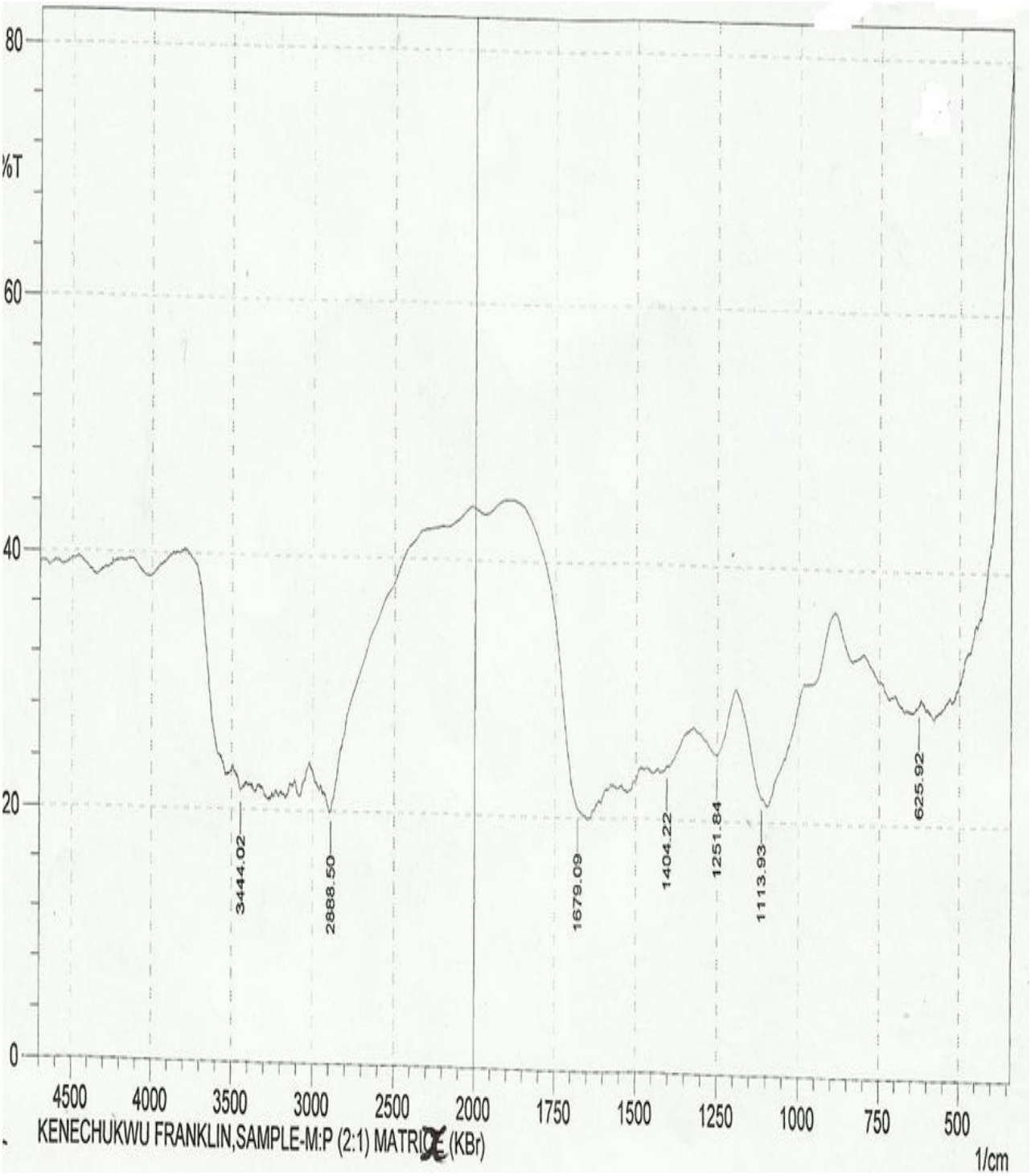


Figure 4. FT-IR spectrum of polymer matrix (2:1).

and 3197.12 cm^{-1} corresponding to aromatic C-H deformation, C-N vibrations, C-O stretching, C=H deformation (CH_3, CH_2), aromatic C=C (conjugated ring), C-H stretching and N-H amide bending, respectively. FT-IR spectra of 2:1 and 3:1 matrices (Figures 4 and 5) showed decrease in the number of peaks due to overlapping of peaks corresponding to 2:0 (mucin) and 0:2 (PEG 4000) matrices. The characteristic differences between the spectra of PEGylated mucin matrices (1:1, 2:1 and 3:1) on one hand and between the spectra of PEGylated

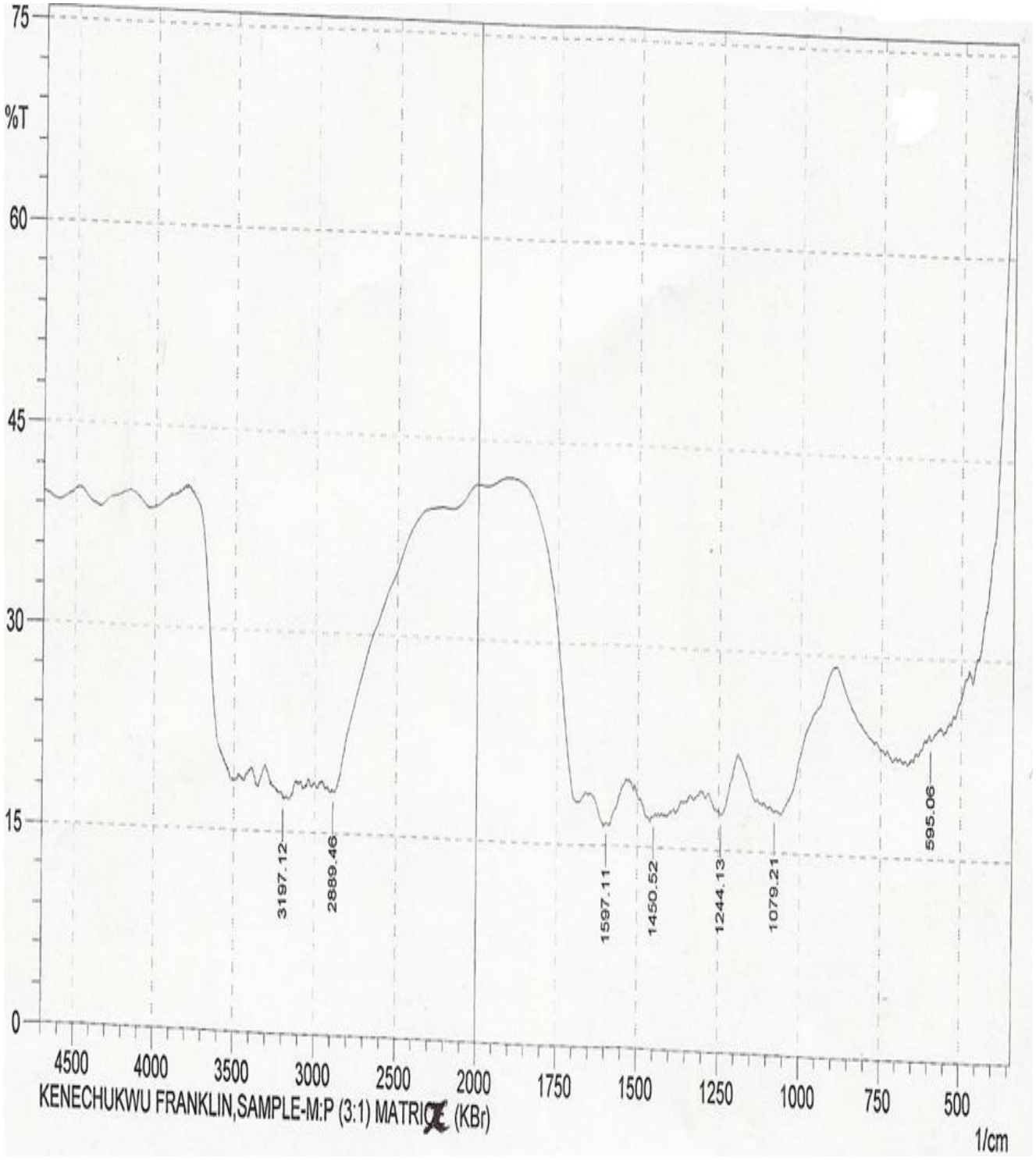


Figure 5. FT-IR spectrum of polymer matrix (3:1).

mucin matrices and those of non-PEGylated mucin (2:0) and non-mucinated PEG (0:2) matrices on the other hand further indicate that new polymeric materials were formed, consistent with a similar report on interaction studies of mucinated cellulose based on FT-IR by Builders et al. (2008).

Although PEGyalted mucin matrices (prepared with PEG 2000 and snail mucin) (Momoh et al., 2010; Momoh et al.,

2011; Momoh et al., 2010) had been characterized with respect to thermal properties by differential scanning calorimetry (DSC) (Momoh et al., 2010), this serves as a basis for further characterization of the interaction between PEG and mucin in PEGylated mucin matrices for drug delivery application. FT-IR spectroscopic analysis can be used to determine the functional groups of the molecules (Sahoo et al., 2012; Sahoo et al., 2012; Sahoo et al., 2011). Therefore, to obtain more information in detail about chemical interaction between PEG and mucin, the FT-IR analysis was carried out on PEGylated mucin matrices prepared with PEG 4000 and snail mucin. The presence of different peaks in the FT-IR spectra of the PEGylated mucin matrices compared with the non-PEGylated mucin (2:0) matrix and non-mucinated PEG 4000 (0:2) matrix indicated the formation of new polymers, which could be employed in drug delivery. Results obtained in this study is in agreement with earlier reports on the drug delivery potential of PEGylated mucin (Momoh et al., 2010). The novelty embodied in this research is that this is the first spectroscopic study to be carried out on PEGylated mucin using PEG 4000 and snail mucin.

Conclusion

The engineering of new polymer biomaterials could be employed to address some problems associated with delivery of some APIs and biomolecules. To obtain new polymer biomaterials that will meet the carrier needs of challenging drug molecules, blending of polymers with desirable properties becomes imperative. In this study, admixtures of PEG 4000 and mucin obtained from African land snail (*A. marginata*) were prepared by combining the two at different ratios. Preliminary spectroscopic characterization was performed on the PEGylated mucin matrices using fourier transform infra-red spectroscopic characterization. Results obtained indicated that a new polymeric carrier was made from admixtures of mucin and PEG 4000 by PEGylation technology. Further solid state spectroscopic charcterization (WAXD and SAXD) studies on PEGylated mucin are currently ongoing in our laboratory.

ACKNOWLEDGEMENTS

This work is a product of research project for the award of M. Pharm. by the Department of Pharmaceutics, University of Nigeria, Nsukka, Nigeria. We wish to thank the National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria, for assistance in FT-IR analyses, as well as Ph. Eur. Carl Roth GmbH + Co.KG Karlsruhhe Germany for provision of PEG 4000 used in the study.

REFERENCES

Abuchowski D, McCoy S, Palczuk C, van Es A, Davis O (1977). Effect of covalent attachment of polyethylene glycol on immunogenicity and circulation life of bovine liver catalase. J. Biol. Chem. 252(11): 3582-3586.

Adikwu MU, Ikejiuba CC (2005). Some physiological and wound healing properties of snail mucin. Boll. Chim. Farm. 144: 1-8.

Adikwu MU, Nnamani PO (2005). Some physiological and toxicological properties of snail mucin. Bioresearch 5: 1-6.

Attama AA, Nwabunze OJ (2007). Mucuna gum microspheres for oral delivery of glibenclamide: *In vitro* evaluation. Acta Pharma 57: 161 - 171.

Bernkop-Schnurch A, Hornof M, Guggi D (2004). Thiolated chitosans. Eur. J. Pharm. Biopharm. 57: 9-17.

Builders PF, Kunle OO, Adikwu MU (2008). Preparation and characterization of mucinated agarose: a mucin-agarose physical crosslink. Int. J. Pharm. 356: 174–180.

Carlstedt I, Lindgren H, Sheehan JK (1983). The macromolecular structure of human cervical-mucus glycoproteins: Studies on fragments obtained after reduction of disulphide bridges and after subsequent trypsin digestion. Biochem. J. 213 (2): 472-435.

Delie F, Blanco-Prieto MJ (2005). Polymeric particulates to improve oral bioavailability of peptide drugs. Molecules 10: 65-80.

Durrer C, Irache JM, Duchene D, Ponchel G (1995). Mucin interactions with functionalized polystyrene latexes. J. Colloid Interf. Sci. 170 (2): 555-561.

Francis MF, Cristea M, Winnik FM (2004). Polymeric micelles for oral drug delivery: Why and how? Pure Appl. Chem. 76(7-8): 1321-1335.

Gillies ER, Frechet JMJ (2004). Development of acid-sensitive copolymer micelles for drug delivery. Pure Appl. Chem. 76(7-8): 1295-1307.

Kodera R, Matsushima S, Hiroto E, Nishimura D, Inada P (1998). PEGylation of proteins and bioactive substances for medical and technical applications. Progress Polym. Sci. 23(7): 1233-1271.

Kreuter J (1991). Peroral administration of nanoparticles. Adv. Drug Deliv. Rev. 7: 71- 86.

Lagarce F, Renaud P, Faisant N, Nicolas G, Cailleux A, Richard J, Menei P, Benoit J-P (2005). Baclofen-loaded microspheres: preparation and efficacy testing in a new rabbit model. Eur. J. Pharm. Biopharm. 59: 449-459.

Lee S, Muller M, Rezman K, Spencer ND (2005). Porcine gastric mucin (PGM) at the water/poly (dimethylsiloxane) (PDMS) interface: Influence of pH and ionic strength on its conformation, adsorption, and aqueous lubrication properties. Langmuir 21(18): 8344-8353.

Momoh MA (2010). Formulation and evaluation of metformin and aqueous leaf extract of *Vernonia amygdalina* loaded polyethylene glycol-mucin particulate drug delivery systems: Unpublished Ph.D Thesis, Department of Pharmaceutics, University of Nigeria, Nsukka.

Momoh MA, Adikwu MU, Ibezim CE, Ofokansi KC, Attama AA (2010). Thermal characterization of PEGylated mucin. Asian Pacif. J. Trop. Med. 458-460.

Momoh MA, Adikwu MU, Ibezim EC (2010). Formulation and evaluation of the bioadhesive drug delivery system based on PEGylated mucin matrices. Asian Pacif. J. Trop. Med. 461 – 464.

Momoh MA, Adikwu MU, Ibezim EC, Attama AA (2011). Effect of metformin and *Vernonia amygdalina* leaf extract loaded PEGylated-mucin formulation on haematological, kidney and liver indices of healthy and Diabetes rats. J. Pharm. Res. 4(10): 3455-3459.

Muller RH, Mader K, Gohla S (2000). Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art. Eur. J. Pharm. Biopharm. 50: 161-177.

Pamujula S, Graves RA, Kishore V, Mandal TK (2004). Preparation and *in vitro* characterization of amifostine biodegradable microcapsules. Eur. J. Pharm. Biopharm. 57: 213-218.

Perez E, Proust JE (1987). Forces between Mica surfaces covered with adsorbed mucin across aqueous solution. J. Colloid Interf. Sci. 118: 182-191.

Ravi-Kumar MNV (2000). Nano and microparticles as controlled drug delivery devices. J. Pharm. Pharm. Sci. 3: 234-258.

Raynal BDE, Hardingham TE, Sheehan JK, Thornton DJ (2003). Calcium-dependent protein interactions in MUC5B provide reversible cross-links in salivary mucus. J. Biol. Chem. 278 (31): 28703-28710.

Sahoo S, Chakraborti CK, Behera PK (2012). FTIR and Raman spectroscopic investigations of a controlled release Ciprofloxacin / Carbopol940 mucoadhesive suspension. Asian J. Pharm. Clin. Res. 5(1): 125-130.

Sahoo S, Chakraborti CK, Behera PK (2012). FTIR and Raman spectroscopic investigations of Ofloxacin / Carbopol940 mucoadhesive suspension. Int. J. PharmTech. Res. 4(1):382-391.

Sahoo S, Chakraborti CK, Mishra SC, Naik S (2011). Analytical characterization of a gelling biodegradable polymer. Drug Invention Today 3(6): 78- 82.

Schnurrer J, Lehr CM (1996). Mucoadhesive properties of the mussel adhesive protein. Int. J. Pharm. 141: 251-256.

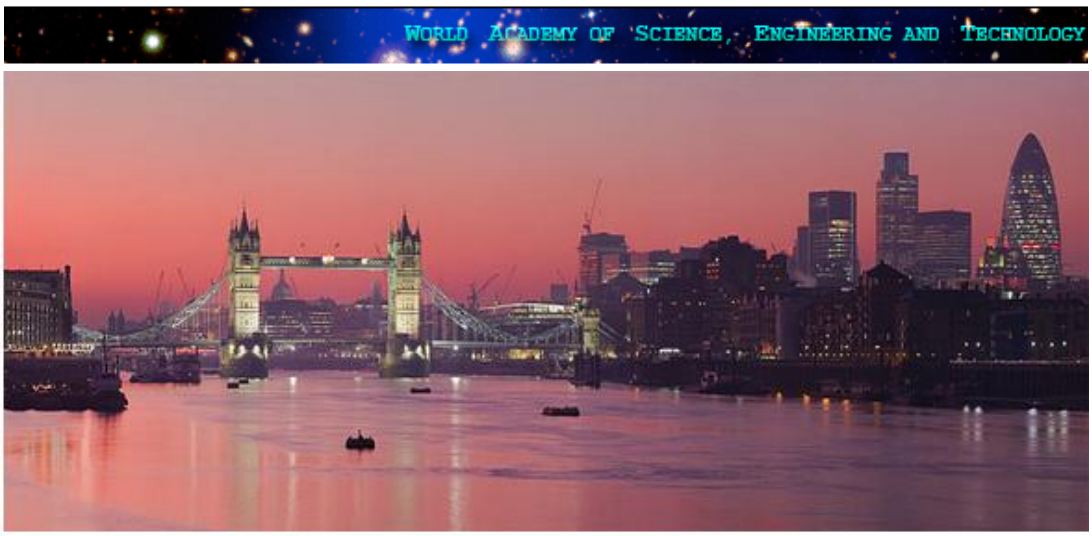
UPCOMING CONFERENCES

International Conference on Molecular Virology and Microbiology, Bangkok, Thailand, 24 Dec 2013

ICMVM 2013 : International Conference on Molecular Virology and Microbiology

Bangkok, Thailand
December 24-25, 2013

International Conference on Biotechnology and Environment Engineering, London, UK, 19 Jan 2014



ICBEE 2014 : International Conference on Biotechnology and Environment Engineering

GB, London
January 20-21, 2014

Conferences and Advert

December 2013
International Conference on Molecular Virology and Microbiology, Bangkok, Thailand, 24 Dec 2013

International Conference on Agriculture and Biotechnology, Kuala Lumpur, Malaysia, 29 Dec 2013

January 2014
International Conference on Biotechnology, Chemical and Environmental Engineering, London, UK, 19 Jan 2014

International Conference on Biotechnology and Environment Engineering, London, UK, 19 Jan 2014

academicJournals

Related Journals Published by Academic Journals

- Journal of Cell and Animal Biology
- International Journal of Genetics and Molecular Biology
- Biotechnology and Molecular Biology Reviews
- African Journal of Microbiology Research
- African Journal of Biochemistry Research
- African Journal of Environmental Science and Technology
- African Journal of Food Science
- African Journal of Plant Science
- Journal of Ecology and The Natural Environment
- Journal of Entomology and Nematology
- Journal of Bacteriology Research
- Journal of Bioinformatics and Sequence Analysis
- Journal of General and Molecular Virology
- International Journal of Biodiversity and Conservation
- Journal of Biophysics and Structural Biology
- Journal of Evolutionary Biology Research
- Journal of Yeast and Fungal Research
- International Journal of Plant Physiology and Biochemistry
- Journal of Brewing and Distilling
- Journal of Computational Biology and Bioinformatics Research
- Journal of Developmental Biology and Tissue Engineering
- Journal of Microbiology and Antimicrobials
- International Journal of Biotechnology and Molecular Biology Research