African Journal of
Biotechnology
Volume 13 Number 22, 28 May, 2014
ISSN 1684-5315

Academic Journals

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.

Editorial Board

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

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.

African Journal of Biotechnology

Table of Contents: Volume 13 Number 22, 28 May, 2014

ARTICLES

Table of Contents: Volume 13 Number 22, 28 May, 2014

academicJournals

Vol. 13(22), pp. 2203-2207, 28 May, 2014
DOI: 10.5897/AJB2014.13623
Article Number: 8EAD3FF44896
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Cytomorphological studies of two mulberry varieties (Moraceae)

K. H. Venkatesh[1]*, S. Shivaswamy[2] and Munirajappa[1]

[1]Department of studies in Sericulture, Bangalore University, Bangalore-56006, India.
[2]Department of Sericulture, Bharati College, Bharati Nagara, Mandya -571422 Karnataka, India.

Received 10 January, 2014; Accepted 29 April, 2014

Two mulberry varieties, namely, S_{34} and Tr-$_{10}$ were selected for cytomorphological studies. Stomatal frequency, somatic chromosome number, ploidy level and meiotic behaviour were studied for these varieties. S_{34} is diploid with 2n=28 and Tr-$_{10}$ is triploid with 2n=42 chromosomes. Meiosis was irregular. Various anomalies like univalents, bivalents, trivalents laggards, loose association and unequal separation of chromosomes were observed in some pollen mother cells (PMCs) studied. Stomatal frequency was found to be lesser in triploid when compared to diploid variety.

Key words: Mulberry (*Morus* spp.), stomatal frequency, diploid, triploid, mitosis, meiosis.

INTRODUCTION

Mulberry is an outstanding bio energy plant of the family Moraceae. In addition to being fed to silkworms (*Bombyx mori* L.) mulberry is used in industry, medicine, aqua-culture, agro-forestry, social forestry and drought prone area development programmes (Dandin et al., 1992; Munirajappa et al., 1995). In sericulture, the most important factor is the cultivation of elite mulberry varieties exhibiting desirable agronomical and commercial traits. It is an established fact that about 60% of the total cost of silk production is attributed to mulberry production alone. Therefore, it is very important to select high yielding varieties with better quality leaves. Most of the cultivated varieties of mulberry are diploids with 2n=28 chromosomes, but few are polyploids (Venkatesh, 2007). For a few Indian species, cytogenetical investtigation was carried out by Kundu and Sharma (1976) and Venkatesh et al. (2013). Cytotaxonomy of the genus *Morus* L. clearly indicates the lacunae. Triploids (2n=42) are developed through natural or controlled hybridization between diploid and tetraploid parents and are considered to be superior than diploids and tetraploids in leaf yield and nutritive qualities of leaf. In the present study, an attempt to understand the stomatal frequency, somatic chromosome number and meiotic behaviour of two different varieties of mulberry have been discussed.

MATERIALS AND METHODS

Mulberry varieties S_{34} and Tr-$_{10}$ were used in the present study and maintained in the germplasm bank attached to Department of Sericulture, Bangalore University, Bangalore. Somatic preparations were made from excised root tips of potted plants. Root tips were collected at 10.15a.m. and pre-treated with 0.002 M 8- hydroxy quinoline for 3 h at 10°C.

*Corresponding author. E-mail: khvenki1972@gmail.com. Tel: +91-80-22961523. Fax: +91-80-23284880.

After thorough washing, root tips were hydrolysed in 1 N HCl for seven minutes at 40°C and then stained with 2% aceto-orcein. Squash preparations were made in 45% acetic acid. Photographs were made on the same day of preparation to ascertain the chromosome number and ploidy level. For meiosis, flower buds of appropriate stages of development were collected between 9.20 to 10.30 a. m. during sunny days and fixed in 1:3 acetic-alcohols for 24 h and preserved in 70% ethanol. Anthers were squashed in 2% aceto-carmine stain. Photo-micrographs were taken using labomed microscope fitted with Nikon cool fix digital Camera. Stomatal frequency was determined by nail polish impression method. Stomatal frequency was calculated by using the formula and expressed as number of stomata/mm^2 (Aneja, 2001; Sikdar et al., 1986).

$$\text{Stomatal frequency} = \frac{\text{Number of Stomata}}{\text{Area of microscopic field}} \times mm^2$$

RESULTS AND DISCUSSION

Variety S_{34}

Variety S_{34} evolved through artificial hybridization between S_{30} and C_{776} at Central Sericultural Research and Training Institute, Mysore. This variety is best suited for rain fed condition. It has better rooting and sprouting abilities and it is capable of thriving well both in temperate and tropical climatic conditions. Stem is green to brown in colour. Leaves are unlobed ovate and light green in colour. The stomatal frequency of this genotype was found to be 190.97/mm^2 (Figure 1a). This genotype revealed 2n=28 somatic chromosomes number (Figure 1b) and it is a diploid genotype. Mitosis was regular with equal separation. Meiotic behaviour was also regular. In metaphase I majority of the pollen mother cells (PMCs) exhibited 14 bivalents are scattered in the cytoplasm without any additional association, of which one bivalent was larger than the others (Figure 1c). Anaphase I showed equal separation of chromosomes were clearly discernible to the respective poles (Figure 1d). Telophase and prophase II were also observed in Figure 1e and f, respectively. Anaphase II was quite abnormal due to the presence of laggards, unequal separation and precocious movement of chromosomes (Figure 1g). Pollen stain ability was found to be 97.23% (Figure 1h).

Variety Tr_{-10}

It evolved through ploypoid breeding technique. It is being cultivated as a perennial bush especially in hilly tract. Stem is green to brown in colour. Leaves are unlobed ovate and dark green in colour. The Stomatal frequency of this genotype was found to be 161.21/mm^2 (Figure 2a). This genotype revealed 2n=42 somatic chromosomes number (Figure 2b) and it is a triploid genotype. Meiosis was irregular. Metaphase I showed loose association and various types of chromosomal associations (Figure 2c). Depending on the pairing of chromosomes, uni, bi, and trivalents showed different configurations. Anaphase I, showed unequal separation of chromosomes (Figure 2d). Majority of the PMCs showed unequal separation of chromosomes at anaphase I. Chances of equal distribution was meagre and 22:20 and 23:19 distribution were common among the PMCs observed. In this variety, the trivalents frequency was high and various types of chromosomal configurations (‘ >-’ ‘---’ and ‘v’) were observed. Different types of chromosomal configurations found in the present study corroborated the findings of Das et al. (1984), Basavaiah et al. (1990) and Venkatesh and Munirajappa (2012) in triploid mulberry varieties. Telophase I was also observed (Figure 2e). Pollen stain ability was found to be 92.23% (Figure 2f).

Cultivars S_{34} and Tr_{-10} have similarity in their adaptation that is, unlobed and light green leaves, good rooting, etc. Triploid forms are better rooting, grow more quickly and posses larger leaves when compared to diploids and tetraploids (Eswar Rao et al., 2000). Stomatal frequency is an important parameter in selecting drought resistant genotype and also belived to regulate leaf yield. The frequency of stomata per unit area is significantly less in triploid compared to diploid. Basavaiah and Murthy (2001) reported that, moisture retention capacity will be higher in those mulberry varieties possessing smaller and lower stomatal frequency. The present findings are also in agreement with the reports of Vijayan et al. (1998). Stomatal frequency also correlated with drought and disease resistant (Hatalli et al., 1993; Nautiyal et al., 1994). Further lesser frequency per unit area is more suitable for rain fed conditions. However, reduction in the internodal and number of stomata per unit area indicates that the increased dosage of genes does not always increase in size but may also reduce it (Dwivedi et al., 1986).

In the present investigation in the genus *Morus* analyzes the chromosome number, ploidy level, meiotic behaviour and stomatal frequency in two different mulberry genotypes. Among these, the present study recorded the S_{34} is diploid (2n=28) and Tr_{-10} is triploid (2n=42) chromosome number of the mulberry varieties studied.

In general meiosis in diploid was regular and it was clear that the gametic chromosomes number was n=14 with regular mega chromosomes. There were no secondary associations and multivalents in metaphase I. Presence of 14 bivalents in most of the PMCs at metaphase I confirms the diploid nature of the variant. The high frequency of bivalents is suggestive of a fair degree of homology between the constituent genomes and allo diploid nature of the variety. One bivalent was found bigger in comparison to others as also reported by Gill and Gupta (1979) in *Morus.* Anaphase I was quite abnormal in triploid variety due to unequal separation of chromosomes, is attributed to irregularities in basic process

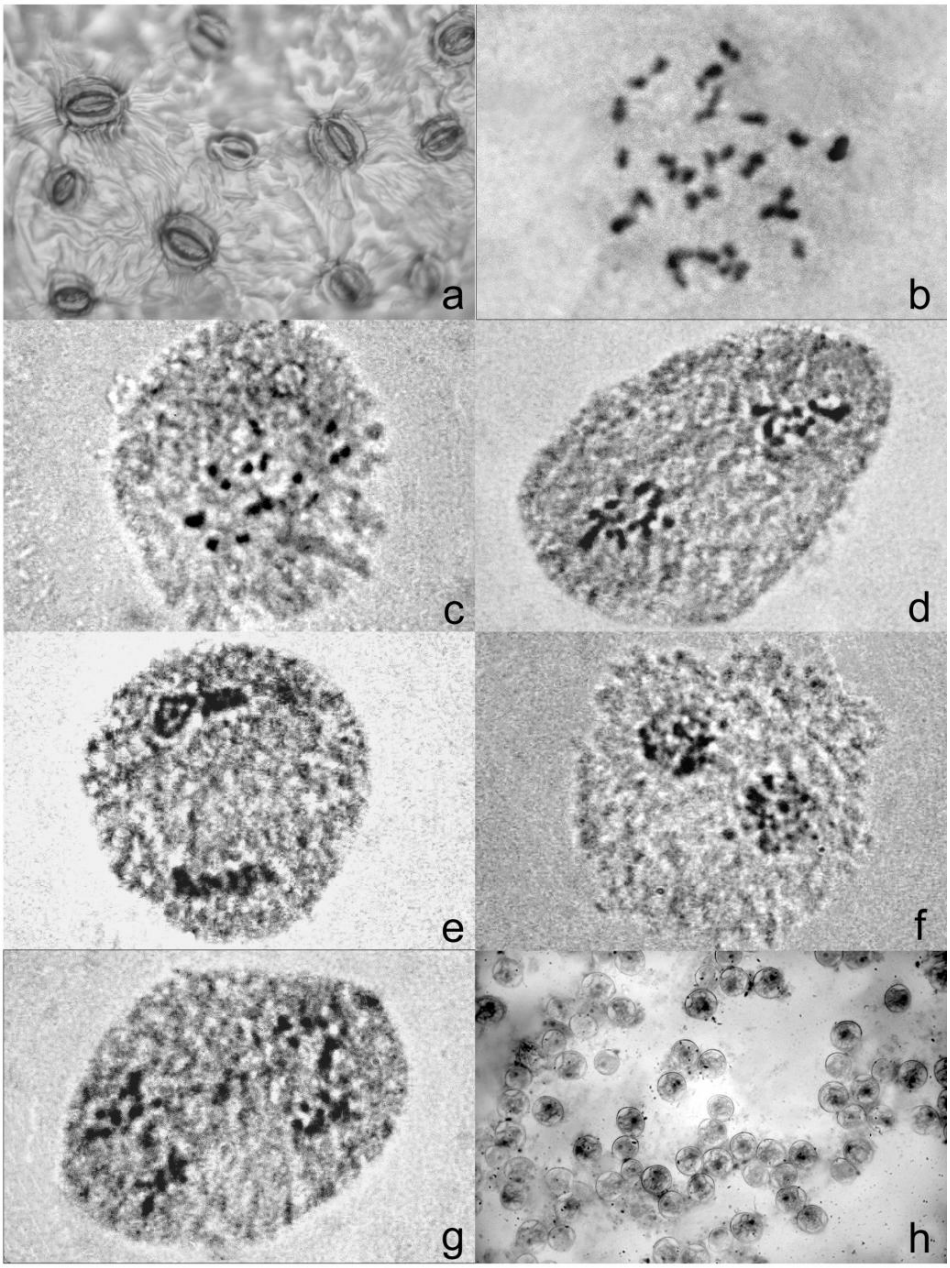


Figure 1. a and b. Stomatal frequency and somatic chromosomes (2n=28) of variety S_{34}, **c.** metaphase I (13 bivalents and one large bivalent scattered in the cytoplasm). **d.** anaphase I, **e.** telophase I, **f.** prophase II, **g.** anaphase II (laggards and unequal separation of chromosomes), h. pollen grains.

like chromosome pairing and alignment. The presence of higher percentages of trivalents in metaphase I is a suggestive of fair degree of homology between the constituent genomes and auto triploid nature of the variety. Meiotic abnormalities such as unequal separation, presence of uni, bi and trivalents, loose association and irregular distribution of chromosomes to different poles have resulted in the size variation of pollen and their low fertility compared to diploids. Reduced pollen fertility observed in triploid here is in confirmation with the findings of Tikader and Dandin (2007). Reduction in pollen fertility in triploid variety can be attributed as multivalents association during synopsis and invariably results in loss of chromatin materials (Gottschalk, 1978). These findings will be much use in establishing a phylogenetic relationship, evolution of mulberry and selecting mother plants for hybridization on the basis of micro morphology, somatic chromosome number and

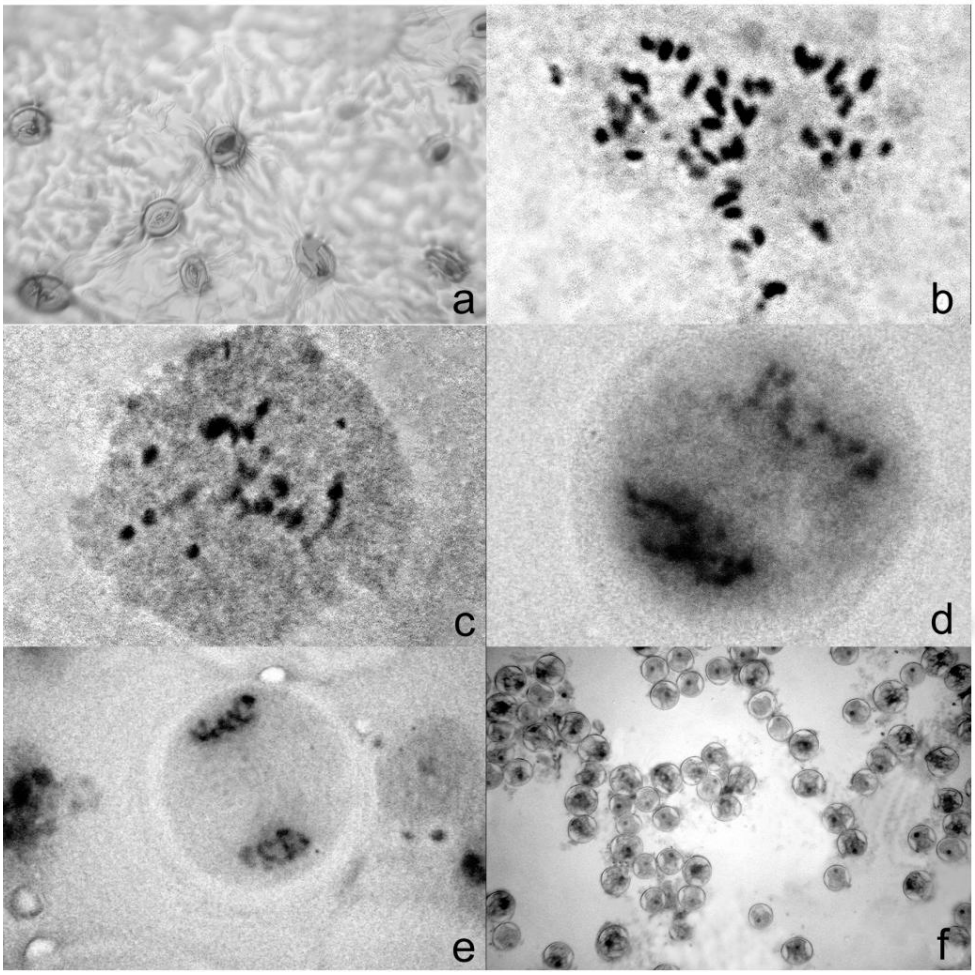


Figure 2a and b. Stomatal frequency and somatic chromosomes (2n=42) of variety Tr-$_{10,}$ **c.** metaphase I (loose association of trivalents), **d.** anaphase I (unequal separation of chromosomes), **e.** telophase I, f. pollen grains.

ploidy level of the mulberry varieties.

REFERENCES

Aneja KR (2001). Experiments in microbiology plant pathology tissue culture and mushroom production technology (3rd edition) New age international publishers, New Delhi.

Basavaiah, Rajan MV, Dandin SB, Suryanarayana N, Sengupta K (1990). Chromosomal association and meiotic behaviour of four triploid varieties of mulberry (*Morus* spp. Cytologia 55: 327-333.

Basavaiah, Murthy TCS (2001). Relation between anatomical features, moisture content and retention capacity of leaf in some mulberry (*Morus* spp.) genotypes of different ploidy. Natl. Semi. Mulb. Seri. Res. November, 26-28, KSSR&DI, Bangalore, India: p. 98.

Dandin SB, Ranganatha SKN, Bongale UD (1992). Mulberry in waste land development programme scale. Indian silk, 26(3):12-14.

Das BC, Verma RC, Sikdar AK (1984). Chromosome association in natural and induced triploids of mulberry *(Morus).* Cell Chromosome Res. 7:60- 61.

Dwivedi MK, Sikdar AK, Dandin SB, Sastry CR, Jolly MS (1986). Induced tetraploidy inmulberry 1, Morphological, anatomical and cytological investigations in cultivar RFS – $_{135}$, Cytologia 51(2):393-401.

Eswar RMS, Mllikarjunappa RS, Dandin SB (2000). "Evaluation of tetraploids and triploids mulberry genotypes for propagation growth and yield parameters" Proceedings of Natl. Conf. Stra. Ser. Res. Devpt., November 16-18 CSR&TI, Mysore, India: 2.

Gill GS, Gupta RC (1979). Cytological studies the sex types of *Morus alba,* L. (*Moraceae*). Curr. Sci. 48(1):35-36.

Gottschalk W (1978). Open problems in polyploidy research, Nucleus 21:91-112.

Hatalli SR, Chetty MB, Koti RV (1993). Interrelationship of Stomatal frequency Interveinal distance and yield under depleting soil moisture regime in wheat genotypes Indian. J. Plant Physiol. 36(3):187-188.

Kundu D, Sharma A (1976). Chromosome studies in some Indian Moraceae in P Kachroo (ed) Recent advances in Botany Bishen Singh Mahendra Pal Singh Dehradum pp. 348-369.

Munirajappa, Ramesh HL, Nijagunaiah R (1995). Revaitalizing dwindling ecosystem through mulberry cultivation. National seminar on Man, Industry and Environment Organised by Dept. of Environmental Science, Bangalore University Bangalore-56, 5th-8th April.

Nautiyal S, Badola HK, Pal H, Negi DS (1994). Plant responses to water stress changes in growth dry matter production Stomatal frequency and leaf anatomy Biol. Plant 36(1): 91- 97.

Sikdar AK, Jolly MS, Susheelamma BN, Giridhar K (1986). Stomatal chloroplast count technique as a tool to ascertain different ploidy

level in mulberry. Indian J. Seric. 25(2)88-90.
Tikedar A, Dandin SB (2007). Pre- breeding efforts to utilize two wild *Morus* spp. Curr. Sci. 92(12):1729-1733.
Venkatesh KH (2007). Cytogenetical investigations in the Genus *Morus* L Ph .D., Thesis Bangalore University Bangalore.
Venkatesh KH, Nijagunaiah R, Munirajappa (2013). Cytogenetical studies in some diploid mulberry varieties (Moraceae). Cytologia 78(1): 69-72.
Venkatesh KH, Munirajappa (2012). Cytogenetical studies in two triploid mulberry varieties J. Cytol Gen. 13(NS):29-34.
Vijayan K, Chakraborti SP, Roy BN, Setua M, Qadri SMH (1998). Cytomorphological characters of a colchicine induced chimeric mutant in mulberry. Cytologia 63:27-31.

academicJournals

Vol. 13(22), pp. 2188-2197, 28 May, 2014
DOI: 10.5897/AJB2014.13644
Article Number: 0C9623644894
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Psychrophilic yeasts and their biotechnological applications - A review

Burhan Hamid[1], Ravinder Singh Rana[2], Deepak Chauhan[2], Poonam Singh[2], Fayaz Ahmad Mohiddin[1]*, Sanjay Sahay[2] and Ishfaq Abidi[3]

[1]Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar-190025, J&K, India.
[2]Government Science and Commerce College, Benazir, Bhopal, Madhya Pradesh, India.
[3]Directorate of Research, SKUAST-K, Srinagar, Kashmir-190025, India.

Received 20 January, 2014; Accepted 6 May, 2014

More than 70% of earth's surface is covered by cold ecosystems and these ecosystems have been colonized by a class of extremophilic microorganisms called as psychrophiles. Thus psychrophiles are true extremophiles that has the ability to live in extremely low temperature conditions. Psychrophilic yeasts are important due to their physiological adaptation at low temperature and they have potential application in biotechnology. Psychrophilic yeast produces cold-active enzymes having numerous applications in textile, medical and pharmaceuticals, fine chemical synthesis, food industry, domestic and environmental applications. Psychrophilic enzymes from yeast have attracted attention of researchers to explore the new application of these enzymes because of their high activity at low and moderate temperatures. The present review describes various immune biotechnological applications of different cold-active enzymes produced by psychrophilic yeast in different industries.

Key words: Extremophilic microorganisms, cold-active enzymes, environmental applications.

INTRODUCTION

Microorganisms which are able to grow at low temperature have been known for long time (Morita, 1966; Farrell and Rose, 1967). Psychrophiles are the microorganisms that have colonized all permanent cold environments. Psychrophilic organisms have been classified in two groups:

i) Which are obligate psychrophiles with optimal growth temperature of ≤ 20°C.
ii) Which are facultative psychrophiles with optimal growth temperature of > 20°C (Stokes, 1963).

Cold-adapted microorganisms can grow at 0°C and are classified as psychrophilic if their optimum and maximum temperatures for growth are ≤15 and ≤ 20°C, respectively, or as psychrotolerant (psychrotrophic) if

*Corresponding author. E-mail: famohiddin@rediffmail.com. Tel: +91-9697349262.

their maximum temperature for growth is above 20°C (Robinson, 2001; Gounot, 1986). Psychrotolerant microbes have an optimum growth temperature between 20 and 40°C, but are also capable of growth at 0°C (Morita, 1975). As the name suggests, these are cold loving microbes that are commonly found in Polar region and also in deep sea, mountains, glaciers, fresh and marine waters, polar and high alpine soils, all together constituting about three-fourth of the biosphere. These organisms produce cold evolved enzymes that are partially able to cope with the reduction in chemical reaction rates induced by low temperatures (D'Amico et al., 2002). The mesophilic yeasts grow between 5 to 35°C. In comparison, the psychrophilic yeasts grow below 5°C and exhibit no growth above 20°C (Shivaji and Bhaskar, 2008). As a group the mesophilic yeasts are the most predominant and constitute the vast majority of the yeasts studied so far as compared to psychrophilic and thermophilic yeasts. Cold-active enzyme might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperature, low thermo stability and unusual specificities (Russell, 2000).

Because of potential biotechnological applications, cold-adapted microorganisms have become increasingly studied in recent years, of the microorganisms most isolated and studied from cold environments, the majority are bacteria, while yeasts constitute a minor proportion (Margesin and Miteva, 2011). Oceans represent 71% of earth's surface and 90% by volume, which are at 5°C. It has been estimated that more than 90% (by volume) of the marine water masses are colder than 4°C (Morita, 1966). Antarctica is considered the coldest and driest terrestrial habitat on Earth. It is covered almost totally with ice and snow, and receives high levels of solar radiation (Holdgate, 1977).

The range of species within a particularly cold habitat reflects many different parameters (for example, primary nutrient, ability to withstand desiccation, pH, salinity) to which an organism must adapt (Blaise et al., 2004).

In this review, we focus on psychrophilic yeast and their cold-active enzymes having biotechnological applications in different industries like; detergent, medical and pharmaceuticals, fine chemical synthesis, food, textile, and domestic industries.

PSYCHROPHILIC YEASTS AND THEIR HABITATS

Psychrophiles are more likely to be found in permanently cold environments such as polar region (Sabri et al., 2001), marine environment and deep water (D'Amico et al., 2006). Psychrophilic yeasts have been isolated from marine waters, Arctic and Alpine glaciers, and Antarctic ecosystems; their occurrence and abundance in these environments have been described (Vishniac, 1999; Díaz and Fell, 2000; Bergauer et al., 2005; Buzzini et al., 2005). Most of the work particularly on psychrophilic yeast and their cold-active enzymes have been reported from Antarctica. The first report of Antarctic yeasts was published 50 years ago (Menna, 1960) current reports have focused on cold-tolerant bacteria and archaea, with yeasts receiving less attention. The occurrence of psychrophilic yeasts has been reported in glacial melt-water rivers originating from glaciers of Argentinean Patagonia (García et al., 2007). Butinar et al. (2007) reported the occurrence of viable yeasts in the different ice layers of Arctic glaciers located in the Svalbard Islands Norway.

In these environments, psychrophilic and psychro-trophic microorganisms are believed to play key roles in the biodegradation of organic matter and the cycling of essential nutrients (Welander, 2005; Lambo and Patel, 2006; Ruberto et al., 2005). Psychrophilic yeasts, particularly *Cryptococcus sp*. have been isolated repeatedly from soil samples and some researchers have described them as the most important life form in Antarctic desert soils (Vishniac and Klinger, 1986). Yeasts dwelling in Antarctic and Sub-Antarctic maritime and terrestrial habitats belong mainly to the *Cryptococcus*, Mrakia,

Candida and *Rhodotorula* genera (Buzzini et al., 2012; Vaz et al., 2011). The presence of organic carbon and nitrogen sources in waters, originated from melting glacier ice, have been demonstrated and the occurrence of yeast strains degrading a variety of organic compounds including polysaccharides, esters, lipids and pectin's have been observed in the yeasts isolated from Alpine glacier environments (Skidmore et al., 2000; Margesin et al., 2002).

Other than Antarctic, psychrophilic yeast *C. capitatum* SPY11 were isolated from the soil of northern region of India Kashmir valley; the yeast was able to grow up to 20°C above which it couldn't grow normally (Hamid et al., 2012). Shivaji and Bhaskar (2008), reported novel psychrophilic yeast *Rhodotorula Himalayans* sp. Nov; isolated from a Roopkund lake of Himalayan mountain range.

BIOTECHNOLOGICAL APPLICATION OF COLD-ACTIVE ENZYMES

Extremophiles are a potent source of extremozymes, which show outmost stability under extreme conditions. Consequently, much attention has been given to the microorganisms that are able to thrive in extreme environments. Thus, biocatalysis using extremophiles as well as extremozymes is rapidly being transformed from an academic science to an industrially viable technology. Each group of the extremophiles has unique features, which can be harnessed to provide enzymes with a wide

range of application possibilities (Adams et al., 1995; Hough et al., 1999). Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1995). Psychrophiles produce cold-adapted enzymes that have high specific activities at low temperatures (Feller and Gerday, 2003). These enzymes have the ability to support transcription and translation at low temperatures (Goodchild et al., 2004). Psychrophilic enzymes isolated from psychrophilic yeasts exhibit high activity at low and moderate temperatures and thus offer potential economic benefits (Allen et al., 2001). Kourkoutas et al., (2002) reported that psychrophilic yeast can be used in low temperature fermentation. Cold-active or cold-adaptive enzymes have attracted great attention as biocatalysts because they have the ability to resist quite unfavorable reaction conditions in industry (Deming, 1998; Singh et al., 2012; Sahay et al., 2013). Psychrophilic and psychrotolerant microorganisms and their unique cold shock and cold-acclimation proteins and enzymes (for example, proteases, lipases and cellulases) having a host of biotechnology applications (Gounot, 1991). Industrially interesting enzymes that are screened from yeasts are lipase / esterase, amylase, cellulase and β-glucosidases (Middelhoven, 1997; Laitila et al., 2006; Kudanga et al., 2007; Strauss et al., 2001; Buzzini and Martini, 2002; Rodríguez et al., 2004).

There are nearly 4000 enzymes known today and of these about 200 are in commercial use (Sharma et al., 2001). As a rule, enzymes produced by microorganisms existing in cold environments display higher catalytic efficiency at low temperatures and greater thermo sensitivity than their mesophilic counterparts (Gerday et al., 1997). Psychrophilic yeast has been reported to be used in low temperature fermentation (Pfeffer et al., 2006; Liu et al., 2006; Kourkoutas et al., 2002). The high activity of psychrophilic enzymes at low and moderate temperatures offers potential economic benefits (Cavicchioli et al., 2002). There is an industrial tendency to treat foodstuffs under mild conditions in order to avoid spoilage, and changes in taste and nutritional value at an ambient temperature. Therefore, cold-active enzymes are used for processing foods, (Margesin and Schinner, 1994; Russell and Hamamoto, 1998; Gerday et al., 2000). Cold-active enzymes with unique molecular adaptabilities (Feller and Gerday, 1997) have opened up potential newer areas of applications (Singh et al., 2012; Sahay et al., 2013). Processes catalyzed by cold-active enzymes have two advantages, they have potential to economise the processes by saving energy (Deming, 1998; Cavicchioli et al., 2002), and they protect the processes from contamination (Gardey et al., 2000). Over 91 basidiomycetous yeasts (belonging to the genera Cryptococcus, Leucosporidiella, Dioszegia, Mrakia, Rhodotorula, Rhodosporidium, Sporobolomyces, Sporidiobolus, Cystofilobasidium and Udeniomyces) have been screened for extra cellular amylolytic, protolytic, lepolytic, esteric, pectinolytic, actenolytic activities (Brizzio et al., 2007). These findings suggest that cold environments of Patagonia (Argentina) may be considered as a potential source of cold adapted yeasts producing industrially relevant cold-active enzymes (Brizzio et al., 2007). The majority of the industrial enzymes are of microbial origin. Several microbes are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches (Kaur et al., 2004; Antranikian, 1992).

The ability to heat-inactivate cold-active enzymes has particular relevance to the food industry where it is important to prevent any modification of the original heat-sensitive substrates and product. This is also of benefit in sequential processes (example, molecular biology) where the actions of an enzyme need to be terminated before the next process is undertaken; with cold-adapted enzymes this might be accomplished by heat inactivation rather than chemical extraction (Russell et al., 1998; Gerday et al., 2000). Cold-active enzymes from psychrophilic yeasts can be applied to the food industry, for clarification of fruit juice at low temperature.

The enzymes from microorganisms are used in various industries such as dairy, food, detergents, textile, pharmaceutical, cosmetic and biodiesel industries, and in synthesis of fine chemicals, agrochemicals and new polymeric materials (Saxena et al., 1999; Jaeger and Eggert, 2002). Several yeast strains have been explored in regards to the biological treatment of industrial and domestic waste water (Thanh and Simard, 1973; Ohno et al., 1991). Katayam et al. (1997) reported psychrophilic yeast *Candida* sp. which was isolated from water samples from Lake Vanda in Antarctica it can be used for the treatment of dissolved organic matter at low temperatures.

In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Akpan et al., 1999; Abu et al., 2005). The properties of cold-active enzymes provide numerous avenues for industrial application; however, specific properties may be improved through enzyme engineering.

Pectinases

Pectinolytic enzymes or pectinases are a heterogeneous group of enzymes that hydrolyze the pectic substances present in plants. They include polygalacturonases (PG), pectin lyase (PL), and pectin esterase (PE) that hydrolyze the glycosidic bonds of pectic substances (Fogarty and Kelly, 1983). Psychrophilic yeast strains *C. cylindricus* and *M. frigida* have been isolated from soil of Abashiri

(Nakagawa et al., 2004). The isolated strains can grow on pectin as the only carbon at below 5°C and showed the activities of several cold-active pectinolytic enzymes (Nakagawa et al., 2004).

Eight cold-adapted polygalactu-ronase producing yeasts have been isolated from frozen environmental samples of Iceland, which belong to *C. larimarini, C. capitatum, C. macerans and C. aquaticus* (Birgisson et al., 2003). It have been demonstrated that yeasts such as *Kluyveromyces wickerhamii* (Moyo et al., 2003) and moulds such as *Aspergillus niger* CH-Y-143 (Aguillar and Huitron, 1990), are capable of producing polygalacturonases constitutively.

The cold-active pectinolytic enzymes (Pectin methylesterase PME, endo-PG and exo-PG) from the newly isolated and identified psychrophilic yeast *C. capitatum* SPY11 and psychrotolerant yeast *R. mucilaginosa* PT1that exhibited 50 to 80% of their optimum activity under some major ecological conditions pH (3 to 5) and temperatures (6 and 12°C) could be applied to wine production and juice clarification at low temperature (Sahay et al., 2013). Pectinolytic enzymes are used for the degradation of pectin compounds in the fruit and vegetable processing industries (Alkorta et al., 1998). Cold-active pectinolytic enzymes are required in wine industries both for extraction and for clarification (Merin et al., 2011). Cold-active pectinases in addition has potential to maintain nutritional value, taste and sensory features (Nakagawa et al., 2002).

Most studies that involve the screening of yeasts for enzyme production target proteinase or pectinase, mainly because these are important enzymes used to clarify fruit juices (Braga et al., 1998). Earlier cold-active pectinase produced by psychrophilic yeast *C. capitatum* strain PPY-1 (Nakagawa et al., 2002) had been reported; these enzymes may be used for processing foods (Margesin and Schinner, 1994; Russell et al., 1998; Gerday et al., 2000). It have been reported that PPY (Pectinolytic-psychrophilic yeast) were examined for pectinolytic activities at 5°C and strains exhibited pectin esterase activities (Nakagawa et al., 2004). Six psychrophilic fungal isolates has been reported earlier from the new geographical region of Jammu and Kashmir, India as a source of cold-active pectinolytic activities of oenological grade (Singh et al., 2012).

Lactases

Lactose is the main part of daily intake carbohydrate. β-Galactosidase hydrolyzes lactose into glucose and galactose, so it's commercially called lactase (Shukla and Wierzbiciki, 1975). Hamid et al. (2013) reported that psychrophilic yeast *C. capitatum* SPY11 and psychro-tolerant yeast *R. mucilaginosa* PT1 strains produce cold-active β-galactosidases that are able to degrade lactose at low temperature; β-galactosidase activity of both strains was found highest at 4°C, thus reflecting the nature of cold active enzymes. The β-galactosidase enzyme produced by these strains will have potential application at low temperature in dairy as well as in biotechnological industries.

Use of cold-active lactase has added advantage to catalyze lactose hydrolysis at storage temperature (that is 4°C) with no extra effort to change place and at the same time no risk of contamination which is possible at higher temperature. A number of important genes coding for cold-active β-galactosidase have been detected in yeast (Nakagawa et al., 2006a, b). Therefore, cold-active lactases have recently been attracting attention, as there is an increasing industrial trend to treat dairy products under mild conditions to avoid spoilage and changes in the taste and nutritional value, and cold-active lactase can be inactivated at a low temperature without heat treatment (Margesin, and Schinner, 1994). The psychrotrophic yeasts (PPY-1) *C. capitatum* with β-galactosidase activity have been isolated (Nakagawa et al., 2002). It has been reported that *A. psychrolactophilus* strains B7, D2 and D5 produce cold-active β-galactosidase (Loveland et al., 1993). Cold-adapted β-galactosidase with high activity levels at low temperatures might prove to be useful for removing lactose from refrigerated milk enabling it to be consumed by lactose intolerant individuals, and for converting lactose in whey into glucose and galactose. *K. lactis*has been used for its industrial potential in the production of β-galactosidase enzyme which could be used to reduce the lactose content of milk (Suarez et al., 1995).

Amylases

Amylases are enzymes that hydrolyze starch molecule to give diverse products including dextrin and progressively smaller polymers made up of glucose units (Pandey et al., 2000; Syed et al., 2009). Amylases have been estimated to comprise approximately 30% of the world's enzyme production (Maarel et al., 2002). These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (Maarel et al., 2002). Though amylases originates from different sources (plants, animals and microorganisms), the microbial amylases are the most produced and used in industry, due to their productivity and thermo stability (Burhan et al., 2003). Fungi, bacteria and yeasts have been reported to produce these enzymes (Salvakumar et al., 1996).

Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical

industry (Maarel et al., 2002; Satyanarayana et al., 2005). Processes catalyzed by cold-active enzymes have two advantages: they have potential to economise the processes by saving energy (Deming, 1998; Cavicchioli et al., 2002), and they protect the processes from contamination (Gardey et al., 2000). Mould amylases are used in alcohol production and brewing industries (Van and Smith, 1968). Amylases are significant enzymes for their specific use in the industrial starch conversion process (Nigam and Singh, 1995).

Amylases have been found in many yeast species (Gupta et al., 2003; De Mot et al., 1984) including *Lipomyces kononenkoae* (Prieto et al., 1995), *Schwanniomyces alluvius* (Wilson and Ingledew, 1982; Moranelli et al., 1982), *Trichosporon pullulans* (De Mot and Verachtert, 1986), *Candida antarctica* (De Mot and Verachtert 1987) and *C. flavus* (Wanderley et al., 2004). A cold active α-amylase from Antarctic psychrophile *Alteromonas haloplanktis* was reported to exhibit maximum α-amylase production at 4°C (Ramachandran et al., 2004; Hayashida and Teramoto, 1986; Moller et al., 2004). A mutant strain of yeast (*S. cerevisiae*) has been found to secrete amylases (Wang et al., 2001).

Proteases

Proteases represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market accounting for about 60% of total worldwide sale of enzymes (Rao et al., 1998). Cold active proteases have found their way into many applications like in industries of detergents, food, textiles, cosmetics, beverages, pharmaceutical, bioremediation and bakery (Hamamoto et al., 1994; Baghel et al., 2005; Anwar and Saleemuddin, 1998; Gupta et al., 2002).

Cold-adapted or low temperature tolerant proteases suit well in waste management in cold environments, where the degradation capabilities of endogenous microflora are reduced due to low temperatures. Probably the largest application of proteases is in laundry detergents, where they help in removing protein based stains from clothes (Banerjee et al., 1999). Psychrotrophic, dimorphic yeast *Candida humicola,* isolated from Antarctic soil, secretes an acidic protease into the medium (Ray et al., 1989). Earlier studies have indicated that yeasts belonging to the genera *Kluyveromyces, Endomycopsis, Cephalosporium, Aureobasidium, Saccharomycopsis, Rhodotorula, Candida* and most sporobolomycetes and trichosporons secrete proteolytic enzymes (Ahearn et al., 1986). Many of these yeasts are probably also psychrotrophic (Ahearn et al., 1986), but the proteolytic enzymes secreted by them has been neither purified nor characterized. Cold-adapted proteases thus can be used to optimize present day industrial processes and for developing future technologies with less energy inputs and process cost by removing the cost of heat inactivation step (Cavicchioli et al., 2002; Deming, 2002; Margesin et al., 2002).

Phytases

Phytase is an enzyme that releases digestible phosphorus, calcium and other nutrients from phytic acid (myo-inositol hexakisphosphate) and thereby, help to reduce environmental phosphorus pollution (Mllaney et al., 2000). Phytases are found naturally in plants and microorganisms, particularly fungi (Stanley, 1961; Somoilova, 1980; Valikhanov et al., 1981; Wang et al., 1980). Several yeast species have been screened for their extracellular phytase activity and it was also reported that yeasts are important source of phytases (Nakamura et al., 2000; Vohra and Stayanarayan, 2001; Wodzinski and Ullah, 1996). Cold-active phytases from psychrophilic yeasts will help in reducing the phosphorus pollution in the cold environments. Earlier reports also attest the stability of yeast phytate (Quan et al., 2001) from *C. krusei.*

Phytase is already used as a supplement in diets for monogastric animals to improve phosphate utilization from phytate, the major storage form of phosphate in plant seeds. In recent years, this class of enzymes has also been found increasingly interesting for use in processing and manufacturing of food for human consumption, particularly because the decline in food phytate results in an enhancement of mineral bioavailability. Different strategies could be applied to optimize phytate degradation during food processing and digestion in the human alimentary tract such as adjustment of more favourable conditions during food processing for the phytases naturally occurring in the raw material, addition of isolated phytases to the production process, use of raw material with a high intrinsic phytate-degrading activity either naturally present or introduced by genetic engineering and the use of recombinant food-grade microorganisms as carriers for phytate-degrading activity in the human gastrointestinal tract (Greiner and Konietzny, 2006). Furthermore, phytases may find application in the production of functional foods or food supplements with health benefits.

Lipases

Lipases are a class of enzymes which catalyze the hydrolysis of long chain triglycerides and constitute the most important group of biocatalysts for biotechnological applications (Joseph et al., 2007). Lipases were first discovered in 1856 by Claude Bernard when he studied the role of the pancreas in fat digestion (Peterson and

Drablos, 1994). Lipolytic enzymes are grouped into three main categories, which are esterases, phospholipases and lipases (Arpigny and Jaeger, 1999). Permanently cold regions such as glaciers and mountain regions are another habitat for psychroplillic lipase producing microorganisms (Joseph, 2006). Microbial lipases are also more stable than their plant and animal derivatives and their production is easier and safer for industrial and research applications (Schmidt-Dannert, 1999).

Although a number of lipase producing sources are available, only a few bacteria and yeast were exploited for the production of cold adapted lipases (Joseph, 2006). Psychrotrophic fungi such as *Rhizopus sp*., *Mucorsp*., have been reported to produce cold active lipases (Coenen, 1997). An extensive research has been carried out in the cold active lipase of *C. antarctica* compared to other psychrophilic fungi. Use of lipase B from *C. antarctica* for the preparation of optically active alcohols has been reported (Rotticci et al., 2001). Lipase from *C. antarctica* has been evaluated as catalyst in different reaction media for hydrolysis of tributyrin as reaction model (Salis et al., 2003). *C. lipolytica*, *G. candidum* and *P. roqueforti* have been isolated from frozen food samples and reported to produce cold active lipases (Alford and Pierce, 1961).

Cold active lypolytic enzymes are currently attracting an enormous attention because of their biotechnological potential (Benjamin and Pandey, 1998). Psychrophilic enzymes are highly approached for different industrial applications; it has been reported that alkaline yeast lipases are preferred because they can work at lower temperatures as compared to bacterial and fungal lipases (Ahmed et al., 2007; Saxena et al., 1999). Various industrial applications of cold-active microbial lipases in the medical and pharmaceuticals, fine chemical synthesis, food industry, domestic and environmental applications have been reported (Joseph et al., 2007). Cold active lipase A and lipase B from *Candida antarctica* have been expressed in *C. antarctica* and *E. coli*, respectively, for their biotechnological applications (Pfeffer et al., 2006; Liu et al., 2006). Research on microbial lipases, has increased due to their great commercial potential (Silva et al., 2005). Cold-active lipases could be a good alternative to mesophillic enzymes in brewing industry and wine industries, cheese manufacturing, animal feed supplements and so on (Collins et al., 2002).

Xylanases

Xylan is the major component of hemicellulose consisting of β-1, 4-linked D-xylopyranosyl residues. The hydrolysis of xylan in plant materials is achieved by the use of a mixture of hydrolytic enzymes including endo-β-1, 4-xylanase and β-D-xylosidase (Polizeli et al., 2005). The importance of xylanase has tremendously increased due to its biotechnological applications for pentose production, fruit-juice clarification, improving rumen digestion and the bioconversion of lignocellulosic agricultural residues to fuels and chemicals (Nigam and Pandey, 2009; Srinivasan and Rele, 1995; Garg et al., 1998).

Xylanase from an Antarctic yeast *C. adeliae* that exhibits optimal growth at low temperature has been reported (Petrescu et al., 2000). The xylanase from *C. adeliae* is less thermostable than its mesophilic homologue when the residual activities are compared, and this difference was confirmed by differential scanning calorimetry experiments. In the range 0 to 20°C, the cold-adapted xylanase displays lower activation energy and a higher catalytic efficiency (Petrescu et al., 2000).

It has been found that yeast, unlike bacteria, can perform certain post translational modifications, such as glycosy-lation which particularly affects the enzymatic activity of recombinant proteins, as demonstrated for the xylanase from yeast *C. albidus* (Runge et al., 1988). Scorzetti et al. (2000) isolated a *C. adeliensis* sp. nov. from Terre Adelie, Antarctica, this produced a cold-active xylanase. Amoresano et al. (2000) reported that a common folding motif might occur within the entire xylanase family isolated from psychrophilic yeast. Alkaliphilic xylanases would also be required for detergent applications where high pHs are typically used (Kamal et al., 2004).Cold-adapted family eight xylanase is more efficient in baking than a commonly used commercial enzyme (Dutron et al., 2004). Xylanase from psychrophilic *Coprinus psychromorbidus* have been reported (Inglis et al., 2000).

CONCLUSION

There are a lot of industrial processes to which cold active enzymes can be applied to improve the quality and the yield of final products. It is important to investigate the production conditions and physico-chemical characteristics of psychrophilic enzymes produced by psychrophilic yeasts. Cold-active enzymes having a set of biochemical and physical properties can be generated for each specific industrial process. These studies can provide valuable tools for biotechnologists and microbiologists to improve microorganisms and make them able to produce efficient cold-active enzymes. More studies are required to find out newer venues of applications as the field of cold active enzymes is yet at infancy.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

Authors thank Madhya Pradesh Council of Science and Technology (MPCST), Bhopal India for providing laboratory facilities and encouragement.

REFERENCES

Abu EA, Ado SA, James DB (2005). Raw starch degrading amylase production by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisae* grown on Sorghum pomace. Afr. J. Biotechnol. 4(8):785-790.

Adams MWW, Perler FB, Kelly RM (1995). Extremozymes: Expanding the limits of biocatalysis. Biotechnology 13: 662-668.

Aguillar G, Huitron C (1990). Constitutive exo-pectinase produced by *Aspergillus* sp. CH-Y-1043 on different carbon sources. Biotechnol. Lett. 12: 655-660.

Ahearn DG, Meyers SP, Nichols RA (1986). Extracellular proteinases of yeasts and yeast like fungi. Appl. Microbiol. 16:1370-1374.

Ahmed A, Delphine P, Alain DC, Yves L, Frederic C (2007). A comparative study on two fungal lipases from *Thermomyces lanuginosus* and *Yarrowia lipolytica* shows the combined effects of detergents and pH on lipase adsorption and activity. Biochem. Biophys. Acta. 1771: 1446-1456.

Akpan I, Bankole MO, Adesemowo AM, Latunde-Dada GO (1999). Production of amylase by *A. niger* in a cheap solid medium using rice band and agricultural materials. Trop. Sci. 39: 77-79.

Alford JA, Pierce DA (1961). Lypolytic activity of microorganisms at low and intermediate temperatures.Activity of microbial lipase at temperatures below 0^{o}C. J. Food Sci. 26: 518-524.

Alkorta I, Garbisu C, Llama MJ, Serra JL (1998). Industrial applications of pectic enzymes: a review. Process Biochem (London). 33: 21-28.

Allen D, Huston AL, Wells LE, Deming JW (2001). Encyclopedia of Environmental Microbiology. 1:1-17.

Amoresano A, Andolofo A, Mcoroaro M, Zocchi I, Petrescu I, Gerday C, Marino G (2000). Structural characterization of a xylane from psychrophilic yeast by mass spectrometry. Glycobiol. 10(5): 452-458.

Antranikian G (1992). Microbial degradation of starch. In: Winkelmann G, Ed. Microbial degradation of natural products. VCH, Weinheim. 27-51.

Anwar A, Saleemuddin M (1998). Alkaline proteases: a review. Bioresour. Technol. 64:175-183.

Arpigny JL, Jaeger KE (1999). Bacterial lipolytic enzymes: classification and properties. Biochem. J. 343:177-183.

Baghel VS, Tripathi RD, Ramteke PW, Gopal K, Dwivedi S, Jain RK, Rai UN, Singh SN (2005). Psychrotrophic proteolytic bacteria from cold environment of Gangotri glacier, Western Himalaya, India. Enzyme Microb. Technol. 36: 654-659.

Banerjee UC, Sani RK, Azmi W and Soni R (1999).Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Process Biochem. 35(1):213-219.

Benjamin S, Pandey A (1998). Candida rugosa lipase: Molecular biology and versatility in Biotechnology. Yeast 14: 1069-1087.

Bergauer P, Fonteyne PA, Nolard N, Shinner F, Margesin R (2005).Biodegradation of phenol and phenol-related compounds by psychrophilic and cold-tolerant alpine yeasts. Chemosphere 59:909-918.

Birgisson H, Delgado O, Garcia AL, Hatti KR, Mattiasson B (2003). Cold-adapted yeasts as producers of cold-active polygalacturonases. Extremophiles 7:185-193.

Blaise GD, Collins T, D'Amico S, Gratia E, Hoyoux A, Marx JC (2004). Some like it cold: biocatalysis at low temperatures. FEMS Microbiol. Rev. 1:25-42.

Braga AA, De Morais PB Linrdi VR (1998).Screening of yeasts from Brazilian Amazon rain forest for extracellular proteinases production. Syst. Appl. Microbiol. 21: 353-359.

Brizzio S, Benedetta T, Virginia G (2007). Extra cellular enzymatic activity of Basidomycetes yeasts isolated from glacial and sub glacial waters of North West Patagonia (Argentina). Can. J. Microbiol. 53:519-525.

Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, Osman G (2003). Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp. isolate ANT-6. Process. Biochem. 38:1397-1403.

Butinar L, Spencer MI, Gunde CN (2007). Yeasts in high Arctic glaciers: the discovery of a new habitat for eukaryotic microorganisms. Antony van Leeuwenhoek 91:277-289.

Buzzini P, Branda E, Goretti M, Turchetti B (2012). Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiol. Ecol. 82:217-241.

Buzzini P, Martini A (2002). Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. J. Appl. Microbiol. 93:1020-1025.

Buzzini P, Turchetti B, Diolaiuti G, D'Agata G, Martini A, Smiraglia C (2005).Culturable yeasts in melt waters draining from two glaciers in the Italian Alps. Ann. Glaciol. 4:119-122.

Cavicchioli R., Siddiqui KS, Andrews D, Sowers KR (2002). Low temperature extremophiles and their applications. Curr. Opin. Biotechnol. 13:253-261.

Coenen TMM (1997). Safety evaluation of lipase derived from Rhizopus oryzae: Summary of toxicological data. Food Chem. Toxicol. 35:315-322.

Collins T, Meuwis MA, Stals I, Claeyssens M, Feller G, Gerday C (2002). A novel Family 8 Xylanase.Functional and Physicochemical Characterization. J. Biol. Chem. 277:35133-35139.

D'Amico G, Palile, Claverie, Tony C, Daphne G, Charle G (2002). Molecular basis of cold adaptation. Philos Trans. R. Soc. Lond. B Biol. Sci. 357(1423):917-925.

D'Amico S, Collins T, Marx JC, Feller G, Gerday C (2006). Psychrophilic microorganisms: challenges for life. EMBO Rep. 7(4):385-389.

De Mot R, Andries K, Verachtert H, (1984). Comparative study of starch degradation and amylase production by ascomycete yeast species. Syst. Appl. Microbiol. 5:106-118.

De Mot R, Verachtert H (1986). Secretion of α-Amylase and multiple forms of glucoamylase by the yeast *Trichosporon pullulans*. Can. J. Microbiol. 32:47-51.

De Mot R, Verachtert H (1987). Purification and characterization of extracellular α-amylase and glucoamylase from the yeast *Candida antarctica* CBS 6678. Eur. J. Biochem. 164:643-654.

Deming JW (1998). Deep ocean environmental biotechnology. Curr. Opin. Biotechnol. 9:283-287.

Deming JW (2002). Psychrophiles and polar regions. Curr. Opin. Microbiol. 5:301-309.

Díaz MR, Fell JW (2000). Molecular analyses of the IGS & ITS regions of rDNA of the psychophilic yeasts in the genus *Mrakia*. Antonie van Leeuwenhoek 77:7-12.

Dutron A, Georis J, Genot B, Dauvrin T, Collins T, Hoyoux A, Feller G (2004). Use of family 8 enzymes with xylanolytic activity in baking. In: World Intellectual Property Organization, PCT, WO 2004/023879 A1.

Farrell J, Rose AH (1967). Temperature effects on microorganisms. In A. H. Rose (ed.). Thermobiology Academic Press London. pp. 147-218.

Feller G, Gerday C (1997). Psychrophilic enzymes: molecular basis of cold adaptation. Cell. Mol. Life Sci. 53:830-841.

Feller G, Gerday C (2003). Psychrophilic enzymes: hot topics in cold adaptation, Nat. Rev. Microbiol. 1:200-208.

Fogarty WM, Kelly CT (1983). "Pectic enzymes," in Microbial Enzymes and Biotechnology, Appl. Science Publishers, London, UK. pp. 131-182.

García V, Brizzio S, Libkind D, Buzzini P, Van Broock M (2007).Biodiversity of cold-adapted yeasts from glacial meltwater rivers in Patagonia (Argentina). FEMS Microbiol. Ecol. 59: 331-341.

Garg AP, Roberts JC, McCarthy A (1998). Bleach boosting effect of cellulase free xylanase of *Streptomyces thermoviolaceus*and its comparison with two commercial enzyme preparations on birchwood Kraft pulp. Enzym. Microb. Biotechnol. 22:594-598.

Gerday C, Aittaleb M, Arpigny JL, Baise E, Chessa JP, Garsoux G,

Petrescu I, Feller G (1997). Psychrophilic enzymes: a thermodynamicchallenge. Biochim. Biophys. Acta 1342:119-131.
Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J (2000). Cold-adapted enzymes: from fundamentals to biotechnology. Trends Biotechnol. 18:103-107.
Goodchild A, Saunders NFW, Ertan H, Raftery M, Guilhaus M, Curmi PMG, Cavicchioli R (2004). A proteomic determination of cold adaptation in the Antarctic archaeon, *Methanococcoides burtonii*. Mol. Microbiol. 53:309-321.
Gounot AM (1986) Psychrophilic and psychrotrophic microorganisms. Experientia 42:1192-1197.
Gounot AM (1991). Bacterial Life at low temperature: physiological aspects and biotechnological implications. J. Appl. Bacteriol. 71: 386-397.
Greiner R, Konietzny U (2006). Phytase for Food Application. Food Technol. Biotechnol. 44(2):125-140.
Gupta R, Beg QK, Lorenz P (2002). Bacterial alkaline proteases: Molecular approaches and Industrial Applications. Appl. Microbiol. Biotechnol. 59:15-32.
Gupta R, Gigras P, Mohapatra H, Goswami VK (2003). Microbial α-Amylase: a biotechnological perspective. Process.Biochem. 38: 1599-1616.
Hamamoto T, Kaneda M, Horikoshi K, Kudo T (1994). Characterization of protease from a psychrotroph, *Pseudomanas fluorescenes* 114. Appl. Environ. Microbiol. 60(10):3878-3880.
Hamid B, Singh P, Mohiddin FA, Sahay S (2013). Partial characterization of cold-active β-galactosidase activity produced by *Cystophallobaidium capatitum* SPY11 and *Rodotorella musloganosa* PT1. Endocytobiosis Cell Res. 24:23-26.
Hamid B, Singh P, Rana RS, Sahay S (2012).Isolation and identification of psychrophilic yeast from the soil of northern region of India. Inventi Rapid: Pharm. Biotechnol. Microbio Vol. 2012, Article ID-Inventi:pbm/40/11.
Hayashida S, Teramoto Y (1986). Production and characteristics of raw-starch-digesting α-amylase from a protease negative *Aspergillus ficuum*mutant. Appl. Environ. Microbiol. 52 1068-1073.
Holdgate MW (1977). Terrestrial Ecosystems in the Antarctic . Philos. Trans. R. Soc. Lond. B Biol. Sci. B1977, 2.Biological Sciences , 279:5-25
Hough DW, Danson MJ (1999). Extremozymes. Curr. Opin. Chem. Biol. 3:39-46.
Inglis GD, Popp AP, Selinger LB, Kawchuk LM, Gaudet DA, McAllister TA (2000).Production of cellulases and xylanases by low-temperature basidiomycetes. Can. J. Microbiol. 46:860-865.
Jaeger KE, Eggert T (2002). Lipases for biotechnology. Curr. Opin. Biotechnol. 13: 390-397.
Joseph B (2006) Isolation, purification and characterization of cold adapted extracellular lipases from psychrotrophic bacteria: Feasibility as laundry detergent additive. Ph.D Thesis. Allahabad Agricultural Institute - Deemed University, Allahabad, India.
Joseph B, Promad W, Ramtake, George T, Nitisha S (2007). Standard revive cold active microbial lipases: A versatile tool for industrial applications. Biotechnol. Microbiol. 2(2):39-48.
Kamal Kumar B, Balakrishnan H, Rele MV (2004) Compatibility of alkaline xylanases from an alkaliphilic *Bacillus* NCL (87-6-10) with commercial detergents and proteases. J. Ind. Microbiol. Biotechnol. 31(2):83-7.
Katayam H, Nishikawa J, Hirayamaja (1997). Biological treatment of dissolved organic matter by Antarctic psychropylic yeast cells. Mizushori Gijutsu (Water Pulification and Liquid Wastes Treatment), 38: 1-4.
Kaur G., Kumar S, Satyanarayana T (2004) Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould Sporotrichum thermophile Apinis. Bioresour. Technol. 94: 239-43.
Kourkoutas Y, Koutinas A, Kanellaki M, Banat I, Marchant R (2002). Continuous wine fermentation using psychrophilic yeast immobilized on apple cuts at different temperatures. Food Microbiol. 19(2-3):127-134.
Kudanga T, Mwenje E, Mandiveja F, Read JS (2007). Esterases and putative lipases from tropical isolates of *Aureobasidium pullulans*. J. Basic Microbiol. 47: 138-147.
Laitila A, Wilhelmson A, Kotavitta E, Olkku J, Home S, Juvonen R (2006). Yeasts in an industrial malting ecosystem. J. Ind. Microbiol. Biotechnol. 33: 953-966.
Lambo A, Patel T (2006). Cometabolic degradation of polychlorinated biphenyls at low temperature by psychrotolerant bacterium *Hydrogenophaga* sp. IA3-A. Curr. Microbiol. 53(1):48-52.
Liu D, Schmid R, Rusnak M (2006). Functional expression of *Candida antarctica* lipase B in the *Escherichia coli* cytoplasm—a screening system for a frequently used biocatalyst. Appl. Microbiol. Biotechnol. 72(5):1024-1032.
Loveland J, Gutshall K, Kasmir J, Prema P, Brenchley JE (1993). Characterization of psychrotrophic microorganisms producing β-galactosidase activities. Appl. Environ. Microbiol. 60: 12-18.
Maarel MJECB, Uitdehaag JCM, Leemhuis H, Dijkhuizen L (2002). Properties and applications of starch-converting enzymes of the a-amylase family. J. Biotechnol. 94:137-155.
Margesin R, Feller G, Gerday C, Russell N (2002). Cold-adapted microorganisms: adaptation strategies and biotechnological potential. In: Encyclopedia of Environmental Microbiology. pp. 871-885.
Margesin R, Miteva V (2011) Diversity and ecology of psychrophilic microorganisms. Res. Microbiol. 162:346-361.
Margesin R, Schinner F (1994). Properties of cold-adapted microorganisms and their potential role in biotechnology. J. Biotechnol. 33:1-14.
Menna ME (1960). Yeasts from Antarctica. J. Gen. Microbiol. 23:295-300.
Merın MG, Mendoza LM, Farıas ME, Ambrosini VIM (2011).Isolation and selection of yeasts from wine grape ecosystem secreting cold-active pectinolytic activity. Int. J. Food Microbiol. 147:144-148.
Middelhoven WJ (1997). Identity and biodegradative abilities of yeasts isolated from plants growing in an arid climate. Antonie van Leeuwenhoek 72:81-89.
Mllaney EJ, Daly CB, Ullah AH (2000). Advances in phytase research. Adv. Appl. Microbiol. 47:157-199.
Moller K, Sharif MZ, Olsson L (2004).Production of fungal α-amylase by *Saccharomyces kluyveri*in glucose-limited cultivations. J. Biotechnol.111:311-318.
Moranelli F, Yaguchi M, Calleja B, Nasim A (1982). Purification and characterization of the extracellular α-amylase activity of the yeast *Schwanniomyces alluvius*. Biochem. Cell Biol. 65:899-907.
Morita RJ (1975). Psychrophilic bacteria.Bacteriol. Rev.39, 144-167.
Morita RY (1966). Marine psychrophilic bacteria oceanoger.Mar. biol. Ann. Rev. 4:105-121.
Moyo S, Gashe BA, Collison EK, Mpuchane S (2003). Optimising growth conditions for the pectinolytic activity of *Kluuyveromyces wickerhamii* by using response surface methodology. Int. J Food Microbiol. 85:87-100.
Nakagawa T, Fujimoto Y, Ikehata R (2006b). Purification and molecular characterization of cold-active β-galactosidase from *Arthrobacter psychrolactophilus* strain F2. Appl. Microbiol. Biotechnol. 72:720-725.
Nakagawa T, Ikehata R, Uchino M (2006a). Cold-active β-galactosidase activity of isolated psychrophilic basidiomycetous yeast *Guehomyces pullulans*. Microbiol. Res. 161:75-79.
Nakagawa T, Nagaoka T, Taniguchi S, Miyaji T, Tomizuka N (2004). Isolation and characterization of psychrophilic yeasts producing cold-adapted pectinolytic enzymes. Lett. Appl. Microbiol. 38:383-387.
Nakagawa T, Yamada K, Miyaji T, Tomizuka N (2002). Cold-active pectinolytic activity of psychrophilic basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. J. Biosci. Bioeng. 94:175-177.
Nakamura Y, Fukuhara H, Sano K (2000). Secreted phytase activities of yeasts. Biosci. Biotechnol. Biochem. 64 (4): 841-844.
Nigam P, Pandey A (2009). Biotechnology for Agro-Industrial Residues Utilisation. Publisher Springer Science Business Media B.V. pp. 1-466.
Nigam P, Singh D (1995). Enzyme and microbial systems involved in starch processing. Enzyme Microb. Technol.17:770-778.
Ohno S, Suzukia, Shiumrak (1991). Treatment of highly concentrated

organic wastewater by yeasts.Yosui to Haisui. J. Water Wastes 33:664-668.
Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000). Advances in microbial amylases. Biotechnol. Appl. Biochem. 31:135-152.
Peterson SB, Drablos V (1994).A sequence analysis of lipases, esterases, and related proteins. In: Lipases-their structure, biochemistry, and application. Ed. P. Woolley, and S.B. Peterson, Cambridge: Cambridge University Press. pp. 23-48.
Petrescu I, Lamotte-Brasseur J, Chessa JP, Ntarima P, Claeyssens M, Devreese B, Marino G, Gerday C (2000). Xylanase from the psychrophilic yeast *Cryptococcus adeliae*. Extremophiles 4(3):137-44.
Pfeffer J, Richter S, Nieveler J, Hansen CE, Rhlid R, Schmid R, Rusnak M (2006). High yield expression of lipase A from *Candida antarctica* in the methylotrophic yeast *Pichia pastoris* and its purification and characterization. Appl. Microbiol. Biotechnol. 72(5):931-938.
Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005). Xylanases from fungi: Properties and industrial applications. Appl. Microbiol. Biotechnol. 67:577-591.
Prieto JA, Bort BR, Martinez J, Randez-Gil F (1995).Purification and characterization of a new α-amylase of intermediate thermal stability from the yeast *Lipomyces kononenkoae*.Biochem. Cell Biol. 73:41-49.
Quan C, Zhang L, Wang Y and Ohta Y (2001).Production of phytase in low phosphate medium by a novel yeast *Candida krusei.* J. Biosci. Bioeng. 92:154-160.
Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G, Pandey A (2004). Coconut oil cake - A potential raw material for the production of α-amylase. Bioresour. Technol. 93:169-174.
Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62:597-635.
Ray MK, Shivaji S, Shyamala N, Rao MB, Bhargava PM (1989). Yeast strains from the Schirmacher Oasis, Antarctica. Polar Biol. 9:305-309.
Robinson CH (2001). Cold adaptation in Arctic and Antarctic fungi. New Phytol. 151:341-353.
Rodríguez ME, Lopes CA, Van Broock M, Valles S, Ramon D, Caballero AC (2004). Screening and typing of Patagonian wine yeasts for glycosidase activities. J. Appl. Microbiol. 9:84-95.
Rotticci D, Ottosson J, Norin T, Hult K (2001). *Candida Antarctica* lipase B: A tool for the preparation of optically active alcohols. Methods Biotechnol. 15: 261-276.
Ruberto L, Vazquez S, Lobalbo A, Maccormack WP (2005). Psychrotolerant hydrocarbon-degrading *Rhodococcus* strains isolated from polluted Antarctic soils. Antarct. Sci. 17(1):47-56.
Runge KW (1988). Postraslational modification during protein secretion.Protein transfer and Organelle Biogenesis. Academic press, San Diego. pp.159-208.
Russell NJ (2000). Towards a molecular understanding of cold activity of enzymes from psychrophiles. Extreamophiles 4.83-90.
Russell NJ, Hamamoto T (1998). Psychrophiles in Extremophiles: Microbial Life in Extreme Environments. Edited by Horikoshi K, Grant WD.New York: Wiley-Liss. pp. 25-45.
Russell RJM, Gerike U, Danson MJ, Hough DW, Taylor GL (1998). Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. Structure 6:351-361.
Sabri A, Bare G, Jacques P (2001). Influence of moderate temperatures on myristoyl-CoA metabolism and acyl-CoA thioesterase activity in the psychrophilic antarctic yeast *Rhodotorula aurantiaca*. J. Biol. Chem. 276(16):12691-12696.
Sahay S, Hamid B, Singh P, Ranjan K, Chauhan D, Rana RS, Chaurse VK (2013) Evaluation of pectinolytic activities for oenological uses from psychrotrophic yeasts, Lett. Appl. Microbiol. 57:115-121
Salis A, Svensson I, Monduzzi M, Solinas V, Adlercreutz P (2003). The atypical lipase B from *Candida antarctica*is better adapted for organic media than the typical lipase from *Thermomyces lanuginosa*. Biochem. Biophys. Acta. 1646:145-151.
Salvakumar P, Ashakumari L, Pandey A (1996). Hydrolytic enzymes by SSF method. J. Ind. Sci. Res. 55:443-452.
Satyanarayana T, Rao JLUM, Ezhilvannan M (2005). α-amylases. In: Enzyme Technology, Pandey A, Webb C, Soccol CR, Larroche C (Eds.), Asiatech Publishers Inc., New Delhi, India. pp. 189-220.
Saxena RK, Ghosh PK, Gupta R, Davidson WS, Bradoo S, Gulati R (1999). Microbial lipases: potential biocatalysts for the future industry. Curr. Sci. 77:101-115.
Schmidt-Dannert C (1999). Recombinant microbial lipase for biotechnological applications. Bioorg. Med. Chem. 7: 2123-2130.
Scorzetti G, Petrescu L, Yarrow D, Fell JW (2000). *Cryptococcus adeliensis sp. Nov.* a xylanase producing basidiomycetous yeast from Antarctica. Antonie van Leeuwenhoek 77(2):153-157.
Sharma R, Chisti Y, Banerjee UC (2001). Production, purification, Characterization and applications of lipases. Biotechnol. Adv. 19:627-662.
Shivaji S, Bhadra B (2008). *Rodotorula himialayensis sp. Nov.,* novel psychrophilic yeast isolated from Roop Kund Lake of Himalayan mountain ranges, India. Extremophiles 12:375-381.
Shukla TP, Wierzbicki LE (1975). Beta-galactosidase technology: a solution to the lactose problem. Food Sci. Nutr. 25:325-356.
Silva EG, Fatima D, Borges M, Medina C, Piccoli RH, Schwan RF (2005).Pectinolytic enzymes secreted by yeasts from tropical fruits. FEMS Yeast Res. 5(9):859-65.
Singh P, Hamid B, Lone MA, Ranjan K, Khan A, Chaurse VK, Sahay S (2012). Evaluation of pectinase activity from the psychrophilic fungal strain Truncatella angustata-BPF5 for use in wine industry. J. Endocytobios Cell Res. 22:57-61.
Skidmore ML, Foght JM, Sharp MJ (2000). Microbial life beneath a high arctic glacier. Appl. Environ .Microbiol. 66:3215-3220.
Somoilova TS (1980). Microbial degradation of inositolphosphates. Dokl. Vses. Akad. Sh. Nauk. 9:17-19.
Srinivasan MC, Rele MV (1995). Cellulase free xylanase from microorganisms and their applications to pulp and paper biotechnology: An overview. Indian J. Microbiol. 35:93-101.
Stanley GH (1961).Phytase, In C.Long, E. J. King, and W. M. Sperry (ed.), Biochemistshandbook. E. and F. N. Spon Ltd., London. pp. 259-262.
Stokes JL (1963). General biology and nomenclature of psychrophilic microorganisms.Recent progress in microbiology VIII. University of Toronto Press, Toronto. pp. 187-192.
Strauss MLA, Jolly NP, Lamberts MG, Van Rensburg P (2001). Screening for the production of extracellular hydrolytic enzymes by *non-Saccharomyces*wine yeasts. J. Appl. Microbiol. 91:182-190.
Suarez FL, Savaiano DA, Levitt MD (1995). Review article: The treatment of lactose intolerance. Alimentary Pharmacology and Therapeutics, December vol. 9, no. 6, p. 589-597.
Syed DG, Agasar D, Pandey A (2009). Production and partial purification of a-amylase from a novel isolate *Streptomyces gulbargensis.* J. Ind. Microbiol. Biotechnol. 36:189-194.
Thanh, Simard (1973). Biological treatment of wastewater by yeasts. J. Water Pollut. Control Fed. 45:674-680.
Valikhanov MN, Abdullaeva MM, Rahlmov MM (1981).Some properties of cottonseed phytase. Biokhimiya 46:100-102.
Van LJM, Smith MB (1968). US Patent 3, 418, and 211.
Vaz ABM, Rosa LH, Vieira MLA, Garcia V, Brandão LR, Teixeira LCR, Moliné M, Libkind D, van Broock M, Rosa CA (2011). The diversity, extracellular enzymatic activities and photoprotective compounds of yeasts isolated in Antarctica. Braz. J. Microbiol. 42:937-947.
Vishniac H, Klinger J (1986). Yeasts in the Antarctic deserts. In Megusar F, Gantar M (eds) Perspectives in Microbial Ecology. Proceedings of the 4th ISME. pp. 46-51.
Vishniac HS (1999). Psychrophilic yeasts. In: Seckbach J (Ed) Enigmatic microorganisms and life in extreme environments. Kluwer, The Netherlands. pp. 317-324.
Vohra A, Stayanarayan T (2001). Phytase production by the yeast Pichia anomala. Biotechnol. Lett. 23:551-554.
Wanderley KJ, Torres FAG, Moraes LMP, Ulhoa CJ (2004). Biochemical characterization of α-amylase from the yeast *Cryptococcus flavus.* FEMS Microbiol. Lett. 231:165-169.

Wang BD, Chem C, Kuo TT (2001). Characterization of a *Saccharomycas cerevisiae* Mutant with over secretion phenotype. Appl. Microbiol. Biotechnol. 55:712-720.
Wang HL, Swain EW, Hasseltine CW (1980).Phytase of molds used in oriental food fermentation. J. Food. Sci. 45:1262-1266.
Welander U (2005). Microbial degradation of organic pollutants in soil in a cold climate. Soil Sediment Contam. 14(3):281-291.
Wilson JJ, Ingledew WM (1982).Isolation and characterization of *Schwanniomyces alluvius* amylolytic enzymes. Appl. Environ. Microbiol. 44:301-307.
Wiseman A (1995). Introduction to Principles. In: A Wiseman (Ed) Handbook enzyme biotechnology. 3rd ed. Padstow, Cornwall, UK:Ellis Horwood Ltd. T.J. Press Ltd. pp. 3-8.
Wodzinski R, Ullah A (1996). Phytase. Adv. Appl. Microbiol. 42: 263-302.

academicJournals

Vol. 13(22), pp. 2231-2239, 28 May, 2014
DOI: 10.5897/AJB2013.13503
Article Number: F67722F44903
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Exogenous application of natural extracts of persimmon (*Diospyros kaki* Thunb.) can help in maintaining nutritional and mineral composition of dried persimmon

Il-Doo Kim[1], Jung-Won Lee[2], Se-Jong Kim[3], Jae-Wook Cho[3], Sanjeev Kumar Dhungana[4], Yang-Sook Lim[3] and Dong-Hyun Shin[4*]

[1]Institute of Agricultural Science and Technology, Kyungpook National University, Daegu, South Korea.
[2]Department of Global Tourism Management, Hanbuk University, Gyeonggi-do, South Korea.
[3]Agricultural Technology Administration, Sangju Persimmon Experiment Station, Sangju, South Korea.
[4]School of Applied Biosciences, Kyungpook National University, Daegu, South Korea.

Received 26 November, 2013; Accepted 16 May, 2014

Persimmon (*Diospyros kaki* Thunb.) fruit is delicious as well as rich in nutritive and medicinal values. The fruit is not available throughout the year and also could not be grown across the world but is liked in many countries. Many people have access only to the dried fruits. Therefore, the demand of quality dry fruit is very high. This study was carried out to investigate the effect of persimmon-peel extracts in maintaining the quality characteristics of dried persimmon. Different treatments applied to the dried fruit were assessed on the basis of physicochemical and organoleptic properties. The findings reveal that dried persimmon sprayed with 10% persimmon-peel extract exhibited the highest overall acceptance value with considerable nutritional and mineral composition as compared to other treatments. The results suggest that application of persimmon-peel extracts could rather be effective to enhance the overall acceptance of dried persimmon fruit together with maintaining its nutritional quality as compared to the commercially available synthetic preservatives.

Key words: Exogenous application, natural extract, nutritional composition, mineral composition, dried persimmon.

INTRODUCTION

Persimmon (*Diospyros kaki* Thunb.) is native to China but has been cultivated in many countries having cold climatic condition. It is a very delicious fruit with different nutrients and phytochemicals such as carbohydrates, organic acids, vitamins, tannins, polyphenols, dietary fiber, carotenoids, etc., which significantly contribute to its taste, color, nutritive and medicinal values (Celik and Ercisli, 2007; Del Bubba et al., 2009; Ebert and Gross, 1985; Gorinstein, 1999). This fruit is seasonal so it is not available throughout the year and also could not be cultivated across the world but is liked in many countries. Therefore, dried fruits could be a good option to them who are living in those areas where persimmon is not produced.

A diet fortified with dry persimmon peel is more efficient than the same diet fortified with dry persimmon pulp. The health-derived benefits are more in the peel due to the presence of carotenoids, polyphenols, ascorbic acid, and dietary fiber as compared to pulp (Gorinstein et al., 1994; 1998). Yokozawa et al. (2007) found the potential benefits of persimmon peel as a valuable source of antioxidant in the diabetic condition which reduced the oxidative stress induced by hyperglycemia. The protective potential of proanthocyanidin from persimmon peel was active against oxidative damage under the aging process since the polymerization of proanthocyanidin plays an important role in retarding aging in a cellular senescence model (Lee et al., 2008).

Different synthetic chemicals have been used as preservative of dried persimmon. To maintain the quality in persimmon, sulfur fumigation is often conducted to prevent surface browning and fungi attacks (Miller, 1984). The hazardous nature of sulfur, however, raises a health risk and food safety concerns (Islam and Hoque, 2013). Sulfur dioxide remained in food causes asthma or allergic reaction to some people. To reduce health risks and maintain essential quality of persimmon during drying, the present study investigated different potential measures. The objective of this research was to develop a safe method for preparing dried persimmon by treating it with natural extracts to eliminate potential health hazards of sulfur. Persimmon fruits were processed with varied concentrations of persimmon-peel extracts and persimmon wine to determine the effect of natural extracts on the quality of the resulting dried fruits. It was expected that the success and adoption of this natural treatment method could play a significant role in strengthening the competitiveness of harmful chemical-free persimmon products.

MATERIALS AND METHODS

Sample materials and chemicals

Persimmon fruits of cultivar *Doongsi*, grown at Sangju Persimmon Experiment Station, Sangju, Korea, were harvested at commercial maturity stage in October 2011. Sulfur powder and prethanol A (commercially available 95% ethanol with chemical formula C_2H_5OH) were purchased from Duksan Pure Chemicals Co. Ltd. (Korea), and red wine containing 15% alcohol produced in 2005 (Cabernet Sauvignon, Australia), was obtained from a local wholesale market in Korea. Glucose, fructose, and sucrose were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

Preparation of persimmon-peel extracts

Persimmon fruits were washed with tap water and peeled off with a knife and sliced into about 1 cm size. A 200 g of sliced peel was extracted in 1800 ml of 50% prethanol A solution for 24 h. The persimmon peel extract was filtered and concentrated by rotary evaporation. The final concentration of persimmon peel extract was adjusted to 10%.

Preparation of dried persimmon sample

The fruits were washed with tap water and peeled off using peeler. The pedicels of peeled fruits were tied with string and hanged for natural drying. Before kept for drying, the fruits were treated as following: T-1, dried persimmon soaked in 70% prethanol A solution for 2 min at 20±1°C; T-2, dried persimmon sprayed with 70% prethanol A solution; T-3, dried persimmon soaked in Medilox (60 ppm Hypochlorous acid) for 2 min at 20±1°C; T-4, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid); T-5, dried persimmon sprayed with 10% persimmon-peel extract; T-6, control sample (dried persimmon containing no added extract); T-7, dried persimmon burned with sulfur powder for 10 min at 20±1°C; T-8, dried persimmon sprayed with 70% prethanol A followed by spraying with 10% persimmon-peel extract; T-9, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid) followed by spraying with 10% persimmon-peel extract; and T-10, dried persimmon sprayed with wine (Alcohol 15%, Cabernet Sauvignon, Australia).

About seven fruits were tied in 1 m long strings and 2 to 3 strings were tied together while hanging for drying. The strings of persimmons were hung under the eaves of a roof where they got plenty of sun and breeze but were protected from rain. As the fruit dried, a white substance of sugar started appearing on the surface. How long it takes to dry depends on the size of the fruit and the environmental conditions. In this experiment, fruits were dried for 28 days and physical, chemical, and organoleptic analyses were carried out.

Proximate analysis

The moisture, crude protein, ash, and crude fiber content of dried persimmon were determined according to AOAC (1995) approved methods 950.01, 976.05, 955.03, and 962.09, respectively. Three replicates of each experiment were conducted to take data with three samples in each replication.

*Corresponding author. E-mail: dhshin@knu.ac.kr. Tel: +82-53-950-5707. Fax: +82-53-958-6880.

Abbreviations: T-1, dried persimmon soaked in 70% prethanol A solution for 2 min at 20±1°C; **T-2,** dried persimmon sprayed with 70% prethanol A solution; **T-3,** dried persimmon soaked in Medilox (60 ppm Hypochlorous acid) for 2 min at 20±1°C; **T-4,** dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid); **T-5,** dried persimmon sprayed with 10% persimmon-peel extract; **T-6,** control sample (dried persimmon containing no added extract); **T-7,** dried persimmon burned with sulfur powder for 10 min at 20±1°C; **T-8,** dried persimmon sprayed with 70% prethanol A followed by spraying with 10% persimmon-peel extract; **T-9,** dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid) followed by spraying with 10% persimmon-peel extract; and **T-10,** dried persimmon sprayed with wine (Alcohol 15%, Cabernet Sauvignon, Australia).

Color measurement

Color measurement of sample was done by using a Chroma Meter CR-300 (Minolta Corp., Japan). Results were recorded using A Minolta calibration plate (YCIE= 94.5, XCIE= 0.3160, YCIE= 0.330) and a Hunter Lab Standard plate (L^*= 97.51, a^*= -0.18, b^*= +1.67) were used to standardize the instrument with D65 illuminant. Color values were measured on three places of outer surface of three samples and the average was taken. Three independent replications were made for data analysis.

Water activity and soluble solids content (°Brix) determination

Water activity (a_w) was measured at 30°C with a Novasina (Samil Ltd., RTD-500, Korea) that allows temperature controlled measurements of a_w. Samples were analyzed for total soluble solids content (SSC) using a digital Refractometer (DR-A1, ATAGO Co. Ltd., Japan) equipped with a Thermostatic water bath set at 20°C. Three replications were made to analyze data with three samples in each replication.

Texture profile analysis

A universal Texture Analyzer TA.XT2 (Stable Micro Systems Ltd., Surrey, England) was used for the texture profile analysis (TPA) (Bourne, 1978) with the probe (P/5 probe, 5 mm diameter). The test speed was 1.0 mm/s, the post test speed 5.0 mm/s, and the distance was 5.0 mm. Samples were tested in triplicates. The following TPA parameters were obtained through the XT.RA dimension software package (SMS, 1992). The mean of three samples was considered for each replication and three replications were made for data analysis. The hardness values were corrected for the different sample areas and expressed as (kg/cm^2).

Determination of free sugar

Free sugars were analyzed using a method of Genard and Souty (1996). A 5 g of each sample was added to 10 ml of distilled water and homogenized using a Homogenizer (Ultra-Turrax T-25, IKA-Labortechnik, Germany), followed by adding 20 ml of distilled water, then by centrifugation at 16,000 × *g* for 30 min. The collected supernatant was filtered through a Sep-Pak C18 cartridge (WAT023501, Waters, USA) and a Millipore 0.45-syringe filter (PVDF, Whatman, Japan). Free sugars were quantified using a high performance liquid chromatography (HPLC) (Model 9300, Younglin, Korea) consisting of a Refractive Index Detector (Triathlon M730D, Younglin, Korea), a column heater set at 85°C, Sugar-Pak (6.5 × 300 mm Alltech, USA), and the mobile phase of deionized-distilled H_2O delivered at 0.5 ml/min. Glucose, fructose, sucrose, and sorbitol, obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA), were used as reference sugars for identification. Manitol was used as the internal standard and the free sugars were expressed as percentage dry weight.

Determination of mineral content

A 0.5 g of sample was mixed with 15 ml of 65% nitric acid (HNO_3). A solution was diluted with 50 mL of distilled water. Essential minerals were determined by Inductively Coupled Plasma (ICP) Emission Spectrophotometer (38 Plus, Jobin Yvon, Co., France) and heavy metals by Atomic Absorption Spectrometer (Varian Spectra, AA-220 FS, Australia) by following Bond et al. (2005). The instrument was calibrated using known standards for each mineral. Average values of two replicates were used for data analysis.

Sensory properties evaluation

Samples prepared with different treatments were rated for color, taste (sweetness, astringency), texture, and overall acceptance, respectively, on the following scale: 1 point= very bad, 2 point= bad, 3 point = moderate, 4 point = good, 5 point = very good. All the sensory properties were evaluated by 10 panelists (5 women and 5 men) randomly selected in Kyungpook National University, Daegu, Korea. The results showed are the average values of 10 evaluations for each sensory property.

Statistical analysis

All the data were expressed as mean±standard deviation. The statistical analyses were performed using the SPSS software. One-way analysis of variance (ANOVA) was used to test statistical differences between treatments. Duncan's multiple range tests were used to examine the differences among treatment means. Statistical significance level was considered at $p<0.05$.

RESULTS AND DISCUSSION

Peels are removed during the processing of dried persimmon fruit. However, reports indicate that persimmon peel contain high nutritional and medicinal values. The 2-methoxy-4-vinylphenol which is a component of persimmon peel has high antioxidant activity lost along with the peels (Fukai et al., 2009). Son et al. (2013) indicate that persimmon peel might possess a potential anti-atherogenic effect. Therapeutic properties of persimmon peel extract show potential antitumor and multidrug resistance-reversing agents (Kawase et al., 2003). Results of this study also showed that exogenous application of persimmon peel extract not only improves the physical and organoleptic properties but also enhance the nutritive value.

Proximate composition of dried persimmon

Proximate attributes such as crude fiber, protein, moisture, and ash of dried persimmons, exposed to various treatments, were determined. The results of each proximate parameter are given in Table 1. Moisture content of T-6 was the lowest (40.9%) while it was the highest in T-9 (45.5%). There was not any significant difference in the crude protein level among different samples (1.16%-1.37%). Crude fiber content ranged from 1.91 (T-9) to 3.11% (T-5), while the crude ash was found highest (1.42%) in T-6 and the lowest (0.8%) in T-4. Evaluation of proximate and nutrient compositions of food products plays a crucial role in assessing their nutritional significance (Kochhar et al., 2006) and devising safer ways to preserve the essential ingredients of the produce (Pandey et al., 2006). World Health Organization has also emphasized on the need and importance of determining proximate and micronutrients analysis of various

Table 1. Proximate composition of dried persimmon prepared with application of different chemicals and/or persimmon peel extracts.

Sample[1)]	Composition (% wet basis)			
	Moisture	Crude protein	Crude fiber	Crude ash
T-1	44.6±0.32[b2)]	1.28±0.12[a]	2.30±0.24[c]	1.08±0.03[d]
T-2	41.7±0.12[e]	1.37±0.21[a]	2.72±0.16[b]	1.12±0.02[d]
T-3	43.8±0.13[c]	1.28±0.17[a]	2.41±0.21[bc]	1.00±0.03[e]
T-4	41.9±0.22[d]	1.29±0.05[a]	2.33±0.19[c]	0.80±0.04[g]
T-5	42.2±0.31[d]	1.20±0.06[a]	3.11±0.20[a]	1.33±0.02[b]
T-6	40.9±0.17[f]	1.22±0.11[a]	2.81±0.18[ab]	1.42±0.02[a]
T-7	42.2±0.19[d]	1.23±0.10[a]	2.16±0.23[cd]	0.91±0.02[f]
T-8	44.3±0.34[b]	1.16±0.09[a]	2.09±0.31[cd]	1.00±0.03[e]
T-9	45.5±0.21[a]	1.18±0.11[a]	1.91±0.21[d]	1.11±0.04[d]
T-10	42.7±0.35[d]	1.24±0.11[a]	2.37±0.11[c]	1.17±0.02[c]

[1)]T-1, dried persimmon soaked in 70% prethanol A solution for 2 min at 20±1°C; T-2, dried persimmon sprayed with 70% prethanol Asolution; T-3, dried persimmon soaked in Medilox (60 ppm Hypochlorous acid) for 2 min at 20±1°C; T-4, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid); T-5, dried persimmon sprayed with 10% persimmon-peel extract; T-6, control sample (dried persimmon containing no added extract); T-7, dried persimmon burned with sulfur powder for 10 min at 20±1°C; T-8, dried persimmon sprayed with 70% prethanol A followed by spraying with 10% persimmon-peel extract; T-9, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid) followed by spraying with 10% persimmon-peel extract; and T-10, dried persimmon sprayed with wine (Alcohol 15%, Cabernet Sauvignon, Australia). [2)]Quoted values are the averages of triplicate experiments. [a-f)]The mean values followed by different superscripts in the same column are significantly different ($p<0.05$).

processed food products. In present study, the proximate parameters were not greatly affected by the various treatments as compared to untreated sample.

Color measurement

The color of the processed persimmon has been known to play an important role in the consumer's acceptability. Table 2 reveals the Hunter's color value of the dried persimmon prepared under different treatment. The control sample (T-6) possessed the highest (43.52) value for lightness while T-2 scored the lowest (30.11). Regarding the redness value, T-6 sample with the highest lightness value showed the lowest (3.28) redness value but it was not true in case of the corresponding lowest lightness value as T-8 which scored the highest (5.68) value for redness.

The yellowness value was highest (4.69) in T-9 and the control sample (T-6) scored the lowest (2.26) yellowness value. T-5 sample which was sprayed with persimmon-peel extract had higher redness and yellowness value than that of T-7 (sample treated with sulfur power). These factors act as a determinant for quality product and are suggested to have a direct impact on the consumer acceptability. Since fruit color changes to yellow or red according to kind and amount of carotenoid as persimmon matures, its dried persimmon surface is more red and better than unripe fruit (Kim et al., 1986).

Water activity and soluble solids content

As shown in Table 3, the soluble solids content (SSC) of samples ranged from 51.2 (T-2 and T-3) to 63.2 (T-5) °Brix. SSC differences among samples were observed significant. The results showed that SSC was higher in persimmon-peel extract sprayed dried than in control sample which might be because of spraying of persimmon-peel extracts.

The water activity (a_w) levels among samples were varied, which ranged 0.85 to 0.94. Water activity is a measure of how efficiently the water present could take part in a chemical reaction. Water activity is also a critical factor that determines shelf life for foods as most of the bacteria do not grow at water activities below 0.91 (Paster, 1968). Thus samples of T-2 (a_w= 0.85), T-3 (a_w=0.90), T-4 (a_w=0.86), T-5 (a_w=0.91), T-6 (a_w=0.87), T-8 (a_w=0.90), T-9 (a_w=0.91) and T-10 (a_w=0.91) were shown to have resistance against microbial attack.

Textural properties of dried persimmon

Textural properties of dried persimmon are presented in Table 4. Hardness value of T-7 sample (4.21 kg/cm^2) was the highest among different samples. T-5 sample revealed moderate value (1.35 kg/cm^2) for hardness whereas T-3 and T-8 samples had 0.88 kg/cm^2. Reports show that high a_w (Kim and Jung, 2011; Chun et al., 2012) and high

Table 2. Hunter's color values of dried persimmons prepared with application of different chemicals and/or persimmon peel extracts.

Sample[1)]	Color value[3)]		
	L (Lightness)	a (Redness)	b (Yellowness)
T-1	32.83±2.10$^{bcd2)}$	5.54±0.06^{a}	3.45±0.02^{c}
T-2	30.11±1.09^{d}	5.19±0.09^{a}	3.42±0.05^{c}
T-3	32.24±0.91^{c}	4.90±0.10^{b}	3.46±0.04^{c}
T-4	33.04±0.81^{c}	4.61±0.19^{bc}	3.55±0.09^{c}
T-5	36.24±2.01^{b}	4.88±0.21^{b}	4.01±0.05^{b}
T-6	43.52±1.51^{a}	3.28±0.31^{d}	2.26±0.06^{d}
T-7	34.56±1.11^{bc}	4.52±0.09^{c}	3.69±0.10^{c}
T-8	32.60±1.92^{bcd}	5.68±0.51^{a}	3.33±0.21^{c}
T-9	33.29±1.81^{bc}	5.19±0.67^{ab}	4.69±0.31^{a}
T-10	31.47±1.00^{cd}	5.52±0.58^{ab}	2.30±0.27^{d}

[1)]Abbreviations are specified in Table 1. [2)]Quoted values are the averages of triplicate experiments; [3)]L, lightness (100, white; 0, black); a, redness (-, green; +, red); b, yellowness (-, blue; +, yellow). [a-f)]The mean values followed by different superscripts in the same column are significantly different ($p<0.05$).

Table 3. Water activity (a_w) and soluble solids content (SSC) of dried persimmon prepared with application of different chemicals and/or persimmon peel extracts.

Sample[1)]	Parameter[3)]	
	A_w	SSC (°Brix)
T-1	0.94 ± 0.02$^{a2)}$	57.8 ± 0.2^{c}
T-2	0.85 ± 0.03^{c}	51.2 ± 0.1^{f}
T-3	0.90 ± 0.01^{b}	51.2 ± 0.2^{f}
T-4	0.86 ± 0.03^{bc}	52.3 ± 0.3^{e}
T-5	0.91 ± 0.01^{ab}	63.2 ± 0.2^{a}
T-6	0.87 ± 0.02^{bc}	55.6 ± 0.3^{d}
T-7	0.92 ± 0.01^{ab}	52.0 ± 0.4^{e}
T-8	0.90 ± 0.03^{ab}	52.3 ± 0.3^{e}
T-9	0.91 ± 0.03^{ab}	60.2 ± 0.2^{b}
T-10	0.91 ± 0.04^{ab}	63.0 ± 0.1^{a}

[1)] T-1, dried persimmon soaked in 70% prethanol A solution for 2 min at 20±1°C; T-2, dried persimmon sprayed with 70% prethanol A solution; T-3, dried persimmon soaked in Medilox (60 ppm Hypochlorous acid) for 2 min at 20±1°C; T-4, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid); T-5, dried persimmon sprayed with 10% persimmon-peel extract; T-6, control sample (dried persimmon containing no added extract); T-7, dried persimmon burned with sulfur powder for 10 min at 20±1°C; T-8, dried persimmon sprayed with 70% prethanol A followed by spraying with 10% persimmon-peel extract; T-9, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid) followed by spraying with 10% persimmon-peel extract; and T-10, dried persimmon sprayed with wine (Alcohol 15%, Cabernet Sauvignon, Australia). [2)]Quoted values are the averages of triplicate experiments. [3)]A_w, water activity; SSC, soluble solids content. [a-f)]The mean values followed by different superscripts in the same column are significantly different ($p<0.05$).

soluble solids content (°Brix) decrease hardness (Özdemir et al., 2009). Results of this study also showed lower hardness value in T-5 than T-6, which might be due to higher a_w and SSC in the former sample (Table 3).

Effect of treatments on free sugar content of dried persimmon

The results of sugar content, as glucose and fructose, of dried persimmon after treatments are shown in Table 5. The glucose levels ranged between 15.50 to 28.90%. The results show that the application of persimmon peel extracts increased the glucose content significantly. Fructose levels were found to have 26.10% in T-5, 19.02% in T-6 and the lowest (14.36%) in T-1. Fructose content of T-5 sample was the highest among the samples.

Total free sugar content of T-5 sample was higher than that of T-6 and showed higher preference by consumers to the persimmon peel extract sprayed dried persimmon over sample without any treatment. Sucrose was not detected in all samples (data not shown). The quantities of sugar in fully mature fruit of persimmon can vary greatly among different cultivars and also it can be affected by the processing methods (Senter et al., 1991).

Mineral contents of dried persimmon

Essential minerals contents of dried persimmons treated with different chemicals and natural extracts are shown in

Table 4. Textural properties of dried persimmon prepared with application of different chemicals and/or persimmon peel extracts.

Sample[1)]	Parameter		
	Hardness (Kg/cm^2)	Cohesiveness	Springiness (%)
T-1	1.06±0.01$^{g2)}$	85.40±0.03^{e}	86.08±0.03^{e}
T-2	1.21±0.02^{e}	93.56±0.03^{a}	93.33±0.02^{b}
T-3	0.88±0.01^{i}	65.79±0.02^{i}	70.26±0.04^{h}
T-4	1.12±0.02^{f}	58.70±0.02^{j}	68.89±0.02^{i}
T-5	1.35±0.02^{d}	92.30±0.02^{b}	95.11±0.02^{a}
T-6	3.36±0.03^{b}	81.41±0.04^{h}	83.45±0.03^{f}
T-7	4.21±0.03^{a}	84.91±0.02^{f}	86.44±0.03^{e}
T-8	0.88±0.01^{i}	86.74±0.03^{d}	88.84±0.03^{c}
T-9	0.99±0.01^{h}	88.38±0.03^{c}	88.08±0.02^{d}
T-10	1.43±0.02^{c}	82.77±0.02^{g}	81.73±0.03^{g}

[1)]T-1, dried persimmon soaked in 70% prethanol A solution for 2 min at 20±1°C; T-2, dried persimmon sprayed with 70% prethanol A solution; T-3, dried persimmon soaked in Medilox (60 ppm Hypochlorous acid) for 2 min at 20±1°C; T-4, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid); T-5, dried persimmon sprayed with 10% persimmon-peel extract; T-6, control sample (dried persimmon containing no added extract); T-7, dried persimmon burned with sulfur powder for 10 min at 20±1°C; T-8, dried persimmon sprayed with 70% prethanol A followed by spraying with 10% persimmon-peel extract; T-9, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid) followed by spraying with 10% persimmon-peel extract; and T-10, dried persimmon sprayed with wine (Alcohol 15%, Cabernet Sauvignon, Australia).[2)]Quoted values are the averages of triplicate experiments. [a-j)]The mean values followed by different superscripts in the same column are significantly different ($p<0.05$).

Table 5. Free sugar content of dried persimmon treated prepared with application of different chemicals and/or persimmon peel extracts.

Sample[1)]	Free sugars (% weight basis)	
	Glucose	Fructose
T-1	17.73±1.31$^{cd2)}$	14.36±1.61^{d}
T-2	22.20±2.00^{bc}	25.53±2.38^{ab}
T-3	15.50±1.21^{d}	15.46±1.31^{d}
T-4	26.95±2.00^{ab}	18.66±0.11^{c}
T-5	28.90±1.09^{a}	26.10±1.51^{a}
T-6	20.12±1.51^{c}	19.02±1.00^{bc}
T-7	24.83±0.99^{b}	21.19±0.92^{bc}
T-8	24.01±1.02^{b}	20.12±1.81^{bc}
T-9	20.58±2.12^{c}	19.02±1.71^{bc}
T-10	24.01±2.01^{bc}	22.29±1.92^{b}

[1)] T-1, dried persimmon soaked in 70% prethanol A solution for 2 min at 20±1°C; T-2, dried persimmon sprayed with 70% prethanol A solution; T-3, dried persimmon soaked in Medilox (60 ppm Hypochlorous acid) for 2 min at 20±1°C; T-4, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid); T-5, dried persimmon sprayed with 10% persimmon-peel extract; T-6, control sample (dried persimmon containing no added extract); T-7, dried persimmon burned with sulfur powder for 10 min at 20±1°C; T-8, dried persimmon sprayed with 70% prethanol A followed by spraying with 10% persimmon-peel extract; T-9, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid) followed by spraying with 10% persimmon-peel extract; and T-10, dried persimmon sprayed with wine (Alcohol 15%, Cabernet Sauvignon, Australia).[2)]Quoted values are the averages of triplicate experiments.[a-d)]The mean values followed by different superscripts in the same column are significantly different ($p<0.05$).

Table 6. Mineral contents of dried persimmon prepared with application of different chemicals and/or persimmon peel extracts.

Sample[1)]	Element (mg/kg)						
	K	Mg	Ca	Na	Fe	Zn	Mn
T-1	4479±6[e2)]	495±5[d]	522±5[b]	77±2[a]	61±2[a]	ND	ND
T-2	4487±7[e]	402±4[h]	440±2[e]	52±1[e]	45±1[d]	ND	ND
T-3	4651±6[d]	430±3[f]	367±3[g]	54±1[e]	51±1[c]	ND	ND
T-4	4378±9[g]	473±4[f]	453±4[d]	69±1[c]	53±2[bc]	ND	ND
T-5	4100±8[h]	392±4[g]	418±5[f]	51±2[e]	48±1[d]	ND	ND
T-6	6312±7[a]	709±3[a]	423±7[f]	61±1[d]	46±1[d]	ND	ND
T-7	4414±6[f]	519±2[b]	525±6[b]	71±2[b]	54±1[bc]	ND	ND
T-8	4406±9[f]	476±5[f]	539±3[a]	68±2[c]	55±1[b]	ND	ND
T-9	4832±8[c]	512±2[c]	538±4[a]	78±2[a]	56±1[b]	ND	ND
T-10	4923±8[b]	483±3[e]	471±5[c]	67±1[c]	53±1[bc]	ND	ND

[1)]Abbreviations are specified in Table 1. [2)]Quoted values are the average of duplicate experiments. [3)]ND, not detected. [a-h)]Means values followed by different superscripts with a column indicate significant differences ($p<0.05$).

Table 6. Heavy metals such as arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg) were not detected in any sample (data not shown). Some essential minerals like zinc (Zn) and manganese (Mn) were also not detected; however, potassium (K), magnesium (Mg), calcium (Ca), sodium (Na) and iron (Fe) were found in varying amounts. The composition of K (6312 mg/kg) and Mg (709 mg/kg) were significantly high in T-6 as compared to other samples. Mineral levels of all samples were shown in the order of K (4100 to 6312 mg/kg) > Mg (392 to 709 mg/kg) > Ca (367 to 539 mg/kg) > Na (51 to 78 mg/kg) > Fe (45 to 61 mg/kg). These results indicate the mineral levels in dried persimmons increase or decrease depending on the treatment. Results reveal that high K and Mg could be obtained in untreated sample, however, levels of Ca, Na, and Fe could be enhanced with application of different chemicals and/or persimmon peel extracts.

Sensory characteristics of dried persimmon

Sensory characteristics of the dried persimmons were determined by color, sweetness, astringency, texture, and overall taste (Table 7). Sensory evaluations showed that T-5, T-8 and T-9 were the highly preferable samples to the consumers. Color score of T-5 sample was the highest among others. The highest score for sweetness was found to T-10 sample and overall taste to T-5. Overall taste of T-5 sample might be enhanced with the application of 10% concentrated persimmon-peel extracts. Sodium metabisulfite treatment gives the best physical, chemical, and microbiological results over the untreated dried persimmon, the latter gives the highest sensory scores (Bölek and Obuz, 2014). However, results of this study shows that use of 10% concentrated persimmon-peel extracts imparted the best overall taste than that of untreated dried persimmon, but beyond 15% extracts, overall taste was negatively affected (data not shown). Results of higher sensory properties signified that application of persimmon-peel extracts on the preparation of dried persimmon could be more effective to enhance the overall acceptance of dried persimmon.

Conclusions

The results of the present study portray beneficial effects of various natural extract treatments and conditions on the dried persimmons. The findings revealed that dried persimmon when sprayed with 10% persimmon-peel extract for 2 min at 20±1°C could exhibit higher acceptance values as compared to other treatments. The impacts of different treatments on the free sugar, color, texture and mineral values were interesting as compared to control. However, further analysis of phytochemicals and other parameters could be useful to understand various regulations happening during the fruit development and processing.

It is evident from these results that application of persimmon-peel extracts in the preparation of dried persimmon could be effective as it avoids the use of health hazardous chemical preservatives and also to some extent restores the nutritive and medicinal properties lost with the peeled out skin before drying.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported in part by Grants from R&D project to overcome Free Trade Agreement (FTA) program

Table 7. Sensory characteristics of dried persimmon prepared with application of different chemicals and/or persimmon peel extracts.

Sample[1)]	Characteristic				
	Color	Sweetness	Astringency	Texture	Overall acceptance
T-1	3.1±0.2[b2)]	3.6±0.1[a]	2.1±0.3[a]	3.5±0.1[a]	3.1±0.1[c]
T-2	3.1±0.1[b]	3.6±0.2[a]	2.1±0.2[a]	3.6±0.3[ab]	3.2±0.2[bc]
T-3	3.2±0.2[b]	3.7±0.1[a]	2.1±0.2[a]	3.6±0.2[a]	3.2±0.1[bc]
T-4	3.2±0.2[b]	3.6±0.2[a]	2.2±0.2[a]	3.5±0.1[a]	3.5±0.2[b]
T-5	4.1±0.1[a]	3.9±0.3[a]	1.3±0.3[b]	3.8±0.2[a]	4.2±0.2[a]
T-6	2.5±0.3[c]	3.2±0.1[b]	2.2±0.1[a]	3.3±0.3[ab]	2.9±0.3[c]
T-7	2.9±0.1[bc]	3.1±0.1[b]	2.2±0.3[a]	3.2±0.1[b]	3.0±0.1[c]
T-8	3.2±0.2[b]	3.9±0.3[a]	1.7±0.4[ab]	3.5±0.2[ab]	4.1±0.1[a]
T-9	3.3±0.3[b]	3.9±0.2[a]	1.8±0.2[ab]	3.6±0.1[a]	4.1±0.2[a]
T-10	3.1±0.2[b]	4.3±0.2[a]	1.2±0.3[c]	3.7±0.2[a]	3.9±0.3[ab]

[1)] T-1, dried persimmon soaked in 70% prethanol A solution for 2 min at 20±1°C; T-2, dried persimmon sprayed with 70% prethanol A solution; T-3, dried persimmon soaked in Medilox (60 ppm Hypochlorous acid) for 2 min at 20±1°C; T-4, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid); T-5, dried persimmon sprayed with 10% persimmon-peel extract; T-6, control sample (dried persimmon containing no added extract); T-7, dried persimmon burned with sulfur powder for 10 min at 20±1°C; T-8, dried persimmon sprayed with 70% prethanol A followed by spraying with 10% persimmon-peel extract; T-9, dried persimmon sprayed with Medilox (60 ppm Hypochlorous acid) followed by spraying with 10% persimmon-peel extract; and T-10, dried persimmon sprayed with wine (Alcohol 15%, Cabernet Sauvignon, Australia). [2)]Quoted values are the mean of triplicate determinations, mean of n = 10 based on 5-point scores (very poor, 1; poor, 2; fair, 3; good, 4; very good, 5). [a-d)]Mean values followed by different superscripts within a column indicate significant differences ($p<0.05$).

2011 in the agriculture and fishery fields of Gyeongsangbuk-Do province, South Korea.

REFERENCES

AOAC (1995). Official Methods of Analysis of AOAC International. 16th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Bölek S, Obuz E (2014). Quality characteristics of Trabzon persimmon dried at several temperatures and pretreated by different methods. Turk. J. Agric. For. doi: 10.3906/tar-1303-41.

Bond B, Fernandez DR, Vanderjagt DJ, Williams M, Huang YS, Chuang LT, Millson M, Andrews R, Glew RH (2005). Fatty acid, amino acid and trace mineral analysis of three complementary foods from Jos, Nigeria. J. Food Compos. Anal. 18:675-690.

Bourne MC (1978). Texture profile analysis. Food Technol. 32(7):62-72.

Celik A, Ercisli S (2007). Persimmon cv. Hachiya (*Diospyros kaki* Thunb.) fruit: Some physical, chemical and nutritional properties. Int. J. Food Sci. Nutr. 18:1-8.

Chun HH, Kim MS, Chung KS, Won MS, Song KB (2012). Dehydration of blueberries using maltodextrin and the physicochemical properties of dried blueberries. Hort. Environ. Biotechnol. 53(6):565-570.

Del Bubba M, Giordani E, Pippucci L, Cincinelli A, Checchini L, Galvan P (2009). Changes in tannins, ascorbic acid and sugar contents in astringent persimmons during on-tree growth and ripening and in response to different postharvest treatments. J. Food Compos. Anal. 22:668-677.

Ebert G, Gross J (1985). Carotenoid changes in the peel of ripening persimmon (*Diospyros kaki*) cv. Triumph. Phytochemist. 24:29-32.

Fukai S, Tanimoto S, Maeda A, Fukuda H, Okada H, Nomura M (2009). Pharmacological activity of compounds extracted from persimmon peel (*Diospyros kaki* THUNB.). J. Oleo Sci. 58(4):213-219.

Genard M, Souty M (1996). Modeling the peach sugar contents in relation to fruit growth. J. Am. Soc. Horti. Sci. 121(6):1122-1131.

Gorinstein S, Kulasek G, Bartnikowska E, Leontowicz M, Zemser M, Morawiec M, Trakhtenberg S (1998). The influence of persimmon peel and persimmon pulp on the lipid metabolism and antioxidant activity of rats fed cholesterol. J. Nutr. Biochem. 9:223-227.

Gorinstein S (1999). Comparative content of total polyphenols and dietary fiber in tropical fruits and persimmon. J. Nutr. Biochem. 10:367-371.

Gorinstein S, Zensler M, Wietz M, Halvey S, Bartnikowska E (1994). Fluorometric analysis of phenolics in persimmon. Biosci. Biotechnol. Biochem. 58:1078-1092.

Islam GMR, Hoque MM (2013). Food safety regulation in Bangladesh, chemical hazard and some perception to overcome the dilemma. Int. Food Res. J. 20(1):47-58.

Kawase M, Motohashi N, Satoh K, Sakagami H, Nakashima H, Tani S, Shirataki Y, Kurihara T, Spengler G, Wolfard K, Molnár J (2003). Biological activity of persimmon (*Diospyros kaki*) peel extracts. Phytother Res. 17(5):495-500.

Kim JH, Kim JC, Ko GC (1986). Orchard Horticulture Outline, Hyangmoonsa, Korea. pp 220-229.

Kim SJ, Jung KM (2011). Effects of the PPO (Polyphenol oxidase) Activity and Total Phenolic Contents on Browning and Quality of Dried-Persimmon According to Maturity Degree of Astringent Persimmon (*Diospyros kaki* Thunb.) 2nd International Conference on Biotechnology and Food Science. IACSIT Press, Singapore.

Kochhar A, Nagi M, Sachdeva R (2006). Proximate composition, available carbohydrates, dietary fibre and anti nutritional factors of selected traditional medicinal plants. J. Hum. Ecol. 19(3):195-199.

Lee YA, Cho EJ, Yokozawa T (2008). Protective effect of persimmon (*Diospyros kaki*) peels proanthocyanidin against oxidative damage under H_2O_2-induced cellular senescence. Biol. Pharm. Bull. 31(6):1265-1269.

Miller EP (1984). Oriental persimmons (*Diospyros kaki*) in Florida. Proc. Fla. Sta. Hort. Soc. 97:310-344.

Özdemir AE, Çandır EE, Toplu C, Kaplankıran M, Yıldız E, Inan C (2009). The effects of hot water treatments on chilling injury and cold storage of 'Fuyu' persimmons. Afr. J. Agric. Res. 4:1058-1063.

Pandey M, Abidi AB, Singh S, Singh RP (2006). Nutritional evaluation of leafy vegetable paratha. J. Hum. Ecol. 19(2):155-156.

Paster T (1968). The HACCP Food Safety Training Manual. John Wiley and Sons, Inc. Hoboken, New Jersey, USA.
Senter SD, Lyon BG, Horvat RJ (1991). Chemical, sensory and aroma volatile profiles of Japanese persimmons grown in the Southwestern United States. 82nd Annual Meeting. pp. 111-120.
SMS (1992). Stable micro systems XT.RA dimension user's guide (Version 3.6). Stable Micro Systems Ltd., Surrey, England.
Son JE, Hwang MK, Lee E, Seo SG, Kim JE, Jung SK, Kim JR, Ahn GH, Lee KW, Lee HJ (2013). Persimmon peel extract attenuates PDGF-BB-induced human aortic smooth muscle cell migration and invasion through inhibition of c-Src activity. Food Chem. 141(4):3309-3316.
Yokozawa T, Kim YA, Kim YH, Lee YA, Nonaka G (2007). Protective effect of persimmon peel polyphenol against high glucose-induced oxidative stress in LLC-PK(1) cells. Food Chem. Toxicol. 45:1979-1987.

academicJournals

Vol. 13(22), pp. 2198-2202, 28 May, 2014
DOI: 10.5897/AJB2014.13703
Article Number: 723EBBA44895
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular markers linked to apomixis in *Panicum maximum* Jacq.

Anna Carolina Bluma-Marques[1], Lucimara Chiari[2]*, Débora Cristina Agnes[3], Liana Jank[2] and Maria Suely Pagliarini[1]

[1]Department of Cell Biology and Genetics, Maringá State University, 87020-900, Brazil.
[2]Embrapa Beef Cattle, Brazilian Agricultural Research Corporation, Brazil.
[3]Anhanguera-UNIDERP University, Campo Grande Brazil.

Received 5 February, 2014; Accepted 8 May, 2014

***Panicum maximum* Jacq. is an important forage grass of African origin largely used in the tropics. The genetic breeding of this species is based on the hybridization of sexual and apomictic genotypes and selection of apomictic F_1 hybrids. The objective of this work was to identify molecular markers linked to apomixis in *P. maximum* to determine easily and at an early stage, the reproductive mode of F_1 hybrids, so to assist the breeding program. A bulked segregant analysis was performed using 184 random amplified polymorphic DNA (RAPD) primers in an F_1 population of *P. maximum* segregating for reproductive mode. Four RAPD markers linked to apomixis were identified and mapped in this population. These markers showed good selection efficiency, ranging from 77.3 to 88%, with 81.3% when analyzed together. Two of these markers were easily transferred to another F_1 population of *P. maximum*. In this population, the selection efficiency was also high for both markers; 84.8 and 90%, when analyzed together. These markers may be used in the assisted selection for reproductive mode in both F_1 progenies of *P. maximum* studied here and in other populations to which they can be transferred.**

Key words: Guinea grass, apospory, reproduction mode, random amplified polymorphic DNA (RAPD), selection efficiency.

INTRODUCTION

Pastures play an important role in the national economy of most countries in the tropics since livestock production depends largely on both quantity and quality of forage crops established for ruminants. Only in Brazil, approximately 171 million hectares are planted to pastures (ABIEC, 2013).The main important cultivated pastures are grasses of African origin, which in general, show great adaptation to the Brazilian climate and soils. Among these grasses, *Panicum maximum* Jacq. stands out due to its good forage quality,high yield, wide

*Corresponding author. E-mail: lucimara.chiari@embrapa.br. Tel: +55 67 3368-2079. Fax: +55 67 3368-2150.

adaptability and ease of establishment (Jank et al., 2008).

Panicum maximum, has both facultative apomixis and obligate sexual modes of reproduction (Savidan, 1980, 1982). Apomixis in *P. maximum* has been characterized as the result of apospory, embryo sac development from a somatic cell, followed by parthenogenesis and development of an embryo from the unfertilized egg cell (Savidan et al., 1989). Sexuality in this species was first discovered by Combes and Pernes (1970) in diploid accessions from East Africa.

For genetic breeding of apomictic species, it is necessary to identify sexual plants to use as maternal genitors in the crosses with apomictic plants, donors of pollen. In general, sexual genotypes in nature are diploid being necessary to undergo chromosome duplication before or after the crosses (Dall'Agnol and Schifino-Wittmann, 2005).

Crosses between sexual tetraploid genotypes of *P. maximum,* obtained from colchicine treatment, and apomictic genotypes have been done since 1971 in the Ivory Coast (Savidan, 1980). The hybrid progenies obtained from these crosses include sexual and apomictic hybrids in the Mendelian proportion of 1:1, demonstrating that apomixis in *P. maximum* is dominant over sexuality and determined by a single gene, the sexual tetraploids being defined as aaaa and the apomictic genotypes as Aaaa (Savidan, 1981).

A large and representative germplasm collection of *P. maximum* was introduced in 1982 through a cooperation-agreement between the Brazilian Agricultural Research Corporation (EMBRAPA) and the French Institute de Recherche pour le Développement (IRD - former ORSTOM - Office de la Recherche Scientifique et Technique d'Outre-Mer) (Jank et al., 2011). Embrapa Beef Cattle Center received from ORSTOM 426 apomictic accessions and 417 sexual genotypes (Jank et al., 2008).

The main thrust of the initial cultivar development efforts in Brazil were focused on the selection of useful commercial genotypes directly from the collection of apomictic accessions and resulted in the release of the cultivars Tanzânia, Mombaça and Massai (Jank et al., 2008).

A hybridization program was also initiated aiming to combine agronomic characteristics of interest and thus increase pasture productivity. After the hybridization, in order to continue the breeding program, the mode of reproduction of these progenies must be identified. Currently, the method used is the characterization of the anatomy of clarified ovaries analyzed under differential interference contrast microscopy. This is a difficult and laborious method, because it involves the harvest of inflorescences in anthesis in the adult plant, and the determination of the anatomical structures can only be made after specific training (Jank, 1995). Therefore, the development of alternative methods to access easier and more precociously the mode of reproduction in *P. maximum* have become extremely important.

A method that stands out for its speed and accuracy is the use of molecular markers that co-segregate with apomixis and can be used precociously in the earlier stages of plant development, with DNA samples extracted from seeds or seedlings (Bhat et al., 2005). This technique showed to be efficient in the apomictic species *Brachiaria* sp. (Pessino et al., 1997), *Pennisetum squamulatum* (Ozias-Akins et al., 1998), *Poa pratensis* (Barcaccia et al., 1998), *Paspalum simplex* (Labombarda et al., 2002; Gualtieri et al., 2006), *P. maximum* (Ebina et al., 2005), *Cenchrus ciliaris* (Gualtieri et al., 2006; Yadav et al., 2012), *Hypericum perforatum* (Barcaccia et al., 2007; Schallau et al., 2010), and *Brachiaria humidicola* (Zorzatto et al., 2010), among others.

The main objective of the present work was to identify molecular markers linked to apomixis in *P. maximum* to determine, easily and in early stages, the reproductive mode of F_1 hybrids, so to assist the breeding program.

MATERIALS AND METHODS

The experiment was conducted in the Laboratory of Plant Biotechnology of Embrapa Beef Cattle, located in Campo Grande, Mato Grosso do Sul State, Brazil.

Plant material

Two F_1 populations, called A and DE, were previously characterized for mode of reproduction by the technique of anatomy of clarified ovaries observed under differential interference contrast microscopy. Population A consisted of 40 apomictic hybrids and 35 sexual hybrids obtained from crossing *P. maximum* S10 (tetraploid sexual genotype) and cv. Tanzânia (tetraploid apomictic accession ORSTOM T58). Population DE consisted of 12 apomictic and 21 sexual hybrids obtained from crossing S12 (tetraploid sexual genotype) and cv. Tanzânia. A Chi-square (χ^2) test was performed on these progenies to verify whether the genetic segregation between apomictic x sexual hybrids fit the expected Mendelian model. Thirty-five accessions from the *P. maximum* germplasm collection from Embrapa were also analyzed (13 sexual genotypes and 22 apomictic accessions), to verify the presence or absence of the linked markers.

Molecular analysis

The DNA was extracted from young leaves by the Bonato et al. (2002) method. DNA purity and concentrations were estimated using a NanoDrop1000 (Thermo) spectrophotometer and on 0.8% agarose gel stained with ethidium bromide (5 $\mu g.mL^{-1}$).

A bulked segregant analysis (Michelmore et al., 1991) was carried out to identify molecular markers linked to apomixis using only population A. Two bulks were prepared, an apomictic bulk (AB) and a sexual bulk (SB), each one containing equimolar quantities of DNA from 10 hybrids characterized by differential interference contrast microscopy. A total of 184 RAPD primers were used for amplification of the bulks (AB and SB) and parents (S10 and

Table 1. Chi-square test (χ^2) for population A segregation and the markers segregation (P<0.05).

Parameter	Observed proportion		Chi- square test (χ^2)	
	Mode of reproduction			
	Apomitic	Sexual	Segregation	χ^2
Anatomical evaluation	40	35	1:1	0.33
Marker				
PM_A01	43	32	1:1	1.61
PM_01	31	41	1:1	2.25
PM_16	36	39	1:1	0.12
PM_U07	27	40	1:1	3.10

Tanzânia). Then, the primers potentially linked to apomixis were applied to the entire population A.

The polymerase chain reactions (PCR) were performed with a final volume of 25 µL, using, 1xTaq DNA polymerase buffer (Invitrogen); 1.5 mM $MgCl_2$ (Invitrogen); 0,2 mM dNTPs (Invitrogen); 0.4 µM primer (Operon Thecnologies); 1.0 U de Taq DNA polimerase (Invitrogen), 30 ng de DNA and H_2O to complete the volume. The thermal conditions consisted of 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 35°C, 2 min at 72°C followed by 7 min at 72°C. The amplification products were separated on a 2% agarose gel, stained with ethidium bromide and photo documented using L.Pix Image systems (Loccus Biotechnology).

Data analysis

The amplification of the potential markers was analyzed as binary, with 1 for presence and 0 for absence of the marker. The binary data was used to confirm the segregation 1:1 of the markers by the chi-square test (χ^2) with 5% probability ($p \geq 0.05$). The linkage analysis was conducted with the GQMOL 1.0.0 computer software (Cruz and Schuster, 2007) using the Kosambi function and a map was constructed. The LOD score used was 3 with a maximum distance of 30 cm. The selection efficiency (SE) of the markers linked to the apomixis locus (Apo locus) was calculated based on the comparison between the phenotypic and the genotypic (markers) according to Silva et al. (2007).

The molecular markers linked to apomixis in population A were also tested on population DE and in some apomictic accessions and sexual genotypes from the germplasm collection, to analyze the transferability of these markers.

RESULTS

The χ^2 test of the apomictic and sexual hybrids from both A (Table 1) and DE (Table 2) populations were not significantly different from the 1:1 ratio, according to the expected Mendelian model. Of the 184 RAPD primers tested on the segregant bulks from population A, 16 did not amplify (8.7%). Of the remaining 168 primers, only 14 (8.3%) amplified polymorphic markers between the bulks. Four of these primers (OP-01, OP-16, OP-A01, and OP-U07) amplified markers that resulted in a good fit considering 1:1 segregation ratio (Table 1).

The linkage analysis revealed that these markers co-segregated with the Apo locus in this population, and that the PM_A01 marker amplified with primer OP-A01, showed the smallest distance, and the marker PM_U07 amplified with primer OP-U07, showed the largest distance (Figure 1).

The selection efficiencies (SE) for PM_A01, PM_01, PM_16 and PM_U07 were 88, 80, 81.3 and 77.3%, respectively, and the SE of the four markers together was 81.3%. After the confirmation of linkage between the four markers and the Apo locus, the amplifications with these four primers were done on population DE. In this case, three markers presented a good fit in the 1:1 segregation ratio and one did not, PM_16 marker (Table 2). However, only two markers co-segregated with the Apo locus by the linkage analysis (PM_A01 and PM_01). For PM_U07 marker the LOD score was lower than 3.0. The two markers were mapped 7.66 and 18.49 cM, respectively (Figure 1).

The SE was also calculated for these two markers in population DE. The PM_A01 marker presented the highest value (90%) and the PM_01 marker presented 84.8%. Together, the markers showed 90% selection efficiency.

As for the accessions from the germplasm collection, four sexual plants amplified none of the markers, being potential genotypes for use in future crosses, where the hybrids will inherit the markers only from the apomictic parent. Eighteen (18) of the 22 apomictic accessions amplified at least one of the markers, which shows that a high percentage of apomictic accessions from the germplasm collection presents the markers.

DISCUSSION

Earlier studies in *P. maximum* proposed a simplex genotype for tetraploid apomicts (Aaaa) and a homozygous recessive genotype for sexual plants, whether diploid (aa) or tetraploid (aaaa) (Savidan, 1981). The

Table 2. Chi-square test (χ^2) for population **DE** segregation and the markers segregation (P<0.05).

Parameter	Observed proportion		Chi- square test (χ^2)	
	Mode of reproduction			
	Apomitic	Sexual	Segregation	χ^2
Anatomical evaluation	12	21	1:1	2.44
Marker				
PM_A01	15	18	1:1	3.66
PM_01	11	22	1:1	1.61
PM_16	10	23	1:1	5.12
PM_U07	15	18	1:1	0.27

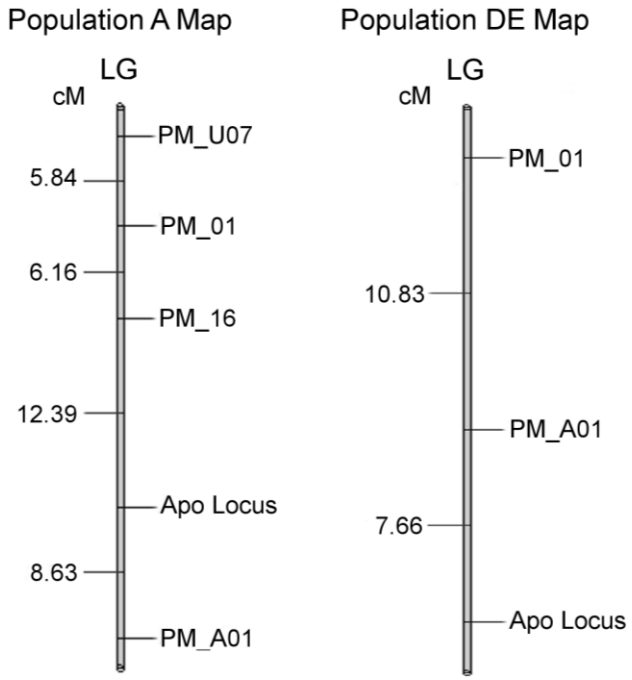


Figure 1. Linkage maps of the two populations of *P. maximum.* On the left is the linkage map of population A and on the right is the linkage map of population DE. On each linkage map the marker name is shown on the right and the estimated map distance is shown on the left.

results presented here for both reproductive modes and molecular markers corroborated this hypothesis, allowing the use of the bulked segregant analysis strategy and RAPD technique to prospect markers linked to apomixis in this species. Four RAPD markers were identified and mapped in population A and two of these were transferable to population DE, without the need to develop Sequenced Characterized Amplified Region Marker (SCARs).

The fact that the other two markers have not been transferred may be due to the small size of population DE. According to Sanglard and Melo (2011), the segregation distortion is most likely an effect caused by the low number of genotypes in the population. One of the four markers which was not transferred had a segregation distortion. Of the others three markers that co-segregated with the apomixis trait in population DE, only two showed LOD score equal or higher than 3.0, which shows that there is a possibility for a thousand times greater of the markers to be linked with the locus of

interest than an independent segregation. Once more, the small size of the DE population may have interfered in the results by not containing enough samples of meiotic events (Cruz and Schuster, 2007).

The identification of the region containing the apomictic locus in *P. maximum* was also attempted previously by Ebina et al. (2005). In their study, they used AFLP and RAPD markers to generate a linkage map and to identify molecular markers tightly linked to apomixis. Only AFLP markers linked to the apomixes trait, none of the RAPD markers linked, however, different RAPD primers were used than in our study.

The SE of the markers (individually or together) in both populations of *P. maximum* studied in this work may be considered high, since they can be assessed in the early stages of plant development, using small amounts of any tissue, and in a large number of individuals. This result demonstrated the potential of the use of these markers for assisted selection of reproductive mode in *P. maximum*.

The availability of a molecular marker linked to the Apo locus in *P. maximum* has immediate implications in the breeding of this commercially very important grass for it provides an efficient tool for rapid, early and reliable screening of the progenies for mode of reproduction without having to wait until flowering and to execute the time-consuming extraction, clarification and examination of ovaries.

In conclusion, this study identified molecular markers linked to apomixis in *P. maximum*. These markers can be used for phenotyping the reproductive mode of F1 hybrids. Each apomictic hybrid derived from crosses between sexual x apomictic plants is potentially a unique cultivar or a hopeful candidate regardless of the heterozigous or homozygous genetic background of the parents. The sexual hybrids can be used in new cycles of crossing and selection.

REFERENCES

ABIEC - Associação brasileira das indústrias exportadoras de carnes. (2013). Brasilian beef perfil 2012. Available at http:// www.abiec.com.br/texto.asp?id=8 (accessed 14 November 2013).

Barcaccia G, Baumlein H, Sharbel TF (2007). Apomixis in St. John's wort: an overview and glimpse towards the future. In Apomixis: Evolution, Mechanisms and Perspectives (ed. E. Horandl, U. Grossniklaus, P. Van Dijk and T. F. Sharbel). pp. 259-280. Vienna: Koeltz Scientific Books.

Barcaccia G, Mazzucato A, Albertini E, Zethof J, Pezzotti M, Gerats A, Falcinelli M (1998). Inheritance of parthenogenesis in *Poapratensis* L.: auxin test and AFLP linkage analyses support monogenic control. Theor. Appl. Genet. 97:74-82.

Bhat V, Dwivedi KK, Khurana JP, Sopory SK (2005). Apomixis: An enigma with potential applications. Curr. Sci. 89: 1879-1893.

Bonato ALV, Valle CB, Jank L, Resende RMS, Leguizamon GOC (2002). Extração de DNA genômico de *Brachiaria* e *Panicum maximum*. Comunicado técnico. 79:1-4.

Combes D, Pernes J (1970). Variations dans lês nombres chromosomiques du *Panicum maximum* Jacq. en relation avec Le mode de reproduction. C. R. Acad. Sci. III. 270:782-785.

Cruz CD, Schuster I (2007). GQMOL: aplicativo computacional para análise de dados moleculares e de suas associações com caracteres quantitativos. Versão 1.0.0.Viçosa: UFV. Available at http://www.ufv.br/dbg/gqmol/gqmol.htm (accessed 20 October 2010).

Dall'Agnol M, Schifino-Wittmann MT (2005). Apomixia, genética e melhoramento de plantas. Rev. Bras. Agrosci. 11:127-133.

Ebina M, Nakagawa H, Yamamoto T, Araya H, Tsuruta I, Takahara M, Nakajima K (2005). Co-segregation of AFLP and RAPD markers to apospory in guineagrass (*Panicum maximum* Jacq.).Grassland Sci. 51:71-78.

Gualtieri G, Conner JA, Morishige DT, Moore D, Mullet JE, Ozias-Akins P (2006). A segment of the apospory-specific genomic region is highly microsyntenic not only between the apomicts *Pennisetum squamulatum* and buffelgrass, but also with a rice chromosome 11 centromeric-proximal genomic region. Plant Physiol. 140:963-971.

Jank L (1995). Melhoramento e seleção de variedades de *Panicum maximum*. In *Simpósio Sobre Manejo da Pastagem, 12.*, Piracicaba, SP, Brazil. Proceedings. Piracicaba: FEALQ. p. 21-58.

Jank L, Resende RMS, Valle CB (2011). Breeding tropical forages. Crop Breed. Appl. Biotechnol. S1:27-34.

Jank L, Resende RMS, Valle CB, Resende MDV, Chiari L, Cançado LJ Simioni C (2008). Melhoramento genético de *Panicum maximum*. In *Melhoramento de Forrageiras Tropicais* (ed. R. M. S. Resende, C. B. Do Valle and L. Jank). pp. 55-87. Campo Grande, MS: Embrapa Gado de Corte.

Labombarda P, Busti A, Caceres ME, Quarin CL, Pupilli F, Arcioni S (2002). An AFLP marker tightly linked to apomixis reveals hemizygosity in a portion of the apomixis-controlling locus in *Paspalum simplex*. Genome 45:513-519.

Michelmore RW, Paran I, Kesseli RV (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA. 88: 9828-9832.

Ozias-Akins P, Roche D, Hanna WW (1998). Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes. Proc. Natl. Acad. Sci. USA. 95:5127-5132.

Pessino SC, Ortiz JPA, Leblanc O, Valle CB, Hayward MD (1997). Identification of a maize linkage group related to apomixis in *Brachiaria*. Theor. Appl. Genet. 94: 439-444.

Sanglard DA, Melo GG (2011). Construção de mapas genéticos: uma das aplicações de maior impacto da tecnologia de marcadores moleculares. Available at http://cdsa.ufcg.edu.br/portal/outras_paginas/artigos/Construcao_Mapas_Geneticos_Contexto_Marcadores_Moleculares.pdf (acessed 23 November 2012).

Savidan YH (1980).Chromosomal and embryological analyses in sexual x apomictic hybrids of *Panicum maximum* Jacq. Theor. Appl. Genet. 57:153-156.

Savidan YH (1981). Naturee thérédité de l'apomixie chez *Panicum maximum* Jacq. PhD thesis.Univervity of Paris, France.

Savidan YH (1982). Embryological analysis of facultative apomixis in *Panicum maximum* Jacq. Crop Sci. 22:467-469.

Savidan YH, Jank L, Costa JCG, Valle, CB (1989). Breeding *Panicum maximum* in Brazil: genetic resources, modes of reproduction and breeding procedures. Euphytica. 41:107-112.

Schallau A, Arzenton F, Johnston AJ, Hahnel U, Koszegi D, Blattner FR, Altschmied L, Haberer G, Barcaccia G, Baumlein H (2010). Identification and genetic analysis of the apospory locus in *Hypericum perforatum* L. Plant J. 62:773-784.

Silva MF, Schuster I, Silva JFV, Ferreira A, Barros EG, Moreira MA (2007).Validation of microsatellite markers for assisted selection of soybean resistance to cyst nematode races 3 and 14. Pesq. Agropec. Bras. 42:1143-1150.

Yadav CB, Anuj, Kumar S, Gupta MG, Bhat V (2012).Genetic linkage maps of the chromosomal regions associated with apomictic and sexual modes of reproduction in *Cenchrus ciliaris*. Mol. Breed. 30:239-250.

Zorzatto C, Chiari L, Bitencourt GA, Valle CB, Leguizamón GOC, Schuster I, Pagliarini MS (2010). Identification of molecular marker linked to apomixis in *Brachiaria humidicola* (Poaceae). Plant Breed. 129:734-736

academicJournals

Vol. 13(22), pp. 2208-2214, 28 May, 2014
DOI: 10.5897/AJB2014.13733
Article Number: E70DF0244897
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Heavy metal and proximate composition associated with the composting of cassava (*Manihot esculenta*) peels used in the cultivation of mushrooms in Ghana

Obodai Mary[1]*, Ofori Hayford[1], Dzomeku Matilda[1], Takli Richard[1], Komlega Gregory[1], Dziedzoave Nanam[1], Mensah Deborah[1], Prempeh Juanita[1] and Sonnenberg Anton[2]

[1]Council for Scientific and Industrial Research (CSIR)-Food Research Institute, PO Box M20, Accra, Ghana.
[2]Plant Research International, Wageningen, Netherlands.

Received 17 February, 2014; Accepted 8 May, 2014

Changes in the heavy metal content and proximate composition during the 28 day composting of cassava peels used in the cultivation of the oyster mushrooms *Pleurotus ostreatus* strain EM-1 was studied. Significant dry weight variations of cellulose, hemicellulose and fat contents were observed from day 0 to 12. Decreases from day 12 to 28 had the values of 15.4, 57.6 and 56.12%, respectively, while lignin, protein and crude fibre values showed a gradual increase from day 0 to 28, with maximum values of 23.73, 49 and 73%, respectively. Cyanide content however showed a reduction from the initial 3.89 to 2.01 mg/L by day 12 and a marginal increase of 16 by day 28. This was however not detected in the mushroom harvested. The levels of heavy metal content in composted cassava peels in decreasing order was iron (Fe), manganese (Mn), zinc (Zn), lead (Pb) and copper (Cu) while that for uncomposted cassava was Fe, Zn, Pb, Mn and Cu. Levels of Cu, Mn, Pd and Zn in mushroom samples analysed were in agreement with reported values in literature. Of all the heavy metals examined, iron accumulated excessively, indicating that *P. ostreatus* strain EM-1 is a good bio-accumulator of Fe.

Key words: Cassava, composting, heavy metals, production.

INTRODUCTION

Production of mushrooms has increased over the years in several countries from a few metric tons to thousands of tons. In China, for example, which is the largest producer of various mushrooms in the world, there has been a 224.3% increase in production over a ten year period (2000-2010) (Li, 2012). These increases in production have grown as a result of the fact that mushrooms are no longer only consumed for its flavor and nutritional benefits but also for functional properties they exhibit. These include among others properties such as being anti-mutagenic, anti-tumoral and anti-viral (Garcia-Lafuente et al., 2011). These functional characteristics are mainly due to their chemical composition (Manzi et al., 2001).

In Ghana, this increase in trend has shown no exception and mushroom production has grown at a steady rate from 120 tons in 2010 to approximately 300 metric tons to date. *Pleurotus ostreatus* mushroom is the main

*Corresponding author. E-mail: obodaime@yahoo.com. Tel: +233 (0) 207 930 703.

species that is produced and is presently cultivated on sawdust. There are however other agricultural by-products available that can be used in mushroom production in both urban and peri-urban areas of Ghana and the subtropics. These include among others, cassava peels, plantain leaves and corn cobs. Among these, cassava peels is the most abundant and has the greatest potential as a substrate for mushroom production. At present, cassava by-product such as peels are in large part unexploited and often discarded along roads and on fields, thus forming a nuisance in the environment.

Cassava (*Manihot esculenta* Crantz) is the sixth most important food crop produced annually globally (FAOSTAT, 2010), and is a staple food for approximately 800 million people (FAO/IFAD, 2000; Lebot, 2009). The annual production of cassava in Ghana is approximately 14.2 million metric tonnes (MT) (FAO, 2013). The peel is a by-product of processing the roots for starch, cassava flour and "gari" (a fermented cassava meal product) constituting 11% of the root, with approximately 3.6 million MT of peels and discharged parts and 400,000 MT (dry matter basis) of peels produced annually (Baah et al., 2011; FAO, 2013).

Cultivation of mushroom in Ghana is carried out on composted sawdust of *Triplochiton scleroxylon* or a combination of *Triplochiton scleroxylon* and *Chlorophora excels*a. Composting is a controlled self-heating, aerobic solid phase biodegradative process of organic materials (Ryckeboer et al., 2003). It is a solid-waste fermentation process, which exploits the phenomenon of microbial degradation and mineralization (Mckinley and Vestal, 1984).

The main purpose of composting to a mushroom grower is to prepare a substrate in which the growth of mushroom is promoted to the practical exclusion of other microorganisms. In several successive steps, microbial communities consume the more easy degradable organic components generating a substrate that is stable and increased in the fibrous components, humified forms and inorganic products, generating heat as a metabolic waste product.

Heavy metal concentration in mushroom is considerably higher than those in agricultural crop plants, vegetables and fruit. This suggests that mushrooms possess a very effective mechanism that enables them to readily take up some heavy metals from the ecosystem (Zhu et al., 2010), due to their dense mycelial system, which infiltrates the substrate (Garcia et al., 2005). The accumulation of heavy metals in mushrooms has been found to be affected by environmental and fungal factors. Environmental factors are organic matter content, pH and metal concentration in soil and fungal factors such as species of mushroom, morphological part of fruit body, developmental stages, age of mycelium, intervals between fructifications and biochemical composition (Radulescu et al., 2010).

This study was conducted to determine for the first time the changes in the heavy metal content and proximate composition during the composting of cassava peels, thus making it suitable for mushroom cultivation in Ghana and the heavy metal contents of mushrooms produced.

MATERIALS AND METHODS

Mushroom culture and spawn preparation

Cultures of *P. ostreatus* (Jacq.ex.Fr.) Kummer strain EM -1 originally from Mauritius and maintained on Malt Extract Agar slants were used to prepare sorghum grain spawn (Oei, 1991).

Cassava substrate preparation

Compost was prepared by the outdoor single-phase solid waste fermentation. Freshly milled dried cassava (*M. esculenta*) peels from a mixture of *Afisiafi* and *Bankye Hemaa* (local names) weighing 193 kg, obtained from the Volta Region of Ghana were mixed with rice bran (10% w/w) and lime (0.5% w/w) and composted as described by Obodai et al. (2007). The mixture was then stacked into a heap of about 0.8 m high and 1.0 m wide at the base and left to compost for 28 days with regular turning every 4 days. Before turning, temperatures were read and samples were taken from the core region of the compost with a pair of sterile forceps and its chemical and cyanide compositions evaluated. All samples were taken in duplicates.

At 28 days of composting, samples of the compost were adjusted to approximately 68 - 70% (Buswell, 1984) and then supplemented with rice bran (12%) and lime (0.5%). The mixtures were bagged, sterilized, incubated and mushrooms harvested as described by Obodai et al. (2007).

Chemical analysis

Samples of composting cassava peels taken at four days intervals from the central portion of the heap were put into sterile bags and quantitative estimation of crude fibre, cellulose, hemicellulose, lignin and fat were carried out using the standard methods as described by AOAC (2005). Lignin and cellulose were determined by acid detergent fibre (ADF) method (AOAC, 2005). Hemicellulose content was estimated by neutral detergent solution using 0.5 g of dried sample (AOAC, 2005). The difference between the acid detergent fibre and neutral detergent fibre gave the value for hemicellulose content. Crude fibre values were determined by AOAC (2005) method and calculated as:

$$\text{Crude fibre} = \frac{\text{Loss in weight on ignition (A - B)}}{\text{Initial sample weight}} \times 100$$

Where, A = Initial weight of sample before ignition, and B = final weight of sample after ignition.

To calculate total nitrogen in the samples, the specimens were dried at 60°C and analysed by the Micro kjeldahl Method (AOAC, 2005). To obtain crude protein value, nitrogen content values were multiplied by a factor of 6.25. For cyanide determinations, the method was in accordance with Obiri et al. (2007).

Heavy metal determination using atomic absorption spectrophotometer (AAS) analysis

Samples of dried uncomposted and composted cassava peel at 28

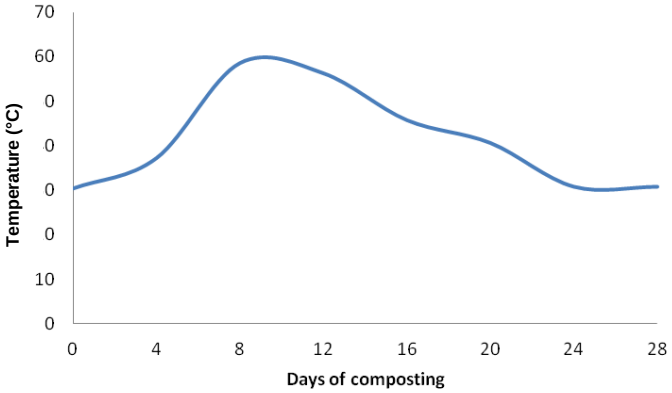


Figure 1. Tempearture changes during composting of cassava peels over a 28 day period.

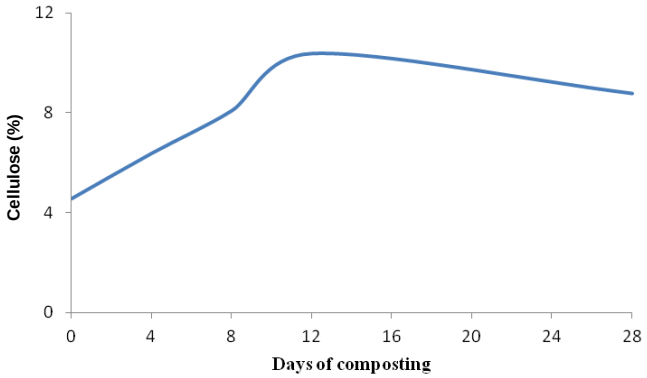


Figure 2. Changes in cellulose content during composting of cassava peels over a 28 day period.

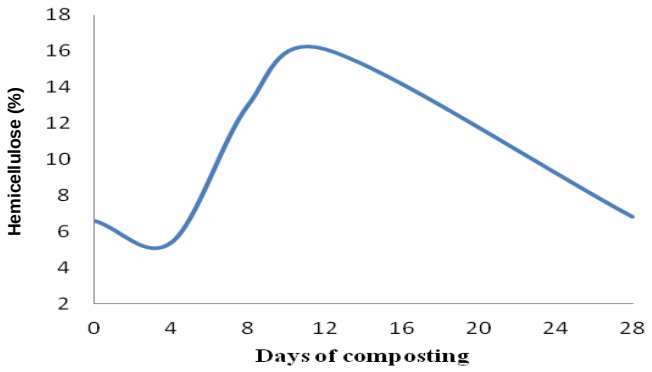


Figure 3. Changes in hemicellulose content during composting of cassava peels over a 28 day period.

days of composting, mycelia from *P. ostreatus* on composted cassava (PEM) after full spawn run, mycelia from *P. ostreatus* on uncomposted cassava (PEM1) after full spawn run, fresh mushroom from composted cassava, and mushroom from uncomposted cassava were milled using stainless steel laboratory blender. The dry ashing method was used for atomic absorption spectrophotometer (AAS) analysis (AOAC, 2005). All glasswares were washed with 1% nitric acid followed by demineralised water. Three grams (3 g) each of the above named samples were weighed into platinum crucibles. The crucible and the test portion were placed in the Muffle furnace at a temperature of 550°C for 8 h. The crucible with ash was put in a desiccator to cool. Five milliliters (5 ml) of nitric acid of mass fraction not less than 65%, having a density of approximately ρ (HNO_3) = 1 400 mg.mL^{-1} was added, ensuring that all the ash came in contact with the acid and the resultant solution heated on hot plate until the ash dissolved. Ten milliliters (10 ml) of 0.1 mol.L^{-1} nitric acid was added and filtered into 50 ml volumetric flask. The resultant solution was topped up to the mark with 0.1 mol.L^{-1} nitric acid. Blank solution was treated the same way as the sample. Buck Scientific 210VGP Flame AAS (Buck Scientific, Inc. East Norwalk, USA) was used to read the absorbance values at appropriate wavelength of the interested metal in the sample solution. Cathode lamps used were copper (Cu) (wavelength 324.8 nm, lamp current 1.5 mA), iron (Fe) (wavelength 248.3 nm, lamp current 7.0 mA), manganese (Mn) (wavelength 279.5 nm, lamp current 3.0 mA), lead (Pb) (wavelength 217.0 nm, lamp current 3.0 mA) and zinc (Zn) (wavelength 213.9 nm, lamp current 2.0 mA). Air/acetylene gas was used for all the analysis. The metal content of the samples were derived from calibration curves made up of minimum of three standards.

Statistical analysis

The standard deviations on mean values of duplicate samples were analysed using Statistical Package for Social Scientist (SPSS, 2005), version 16.0.

RESULTS AND DISCUSSION

During the composting of the cassava peels, various changes in the chemical components of the peels were observed. Temperatures within the heap became stable from day 24 to 28 (Figure 1). Significant increases of cellulose, hemicellulose and fat contents were observed up to day 12 (10.37, 16.1 and 9.39%, respectively) after which there were gradual declines of 15.4, 57.6 and 56.12%, respectively (Figures 2 to 4). The increase in cellulose, hemicellulose and lignin during the first 12 days will likely due to the consumption of starch by microorganisms. The decrease of these compounds (cellulose and hemicelluloses) in subsequent days indicate that when starch is removed mostly, microorganisms start to degrade also the (hemi) cellulose. Lignin, protein and crude fibre values showed a gradual increase from day 0 to 28, with a maximum value of 23.73, 49 and 73%, respectively (Figures 5, 6 and 7). These changes could be due to the type of microorganisms present in the substrate. Presumably, antibiosis was at play in the composting cassava peel by-product. The composting process involves microbial activity, chemical reactions, aeration, temperature and nutritional factors. Previous work carried out by Obodai et al. (2011) on decomposing sawdust of *Triplochiton scleroxylon* used in the cultivation of *P. ostreatus* showed

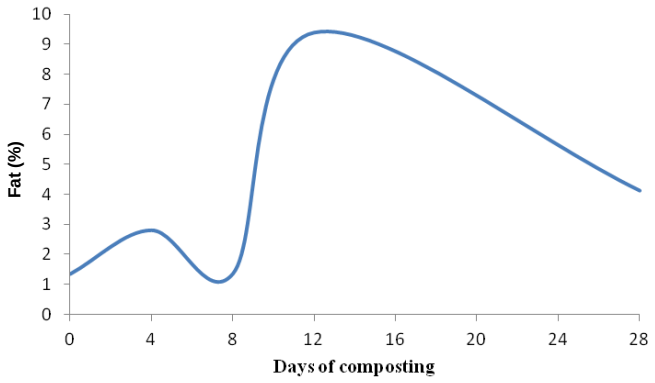


Figure 4. Changes in fat content during composting of cassava peels over a 28 day period.

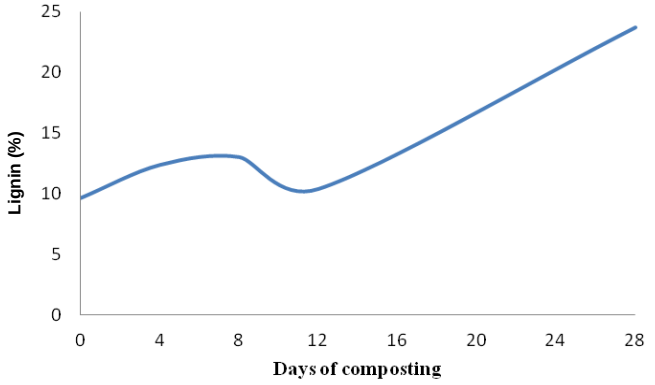


Figure 5. Changes in lignin content during composting of cassava peels over a 28 day period.

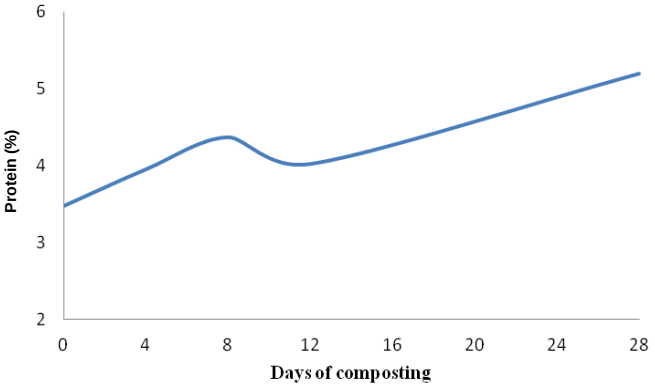


Figure 6. Changes in protein content during composting of cassava peels over a 28 day period.

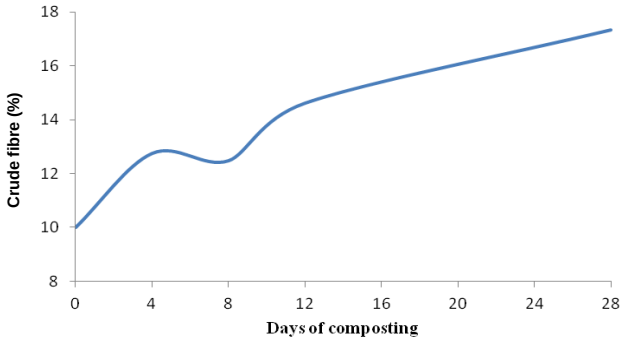


Figure 7. Changes in crude fibre content during composting of cassava peels over a 28 day period.

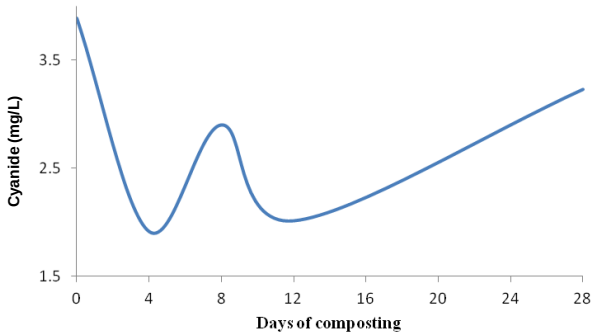


Figure 8. Changes in cyanide content during composting of cassava peels over a 28 day period.

decreasing amounts of cellulose, hemicelluloses and crude fibre as compared to lignin with increasing days of composting and attributed it to these components being easily degradable by fungi and bacteria present as compared to lignin a polymer of aromatic compounds which is very resistant and relatively difficult for cellulolytic organisms to decompose (Insam and de Bertoldi, 2003).

Cyanide however showed a reduction from the initial 3.89 to 2.01 mg/L by day 12 and a marginal increase of 16% by day 28 (Figure 8). This marginal increase in cyanide can probably be attributed to the microorganism at play during that period of composting.

Heavy metals

Metals such as iron (Fe), copper (Cu), zinc (Zn) and manganese (Mn) are essential metals since they play an important role in biological systems, whereas aluminium (Al) and lead (Pb) are non-essential metals as they are toxic even in traces (Unak et al., 2007). The essential metals can also produce toxic effects when the metal intake is excessively elevated (Al-Khlaifat and Al-Khashman, 2007; Gopalani et al., 2007). Five metals namely, Cu, Fe, Mn, Pb and Zn were analysed in *P. ostreatus* mushroom and in the substrates on which they

Table 1. Heavy metal contents ($mgkg^{-1}$) of cassava peel substrates and mushrooms.

Sample	Cu	Fe	Pb	Mn	Zn
Uncomposted cassava	3.7 ± 0.08[a]	380.22 ± 8.84[b]	21.3 ± 2.63[b]	20.24 ± 1.09[a]	24.06 ± 1.09[b]
Composted cassava	6.96±1.33[a]	592.16±37.97[d]	30.97±1.56[c]	42.46±0.47[b]	38.58±0.92[c]
PEM + composted cassava only	8.14±0.52[a]	388.39±11.23[b]	11.54±3.81[a]	25.48±0.47[a]	34.07±0.66[c]
PEM on uncomposted cassava	5.44±1.07[a]	496.82±19.40[c]	32.58±4.65[c]	36.16±3.29[b]	23.57±0.53[b]
Mushroom from composted cassava	28.72±4.67[c]	70.50±20.89[a]	8.34±2.17[a]	63.23±7.58[c]	13.68±0.57[a]
Mushroom from uncomposted cassava	17.44±0.20[b]	86.67±2.89[a]	3.75±0.24[a]	45.68±2.23[b]	12.03±0.62[a]

Results presented as mean concentrations ± standard deviations. Superscript figures in the same column represent significant or insignificant differences at $p \leq 0.05$ (ANOVA, Duncan test, $p \leq 0.05$). Results are on dry weight basis. Cu = copper; Fe = iron; Pb = lead; Mn = manganese; Zn = zinc.

were grown. Of all the heavy metals examined, Fe accumulated more excessively than Cu, Pb, Mn and Zn. The mean concentration of Fe was 380±8.84, 592.16±37.97, 388.39±11.23, 496.82±19.40, 70.50±20.89, 86.67±2.89 mg/kg (dry weight basis) (Table 1) for uncomposted cassava peels, composted cassava peels, *P. ostreatus* mycelium (PEM) on composted cassava peels, PEM1 on uncomposted cassava peels, mushroom harvested from composted cassava peels and mushroom harvested from uncomposted cassava peels, respectively.

The maximum and minimum Fe values determined in the mushroom samples were 86.67±2.89 and 70.50±20.89 mg/kg, respectively which far exceed the limit of 15 mg/kg set by WHO (1982). Iron (Fe) deficiency anemia for instance affect one third of the worlds population. On the other hand, excessive intake of iron is associated with an increase risk of colorectal cancer (Senesse et al., 2004).

The high values of Fe recorded in the mushrooms can be attributed to the Fe content in the cassava peels before and after composting (Table 1). Also it has been reported that the part of the mushroom analysed, stipe or cap and the age of the mushroom plays a major part in the amount of heavy metal content detected (Radulescu et al., 2010).

Statistically, there was no significant difference in the level of Fe determined in mushroom from composted and uncomposted cassava peels at $p \leq 0.05$. Iron levels reported in this study were however far lower than those reported by other authors. Sesli and Tùzen (1999) reported values such as 31.3-1,190 mg/kg, Isiloğlu et al. (2001) had values such as 180-407 mg/kg and 146-835 mg/kg was reported by Tùzen (2003).

Copper is an essential constituent of some metalloenzymes and is required in haemoglobin synthesis and in the catalysis of metabolic growth (Silvestre et al., 2000). Copper concentrations determined ranged from 3.7±0.08 to 28.72±4.67 mg/kg which are below the safe limit set by World Health Organization (WHO) (40 mg/kg) as copper in foods (WHO, 1982). At $p \leq 0.05$, there was significant difference between the concentrations of Cu in mushroom from composted cassava and mushroom from uncomposted cassava. Cu levels in mushrooms reported in literature are 4.71-51.0 mg/kg (Tùzen et al., 1998), 13.4-50.6 mg/kg (Soylak et al., 2005) and 12-181 mg/kg (Tùzen, 2003).

Lead (Pb) is toxic even at trace levels (Dobaradaren et al., 2010) and the impairment related to Pb toxicity in humans include abnormal size and haemoglobin content of the erythrocytes, hyper stimulation of erythropoisis and inhibition of haem synthesis according to Vonugopal and Lucky (1975). The maximum and minimum values of Pb concentrations determined in the mushrooms were 8.34±2.17 and 3.75±0.24 mg/kg on composted and uncomposted cassava peels, respectively, which were below the 10.0 mg/kg limit that has been set by WHO as Pb content in raw plant materials (WHO, 1982). However, the difference in Pb levels between the mushroom from composted cassava and mushroom from uncomposted cassava was statistically not significant at $p \leq 0.05$. Pb levels reported in literature are 0.75-7.77 mg/kg (Tùzen et al., 1998), 0.40-2.80 mg/kg (Svoboda and Kolac, 2003) and 1.43-4.17 mg/kg (Tùzen, 2003).

According to Unak et al. (2007), Mn is an essential metal and it plays an important role in biological systems such as its presence in metalloproteins. The highest and lowest Mn concentrations determined in both mushrooms from composted cassava and mushroom from uncomposted cassava were 63.23±7.58 and 45.68±2.23 mg/kg, respectively which are far below the toxicity limit between 400-1000 mg/kg of Mn in plant. However, at $p \leq 0.05$, the difference in the levels of Mn between mushroom from composted cassava peels and mushroom from uncomposted cassava peels is significant. Varying ranges of Mn values have been reported such as 14.5-63.6 mg/kg (Isiloğlu et al., 2001), 12.9-93.3 mg/kg (Tùzen, 2003), 14.2-69.7 mg/kg (Soylak et al., 2005).

Zinc (Zn) is an essential metal and a component of a wide variety of different enzymes in which it is involved in catalytic, structural and regulatory roles. It constitutes about 33 ppm of adult body weight and is essential as a constituent of many enzymes involved in a number of physiological functions, such as protein synthesis and

energy metabolism (Ma and Betts, 2000). WHO has recommended permissible limit of Zn in foods such as 60 mg/kg (WHO 1982). The maximum and minimum concentration of Zn determined in mushroom samples were 13.68±0.57 and 12.03±0.62 mg/kg, which are below the safe limit of Zn in food set by WHO. There was no significant difference in the concentration of Zn in mushroom from composted cassava peels and mushroom from uncomposted cassava peels at $p \leq 0.05$. The order of heavy metal accumulation in mushrooms harvested from both composted and uncomposted cassava peels was Fe>Mn>Cu>Zn>Pb while it was Fe>Zn>Pb>Mn>Cu, Fe>Mn>Zn>Pb>Cu, Fe>Zn>Mn>Pb>Cu, Fe>Mn>Pb>Zn>Cu for uncomposted cassava peels, composted cassava peels, PEM on composted cassava peels, PEM1 on uncomposted cassava peels, respectively. Of all the heavy metals examined, Fe was accumulated excessively than Cu, Pb, Mn and Zn indicating that *P. ostreatus* strain EM-1 is a good bio-accumulator of iron.

Conflict of Interests

The author(s) have not declared any conflict of interests

ACKNOWLEDGEMENT

This work was funded by the Gains from losses of Root and Tuber Crops (GRATITUDE), an EU funded project

REFERENCES

AOAC (2005). Official methods of analysis of AOAC International, 18th Edition. AOAC International, Gaithersburg, Maryland, USA.

Al-Khlaifat AL, Al-Khashman OL (2007). Atmospheric heavy metal pollution in Aqaba city Jordan using *Phoenix dactylifera* L. leaves. Atmos. Environ. 41:8891-8897.

Baah J, Tait RM, Tuah AK (2011). Selecting browse plants to supplement cassava peel-based diet for peri-urban small ruminants. Small Ruminant Res. 96:36-40.

Buswell JA (1984). Potentials of spent mushroom substrates for bioremediation purposes. Compost 2:31-35.

Dobaradaren S, Kaddafi K, Nazmara S, Ghaedi H (2010). Heavy metals (Cd, Cu, Ni, and Pb) content in fish species of Persian Gulf in Bushehr Port, Iran. A. J. Biotech. 32:6191-6193.

FAO (2013). MAFAP- SPAAA: Monitoring African foods and Agricultural policies: Analysis of incentives and disincentives for cassava in Ghana http://www.fao.org/fileadmin/templates/mafap/documents/technical_notes/GHANA/GHANA_Technical_note_CASSAVA_EN_Feb 2013.pdf

FAO/IFAD (2000). The World Cassava Economy:Facts, Trends and Outlooks, Food and Agriculture Organization of the United Nations and International Fund for agricultural Development. Rome.

FAOSTAT (2010). Food and agricultural Commodities production. Food and Agricultural Organization of the United Nations Statistics Database.

García MA, Alonso J, Melgar MJ (2005) *Agaricus macrospourus* as Potencial Bioremediation Agent in Compost Material Contaminated with Heavy Metals. J. Chem. Tech. Biotech. 80:(3)-325-330.

Garcia-Lafuente A, Moro C, Villares A, Guillamón E, Rostagno MA, D'Arrigo M, Martínez JA (2011). Mushrooms as a source of anti-inflammatory agents. Am. J. Commun. Psychol. 48(1-2):125-141.

Gopalani M, Shahare M, Ramteke DS, Wate SR (2007). Heavy metal content of potato chips and biscuits from Nagpur City, India. B. Environ. Contam. Tox. 79:384-387.

Insam H, de Bertoldi M (2003). Microbiology of the composting process. In: Golueke, C., Bidlingmaier, W., de Bertoldi, M. and Diaz, L. (Eds). Comp. Sci. Tech. 25-47. Elsevier Science Ltd.

Isıloğlu M, Yilmaz F, Merdivan M (2001). Concentrations of trace elements in wild edible mushrooms. Food Chem. 73: 169-175.

Lebot V (2009). Tropical Root and Tuber crops: Cassava, Sweet Potato, Yams and Aroids, CABI, Wallingford, UK.

Li Y (2012). Present development situation and tendency of edible mushroom industry in China. Mush. Sci. 18:3-9.

Ma J, Betts NM (2000). Zinc and Copper intakes and their major food sources for older adults in the 1994-96 continuing survey of food intakes by individual 9CSF-II). J. Nutr. 130:2838-2843.

Manzi P, Aguzzi A, Pizzoferrato L (2001). Nutritional value of mushrooms widely consumed in Italy. Food Chem. 73: 321-325.

McKinley VL, Vestal JR (1984). Biokinetic analysis and succession of microbial activity in decomposition of municipal sewage sludge. App. Environ. Microb. 47:933-941.

Obiri S, Dodoo D K, Okai-Sam F, Essumang D K (2007). Determination of free cyanide and total cyanide concentrations in surface and underground waters in Bogoso and its surrounding areas in Ghana. Bull. Chem. Soc. Ethiop. 21(2):213-220.

Obodai M, Amoa-Awua W, Odamtten GT (2011). Physical, chemical and fungal phenology associated with the composting of 'wawa' sawdust (*Triplochiton scleroxylon*) used in the cultivation of oyster mushrooms in Ghana. Int. Food Res. J. 17:229-237.

Obodai M, Dzomeku M, Awotwe B, Takli RK, Narh D (2007). Manual on mushroom cultivation technology in Ghana. CSIR-FRI Technical Report

Oei P (1991). Manual on mushroom cultivation: techniques, species and opportunities for commercial application in developing countries. CTA, Wageningen, The Netherlands

Radulescu C, Stihi C, Popescu I V, Busuioc G, Gheboianu AI, Cimpoca VG, Dulama ID, Diaconescu M (2010). Determination of heavy metals content in wild mushrooms and soil by EDXRF and FAAS techniques. Ovidus Uni. Ann. Chem. 21(1):9-14.

Ryckeboer J, Mergaert J, Vaes K, Klammer S, Clercq DE, Coosemans D, Insam JH, Swings J (2003). A survey of bacteria and fungi occurring during composting and self-heating processes. Ann. Microb. 53(4):349-410.

Senesse P, Meance S, Cottet V, Faivre J, Boutron-Ruault MC (2004). High dietary iron and copper and risk of colorectal cancer: a case – control study in Burgundy, France. Nutr cancer. 49:66-71.

Sesli E, Tuzen M (1999). Levels of trace elements in the fruiting bodies of macrofungi growing in the east Black sea region of Turkey. Food Chem. 65(4):453-460.

Silvestre MD, Lagarda MJ, Farra R, Martineze-Costa C, Brines J (2000). Copper, iron and zinc determination in human milk using FAAS with microwave digestion. Food Chem. 68:95-99.

Soylak M, Saracoglu S, Tüzen M, Mendli D (2005). Determination of trace metals in mushroom samples from Kayseri, Turkey. Food Chem. 92:649-652.

SPSS 16 for Windows (2005). SPSS 16 for Windows. Chicago. Illinois, USA

Svoboda L, Kalac P (2003). Contamination of two edible *Agaricus* spp. mushrooms growing in a town with cadium, lead and mercury. Bull. Environ. Contam. Toxicol. 71:123-130.

Tüzen M (2003). Determination of heavy metals in soil, mushroom and plant samples by atomic absorption spectrometry. Micro Chem. J. 74:289-297.

Tüzen M, Özdemir M, Demirbas A (1998). Study of heavy metals in some cultivated and uncultivated mushrooms of Turkish origin. Food Chem. 63 (2):247-251.

Unak P, Lambrecht FY, Biber FZ, Darcan S (2007). Iodine measurements by isotope dilution analysis in drinking water in Western Turkey. J. Radioanalytical Nuclear Chem. 273:649-651.

Vonugopal B, Lucky T (1975). Toxicity of non-radioactive heavy metals and their salts in heavy metals toxicity, safety and hormology. (Ed.) F Coulston. Academic Press, Georg Thieme, Stuttgart, New York.

World Health Organaiztion (WHO) (1982). Evaluation of Certain Foods Additives and Contaminants (Tweenty-Six Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report series, No. 683 Geneva.

Zhu F, Qu L, Fan W, Qiao M, Hao H, Wang X (2010). Environmental Monitoring Assessment. DOI 10.1007/s10661.01-1728-5

academicJournals

Vol. 13(22), pp. 2215-2219, 28 May, 2014
DOI: 10.5897/AJB2014.13650
Article Number: 7A4CFE944898
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluating the production of *Ganoderma* mushroom on corn cobs

I. S. E. Ueitele[1]*, N. P. Kadhila-Muandingi[2] and N. Matundu[2]

[1]Zero Emissions Research Initiative, University of Namibia, Namibia.
[2]Department of Biological Sciences, University of Namibia, Namibia.

Received 22 January, 2014; Accepted 2 May, 2014

Mushroom substrates are organic materials which mushroom mycelia can digest and which support growth, development and fruiting of mushrooms. Without good substrates, satisfactory yields of mushrooms will not be obtained. Consequently, finding good substrates on which to grow *Ganoderma lucidum* is of great importance to the medicinal world, and for mushroom farming in Namibia. The objective of this research was to determine the possibility of growing *G. lucidum* mushrooms on corn cobs as a substrate base in order to see if corn cobs can replace saw dust as a substrate of *Ganoderma* cultivation in Namibia. The corn cobs and saw dust (control) were sterilized, inoculated under aseptic conditions in clear plastic bags and incubated at room temperature. Fruit bodies were observed within 30 to 50 days, results considerably shorter compared to when using saw dust, which can take up to four months before mushrooms are obtained. This study demonstrated that corn cobs can be used as a substrate if supplemented with nutrients to support growth of the basidiocarp.

Key words: *Ganoderma*, corn cobs, substrate, mushrooms, sawdust, basidiocarp.

INTRODUCTION

Ganoderma lucidum, a medicinal mushroom, is among the most popular herbal medicines in East Africa that has been used to modulate immune functions, inhibit tumor growth and in the treatment of chronic conditions like hypertension and hyperglyceamia (Jan et al., 2011). Mushroom cultivation techniques which use methods such as submerged culture to obtain mycelium have been described during the last 15 to 20 years while solid culture is used to obtain fruiting body or basidiocarp on several types of substrate and by monitoring important growth parameters including temperature, relative humidity and pH (Gurung et al., 2012; Erkel, 2009). The organic materials which can be digested by mushroom mycelia and support growth, development and fruiting of mushrooms are called substrate (Kadhila-Muandingi and Mubiana (no date)). Hence, without good substrates, satisfactory yields of mushrooms will not be obtained. Erkel (2009) wrote that for most medicinal mushrooms of the basic substrate is hardwood sawdust (75 to 80%) from deciduous trees like maple and elm, supplemented with wheat bran, although rice bran, rice husks, coconut fiber, peanut hulls, corn, sorghum and sugarcane bagasse and rice bran, ground corn and ground sorghum were found to be good supplements for substrate

*Corresponding author: E-mail: iueitele@unam.na.

Table 1. The biological efficiency obtained from corn cobs and saw dust.

Parameter	Corn cob	Sawdust
Weight of Substrate (kg)	3.80	3.30
Weight of Mushrooms harvested (kg)	0.202	0.00172
Biological efficiency (BE) in % [Weight of mushrooms/ weight of substrate x 100%]	5.32	0.05

mixture. In Nepal, *G. lucidum* had been cultivated on four different substrate mixtures with sawdust 70 to 90% supplemented with rice bran 10%, corn meal 20% and wheat bran 10% and wheat bran 12% (Gurung et al., 2012). In another study, corn cobs were used as a lignocellulosic substrate for the production of lignin peroxidase by *G. lucidum* (Mehboob et al., 2011). From the above examples, it appears that corn cobs have successfully been used in the cultivation of Ganoderma, but mostly as a supplement to a substrate base of sawdust or to grow mycelia instead of cultivating the basidiocarp.

Maize is the major commercial crop produced in Namibia (Mushendami et al., 2008). The cultivation of *G. lucidum* on corn cobs, which are an agricultural waste, could increase the availability of substrates in Namibia, and thus increase its production. Namibia is a dry and arid country. Trees that can provide good quality sawdust are mostly found in the Kavango region (http://www.openafrica.org/route/Kavango-Open-Africa-Experience), making it expensive to transport sawdust to be used in mushroom cultivation. Finding alternate substrates on which to grow *G. lucidum* will help to cut on transportation costs and allow people to use corn cobs which are more widely available (Philippoussis et al., 2003). The objective of this research was to determine the possibility of growing *G. lucidum* mushrooms on corn cobs as a substrate base in order to see if corn cobs can replace saw dust as a substrate of Ganoderma cultivation in Namibia.

METHODOLOGY

Preparation of culture

Ganoderma lucidum culture was obtained from the ZERI laboratory at the University of Namibia and used to multiply more cultures. Potato dextrose agar was prepared according to instructions on label. The pure culture was multiplied by aseptically cutting small blocks and placing them on the fresh potato dextrose agar in Petri dishes. The Petri dishes were covered and sealed with Para film to prevent contamination. After 6 days, the mycelia had covered most of the surface of the agar.

Development of spawn

Dry wheat grains weighing 253 g were soaked in water overnight before the grains were removed; rinsed and excess water was drained. The grains were mixed with 1.5% of lime to alter the pH and packed in plastic bottles. The bottles were half-filled and closed lightly to autoclave at 121°C for 15 min. The grains were cooled and inoculated with 3 to 4 pieces of the pure culture obtained after which the bottles were stored in a cardboard box at room temperature to allow for mycelia growth. After 2 weeks, the grains were fully invaded by mycelia and the spawn was ready for substrate inoculation.

Substrate preparation

Mushroom substrates are organic materials that support the growth, development and fruiting of the mushroom mycelia. The substrates used were corn cobs and saw dust (control). The corn cobs and saw dust were weighed and soaked in water overnight (Table 1). The next day, the water was drained off and a handful of wheat bran was mixed with both substrates and substrates were packed into autoclavable plastic bags and autoclaved for 15 min at 121°C. The prepared substrates were transferred to smaller 'houtsak' plastic bags after cooling and inoculated with pure Ganoderma spawn using aseptic techniques. Finally, the bags were transferred to the dark room to allow mycelia growth. This step was replicated on four different days.

Fruiting phase

Once the bags were fully invaded by mycelium they were taken to the fruiting room where temperature and humidity was controlled to allow fruiting of the mushrooms. A blade was used to make openings on the bags to allow mushroom pinning. Daily watering of the fruiting room was necessary to raise the humidity and lower the temperature.

RESULTS

Pure cultures

A thick white carpet of pure Ganoderma culture was obtained after six days (Figure 1). Some plates had to be discarded because they showed signs of Trichoderma contamination.

Development of spawn

Pure spawn was obtained within two weeks of inoculating the grains (Figure 2).

Figure 1. Ganoderma pure culture.

Figure 2. Pure spawn.

Fruiting phase

Fruiting has been observed in corn cobs substrate but none in some bags or poor fruiting in other saw dust bags (Figures 3 and 4).

DISCUSSION

Ganoderma cultivation at the University of Namibia is usually done using hardwood sawdust or woodchips as substrate. However, it is a long process which takes up to 5 months before mushrooms can be obtained. Thus, there is a need to find alternative substrate which can support the growth of this important mushroom. Saw dust was used as a control to compare the growth of Ganoderma on corn cobs. The results observed indicated that mycelia invasion of the corn cobs was faster than in saw dust (Figure 5) and some of the bags produced very small mushrooms; according to Kadhila-Muandingi and Mubiana (no date), this may occur when a number of mushrooms are growing from one bag at the same time and there is a lack of nutrients in the substrates. Previous studies indicated that corn cobs have been used as supplement for growth of *G. lucidum* in combination with other substrates such as rice bran and saw dust (Lakshmi, 2013), but has not been used as a base substrate on its own.

In this study, Ganoderma grew on corn cobs with a BE of 5.32% while saw dust had a minimal BE of 0.05%. In previous studies, Erkel (2009) grew Ganoderma on Poplar, Beech and Oak sawdusts and obtained an average BE of 15.09% while Azizi et al. (2012) achieved a BE of 12.89% on Hornbeam sawdust and a high 18.68% on Poplar sawdust. Veena and Pandey (2010) reported a BE ranging from 4 to 13% when they cultivated *G. lucidum* on locally available saw dust in India. The yield of *G. lucidum* on both saw dust and corn cobs obtained in this study was very low, when compared to the previous studies. There is therefore need for continuous efforts to be made in order to find suitable substrate combinations and supplements to increase yield of mushrooms from locally available agro-industrial substrates in Namibia. The substrates were exposed to the same environmental conditions, thus it can be assumed that the differences in the yield of mushrooms obtained was caused by differences in the substrates. Corn cobs have a higher ability to retain moisture compared to sawdust which dries out much faster. Additionally, substrates which have a high cellulose and lower lignin content allow mushrooms to pin faster than those which have high lignin and low cellulose. This is because high cellulose substrates have carbon available for rapid breakdown during mycelia growth whilst lignin takes longer to decompose. Corn cobs and wheat straw contain higher cellulose, crude proteins and moisture content than saw dust and sugar cane bagasse (Onyango et al., 2011). In the Namibian context, corn cobs is an ideal substrate for Ganoderma mushroom cultivation, especially considering that it is a farm produce which is inexpensive and widely available (Samuel and Eugene, 2012). The quality and yield of mushrooms depend on different factors such as the quality of spawn used, type of substrate, climatic factors and nutrients supplemented to the substrate before and at the time of fruiting (Khare et al., no date). It is recommended that corn cobs can be used as a substrate if supplemented

Figure 3. Corncobs (L) and sawdust (R) growing in bags.

Figure 4. Ganoderma growing on maize cobs.

with nutrients to support growth of the basidiocarp.

It is also recommended that quality assurance tests be done on the mushrooms obtained to ensure that their bioactive compound content are the same as mushrooms cultivated on hardwood sawdust. Additionally, the content of lignocellulose on corn cobs and saw dusts should be evaluated and, it is advised that materials containing corn cobs as supplement should be another control, in order to

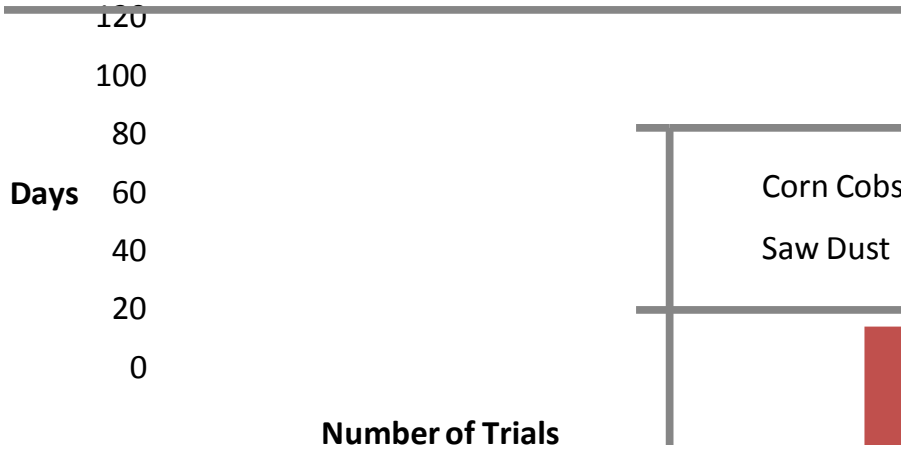


Figure 5. Graph showing the number of days it took for corn cobs and saw dust to grow.

indicate the advantage of corn cobs more clearly.

Conclusion

The objective of this research was to determine the possibility of growing *G. lucidum* mushrooms on corn cobs as a substrate base in order to see if corn cobs can replace saw dust as a substrate of Ganoderma cultivation in Namibia. In the results obtained, corn cobs had a BE of 5.32%, which was higher than the saw dust BE of 0.05%. It can be concluded that corn cobs have the potential to be used as alternate substrate for Ganoderma mushroom cultivation in Namibia.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was funded by the Zero Emissions Research Initiative (ZERI) Project at the University of Namibia.

REFERENCES

Azizi M, Tavana M, Farsi M, Oroojalian F (2012). Yield performance of Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum*(W. Cart.:Fr.) P. Karst. (higher Basidiomycetes), using different waste materials as substrates. Int. J. Med. Mushrooms 14(5):521-527.

Erkel EI (2009). The effect of different substrate mediums on yield of *Ganoderma lucidum* (Fr.) Karst. J. Food. Agric. Environ. 7(3&4):841-844.

Gurung OK, Budathoki U, Parajuli G (2012). Effect of different substrates on the production of *Ganoderma lucidum* (Cart.:Fr.) Karst. Our Nature 10:191-198.

http://www.openafrica.org/route/Kavango-Open-Africa-Experience. Accessed 17 March 2014.

Jan RH, Lin TY, Hsu YC, Lee SS, Lo SY, Chang M, Chen LK, Lin YL (2011). Immuno-modulatory activity of *Ganoderma lucidum*-derived polysaccharide on human monocytoid dendritic cells pulsed with Der p 1 allergen. BMC Immunol. 12:1-10.

Kadhila-Muandingi NP, Mubiana FS (no date). Mushroom cultivation: A beginners guide. The University of Namibia, Windhoek, Namibia.

Khare KB, Achwanya OS, Mutuku JM, Guthuru EM, Ombiri J (no date). Development and Biotechnology of Pleurotus mushroom Cultivation. Department of Biological Sciences, Egerton University, Egerton, Kenya.

Lakshmi SS (2013).Comparative study on mycelia growth rate of *Ganoderma lucidum* and *Pleurotus flabellatus* on agro-wastes. Int. J. Adv. Res. 1:199-203.

Mehboob N, Asad MJ, Imran M, Gulfraz M, Wattoo FH, Hadri SH, Asghar M (2011). Production of lignin peroxidase by *Ganoderma leucidum*(sic)using solid state fermentation. Afr. J. Biotechnol. 10(48):9880-9887.

Mushendami P, Biwa B, GaomabII M (2008). Unleashing the potential of the Agricultural sector in Namibia.Bank of Namibia Research Department, Windhoek, Namibia.

Onyango BO, Palapala VA, Arama PF, Wagai SO, Otieno CA (2011). Nutritional analysis of some composted and non-composted agricultural substrates used for production of Kenyan native Wood Ear Mushrooms [*Auricularia auricular* (L. Ex Hook.) Underw.]. Am. J. Food. Technol. DOI:10.3923/ajft.2011.

Samuel AA, Eugene TL (2012). Growth performance and yield of oyster mushroom (*Pleurotus Ostreatus* [sic]) on different substrate composition in Buea South West Cameroon. Sci. J. Biochem. Article ID sjbch-139, 6 Pages, 2012. doi: 10.7237/sjbch/139

Veena SS, Pandey M (2010). Effect of spawn substrate and spawn rate on cultivation of *Ganoderma lucidum*. J. Mycol. Pl. Pathol. 40(1):158-161.

academicJournals

Vol. 13(22), pp. 2220-2226, 28 May, 2014
DOI: 10.5897/AJB2013.13487
Article Number: 9FF8DB944900
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Citric acid production and citrate synthase genes in distinct strains of *Aspergillus niger*

Lívia Martinez Abreu Soares Costa[1]*, Sara Maria Chalfoun Souza[2], Priscilla silva de abreu[2], Sabrina Carvalho Bastos[2], Marilza Neves do Nascimento[3] and Aline Cristina Teixeira Mallet[2]

[1]Departamento de Biologia, UFLA Campus Universitário, CEP 37200-000, Caixa Postal 3037 Lavras MG Brasil.
[2]Departamento de Ciências dos Alimentos, UFLA Campus Universitário CEP 37200-000 Caixa Postal 3037 Lavras MG Brasil.
[3]Departamento de Biologia, UEFS Campus UniversitárioCEP 44.036-900 Caixa Postal 252-294 Feira de Santana BA Brasil.

Received 19 November, 2013; Accepted 16 May, 2014

Citric acid is an important organic acid, multifunctional with a wide array of uses. The objectives of this study were the isolation and selection strains of the genus *Aspergillus*, investigating the solubilization of phosphate of these isolates, verifying the expression rate of genes involved in the identification of isolates, and efficiency of citrate synthase in the citric acid production. To verify the mechanisms of the microorganisms in solubilizing phosphate into the medium in the citric acid production, Araxá rock phosphate was utilized. Further, citric acid was evaluated in two culture media, namely BD and SA. The amplification by the polymerase chain reaction was done by using primer ITS region and citrate synthase (P1/P2). The isolates of *Aspergillus niger* were efficient to solubilize phosphate. The isolate *A. niger* 00118 stood out in the solubilization with an increase as high as five times the amount of soluble phosphorus when compared with the control treatment. The results of citric acid production highlighted better influence conditions of the culture medium Sabouraud (SA) on its production. The P1/P2 primer was sensitive in distinguishing the isolates *A. niger* 00116, *A. niger* 00104, *A. niger* 00098 and *A. niger* 00118 in the analysis of citrate synthase enzyme genes.

Key words: Biotechnology, enzyme, strains.

INTRODUCTION

Citric acid is the leading constituent of citrus fruits, currently one of the most important organic acids produced by microbial pathway. Because of its characteristics, it is widely utilized in food industry (acidulants, flavorings, antioxidants), in the pharmaceutical industry (buffering, scavenger, chelating) and others. Its importance is due to the characteristics (such as palatability, low toxicity) that it is easily assimilated by

*Corresponding author. E-mail: livinhamartinez@dbi.ufla.br. Tel: +55 3588542051.

the organism. With the use of microbiological process technique, it was possible to obtain acid by reducing the obtaining cost and greatly increasing the use in industrial scale.

The metabolic pathway established involved in the citric acid biosynthesis includes citrate synthase and this enzyme is most time, considered fundamental in the citric acid cycle, maybe as the first step of the cycle. The enzyme catalyzes the reaction of condensation of acetate and acetyl coenzyme A with an oxaloacetate molecule to form citrate (Papagianni and Mattey, 2007).

As the accumulation of citric acid by *A. niger* is accompanied by the action and activation or disappearance and reduction in the activity of some Krebs cycle enzymes, the utilization of molecular techniques may be an alternative viable for the characterization and identification of the gene, which codifies for the citrate synthase in lactic acid production by *A. niger* and with the use of species-specific primers of the genus, it is possible to improve the detection techniques to the desirable extent of performing this operation directly in selected isolates, making the process faster.

From among the molecular techniques which stand out, those ground on the DNA sequences amplification by the polymerase chain reaction (PCR) are included. The polymerase chain reaction is a highly sensitive technique by means of which small amounts of unique DNA or RNA sequences may be enzymatically amplified till millions of copies of the target sequence may be obtained (Gandra et al., 2008).

A number of microorganisms, including both bacteria and fungi, possess the capacity of solubilizing inorganic phosphates by means of different mechanisms. The solubilization of inorganic phosphates takes place through the decrease of the pH value caused by the microbial production of organic or inorganic acids releasing soluble phosphate (Barroso and Nahas, 2005). In this context, the objectives of this study were isolation and selection of strains of the genus *Aspergillus*, examining the solubilization of phosphate of these isolates, verifying the level of expression of genes involved in the identification of isolates and efficiency of citrate synthase with the citric acid production aiming at the selection of superior isolates for industrial production.

MATERIALS AND METHODS

Microorganisms

By the direct plating method, samples of soil, fruits, grains and breads were utilized for selection of *A. niger* for further purification. The characterization and the identification of the species of the genus *Aspergillus* was conducted on the basis of the taxonomy of Pitt and Hocking (1997).

From these, 12 isolates of *A. niger* were selected (00098, 00100, 00102, 00104, 00106, 00107, 00108, 00114, 00116, 00118, 00119, 00124) with best performance as to the capacity of solubilizing phosphate and producing citric acid.

Efficient phosphate solubilization and acid citric production

To verify the mechanism of phosphate solubilization of the isolates, Araxá rock phosphate was utilized, which was added into dose of 3 g/100 ml into liquid culture medium GL (glucose, yeast extract). Aliquots of 1 ml of suspension of the fungi 10^8 CFU (cells ml^{-1}) were transferred, singly, into a 250 ml Erlenmeyer (flasks) containing 100 ml of liquid culture medium and 3 g of phosphate as a source of phosphorus (P). The culture medium plus the phosphate with no inoculum was established as controls (Control 1) and the culture medium (Control 2). The treatments were inoculated at 28°C under stirring of 190 rpm for eight days, three replications being utilized. To quantify the soluble phosphorus (P) (deletion, it was earlier), the colorimetric method of Murphy and Riley (1962) was used. The pH was also determined according to the Association of Official Agricultural Chemists (AOAC) technique (1992).

Citric acid production

Citric acid was determined in two sorts of culture media, Sabouraud (SA) and Potato Dextrose (BD). Components BD medium include potato extract (200 g/500 ml), dextrose (20 g) and distilled water (1000 ml). SA media components include casein hydrolyzate (5 g), peptic hydrolyzate of animal tissue (5 g), glucose (40 g) and distilled water (1000 ml). Aliquots of 1 ml of suspension at 10^8 CFU (cells ml^{-1}) of each of the 12 isolates of *A. niger* in the two culture media (BD and SA) were inoculated into Erlenmeyer flasks of 125 ml containing 50 ml of culture medium. The treatments were incubated at 28°C under stirring of 140 rpm for 7 days; three replications were utilized. After the incubation period, citric acid in the medium with the different isolates of *A. niger* was determined.

Statistical analysis

For the soluble phosphorus analysis, the experimental design utilized was completely randomized with 14 treatments and three replications. For citric acid, the experiment was also conducted according to a completely randomized experimental design, nevertheless, the treatments were arranged in conformity to a factorial scheme 12×2 with 12 isolates of *A. niger* and two culture media (BD and SA) amounting to 24 treatments. The media obtained were compared by the Tukey test at 5% of probability, utilizing the SISVAR program (Ferreira, 2011).

DNA extraction and amplification

For DNA extraction, the samples of the isolates were ground in liquid nitrogen and approximately 1 g of each samples/isolate was taken, and for each replication used, 0.04 g was weighted for DNA extraction. DNA extraction of the samples coming from the 12 isolates of *A. niger* was done by means of *Wizard*™ Genomic DNA Purification Kit (Promega, Madison, WI, USA).

For amplifications by the polymerase chain reaction (PCR), the pairs of primers of the sequences Internal Transcriber Spacer 1 and 4 (ITS 1 and ITS 4) (TCCGTAGGTGAACCT GCGG/ TCC TCCGCTTATTGATATGC) reported by Mirhendi et al. (2007) were utilized to amplify regions of the rDNA of the 12 isolates of *A. niger* and for analysis of the citrate synthase gene in the isolates, the pairs primer (GCGAATTCATGTCTACCGGCAAGGCCAAGTCC/GCCCCGGGT CATTT ACAGCTTAGCACC), reported by Kirimura et al. (1999) were utilized. The gel was stained with red gel and the PCR products were observed in an UV transilluminator.

Table 1. Efficient solubilization of phosphate Araxá by isolates of *Aspergillus niger* in liquid medium.

Strain	Soluble phosphorus (mg/kg)	pH
A. niger 00118	77.23± 0.81[a]	1.87± 0.01[a]
A. niger 00104	66.41± 0.65[b]	1.91± 0.02[a]
A. niger 00114	56.84± 0.72[c]	2.01± 0.01[b]
A. niger 00116	51.12± 0.69[d]	2.11± 0.04[c]
A. niger 00098	47.66± 0.54[e]	2.27±0.05[d]
A. niger 00119	42.04± 0.93[f]	2.33± 0.01[e]
A. niger 00107	35.24± 0.35[g]	2.41± 0.01[f]
A. niger 00108	33.94±0.65[h]	2.52±0.06[g]
A. niger 00124	28.54± 0.82[i]	2.97±0.01[i]
A. niger 00106	26.42± 0.63[j]	3.04± 0.02[i]
A. niger 00100	25.37± 1.52[l]	2.97± 0.01[h]
A. niger 00102	19.29±0.25[m]	3.21± 0.03[j]
Control 1	14.69±0.64[n]	6.85± 0.01[l]
Control 2	1.13± 0.84[o]	7.02± 0.01[m]

Means followed by the same small letter in the column do not differ from one another by the Tukey test at 5% of probability.

RESULTS AND DISCUSSION

Efficient solubilization of the phosphate

In liquid medium, all the 12 isolates of *A. niger* presented additions in the amount of soluble phosphorus with Araxá rock phosphate. The isolates reduced pH of the liquid medium in relation to the control with no inoculation. The results are shown in Table 1.

That solubilization of phosphate related with the decrease of pH demonstrates the possible solubilization mechanism and acid production. The study of Chuang et al. (2007) indicated the significant and positive correlation between phosphate and acidity. For solubilization to exist, there is need of acid production. That same mechanism was also related in the report of Vassilev et al. (2006), citric acid being considered as a strong solubilizing agent.

According to Barroso and Nahas (2005), increasing titrable acidity, decrease in the values of final pH occurs which correspond to the increase of the soluble phosphate content. In general, isolate *A. niger* 00118 stood out from the other treatments with the greatest amount of soluble phosphorus and lowest pH, increasing on the average about as many as 5 the amount of soluble phosphorus in the medium when compared with Control 1.

That result can be ascribed to the best adaptation of the isolate to the culture medium highlighting an increased capacity to produce acidifying metabolites for phosphate solubilization. That indicates that the solulibizing capacity differs among the different isolates and that one can be better than the other.

Citric acid production

As regards the citric acid production, variation among the isolates was found and that of *A. niger* 00118 was the most efficient attaining a concentration of 18.42 g/(100 g) of citric acid in SA medium and 8.62 g/(100 g) in BD medium; being superior to the production obtained among the isolates studied. The results are shown in (Table 2).

In the work of Soccol et al. (2006), the production of citric acid by submerged fermentation by utilizing strains of *A. niger* and control of concentration of sugar, metals and oxygen obtained concentrations of about 14.98 g/L of citric acid. Prado et al. (2005), with the fermentation in the solid state of cassava bagasse utilizing also *A. niger* obtained an accumulation of citric acid corresponding to 26.9 g/(100 g).

The production of citric acid was greater in the SA medium than in BD medium. The results presented highlighted the influence of the conditions of the medium on citric acid production. The fact can be explained by the difference of the sugar and protein concentration present in the SA medium as glucose and peptone, making greater energy for the isolates available with the best sources of carbon and nitrogen. This occurrence is in accordance with Soccol et al. (2006), who observed poor output of citric acid owing to the fact that the sugar concentration is low in the medium.

The pH values of the 2 media studied had a small difference, the lowest being for those of the BD medium and the highest values for the ones of the SA medium. The results do not implicate in the linking between pH a citric acid production. The results are shown in (Table 3).

Table 2. Citric acid production by isolates of *Aspergillus niger* in submerged cultivation.

Strain	Citric acid BD medium (g/100 g)	Citric acid SA medium (g/100 g)
A. niger 00098	7.81± 0.04Ba	12.34± 0.01Eb
A. niger 00100	1.97± 0.06La	7.56± 0.02Hb
A. niger 00102	2.02± 0.02La	7.15± 0.04Ib
A. niger 00104	7.69± 0.02Ca	12.83± 0.06Cb
A. niger 00106	3.06± 0.03Ia	10.15± 0.06Gb
A. niger 00107	4.72± 0.02Ga	11.25± 0.08Fb
A. niger 00108	4.02± 0.06Ha	11.21± 0.03Fb
A. niger 00114	6.47± 0.08Da	13.63± 0.04Bb
A. niger 00116	6.33± 0.04Ea	12.82± 0.08Cb
A. niger 00118	8.62± 0.06Aa	18.42± 0.09Aa
A. niger 00119	5.25± 0.02Fa	12.47± 0.02Db
A. niger 00124	2.24± 0.03Ja	6.65± 0.02Jb

Means followed by the same capital letter in the column and small letter in the row do not differ from one another by the Tukey test at 5% of probability.

Table 3. Influence of pH in citric acid production by isolates of *Aspergillus niger* in submerged cultivation.

Strain	pH (BD medium)	pH (SA medium)
A. niger 00098	1.99± 0.01Da	2.47± 0.04Gb
A. niger 00100	2.30± 0.01Ga	2.96± 0.03Ib
A. niger 00102	2.44± 0.01Ha	3.02± 0.06Jb
A. niger 00104	1.81± 0.01Ba	2.16± 0.02Db
A. niger 00106	2.04± 0.02Ea	2.61± 0.04Hb
A. niger 00107	1.90± 0.01Ca	2.07± 0.02Cb
A. niger 00108	2.14± 0.02Fa	2.41± 0.01Fb
A. niger 00114	2.03± 0.01Ea	2.33± 0.02Eb
A. niger 00116	1.92± 0.02Ca	1.91± 0.02Ba
A. niger 00118	1.74± 0.04Aa	1.85± 0.02Ab
A. niger 00119	2.01± 0.02Da	2.45± 0.05Gb
A. niger 00124	2.32± 0.01Ga	2.97± 0.02Ib

Means followed by the same capital letter in the column and small letter in the row do not differ from one another by the Tukey test at 5% of probability.

The findings of Max et al. (2010) suggest that the variations in the pH values do not present significant effect for citric acid production with strains of *A. niger*. In the same way, Levinson et al. (2007), in a study with *Yarrovia lipolytica*, also found that pH does not promote significant alterations in the accumulated amount of citric acid.

In spite of all the 12 isolates solubilizing phosphate, one can realize that a somewhat higher citric acid production in the isolates (*A. niger* 00118, *A. niger* 00104, *A. niger* 00114, *A. niger* 00098, *A. niger* 00116) who presented a higher amount of soluble phosphorus and the isolates (*A niger* 00124, *A. niger* 00102, *A. niger* 00100) which had less citric acid showed less efficiency in solubilizing phosphate. Those data demonstrate the relationship between the efficiency of phosphorus solubilization and citric acid production. Therefore, greater understanding of the diversity, efficiency and capacity of microorganisms of solubilizing phosphate can further both the selection and identification of isolates potentially useful to produce citric acid.

Amplification by polymerase chain reaction (PCR)

The DNA of the isolates were extracted and amplified efficiently, nevertheless, the control (line 13) was not amplified for the ITS region. The sizes of the amplified fragments was of approximately 600 pairs of bases (bp) for all the isolates and were viewed at the same position

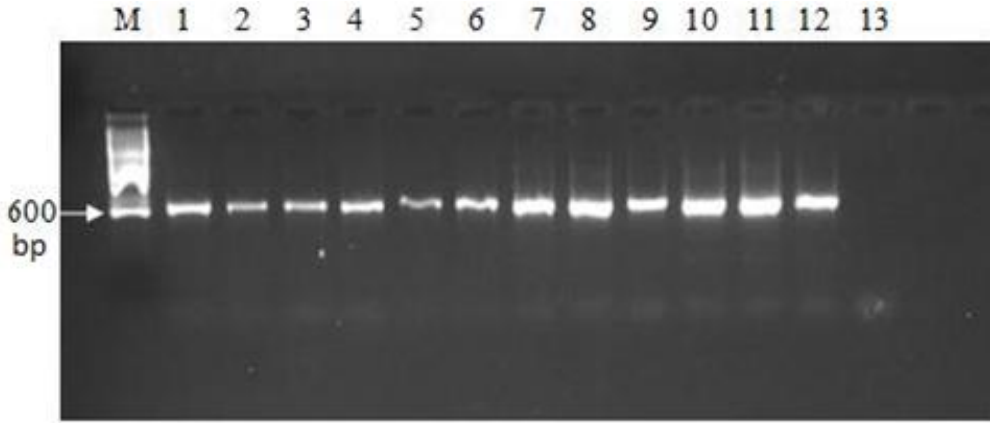


Figure 1. Product of PCR amplification of genomic DNA on agarose gel of the isolates of Asper*gillus niger*. Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 represent the amplification of the genomic DNA of pure cultures of isolates of *A. niger*: 00116, 00108, 00107, 00104, 00106, 00114, 00119, 00100, 00102, 00098, 00118, and 00124, respectively and line 13 represents the control, utilizing primer ITS 1/ITS 4; line M is the molecular marker (1 Kb AMRESCO).

on the agarose gel considered of same size (Figure 1).

The 12 isolates of *A. niger* had amplified only fragments of the genomic DNA corresponding to ITS 1 and ITS 4 region with bands of about 600 pairs of base. The primers ITS 1 and ITS 4 were utilized also to warrant the quality of DNA. According to Barrocas et al. (2012) and Phan et al. (2002), the regions of ITS of rDNA have been widely utilized to distinguish and detect closely related fungal species. According to White et al. (1990), the ITS region is easily amplified, since it is comprehended between 600 and 800 pairs of base.

In the work by Menezes (2010) by means of molecular methods of DNA analysis, it was possible to distinguish species, since they detect the polymorphism existing among the sequences of nucleotides of the organisms. They exposed also in their results, the idea that the identification of species, based upon only morphological and cultural characteristics have limitations, since those are influenced by environment, altering the phenotype of fungal isolates. According to Hinrikson et al. (2005), in addition, the morphology tests are generally difficult and need staff skilled in mycology. González-Salgado et al. (2005), in a distinguishing work of *A. niger* with other species of *Aspergillus*, determined that their PCR assays based on the ITS region were both highly sensitive and unique and represent a good tool for the detection of species.

The pair of P1/P2 of citrate synthase was unique to distinguish isolates of *A. niger* in the enzyme activity. Only genomic DNA of isolates *A. niger* 00116, *A. niger* 00104, *A. niger* 00098 and *A. niger* 00118 were amplified with primers P1/P2. The size of the fragments was of about 920 pairs of bases and were viewed in the same position on the agarose gel considered of same size, the control (Line 13) being negative for amplification (Figure 2).

There are few works in the literature which define a correlation of citrate synthase of *A. niger* with citric acid production. Jaklitsch et al. (1991) in their study with enzymes involved in the citrate production by *A. niger*, evaluated citric-acid producing strains and all presented citrate synthase activity.

The tricarboxlic acid cycle plays an important role in the citric acid production by *A. niger*. On the basis of the activity of the enzymes present in fungal extract, the reaction seems to have enough natural capacity for the accumulation of citric acid (Ratledge, 2000) and an overexpression of the gene corresponding to citrate synthase, therefore, it has no effect upon the citric acid accumulation rate (Ruijter et al., 2000). So, the alterations in the citrate synthase, which occur along fermentation, should not have consequences upon citric acid production (Karaffa and Kubicek, 2003).

All the 12 isolates of *A. niger* surveyed for the analysis of genes of the citrate synthase enzyme, were selected for producing citric acid in liquid medium and only 4 of those isolates amplified their fragments for citrate synthase demonstrating a variation in the presence of the enzyme among them, that difference may be related with the fact of the isolates consisting of different sources and origins. The citrate synthase activity of isolated *A. niger* 00116, *A. niger* 00104, *A. niger* 00098, *A. niger* 00118 can be correlated with the efficient production of citric these isolates.

The citrate synthase activity of the enzyme can be considered for the natural production and accumulation of citric acid by *A. niger*, therefore, amplification of DNA is positive for the selection of different strains of microorganisms. It is necessary that further studies are developed using an induction of the production of citric acid which is used in methods to quantify the expression of citrate synthase with increased production of citric acid.

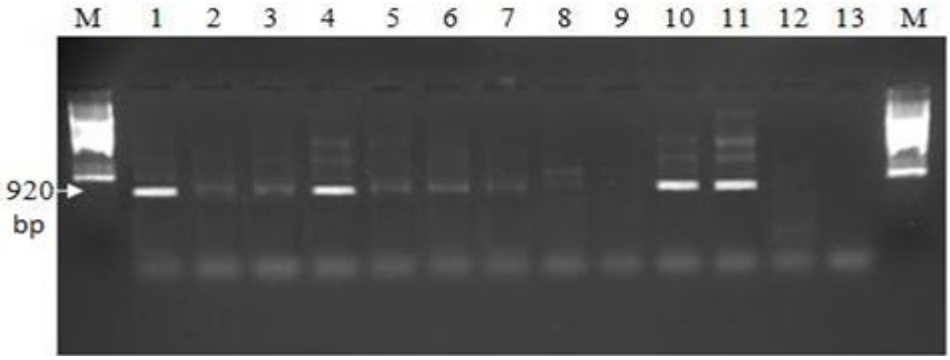


Figure 2. Product of PCR amplification of genomic DNA on agarose gel of the isolates of *Aspergillus niger*. Lines 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 represent the amplification of the genomic DNA of pure cultures of isolates of *A. niger*: 00116, 00108, 00107, 00104, 00106, 00114, 00119, 00100, 00102, 00098, 00118, and 00124, respectively and Line 13 which represents the control utilizing the pair of unique primers (P1/P2) of the citrate synthase of *Aspergillus niger*. Line M, Molecular marker (1 Kb AMRESCO).

As the real-time PCR is molecular specific, sensitive and reproducible, it may contribute to the understanding of the relationship between the enzymes of the Krebs cycle and the production of citric acid by *A. niger* and establish the biochemical mechanism and control of enzymes that inhibit or promote the process of accumulation of citric acid biosynthesis by enabling the optimization of citric acid production on an industrial scale.

Conclusions

Citric acid was one of the mechanisms of solubilizing phosphate by the isolates of *A. niger*. Isolate *A. niger* 00118 was the most efficient in the production for citric acid in the two media studied. The base sequencing of the ITS region was a simple and fast method to aid in the quality of the amplification of the DNA of the isolates of *A. niger* studied. Since the citrate synthase activity of the enzyme can be considered for the natural production and accumulation of citric acid by *A. niger*, therefore, amplification of DNA is positive for the selection of different strains of microorganisms.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKOWLEDGEMENTS

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) are ackowledged for the financial support.

REFERENCES

Association of Official Agricultural Chemists (AOAC) (1992). Official methods of analysis of the Association of the Agricultural Chemists. 12 ed. Washington: A.O.A.C. USA.

Barrocas EN, Machado JC, Almeida MF, Botelho LS, Pinho EVRV (2012). Sensibility of the PCR technique in the detection of Stenocarpella sp. associated with maize seeds. Rev. Bras. Sement. 34:218-224.

Barroso CB, Nahas E (2005). The status of soil phosphate fractions and the ability of fungi to dissolve hardly soluble phosphates. Appl. Soil Ecol. 29:73-83.

Barroso CB, Nahas E (2008). Solubilização do fosfato de ferro em meio de cultura. Pesq. Agro. Bras. 43:529-535.

Chuang CC, Kuo YL, Chao CC, Chao WL (2007). Solubilization of inorganic phosphates and plant growth promotion by *Aspergillus niger*. Biol. Fertili. Soil 43:575-584.

Ferreira DF (2011). Sisvar: a computer statistical analysis system. Cienc. Agrotecnol. 35:1039-1042.

Gandra EA, Gandra TKV, Mello WS, Godoi HS (2008). Técnicas moleculares aplicadas à microbiologia de alimentos. Acta Scient. Technol. 30:109-118.

González-Salgado A, Patiño B, Vázquez C, Gonzáles-Jaén MT (2005). Discrimination of *Aspergillus niger* and other *Aspergillus* species belonging to section *Nigri* by PCR assays. FEMS Microbiol. Letter 245:353-361.

Hinrikson HP, Steven FH, Timothy JL, David WW, Christine JM (2005). Assessment of ribosomal large-subunit D1–D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. J. Clin. Microbiol. 43:2092-2103.

Jaklitsch WIM, Kubfcek CP, Scrutton MC (1991). Intracellular location of enzymes involved in citrate production by *Aspergillus niger*. Can. J. Microbiol. 37:823-827.

Karaffa L, Kubicek CP (2003). *Aspergillus niger* citric acid accumulation: do we understand this well working black box. Appl. Microbiol. Biotechnol. 61:189-196.

Kirimura K, Yoda M, Ko I, Oshida Y, Miyake K, Usami S (1999). Cloning and sequencing of the chromosomal DNA and cDNA encoding the mitochondrial citrate synthase of *Aspergillus niger*. J. Biosci. Bioeng. 88:237-243.

Levinson WE, Kurtzman CP, Kuo TM (2007). Characterization of *Yarrowia lipolytica* and related species for citric acid production from glycerol. Enzym. Microbial. Technol. 41:292-295.

Max B, Salgado JM, Rodríguez N, Cortés S, Converti A, Domínguez JM (2010). Biotechnological production of citric acid. Braz. J. Microbiol. 41:862-875.

Menezes JP (2010). Variabilidade genética na região its do rDNA de isolados de trichoderma spp. (Biocontrolador) e Fusarium oxysporum f. sp. Chrysanthemi. Ciênc. Agrotecnol. 34:132-139.

Mirhendi H, Diba K, Kordbacheh P, Jalalizand N, Makimura K (2007). Identification of pathogenic *Aspergillus* species by a PCR-restriction enzyme method. J. Medical. Microbiol. 56:1568-1570.

Murphy J, Riley JP (1962). A modified single solution method for determination of phosphate in natural waters. Analytic Chemis. Acta. 27:31-36.

Papagianni M, Mattey M (2007). Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. Biotechnol. Advanc. 25:244-263.

Phan HTT, Ford R, Bretag T, Taylor PWJ (2002). A rapid and sensitive polymerase chain reaction (PCR) assay for detection os *Ascochyta rabiei*, the cause of ascochyta blight of chickpea. Austral. Plant. Pathol. 31:31-39.

Pitt JI, Hocking AD (1997). *Fungi* and food spoilage. London. Chapman & Hall. p.593.

Prado FC, Vandenberghe LPS, Soccol CR (2005). Relation between citric acid production by solid-state fermentation from cassava bagasse and respiration of *Aspergillus niger* LPB 21 in semi-pilot scale. Braz. Arch. Biol. Technol. 48:29-36.

Ratledge C (2000). Look before you clone: a comment on 'properties of *Aspergillus niger* citrate synthase and effects of citA overexpression on citric acid production' by Ruijter, Panneman, Xu and Visser. FEMS Microbiol. Letter. 189:317-319.

Ruijter GJG, Panneman H, Xu DB, Visser J (2000). Properties of *Aspergillus niger* citrate synthase and effects of citA overexpression on citric acid production. FEMS Microbiol. Letter. 184:35-40.

Soccol CR, Vandenberghe LP, Rodrigues C, Pandey A (2006). New perspectives for citric acid production and application. Food Technol. Biotechnol. 44:141.

Vassilev N, Medina A, Azcon R, Vassileva M (2006). Microbial solubilization of rock phosphate on media containing agro-industrial wastes and effect of the resulting products on plant growth and P uptake. Plant Soil. 287:77-84.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequences of fungal ribossomal RNA genes for phylogenetics. In: INNIS, M. A. et al. (Ed.). PCR protocols: a guide to methods and applications. San Diego Academic. pp.315-322.

academicJournals

Vol. 13(22), pp. 2227-2230, 28 May, 2014
DOI: 10.5897/AJB2013.12836
Article Number:
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Biodegradation of norfloxacin by *Penicillium frequentans* isolated from polluted soil

Cong-Liang Zhang*, Shu-Jie Cui, Bao-Ying Li and Yan Wang

College of Chemical Engineering and Energy, Zhengzhou University, Zhengzhou, Henan 450001, P. R. China.

Received 24 May, 2013; Accepted 16 May, 2014

One norfloxacin-degrading fungi was isolated from soil contaminated by norfloxacin and preliminary identified as *Penicillium frequentans*. Indoor simulative degradation experiments were carried out to investigate the biodegradation kinetics of norfloxacin with or without NFX3 in soil. The results indicate that the biodegradation of norfloxacin were both in accordance with first-order kinetics equation whether the NFX3 existed or not, and the microbial degradation rate constant of norfloxacin with NFX3 in soil was 0.0121 day^{-1}.

Key words: Norfloxacin, soil, biodegradation, microbial consortium.

INTRODUCTION

Quinolones are known as a group of synthetic organic antibiotics, and extensively used in agriculture to prevent diseases in livestock and treat illness; therefore, soil and groundwater body have been badly contaminated (Kay et al., 2005). Some measures revealed that the dominant veterinary drugs used in China are antimicrobial drugs, especially sulfonamides, macrolides and quinolones (Yu et al., 2012). Nowadays, systematic studies on the accumulation, transportation, and transformation of veterinary drugs in aquatic and terrestrial environment, as well their effects on various organisms are still scarce.

Consequently, it is critical to investigate the environmental behavior of veterinary drugs, which would be helpful for accessing the security of veterinary drugs utilized in aquatic and terrestrial environment, and modifying the contaminated soil. In predicting the transport of quinolones in the environment, and assessing their risk to terrestrial and aquatic ecosystems, it is necessary to know the biodegradation data of quinolones, but only a limited amount of biodegradation data of quinolones have been reported in the literature (Baran et al., 2006; Peng et al., 2006). In this study, biodegradation for norfloxacin in soil was carried out to investigate the biodegradation kinetics.

MATERIALS AND METHODS

Norfloxacin (NFLX), obtained from Daming Biotechnology Co. Ltd., China was further purified by recrystallization from aqueous solutions. After filtration and drying, its purity was determined by UV spectrometry (type UV-2401PC, Shimadzu Co. Ltd, China), to be 0.996 in mass fraction. H_2SO_4 and NaOH used in experiments were all analytical reagents.

The soil sample was collected from Xingyang in China. Its physical and chemical properties show that the pH value is 7.82, which indicate that it is alkaline and the organic matter content is not small (11.0 g/kg).

*Corresponding author. E-mail: zhangcl201@zzu.edu.cn. Tel: +86 0371 67781062.

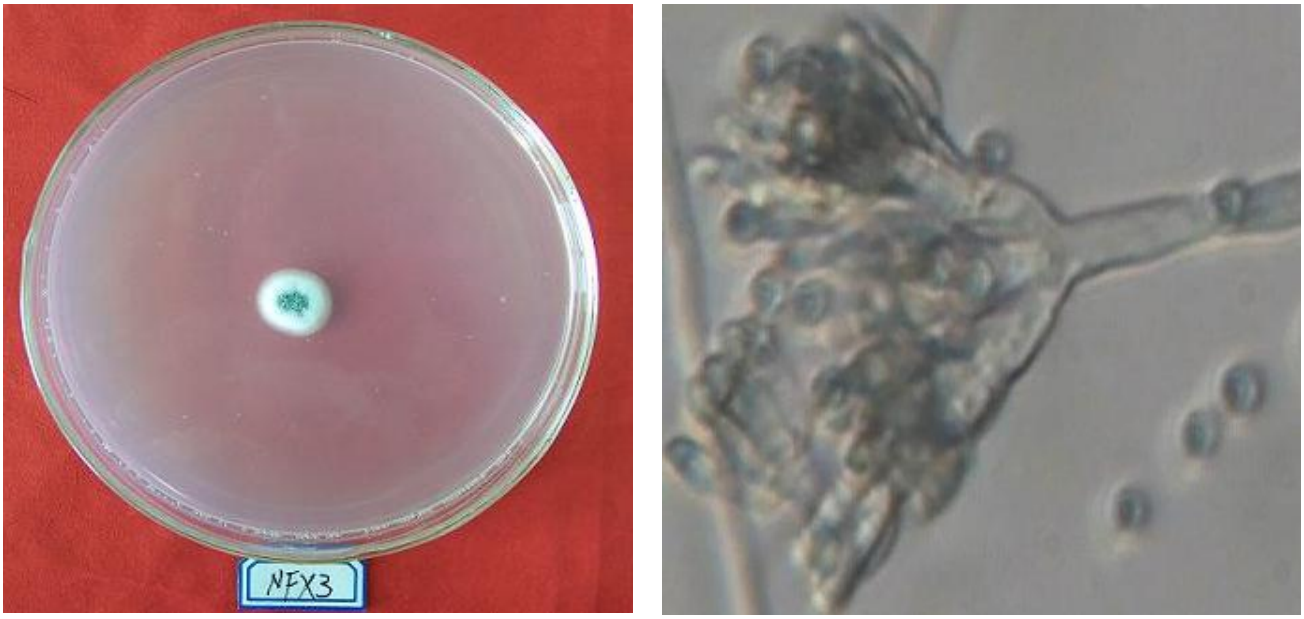


Figure 1. Colony shape feature and microscopic observation (10×40 times) on bacteria isolated.

Isolation and identification of bacteria

Ten grams of the norfloxacin contaminated soil sample was added to 90 ml sterile distilled water, and the suspension was shaken vigorously at 303.15 K and 120 r/min for 30 min. Serial dilutions (10^{-2}-10^{-4}) were prepared using sterilized distilled water, and 0.1 ml aliquots were inoculated in Petri dishes that contained potato dextrose agar medium (glucose, 20.0 g; potato extract, 200.0 g; agar, 20.0 g; distilled water, 1000 ml), beef extract peptone agar medium (beef extract, 5.0 g; peptone, 10.0 g; NaCl, 5.0 g; agar, 20.0 g; distilled water, 1000 ml; pH 7.2-7.4) and Gause I agar medium (soluble starch, 20.0 g; KNO_3, 1.0 g; NaCl, 0.5 g; $K_2HPO_4 \cdot 3H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 20.0 g; distilled water, 1000 ml). The pH of the media was adjusted to 7.0-7.2 with 0.1 mol/L HCl prior to sterilization (YXQ-LS-30S vertical pressure steam sterilizer, Shanghai Boxun Medical Equipments Co. Ltd., China) at 394.15 K for 20 min. Each medium was supplemented with 10.0 mg/L of norfloxacin to inhibit the growth of Gram-negative bacteria and fungi, respectively.

Following inoculation, the plates were incubated at 303.15 K for up to seven days, and colonies were purified without antibiotics by streaking onto the respective agar medium from which the colonies were isolated, and selected isolates of bacteria were preserved on nutrient agar medium at 277.15K for further experiments. Microbial selection was based on colony morphology, color and presence of diffusible pigments according to Bergey's manual of systematic bacteriology (Malghani et al., 2009).

The selected strain was transferred to mineral salt medium (MSM) (Herman and Frankenberger, 1999) containing 10.0 mg/L norfloxacin in a 250 ml Erlenmeyer flask and incubated on an orbital shaker (Shanghai Yuejin Medical Apparatus Factory) at 303.15 K and 120 r/min for 3-5 days. Then, growth was observed and the culture (1 ml) was shifted to fresh MSM containing 20.0 mg/L norfloxacin. In the same way, the culture was transferred to serially increasing concentrations of norfloxacin up to 30.0 mg/L.

Degradation experiments

Nearly 4.0 g of each soil sample was added to 250 ml conical flask with plug. Following, norfloxacin solution was added up to 50 mg/kg; 1 ml NFX3 bacterial suspension was added or not; appropriate amount of water was reentered up to 60% of maximum water holding capacities in field. Then lucifuge degradation experiments enclosed in constant temperature incubator (Shanghai Yuejin Medical Apparatus Factory) at 298.15K was processed. Thereafter, each soil was sampled at intervals (0, 5, 10, 20, 30, 40 and 60 d), with 100 ml 0.1 mol/L NaOH extracting solution being affiliated to oscillate (140 r/min) and extract at 298.15 K for 24 h. After centrifugal separating (4000 r/min; type LD4-2A, Beijing Medical Appliance Factory), supernatant liquor was taken to determine the norfloxacin concentration using UV spectrophotometry (Diaz-Cruz et al., 2003), and residual quantity of norfloxacin in soil was calculated.

RESULTS AND DISCUSSION

Isolation and identification of bacteria

Five different microorganisms were isolated from the norfloxacin-exposed soil samples by the above mentioned method. All isolates were fungi and had high tolerance to norfloxacin. Among them, strain NFX3 was preliminary identified as *Penicillium frequentans*, and further confirmation will be made by sequencing of their 16 SrRNA gene. The colony shape feature and microscopic observation are shown in Figure 1.

Degradation of norfloxacin with or without NFX3 in soil

Based on the degradation curves of norfloxacin and their correlation coefficients (r = 0.95-0.99), its degradation process could be described by the first order kinetic

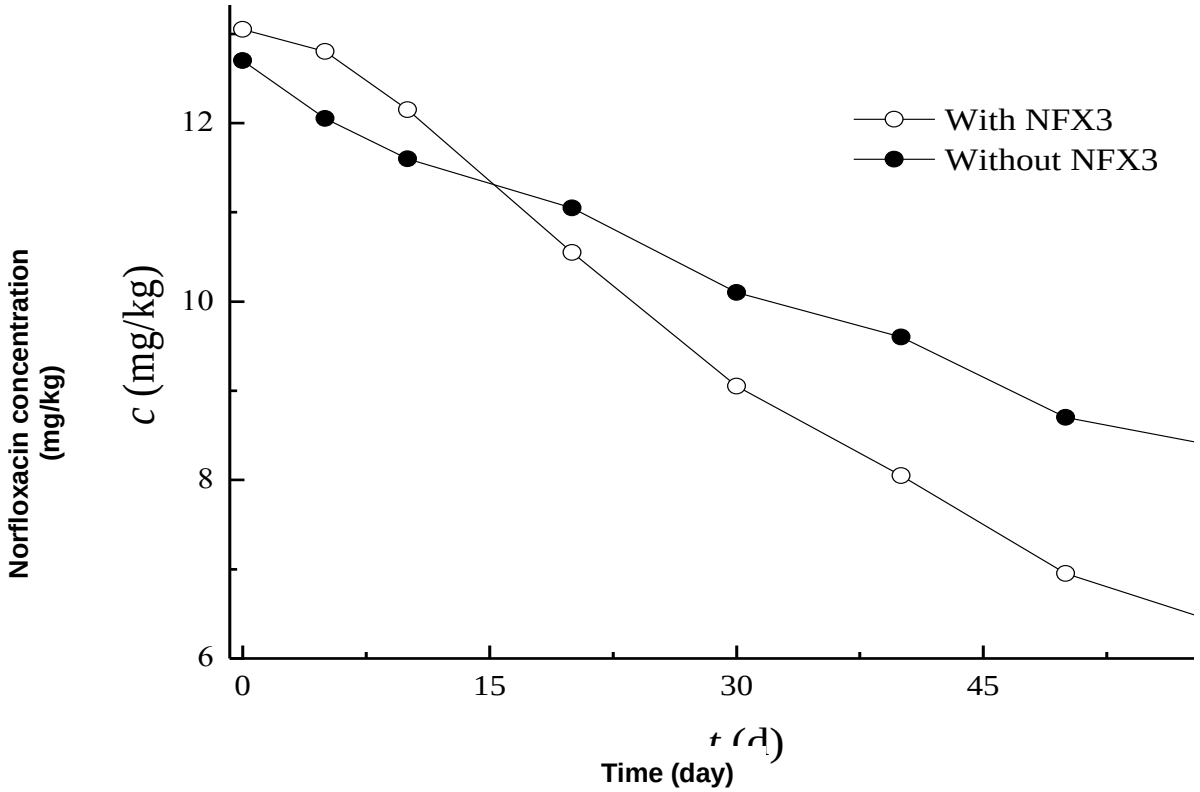


Figure 2. Degradation dynamics of norfloxacin with or without NFX3 in soil. *c* = norfloxacin concentration, *t* = time (day).

Table 1. Kinetic parameters of norfloxacin degradation with or without NFX3 in soil at 298.15 K.

Treatment	$\ln(c_0/c)=kt$	*r*	*k* (day^{-1})	$t_{½}$ (day)
Without NFX3	$\ln(c_0/c) = 0.0072t$	0.9943	0.0072	96.3
With NFX3	$\ln(c_0/c) = 0.0121t$	0.9935	0.0121	57.3

c = norfloxacin concentration; c_0 = initial norfloxacin concentration; *k* = degradation rate constant; *t* = time (day), $t_{1/2}$ half-life (day).

equation (Zhang et al., 2004).

$$\ln \frac{c_0}{c} = kt \quad (1)$$

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

Where, *c* is the norfloxacin concentration at *t*, mg/kg, c_0 is the initial norfloxacin concentration, mg/kg, *k* is the degradation rate constant, day^{-1}, *t* is time, day, $t_{1/2}$ is the half-life, day.

The degradation dynamics of norfloxacin with or without NFX3 in soil at 298.15K is shown in Figure 2 and Table 1. It is shown in Table 1 that the biodegradation of norfloxacin with or without NFX3 were both in accordance with first-order kinetics equation, and the microbial degradation rate constant of norfloxacin with NFX3 in soil was 0.0121 day^{-1}, which is probably dependent on stability and bacterial inhibition for norfloxacin in soil (Bel et al., 2009). From Figure 2, the degradation efficiency of norfloxacin with NFX3 was slightly lower than without NFX3 in the first 15 day, which may be that the NFX3 still could not meet the new living environment; but 15 days later, the former was significantly higher than the latter, NFX3 could have been domesticated and adapted to the soil environment. Faster degradation of norfloxacin occurred after the exponential growth phase, probably due to acetate consumption since the presence of such easily degradable carbon source allowed the increase in the biomass of the degrading strain, accelerating the biodegradation process. The degradation efficiency of norfloxacin with or without NFX3 in soil at 60 day reached 51.3 and 34.2%, respectively, while the initial norfloxacin

concentration was 13.0 g/kg. The results showed that NFX3 promoted significantly norfloxacin degradation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by the Henan Province Natural Science Foundation of China (Project No. 0611033400).

REFERENCES

Baran W, Sochacka J, Wardas W (2006). Toxicity and biodegradability of sulfonamides and products of their photocatalytic degradation in aqueous solutions. Chemosphere 65(8):1295-1299.

Bel ED, Dewulf J, Witte BD, Langenhove HV, Janssen C (2009). Influence of pH on the sonolysis of ciprofloxacin: Biodegradability, ecotoxicity and antibiotic activity of its degradation products. Chemosphere 77(2):291-295.

Diaz-Cruz MS, de Alda MJL, Barcelo D (2003). Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. Trac-Trend Anal. Chem. 22(6):340-350.

Herman DC, Frankenberger WT (1999). Bacterial reduction of perchlorate and nitrate in water. J. Environ. Qual. 28(3):1018-1024.

Kay P, Blackwell PA, Boxall ABA (2005). A lysimeter experiment to investigate the leaching of veterinary antibiotics through a clay soil and comparison with field data. Environ. Pollut. 134(2):333-341.

Malghani S, Chatterjee N, Hu XY, Zejiao L (2009). Isolation and characterization of a profenofos degrading bacterium. J. Environ. Sci. 21(11):1591-1597.

Peng XZ, Wang ZD, Kuang WX, Tan JH, Li K (2006). A preliminary study on the occurrence and behavior of sulfonamides, ofloxacin and chloramphenicol antimicrobials in wastewaters of two sewage treatment plants in Guangzhou, China. Sci. Total Environ. 371(1-3):314-322.

Yu H, Mun H, Hu Y (2012). Determination of fluoroquinolones, sulfonamides, and tetracyclines multiresidues simultaneously in porcine tissue by MSPD and HPLC-DAD. J. Pharm. Anal. 2(1):76-78.

Zhang W, Yu YL, Lin KF, Li SN, Wu JL, Fan DF (2004). Microbial degradation of abamectin in soil. Chin. J. Appl. Ecol. 15(11):2175-2178.

academicJournals

Vol. 13(22), pp. 2240-2247, 28 May, 2014
DOI: 10.5897/AJB2014.13708
Article Number: F8CF54844904
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Bioassay-guided investigation of *Lonchocarpus cyanescens* benth leaves extracts for antioxidant activities

Babatunde Samuel [1, 2]*, Oluwatoyin Adigun[2] and Oluwatosin Adaramoye[3]

[1]Indian Institute of Integrative Medicine, Bioorganic Chemistry Laboratory, Natural Product Chemistry unit, Jammu, India.
[2]Pharmaceutical Chemistry Department, Faculty of Pharmacy, University of Ibadan, Nigeria.
[3]Biochemistry Department, Faculty of Basic Medical Sciences, University of Ibadan, Nigeria.

Received 8 February, 2014; Accepted 16 May, 2014

Antioxidants have been reported to prevent oxidative damage caused by free radicals and can be used to ameliorate conditions in cardiovascular and inflammatory diseases. Ethnobotanical study revealed that the leaves of *Lonchocarpus cyanescens* are traditionally used in Africa to treat ulcer and arthritis. This study investigates the antioxidant activities of its extract and fractions. Acetone leaf extract of *L. cyanescens* was screened for 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging activity, ferric reducing power (FRAP), total phenol content and total flavonoid content using catechin as standard antioxidant. Bioguided column chromatographic separation was carried out and the resultant fractions were screened for antioxidant activities. Preliminary spectroscopic profile of the most active fraction was obtained. DPPH and FRAP methods showed that *L. cyanescens* had antioxidant activity which correlated with its phenolic and flavonoid contents. There was a higher correlation of the total phenol/flavonoid content to the antioxidant activity by the DPPH method [r2 = 0.9906, 9926 respectively] than the FRAP method [0.8635, 8840 respectively]. Bioactivity guided fractionation identified fraction F5 as the most active. ^{1}H and Infrared spectra indicated that the most active fraction contained flavonoids. Comprehensive *in-vivo* studies and toxicity profile of the extract will be required before considerations for development as a phyto-drug.

Key words: *Lonchocarpus cyanescens*, antioxidant, DPPH, FRAP, phenol, flavonoid.

INTRODUCTION

Free radicals formed in the body are chemical species that possess an unpaired electron in the outer valance shell of the molecule. This makes them highly reactive and can complex with major macromolecules including proteins, lipids, carbohydrates and DNA (Evans and Halliwall, 1999). Free radicals may either be oxygen derived (reactive oxygen species, ROS) or nitrogen derived (RNS, reactive nitrogen species) (Devasagayam and Kesavan, 2003). An imbalance between antioxidants and ROS results in oxidative stress (Mohammed et al., 2012). Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, ulcer, arthritis and neurodegenerative disorders

(Peterhans, 1997; Ames et al., 1993).

Studies on medicinal plants and vegetables strongly supported this idea that plant constituents with anti-oxidant activity are capable of exerting protective effects against oxidative stress in biological systems and other health related benefits (Mali et al., 2004; Chaudhary et al., 2004). Recently, there has been growing interest in natural antioxidants of plant origin because they have greater application in the food industry for increasing the stability and shelf life of food products.

Lonchocarpus cyanescens (Schumach. and Thonn.) Benth is a shrub of twinning habit, belonging to the legumes family Fabaceae (Ogungbaro, 2010). Deciduous scandent shrub up to 4 m tall (in cultivation usually up to 2.5 m) or liana up to 20 m long; bark grey to very pale brown, slash yellowish; branchlets silky when young. It is widespread in Western Africa such as Cameroon, Ivory Coast, Sierra Leone, Benin, Nigeria, Togo and Guinea. It is also cultivated, particularly in Sierra Leone and Ghana. The plant is commonly known as West African indigo. The leaves of *L. cyanescens* is traditionally used for the treatment of diseases like arthritis, ulcer, intestinal disorder, dysentery, psychosis and leprosy (Ismot et al., 2013; Mubo et al., 2012; Iwu and Ayanwu, 1982). There is scanty scientific data to support the folkloric use of this plant in many of these disease states. The current study was designed to investigate the antioxidant activities of *L. cyanescens* using *in vitro* assay models which could possibly suggest the pharmacological basis of some of its local applications.

MATERIALS AND METHODS

General methods

Nuclear magnetic resonance (NMR) data were obtained on a Bruker 500 MHZ model using deuterated methanol as solvent and tetra-methyl silane [TMS] as internal standard. Infrared data was obtained from a Bruker Fourier transform infrared (FT-IR) spectro-photometer model Vector 22. Silica gel 60 - 120 mesh was used for the Bioguided separation of bioactive extract. All Spectral data were obtained from the Facilities of Indian Institute of Integrative Medicine, Jammu, India.

Plant material

Fresh leaves of *L. cyanescens* were identified and collected from the University of Ibadan Botanical Garden by Mr. M. K. Owolabi of the Department of Botany (Curator of the Botanical garden of the University of Ibadan). The leaves were air dried and milled into the University of Ibadan). The leaves were air dried and milled into fine powders and kept in airtight containers until use.

Extraction of plant

Powdered plant material (350 g) was extracted with 3.5 L of acetone by cold maceration with intermittent shaking for a period of 72 h. The extract was filtered through Whatman No.1 filter paper and concentrated to a minimum volume of solvent at 45°C with rotary evaporator. The extract was finally concentrated under a stream of cold air to dryness. The weight of the dried extract was determined and then stored in the refrigerator until use.

Thin layer chromatography (TLC) analysis of extracts

The extracts were analyzed using TLC F254 with the following solvent systems: hexane: ethyl acetate (HE) (7:3), chloroform: methanol (CM) (9:1) and chloroform: ethyl acetate: formic acid (CEF) (5:4:1). Compounds were visualized by spraying with vanillin-sulphuric acid spray reagent (0.1 g vanillin, 28 ml methanol and 1 ml sulphuric acid). The plates were heated at 110°C for 5 min to view the spots.

Antioxidant assays

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

Free radical scavenging activity was evaluated using catechin as standard antioxidant. The radical scavenging activity was measured using the stable radical DPPH according to the method described by Hatano et al. (1988). Various concentrations of the extracts were added to 4 ml of methanol solution of DPPH (1 mM, 1 ml). The mixture was shaken and left for 30 min at room temperature in the dark and the absorbance was measured with a spectrophotometer at 517 nm. All determinations were performed in triplicate. The antioxidant activity was calculated as the percent inhibition caused by the hydrogen donor activity of each sample according to the following:

$$\text{DPPH scavenged (\%)} = \frac{\text{A control} - \text{A test}}{\text{A control}} \times 100$$

Where, A control = absorbance of control and A test = absorbance of test sample.

Ferric reducing power (FRAP) assay

This was determined according to the method of Oyaizu (1986). The extract or standard (10-1000 µg) in 1 ml of distilled water was mixed with phosphate buffer (pH 6.6) and potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%, 2.5 ml) was added to the mixture. A portion of the resulting

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

Abbreviations: DPPH, 2,2-Diphenylpicrylhydrazyl**; FRAP,** ferric reducing power**.**

Table 1. Antioxidant activity of *L. cyanescens* extract by DPPH method.

Concentration (µg/ml)	% Antioxidant activity	
	L. cyanescens	Catechin
500	14.83 ± 0.386	17.38 ± 1.655
750	22.75 ± 0.425	21.01 ± 0.850
1000	41.87 ± 2.790	45.95 ± 1.874
1500	63.89 ± 2.730	70.73 ± 3.776

Values are expressed as Mean ± SEM, n=3. Similar activity pattern between *L. cyanescens* extract and catechin was at p>0.05

mixture was mixed with $FeCl_3$ (0.1%, 0.5 ml) and the absorbance was measured at 700 nm ina spectrophotometer. Higher absorbance of the reaction mixture indicated reductive potential of the extract.

Total phenolic content (TPC)

Total phenolic content was determined using the Folin-ciocalteu assay, following the method of Singleton and Rossi (1965). In total, 10 to 1000 µg of the extract (1 mg/ml) was added to 1 ml of Folin ciocalteu reagent. It was allowed to stand for 3 min after mixing. Saturated Na_2CO_3 solution (11 ml) was added and the solution was made up to 10 ml with distilled water. The final mixture was mixed and then incubated for 90 min in the dark at room temperature. A reagent blank was prepared using distilled water. The absorbance was measured spectrophotometrically at 725 nm. Total phenolic values are expressed in terms of catechin equivalents (CE) in milligrams per gram plant extract.

All determinations were made in triplicate. Correlation plots of total phenolic content against the antioxidant activities by DPPH and FRAP models were obtained.

Total flavonoid content (TFC)

The total flavonoid content in extracts was determined according to Jia et al. (1999), briefly, to various concentrations of distilled water and sample, $NaNO_2$ (75 µl, 5%) was added and left to stand for 5 min. Hydrated aluminium chloride (10%, 150 µl) was added and left to stand for another 6 min. NaOH (500 µl, 1 M) and 275 µl of distilled water were added and mixed. The colour intensity was read spectrophotometrically at 500 nm. All determinations were performed in triplicate.

Total flavonoid values are expressed in terms of catechin equivalents (CE) per gram of plant extract from a standard curve. Correlations plots of total flavonoid content against its antioxidant activities by DPPH and FRAP models were obtained.

Column chromatographic separation of extracts

Dried acetone extract (3 g) was separated into different fractions by column chromatographic technique with silica gel as stationary phase. A wet column packing was done and the separation was carried out with hexane and ethyl acetate in the gradient of 90:10 (hexane: ethyl acetate) to 100% ethyl acetate and finally washed with methanol.

The pattern of the separation was monitored by thin layer chromatographic fingerprint and similar patterns were pooled together to obtain six fractions (F1, F2, F3, F4, F5 and F6). ^1H and infrared spectral data were obtained for the most active fraction (F5) to determine the nature of compound it contained.

Bioassay of fractions

Antioxidant properties of the different fractions were determined by measuring the free radical scavenging activity using the stable radical (DPPH) according to the method described above. The comparative antioxidant activities of the fractions were obtained from a statistical plot of their activities at different concentrations.

Statistical analysis

The results are given as mean ± standard error of mean (SEM). The experiments were done in triplicate. Student t-test was used for comparison of two mean values and one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test was applied in the analysis of data with more than 2 mean values. $P < 0.05$ was taken as significant using GraphPad Prism 5 software.

RESULTS

350 g of dried *L. cyanscens* extract yielded 26 g of dried extract (7.4%). The mobile system of hexane: ethyl acetate (5:5) (H: E) was most appropriate for the study.

Antioxidant assay

As shown in Tables 1 and 2, the antioxidant activity of the extract was concentration-dependent. Scavenging effects of *L. cyanescens* on DPPH radical increased with increase in concentration. At 0.50 mg/ml, scavenging effect of *L. cyanescens* was 14.83% while catechin was 17.38%. At 1.5 mg/ml, scavenging effect of *L. cyanescens* was 63.89% and that of catechin was 70.73%. A similar pattern was obtained in the FRAP method as shown in Table 2. *L. cyanescens* extract

Table 2. Ferric reducing-antioxidant power (FRAP) capacity of *L. cyanescens* extract.

Concentration (µg/ml)	*L. cyanescens*	Catechin	T- test (P value)
100	0.069 ± 0.003	0.071 ± 0.008	0.188
500	0.221 ± 0.002	0.314 ± 0.016	0.878
750	0.340 ± 0.020	0.483 ± 0.036	0.032
1000	0.435 ± 0.008	0.610 ± 0.011	0.008

Values are expressed as Mean ± SEM, n=3 in each group. Similar activity pattern between *L. cyanescens* extract and catechin was at p>0.05.

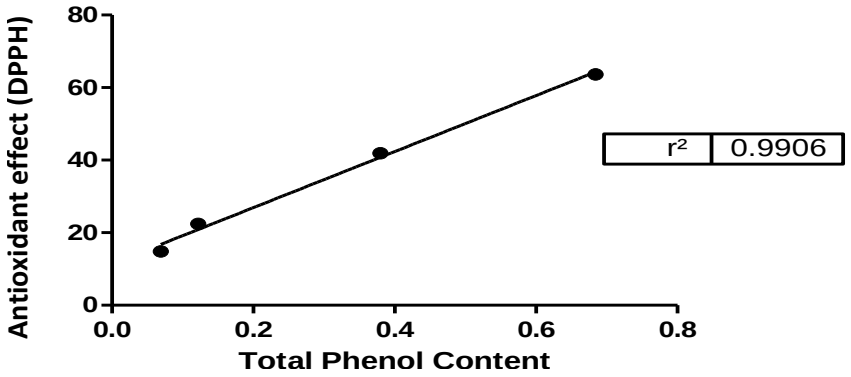


Figure 1. Correlation between DPPH and TPC of *Lonchocarpus cyanescens* benth leaves extracts.

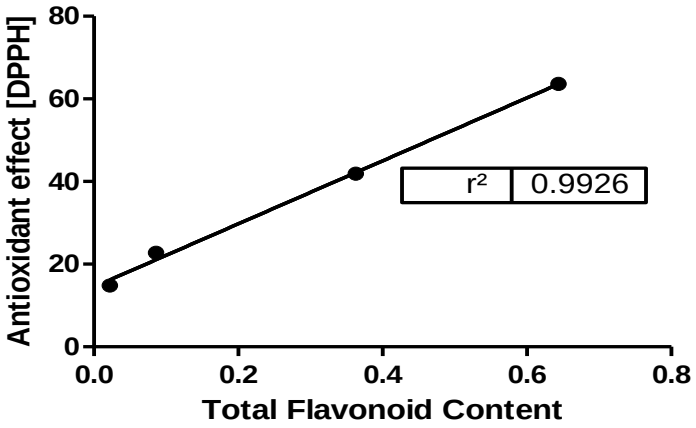


Figure 2. Correlation between DPPH and TFC of *Lonchocarpus cyanescens* benth leaves extracts.

showed comparative antioxidant activity to catechin in DPPH and FRAP methods P>0.05.

Total phenolic content (TPC) and total flavonoid content (TFC)

TPC and TFC of *L. cyanescens* obtained were correlated with the antioxidant activities (DPPH and FRAP). The correlations for TPC and TFC with DPPH are shown in Figures 1 and 2. We observed a strong correlation between antioxidant activity (DPPH method) of the extract and phenol and the flavonoid contents (Figures 1 and 2) with a correlation coefficient of 0.9906 and 0.9926, respectively. While the same pattern was observed with

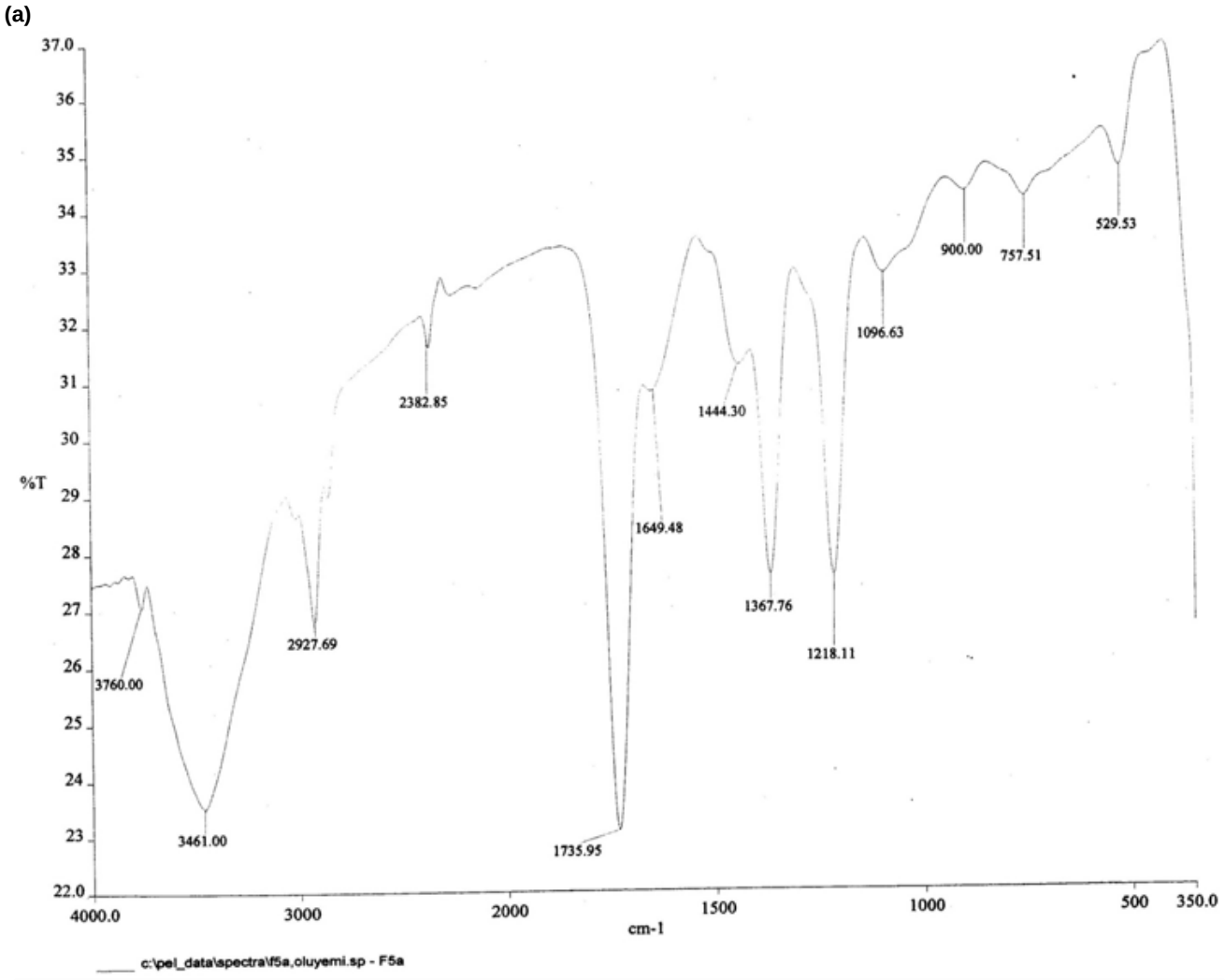


Figure 3a. IR spectra of the most active fraction of *L. cyanescens* [F5].

the FRAP method, there was a lower correlation to the total phenol and flavonoid content observed (0.8635 and 0.8840, respectively) than in the DPPH method.

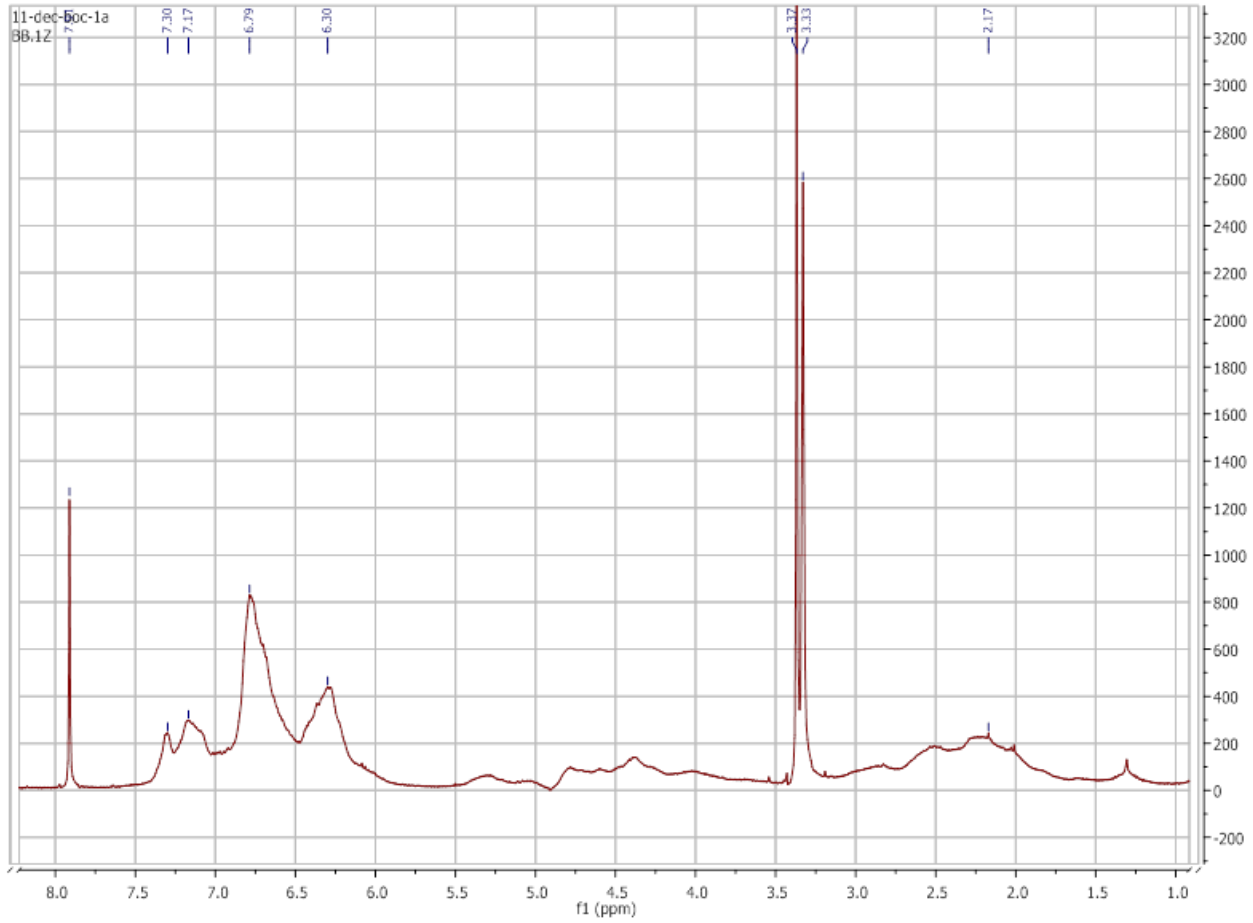
Activity of chromatographic fractions

As shown in Figure 4, the most active fraction was F5. There is a significant difference between the activity of F5 and the other inactive fractions such as F2 and F3 ($p < 0.05$). Fraction F6 also showed significant activity. ^{1}H NMR spectra showed downfield signals between 6 and 8 ppm, while Infrared spectra indicated the following functional groups - OH, C=O and C-O as shown in Figure 3. These signal patterns are associated with flavonoids.

DISCUSSION

DPPH free radical method is based on the determination of the concentration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) at steady state in solution. After the addition of the antioxidant, the concentration of DPPH which absorbs at 517 nm is reduced by the antioxidant; while in the ferric reducing antioxidant power (FRAP) assay, the antioxidant principle is based on the ability of the antioxidant to reduce ferric (III) ions to ferrous (II) ions. The two methods are fast and sensitive methods that are readily applicable in the study of plant extracts for their antioxidant activities in the search for bioactive compounds against diseases associated with oxidative stress (Azizah et al., 2010). The antioxidant activities of

(b)



^{1}H NMR Spectrum

Figure 3b. ^{1}HMR spectra of the most active fraction of *L. cyanescens* [F5].

the extract which is comparable to catechin in both studies highlighted its potential. This is because catechin related compounds have a central place in antioxidant and cancer related research (Menon et al., 1999).

Plant polyphenols which constitute a diverse group of compounds (flavonols, flavanol, stilbenes, phenolic acids, e.t.c) possess an ideal structure for free radical scavenging activity. This family of molecules has a remarkable pharmacological importance as therapeutic agents. They have been used directly or through some of their derivatives in various disease conditions including cancer, arthritic condition, among others (Gomes et al., 2012; Izzi et al., 2012). The fact that they have antioxidant activities by acting through several ways is central in the explanation of their diverse pharmacological actions (Villaño et al., 2007). Very good correlation observed between the phenolic/flavonoid contents and the antioxidant activity confirmed that the observed activity in *L. cyanescens* extract was majorly due to the flavonoid content. The most active fraction as indicated from preliminary spectroscopic investigations (IR and ^{1}NMR) as shown in Figure 3 contained essentially phyto-phenolic compounds (flavonoids). Our findings possibly account for the use of this plant in the treatment arthritic conditions and ulcers (Ismot et al., 2013; Iwu and Ayanwu, 1982).

While there is no report on any characterized flavonoid from this specie [*L. cyanescens*], studies on other species of *Lonchocarpus* genus have led to the isolation of some phyto-phenolic compounds. Jean-Robert et al. (2001) previously isolated some phenolic compounds from *Lonchocarpus chiricanus* which include chiricanine. Cassidy and Setzer (2010) investigated the anti-cancer potential of some flavonoids from *Lonchocarpus haberi*.

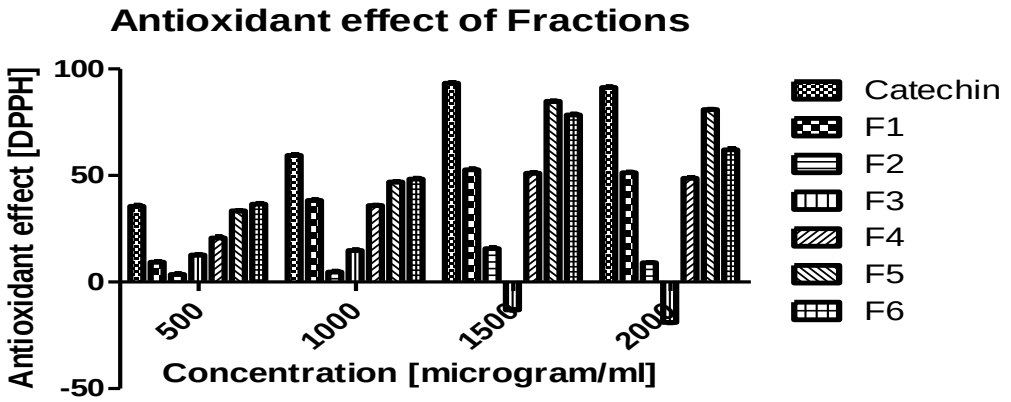


Figure 4. Comparative antioxidant activities of chromatographic fractions by DPPH method.

Such related compounds isolated from the other species in this genus are possibly associated with the antioxidant activity we have observed in the extract of *L. cyanescens.*

In the correlation studies, a better correlation was observed with the DPPH than the FRAP model. The mode of action of the two methods are closely related but several authors have found their sensitivity slightly different, although the difference in the two methods is not statistically significant which is similar to what we observed in this study (Christina et al., 2011).

The fact that more than one fraction (fractions 5 and 6) showed good activity suggests that the mode of action is possibly synergistic. There are possibly several phyto-phenolic compounds in this extract, since our correlation studies showed the antioxidant activities are essentially through this class of compounds. Previous studies on the bioactivity of *Ochna pretoriensis* indicated a similar synergistic pattern (Makhafola et al., 2012). Comparative antioxidant activity of the whole extract and the most active fraction of *L. cyanescens* were 14.83, 41.87, 63.89 and 33.37, 46.79, 84.80 respectively at 500, 1000 and 1500 µg/ml. Some extracts are better applied as whole, but that does not diminish the importance of isolating bioactive compounds from whole extracts because of the potential of obtaining lead compounds for the design and synthesis of drug candidates with increased activity and reduced toxicity.

Conclusion

This study shows that *L. cyanescens* extract have significant antioxidant and free radical scavenging activities. Antioxidant-rich plant extracts serves as sources of neutraceuticals that alleviate oxidative stress and therefore prevent or slow down degenerative diseases. This possibly explains the basis for the local application of this plant in patho-physiological conditions associated with oxidative stress which include ulcer, arthritis, neurodegenerative disorders, among others. ^{1}H and Infra-red spectra indicated the presence of flavonoid in the most active fraction. Further studies are required on the *in vivo* antioxidant activity and toxicity profile of these extract and fractions before its consideration as a phyto-drug.

Conflict of Interests

The author(s) have not declared any conflict of interests

REFERENCES

Ames BN, Shigenega MK, Hagen TM (1993). “Oxidants and the degenerative diseases of ageing” Proc. Nat. Acad. Sci. 90:7915-7922.

Azizah O, Abbe MMJ, Kong KW, Amin I, Nawalyah AG, Ilham A (2010). Epicatechin content and antioxidant capacity of cocoa beans from four different countries. Afr. J. Biotechnol. 9(7): 1052-1059.

Cassidy CE, Setzer WN (2010). Cancer-relevant biochemical targets of cytotoxic Lonchocarpus flavonoids: a molecular docking analysis. J. Mol. Model. 16(2):311-326.

Chaudhary SR, Chavan MJ, Gaud RS (2004). Anti inflammatory and Analgesic activity of *Capparis zeylanica* root extracts. Indian J. Nat. Prod. 20(1): 36-39.

Christina MM, Liviu AM, Daniel SD, Lavinia B (2011). Correlation between Polyphenolic Profile and Antioxidant Activity of Propolis from Transylvania. Anim. Sci. Biotechnol. 44(2):100-103.

Devasagayam TPA, Kesavan PC (2003). “Radio protective and antioxidant action of caffeine: mechanistic considerations, Ind. J. Exp. Biol. 41:267-269.

Evans P, Halliwall B (1999). “Free radicals and hearing” Ann N. Y. Acad. Sci. 884:19.

Gomes A, Couto D, Alves A, Dias I, Freitas M, Porto GA, Duarte JA, Fernandes E (2012)."Trihydroxyflavone with antioxidant and anti-inflammatory efficacy". BioFactors 38(5):378-386.

Hatano T, Edmatsu R, Mori A, Fujita Y, Yasuhara E (1989). Effects of tannins and related polyphenols on superoxide anion radical and on

1,1-diphenyl-2-picrylhydrazyl radical. Chem. Pharm. Bull. 37:2016-2023.
Ismot TA, Mubo AS, Solomon U (2013). Antipsychotic property of solvent-partitioned fractions of *Lonchocarpus cyanescens* leaf extract in mice. J. Basic Clin. Physiol. Pharmacol. pp. 1-6.
Iwu MM, Anyanwu BN (1982). “Phytotherapeutic Profile of Nigerian Herbs, 1: Anti-Inflammatory and Anti-Arthri- tic Agents,” J. Ethnopharmacol. 6(3):263-274.
Izzi V, Masuelli L, Tresoldi I, Sacchetti P, Modesti A, Galvano F, Bei R (2012). "The effects of dietary flavonoids on the regulation of redox inflammatory networks". Frontiers in bioscience (Landmark edition) 17(7):2396-2418.
Jean-Robert L, Andrew M, Mahabir PG, Kurt H (2001). Five New Prenylated Stilbenes from the Root Bark of *Lonchocarpus chiricanus*. J. Nat. Prod. 64:710-715.
Jia Z, Tang M, Wu J (1999). The determination of Flavonoid contents in mulberry and their scavenging effect on superoxide radicals. Food Chemistry 64:555-559.
Makhafola TJ, Samuel BB, Elgorashi EE, Eloff JN (2012). Ochnaflavone and Ochnaflavone 7-*O*-methyl Ether: two Antibacterial Biflavonoids from *Ochna pretoriensis* (Ochnaceae). Natural Products Communications 7(12):1601-1604.
Menon LG, Kuttan R, Kuttan G (1999). Anti-metastatic activity of curcumin and catechin. Cancer Lett. 141(1-2):159-65.tr
Mohammed FA, Srinivasa R, Shaik RA, Mohammed I (2012). Pytochemical studies and antioxidant activity of Mella azedarach linn leaves by DPPH Scavenging Assay. Int. J. Pharmaceutical Appl. 3:271-276.
Mubo AS, Solomon U, Esther TS (2012). Antipsychotic property of aqueous and ethanolic extracts of *Lonchocarpus cyanescens* (Schumach and Thonn.) Benth. (Fabaceae) in rodents. 66 (1):127-132.
Ogungbaro ST (2010). *In-vitro* study of the effects of ethanolic extract of *Lonchocarpus cyanescens* and *Crassocephalum crepidioides* leaf extracts on monoamine oxidase activity from rat brain; University of Agriculture, Abeokuta thesis.
Oyaizu M (1986). Studies on product of browning reaction prepared from glucose amine. Japan J. Nutr. 44:307-315.
Peterhans E (1997). “Oxidants and antioxidants in viral diseases; disease mechanisms and metabolic regulation. J. Nutr. 127:962.
Singleton VL, Rossi JA (1965). Colorimetry of total Phenolics with phosphotungstic acid reagents. American J. Enol. Vitic. 16:144-158.
Villaño D, Fernández-Pachón MS, Moyá ML, Troncoso AM, García-Parrilla MC (2007) Radical scavenging ability of polyphenolic compounds towards DPPH free radical. Talanta. 71(1):230-235.

academicJournals

Vol. 13(22), pp. 2248-2259, 28 May, 2014
DOI: 10.5897/AJB2013.13479
Article Number: 4D0173944905
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

High xylanase production by *Trichoderma viride* using pineapple peel as substrate and its apllication in pulp biobleaching

Fortkamp, D. and Knob, A.*

Department of Biological Sciences, Midwest State University. Camargo Varela de Sá Street, 03, zip code 85040-080, Guarapuava, PR, Brazil.

Received 18 November, 2013; Accepted 16 May, 2014

Xylanases are hydrolases which depolymerize the xylan components present in plants cell wall. Commercial applications for these enzymes include its use in the pulp bleaching, food and animal feed industries, among others. Recently, there is a great interest on the exploitation of agro-industrial wastes as low-cost raw materials for value-added compounds production, as xylanolytic enzymes for industrial applications. This is the first report about the xylanase production using pineapple peel as substrate. The xylanase production by *Trichoderma viride* was optimized and the enzymes produced were biochemically characterized. Additionally, the effect of these enzymes on pulp biobleaching process was evaluated. High xylanase production was obtained with pineapple peel at 2% concentration, for seven days, in stationary cultivation at 28°C, in pH 7.5. Xylanases were more active at 50°C, pH 6.0-6.5 remaining stable at pH 5.0-5.5. *T. viride* xylanase was stable at 40°C, showing the half-life ($T_{1/2}$) value of 255 min. The enzyme was remarkably stimulated by Mg^{2+} and Zn^{2+}, while Pb^{3+} and Hg^{2+} were strong inhibitors of the xylanase activity. This work shows the ability of the filamentous fungus *T. viride* to produce high levels of xylanases using pineapple peel as substrate, an inexpensive and abundant agro-industrial waste. Therefore, the reduction on kappa number achieved in this investigation revealed the application potential of these enzymes in biobleaching process.

Key words: Agro-industrial wastes, filamentous fungi, submerged fermentation, pulp pretreatment, xylanolytic enzymes.

INTRODUCTION

After cellulose, xylan is the most abundant polysaccharide present in wood, agricultural and several agro-industrial wastes. This complex heteropolysaccharide consists of a main chain of 1,4-β-D-xylose monomers which is partially acetylated and substituted in different degrees by a variety of side chains, mainly single α-D-glucuronosyl and α-L-arabinosyl units (Collins et al., 2005; Wakiyama et al., 2008). Due to its structural

*Corresponding author. E-mail: knob@unicentro.br. Tel: 55-42-36298133. Fax: 55-42-36211090.

complexity, several hydrolases are required for complete degradation of xylan. The main enzymes involved in xylan degradation are the endo-β-(1,4)-xylanases. These enzymes cleave the internal β-(1→4) bonds in the xylan backbone at non-modified residues, yielding different chain length substituted xylooligosacharides (Zhang et al., 2007; Sharma and Kumar, 2013).

Recently, xylanases have been extensively studied due to their industrial applications (Buthelezi et al., 2011). An example is the bioconversion of lignocellulosic residues in their constituent sugars. These enzymes have attracted attention due to their role in the production of xylose which can be converted into bioethanol and xylitol (Laxmi et al., 2008).

Other industrial applications include the improvement of digestibility of animal feedstock, clarification of wines and juices and the baking of rye to improve the bread volume and crumb structure (Romanowska et al., 2006; Khandeparker and Numan, 2008; Gupta and Kar, 2009; Albert et al., 2011). In the last years, the use of xylanases as bleaching agents of wood kraft pulps has been considered the main industrial application of xylanolytic enzymes. Many studies have demonstrated that the pulp treatment with xylanases promotes the release of lignin from the pulp, thereby reducing the chlorine required in this process (Khandeparker and Numan, 2008; Gupta and Kar, 2009; Albert et al., 2011).

The agro-industrial residues are composed of lignocellulosic material and are generated in large quantities in agribusiness, food, wood, pulp and paper industries, among others. These wastes contain many high-value constituents, such as carbohydrates and fibers.

Also, current trends emphasize that agro-industrial wastes have potential for microbial enzymes production. However, these potentially valuable materials are often disposed in the environment without an adequate treatment, increasing environmental damage (Dashtban et al., 2009).

The global market for enzymes is expected to reach 8.0 billion in 2015 (Li et al., 2012). However, the production cost of xylanases is the major factor limiting its use, requiring the development of low cost production systems (Buthelezi et al., 2011). According to Widjaja et al. (2009) and Ali et al. (2012), the cost of the industrial enzymes can be decreased by the establishment of the optimal conditions for its production, including the use of agro-industrial wastes as substrates.

Pineapple waste is one of the agro-industrial residues which represent a serious environmental pollution (Rani and Nand, 2004). The peel is rich in cellulose, hemicellulose and other carbohydrates with a substrate potential generation of methane by anaerobic digestion. Brazil is the second largest pineapple producer in the world, reaching a volume of 2.318.120 tons (FAOSTAT, 2013). Thus, a large amount of pineapple peel waste is generated over the years. However, the pineapple peel waste has received little attention and its use is limited mainly in animal feed (Tran, 2006; Paengkoum et al., 2013). *Trichoderma viride* is an excellent biocontrol agent (Reena et al., 2013), cellulolytic microorganism (Mojsov, 2010) and xylanase producer (Goyal et al., 2008; Simões et al., 2009).

The aims of this study were to produce xylanases from a *T. viride* strain on agro-industrial wastes and bio-chemically characterize these enzymes. Additionally, the potential application of *T. viride* xylanases in pulp bleaching process was evaluated.

MATERIALS AND METHODS

Fungal strain and culture conditions

T. viride J40 was isolated from the Atlantic forest soil on Ecologic Station Juréia-Itatins, located in São Paulo State, Brazil. The strain belongs to the culture collection of the Environmental Studies Center - CES/UNESP, Brazil.

The fungus was propagated on Vogel's solid medium (Vogel, 1956) containing 1.5% (m/v) glucose and 1.5% (m/v) agar, at 28°C, during 7 days for conidia production. After, the strain was maintained at 4°C. Submerged fermentation was prepared in 125 ml flasks containing 25 ml of the Vogel's medium, pH 6.5, supplemented with 1% carbon source and inoculated with 1.0 ml conidia suspension (7×10^7 conidia/mL) to each flask. The cultures were incubated at different conditions for the xylanase production optimization. After incubation, cultures were filtered by vacuum through filter paper Whatman number 1. The crude filtrate was assayed for extracellular activity and total secreted protein. All cultures were carried out in duplicate to verify the reproducibility and the results are presented through mean values.

Enzyme assay

For xylanase assay, 20 µL of the crude filtrate, 480 µL of the McIlvaine buffer pH 6.5 and 750 µL of 1% birchwood (*Betula* spp.) xylan solution prepared in the same buffer were incubated for 5 min at 50°C. After pre-determined periods, the reaction was stopped by the addition of 250 µL of 3,5-dinitrosalicylic acid. The reducing sugars liberated were measured according to Miller (1959), using xylose as standard. For this, the sample was heated at 100°C for 5 min and allowed to cool to room temperature in ice bath. After that, 2.5 mL of distilled water was added and the absorbance was determined in spectrophotometer at 540 nm. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugar per min, under assay conditions. Specific activities were expressed as enzyme units per milligram of secreted protein. All determinations were developed in triplicate and the results were presented as mean values.

Total protein determination

In order to determine the fungal growth, the intracellular protein was measured. The mycelium was frozen and macerated with sand in McIlvaine buffer pH 6.5. The slurry was centrifuged at 3.900 x *g* and the supernatant was used as an intracellular protein source. As previously mentioned, the crude filtrate was used as a source of extracellular protein. In both cases, the total protein concentration was determined by modified Bradford method (Sedmak and Grossberg, 1977), using bovine serum albumin (BSA) as standard.

For this, the sample appropriately diluted was mixed with the Bradford reagent. The samples were then incubated at room temperature for 5 min. After that, the absorbance was determined in spectrophotometer at 595 nm. All determinations were developed in triplicate and the results were presented as mean values.

Culture conditions for xylanase production

Agro-industrial wastes

The agricultural wastes wheat bran (*Triticum* spp.), apple peel (*Malus* spp.), brewer's spent grain (*Hordeum vulgare*), passion fruit peel (*Passiflora edulis*), orange peel (*Citrus sinensis*), rice peel (*Oryza sativa*), soybean peel (*Glycine max*) and pineapple peel (*Ananas comosus*) were obtained locally. The residues were prepared by exhaustive washing with distilled water, dried at 80°C for 24-48 h and milled (35 mesh).

Enzyme production on different carbon sources

In order to verify xylanase induction, the Vogel's liquid medium was supplemented with various dried substrates as sole carbon source at a concentration of 1% (w/v). The inoculated flasks were incubated at 28°C under stationary condition, for five days. Xylanase activity was determined as described previously. After the selection of the best agro-waste for xylanase production, some concentrations of this carbon source were evaluated from 0.5 to 3.0% (w/v).

Effect of incubation period, initial pH and temperature on xylanase production

The incubation period influence on xylanase production was evaluated in stationary culture for 9 days. The effect of initial pH on the enzyme production was evaluated from 3.0 to 9.0 and the cultivation temperature influence was verified from 20 to 30°C. The initial pH values were adjusted by the addition of 1.0 M sodium hydroxide or hydrochloric acid solutions.

Enzyme characterization

Optimum pH and temperature xylanase activity

The optimum temperature was determined by measuring the activity at temperatures ranging from 25 to 75°C in McIlvaine buffer pH 6.5. In order to establish the optimum pH of the enzyme, the relative activity was determined in different pH values, using McIlvaine buffer pH 3.0-8.0, at the optimum temperature.

Xylanase thermostability and pH stability

The thermal stability was measured by incubating the crude filtrate for different periods at 40, 45 and 50°C. Following incubation, the enzyme solution was frozen and the remaining activity was determined. For pH stability assays, the culture filtrate was diluted (1:2 v/v) in McIlvaine buffer for pH range from 3.0-8.0. The samples were incubated at 4°C for 24 h. After this period, the residual xylanase activity was assayed under optimal conditions.

Effect of ions and other substances on xylanase activity

The enzymatic activity was measured in presence of different ions ($BaCl_2$, $MgSO_4$, $ZnSO_4$, NH_4Cl, $Co(NO_3)_2$, $MnSO_4$, $CuSO_4$, $CaCl_2$, sodium citrate, NaCl, $Pb(CH_3COO)_2$ and $HgCl_2$ and substances [glycerol, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol] at 2 and 10 mM concentrations. The enzyme assay was performed in optimal conditions and the relative activities were expressed as a percentage against the control.

Optimization of kraft pulp pretreatment with *T. viride* xylanases

Xylanases produced by *T. viride* under optimized submerged fermentation conditions were used for biobleaching of *Eucalyptus grandis* kraft pulp in order to evaluate its potential use in this process. The optimization of kraft pulp pretreatment was carried out in completely randomized block design with different enzyme dosages, temperature, reaction time and pH as individual treatments, in triplicate. For this, the oxygen pre-bleached pulp at 10% (w/v) consistency was pretreated with *T. viride* crude extract in polyethylene bags, with a xylanase dosing of 10 U/g oven dried pulp, at 50°C, in pH 6.0, for 60 min, except the variable parameter. An untreated pulp sample was also incubated simultaneously under these conditions and used as control.

After the treatment, the cellulose pulps were filtered on a Büchner funnel, rinsed with 200 mL of distilled water and used for pulp properties determination. The optimum pretreatment conditions were determined by measuring kappa number and viscosity parameters. These procedures were conducted according to the standard methods of Technical Association of the Pulp and Paper Industry (TAPPI test methods, 1996). The data were submitted to one-way analysis of variance and compared through the Tukey test, using the Statistical Analysis Software (SAS).

RESULTS AND DISCUSSION

Influence of the carbon source and its concentration on the xylanase production

The use of agro-industrial wastes as carbon source in the growth medium is an alternative to reduce the costs and increase the enzyme production. Thereby, the xylanase production by *T. viride* using different agro-industrial wastes was investigated (Table 1). The carbon source that induced the highest enzymatic activity and fungal growth was pineapple peel, corresponding to 35.12 U/mL and 20.38 mg protein, respectively. *T. viride* was also able to produce high levels of xylanase in the presence of wheat bran and brewer's spent grain, corresponding to 29.39 and 28.64 U/ml. At the present date, only cellulose production using pineapple peel as substrate was described in the literature (Folakemi et al., 2008; Saravanan et al., 2013). Meenakshi et al. (2008) found maize straw as the best inducer for xylanase production by other *T. viride* strain, reaching 6.24 U/ml, whereas sugarcane bagasse and wheat bran were established as the best substrates for xylanase production by distinct *Trichoderma reesei* strains (Irfan and Syed, 2012; Kar et al., 2013).

Rice peel provided minimal fungal growth, with intermediate levels of xylanase produced (9.95 U/ml). No significant levels of xylanase were observed with orange peel when compared to the other lignocellulosic

Table 1. Influence of agro-industrial wastes on xylanase production by *T. viride*.

Carbon source (1% w/v)	Intracellular protein (mg)	Enzymatic activity (U/mL)	Specific activity (U/mg extracellular protein)
Wheat bran	13.05 ± 0.89	29.39 ± 1.63	31.48 ± 2.78
Apple peel	22.56 ± 2.52	14.27 ± 1.14	13.51 ± 1.62
Brewer's spent grain	8.25 ± 2.15	28.64 ± 2.26	38.13 ± 6.65
Passion fruit peel	20.04 ± 1.09	11.08 ± 1.14	14.32 ± 0.89
Orange peel	9.75 ± 0.61	1.53 ± 0.15	2.06 ± 0.21
Pineapple peel	20.38 ± 1.58	35.12 ± 1.27	24.79 ± 1.32
Rice peel	1.53 ± 0.02	9.95 ± 0.59	33.52 ± 5.85
Soybean peel	2.89 ± 0.12	15.21 ± 1.02	12.74 ± 0.85

Average and standard deviation of two cultures.

Table 2. Effect of pineapple peel concentration on xylanase production by *T. viride*.

Carbon source (% w/v)	Intracellular protein (mg)	Enzymatic activity (U/mL)	Specific activity (U/mg extracellular protein)
0.5	7.75 ± 0.39	10.68 ± 0.11	21.83 ± 3.04
1.0	20.38 ± 1.58	35.12 ± 1.27	24.79 ± 1.32
1.5	19.57 ± 0.64	45.36 ± 1.57	27.88 ± 2.06
2.0	35.35 ± 1.73	48.05 ± 1.89	23.56 ± 1.2
2.5	14.27 ± 0.17	36.47 ± 2.71	14.5 ± 0.78
3.0	8.49 ± 0.89	31.77 ± 1.78	19.01 ± 1.27

Average and standard deviation of two cultures.

materials, although *T. viride* has shown a good development in this substrate. The differences verified on xylanases levels produced with various agro industrial wastes are possibly related on distinct composition and the accessibility of the substrates. Considering the high xylanase production obtained with pineapple peel, this waste was selected for the subsequent optimization experiments.

The most efficient concentration of pineapple peel to induce *T. viride* xylanase production and fungal growth was 2% (w/v), with 48.05 U/ml and 35.35 mg protein, respectively (Table 2). High xylanase levels were produced by *Trichoderma atroviride* when 3.0% (w/v) of untreated sugarcane bagasse was used as substrate, after 3-4 days of fermentation (Grigorevski-Lima et al., 2013). Sanghvi et al. (2010) found that an increase in concentration of substrate lead to a decrease in xylanase activity produced by *Trichoderma harzianum.* According to Gupta et al. (2009), a high substrate concentration let to increased viscosity, which influenced medium components and oxygen transfer.

Effects of culture conditions on xylanase production

Culture conditions are essential for the successful production of an enzyme. For this reason, optimization of these parameters is important for a process improvement. In stationary culture, with pineapple peel as carbon source, the highest extracellular xylanase production as units per volume and specific activity were obtained in 7-day-old cultures, corresponding to 54.15 U/mL and 37.33 U/mg protein, respectively (Figure 1a). The maximal fungal growth measured by the intracellular protein concentration was also observed in 7^{th} day, corresponding to 56.85 mg of protein (not shown). Likewise, Kar et al. (2006) verified that xylanase production by *T. reesei* SAF3 exhibited a similar increase following the biomass peak. In addition, Norazlina et al. (2013) achieved a gradual increase of xylanase levels produced by *Trichoderma* sp. from the second day to the sixth day of the fermentation process.

Sanghvi et al. (2010) observed that the xylanase production by *T. harzianum* with wheat straw increases up to 12 days of cultivation. Generally, a further increase in incubation time resulted in a decrease in enzyme production. The decline in xylanase production observed in the present study may be due to the susceptible portion of xylan molecules that are rapidly digested and only the crystalline portions remains (Jing-Min et al., 1998).

Temperature and pH are important environmental

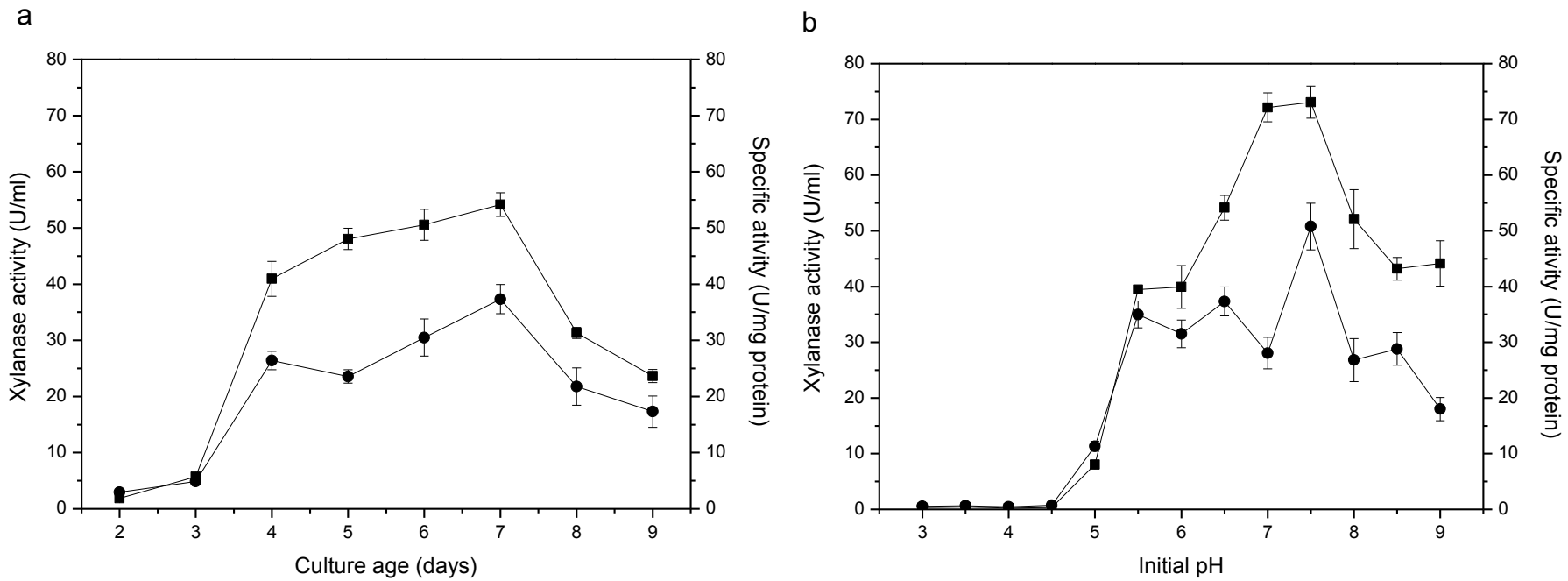


Figure 1. Time-course (a) and initial pH (b) effect on xylanase production by *T. viride*. (■) xylanase activity (U/mL); (●) specific xylanase activity (U/mg of protein). Culture conditions: Vogel medium with pineapple peel 2% (w/v), at 28°C. **(a)** pH 6.5; **(b)** stationary condition for seven days.

parameters that determine growth rates of microorganisms and significantly affect the level of xylanases produced. The influence of pH on *T. viride* xylanase production is showed in Figure 1b. Xylanase production was verified in all pH evaluated. The highest activity in units per volume was observed in pH from 7.0 to 7.5 (72.15 and 73.09 U/ml, respectively). Similarly, the maximal fungal development was verified in the same pH range, corresponding to 59.46 mg protein and 58.03 mg protein (not shown). The xylanase production at neutral pH values has been reported previously in *T. reesei* cultivated on the xylan based media (Bailey et al., 1993; Xiong et al., 2004). On the other hand, the optimum pH value for the growth and xylanase production by *Trichoderma* sp. T-1 and T-2 were found to be 5.5 and 5.7, respectively (Mohan et al., 2011). According to Bailey et al. (1993), the optimal pH medium for xylanase production depends not only on the fungal strain considered, but also on the nature of the carbon source in the cultivation medium.

The effect of temperature on xylanase production by *T. viride* is presented in Table 3. The maximum value of xylanase production and highest specific activity were verified at 28°C, corresponding to 73.09 U/mL and 50.78 U/mg protein. It is remarkable that the optimum temperature for the fungal growth and xylanase production correspond to the environmental temperature which the fungus was initially isolated. Meenakshi et al. (2008) achieved the maximum xylanase production by *T. viride* at 25°C, whereas *Trichoderma* sp. showed enhanced xylanase production at 30°C (Pang et al., 2006).

Significantly higher levels of xylanase were obtained after optimization in this study, corresponding to 73.09 U/mL. The production levels of xylanase verified in this work are higher than many reported in the literature with other agro-industrial wastes. The titers of xylanase produced by a *T. virde* strain was grown in a medium containing maize straw corresponding to

Table 3. Effect of different temperatures on xylanase production by *T. viride*.

Temperature (°C)	Intracellular protein (mg)	Enzymatic activity (U/mL)	Specific activity (U/mg extracellular protein)
20	21.57 ± 0.61	36.62 ± 2.05	14.10 ± 1.09
25	28.43 ± 0.51	37. 25 ± 2.83	16.85 ± 2.20
28	58.02 ± 2.35	73.09 ± 2.87	50.78 ±3.25
30	50.64 ± 3.76	49.46 ± 0.98	31.08 ± 1.24

Average and standard deviation of two cultures.

4.6 U/mL (Goyal et al., 2008). *T. harzianum* presented maximum xylanase production of 26.5 U/mL, upon induction with melon peel (Seyis and Aksoz, 2005). On the other hand, when *T. atroviride* was cultivated in sugarcane bagasse, a xylanase titer of 61.3 U/mL was observed (Grigorevski-Lima et al., 2013). Pineapple peel constitutes a renewable resource and can serve as an abundant and inexpensive carbon source. As a result, the use of the above-mentioned waste in the production of xylanase by *T. viride* would decrease the cost of production in an environmentally sound manner.

The crude filtrate from *T. viride* produced with pineapple peel obtained under optimized conditions was evaluated for the presence of cellulolytic enzymes. It was cellulase-free as it exhibited negligible cellulase activity, suggesting that the production of cellulolytic and xylanolytic enzymes is under separate regulators control (Biely et al., 1993). Similarly, no detectable cellulase activity was noted during whole cultivation period of *T. reesei* on xylan containing enriched medium (Kar et al., 2006) and when this fungal specie was cultivated in presence of rice straw (Soroor et al., 2013). However, according to Goswami and Pathak (2013), fungal xylanases generally are associated with cellulases. Thus, the absence of cellulase activity in the crude extract of *T. viride* is an important feature, which enables its application in industrial process which cellulase activity is undesirable, such as pulp, paper and textile industries.

Biochemical properties of *T. viride* xylanase

Xylanase from *T. viride* showed maximal activity at 50°C (Figure 2a). This temperature has been reported as optimal for xylanases from many fungal species, including *Trichoderma* spp. (Irfan and Syed, 2012; Lopes et al., 2013). Conversely, the optimal temperature for *T. harzianum* xylanases was 60°C (Seyis and Aksoz, 2005; Ahmed et al., 2012).

T. viride xylanase exhibited optimal activity at pH 6.0-6.5 (Figure 2b). Most xylanases presents optimal activity in pH between 5.0 and 7.0 (Madlala et al., 2001) and among the acidophilic xylanases, majority of them showed high activity only under slight acid conditions, as verified in this study. Irfan and Syed (2012) determined pH 5.0 as optimal for xylanases produced by *T. viride.* The same was observed for *T. harzianum* xylanases (Ahmed et al., 2012).

The enzyme stability remains a critical aspect for its biotechnological applications. For this reason, thermal and pH stability of xylanase produced by *T. viride* were investigated. The enzymatic preparation was incubated without substrate at 40, 45 and 50°C. *T. viride* xylanase was stable at 40°C, showing the half-life ($T_{1/2}$) value of 255 min (Figure 3a). This enzyme is more thermostable that other fungal xylanases, such as those from *Trichoderma longibrachiatum* strains (Chen et al., 1997; Medeiros et al., 2003).

The xylanase produced by *T. viride* was stable in acid conditions (Figure 3b). High stability (93.88 and 85.25%) was observed in pH 5.0 and 5.5, while low residual activity (less than 50%) was observed at pH 3.0, 7.5 and 8.0. In the range of pH 6.5-7.0, around 50% of its initial activity was maintained. Usually, microbial xylanases are stable over a wide pH range (3-10) (Kulkarni et al., 1999). Irfan and Syed (2012) verified that xylanases produced by another *T. viride* strain showed pH stability in the range 4.0-70, whereas *T. reesei* xylanase showed stability at pH values of 3.0 to 7.5 (Huang et al., 2013). The biochemical properties exhibited by *T. viride* xylanase such as optimum activity in slight acid conditions and pH stability are attractive for some industrial applications, such as in pulp and paper industry, in which optimal activity at pH 6.0 is required (Polizeli et al., 2005; Ahmed et al., 2012).

In order to verify the effect of substances on xylanase activity, the crude filtrate was incubated in the presence of several metallic ions and chemical reagents, at 2 and 10 mM concentrations (Table 4). *T. viride* xylanase was remarkably stimulated when incubated with Mg^{2+} and Zn^{2+}. The ion Mg^{2+} also activated the xylanase from another *T. viride* strain (Irfan and Syed, 2012), whereas *Trichoderma* sp. xylanase was activated by Zn^{2+} (Sathiyavathi and Parvatham, 2013). The slight activation of *T. viride* xylanases observed in the presence of Ba^{2+} and NH_4^+ may be explained by the enzymatic structure stabilization by these ions. The requirement of monovalent cations for activities of a number of different enzymes has been reported in the literature. However, NH_4^+ activation is a property not common to all previously described *Trichoderma* spp. xylanases.

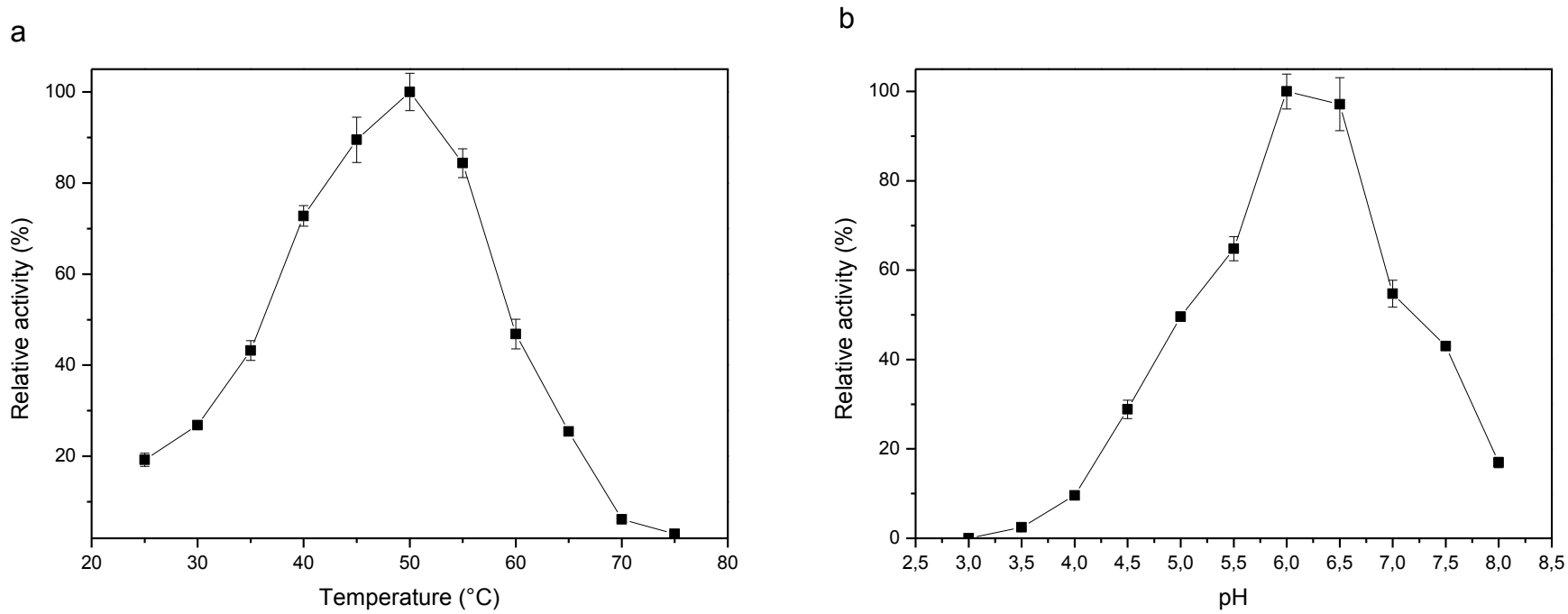


Figure 2. Influence of temperature (a) and pH (b) on xylanase activity from *T. viride*. Culture condition: Vogel medium with 2% pineapple peel (w/v) under stationary condition for 7 days, pH 7.5 at 28°C. **(a)** Xylanase activity was assayed with McIlvaine buffer pH 6.0 and **(b)** with McIlvaine buffer from pH 3.0 to 8.0, at 50°C.

T. viride xylanase showed enhanced activity in the presence of the reducing agent β-mercaptoethanol. This fact can be explained by preventing the oxidation of sulfidryl groups by this thiol group-protecting agent. Medeiros et al. (2003), Franco et al. (2004) and Soroor et al. (2013) also verified the involvement of cysteine residues in the maintenance of enzyme active conformation in *T. longibrachiatum*, *T. harzianum* and *T. reesei* xylanases, respectively.

Cu^{2+} and Hg^{2+} were strong inhibitors of xylanase activity, while Ca^{2+}, sodium citrate, Na^{+}, glycerol and Pb^{3+} had a moderate inhibitory effect. Similarly, *T. reesei* xylanases were inhibited by Cu^{2+} and Hg^{2+} (Soroor et al., 2013). The inhibition by Hg^{2+} seems to be a general property of xylanases, indicating the presence of thiol groups of cysteine residues in enzyme active sites or around them. Cu^{2+} ions are known to catalyze auto-oxidation of cysteines, which leads to the formation of intramolecular and intermolecular disulphide bridges (Vieille and Ziekus, 2001). The inhibitory effect of Cu^{2+} could be explained by the presence of sulfhydryl group in the catalytic center of these enzymes.

T. viride xylanase was also inhibited by SDS and EDTA, indicating that hydrophobic interactions are important for tertiary structure maintenance and metals ions are required for its action, respectively. Likewise, the xylanases from another *T. viride* strain also were inhibited by EDTA, exhibiting 26% of initial xylanase activity at 10 mM concentration (Irfan and Syed, 2012). In contrast, EDTA at a 5 mM concentration has a weak inhibitory effect on *T. reesei* xylanases (Soroor et al., 2013).

Kraft pulp biobleching using *T. viride* xylanases

In recent years, the use of xylanase in pulp

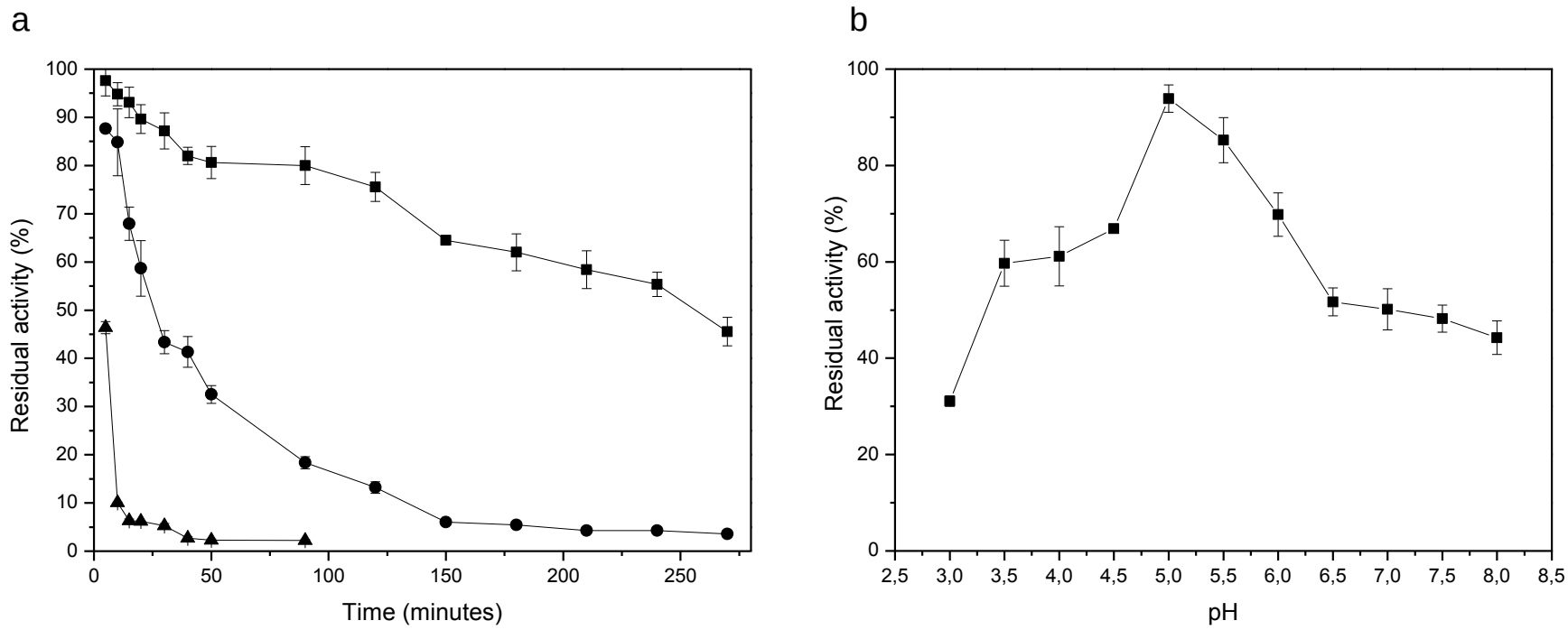


Figure 3. Thermal (a) and pH (b) stability of xylanase activity from *T. viride*. **(a)** The enzymatic preparation was incubated at (■) 40, (●) 45 and (▲) 50 without substrate. **(b)** The enzymatic preparation was incubated without substrate with McIlvaine buffer from pH 3.0 to 8.0 at 4°C for 24 h. In both assays, the residual xylanase activity was assayed with McIlvaine buffer, pH 6.0 at 50°C.

pretreatment has been given special attention as it may reduce the chlorine compounds consumption by up to 30%, so that a 15-20% reduction in organochlorines in the effluents can be achieved (Polizeli et al., 2005). For this reason, the application of *T. viride* crude extract in kraft pulp biobleaching was investigated and optimized. During optimization, the pulp properties kappa number and viscosity were determined. A decrease in kappa number and viscosity maintenance would indicate a better efficiency of the xylanase treatment.

The effect on enzyme dosage on the pretreatment process was investigated in pH 6.0, at 50°C, for 60 min. The data in Table 5 reveal that the least mean kappa number was verified with enzyme dosage of 10 U/g dried pulp (12.99), followed by 15 (13.02) and 20 U/g dried pulp (13.01). However, these values are not statically different. Similarly, Nagar et al. (2013) did not found any differences in kappa number between treatments using 10, 12.5 and 15 U/g dried pulp. These results are in accordance with several studies that established an enzyme dosage of 10 U/g dried pulp as optimum for pretreatment of hardwood kraft pulp (Dhiman et al., 2009; Garg et al., 2011). Conversely, the best performance of *T. longibrachiatum* and *Trichoderma* sp. xylanase preparations were obtained with an enzyme dosage of 25 and 40 U/g dried pulp, respectively (Medeiros et al., 2007; Sathiyavathi and Parvatham, 2013).

The influence of temperature on the pulp pretreatment was investigated with enzyme dosage of 10 U/g dried pulp, pH 6.0, for 60 min (Table 5). The highest decrease in resulting kappa number was observed at 50-55°C. Many studies have reported temperatures in the range of 50-60°C as optimum for pulp bleaching (Nagar et al.,

Table 4. Effect of different substances on xylanase from *T. viride*.

Substance	Xylanase activity (%)	
	Concentration	
	2 mM	10 Mm
Control	100	100
$CuSO_4$	90.53 ± 3.31	19.96 ± 1.57
$ZnSO_4$	104.86 ± 4.39	140.25 ± 1.69
$MnSO_4$	111.53 ± 3.61	80.03 ± 2.25
$BaCl_2$	100.44 ± 4.13	108.48 ± 1.89
$CaCl_2$	77.11 ± 4.17	65.4 ± 2.15
NH_4Cl	103.56 ± 2.25	106.81 ± 4.49
NaCl	74.52 ± 2.84	70.74 ± 3.61
SDS	86.07 ± 0.82	65.3 ± 1.25
$MgSO_4$	133.02 ± 1.38	114.11 ± 2.75
Sodium citrate	74.35 ± 4.23	69.02 ± 4.88
$Co(NO_3)_2$	106.54 ± 0.78	92.94 ± 2.25
$HgCl_2$	11.75 ± 0.44	0
$Pb(CH_3COO)_2$	78.34 ± 4.52	58.34 ± 3.21
EDTA	68.04 ± 2.63	66.16 ± 0
β-mercaptoethanol	131.74 ± 1.84	148.61 ± 2.78

Table 5. Pretreatment optimization of kraft pulp with *T. viride* xylanases.

Enzyme dose (U/g dried pulp)	Kappa number	CTS (%)
Control	16.67[a]	19.22[a]
5	14.60[b]	19.20[a]
10	12.99[c]	19.19[a]
15	13.02[c]	19.23[a]
20	13.01[c]	19.17[a]
Temperature (°C)		
45	14.60[a]	19.22[a]
50	12.99[c]	19.19[a]
55	13.27[bc]	19.17[a]
60	14.16[ab]	19.23[a]
Time (min)		
0	16.63[a]	19.23[a]
60	12.99[b]	19.19[a]
120	11.93[c]	19.18[a]
180	11.91[c]	19.18[a]
pH		
5.5	13.46[a]	19.23[a]
6.0	12.99[ab]	19.19[a]
6.5	11.81[c]	19.17[a]
7.0	12.09[bc]	19.19[a]

2013; Dhiman et al., 2009; Garg et al., 2011). Savitha et al. (2009) reported that 60°C was the optimum temperature for attaining maximum kappa number reduction of waste paper pulp with the purified xylanase from *T. harzianum*.

The effect of xylanase pretreatment time is also shown

in Table 5. It was investigated with enzyme dosage of 10 U/g dried pulp, pH 6.0, at 50°C. The maximum efficiency of the xylanases in this process was achieved after 120 min of incubation, corresponding to a reduction in mean kappa number from 16.63 to 11.93. It can also verify that a longer period of incubation did not enhance the pretreatment efficiency, significantly. Likewise, some authors have been determined 120 min as the biobleaching optimal incubation time (Sandrim et al., 2005; Ko et al., 2011). Sathiyavathi and Parvatham (2013) verified that the kappa number was reduced significantly with increase in reaction time up to 3 h when *Trichoderma* sp. crude extract was used, whereas other researcher have verified maximal efficiency after 180 min of incubation (Shindu et al., 2006; Kiddinamoorthy et al., 2008; Kumar et al., 2009).

The pH influence on pulp treatment was evaluated with an enzyme dosage of 10 U/g dried pulp, at 50°C, for 60 min of incubation (Table 5). Greater bleaching efficiency was verified at pH values of 6.5-7.0, corresponding a kappa number of 12. These results are in accordance with other studies in literature, which related the optimum pH in the range of 6.0-8.0 (Sandrim et al., 2005; Ko et al., 2011; Sangui et al., 2009).

In addition, it can be observed in all treatments that the pulp viscosity was not significantly modified (Table 5), indicating the maintenance of pulp integrity, due to absence of cellulolytic enzymes in crude filtrate, as previously mentioned. This is a crucial aspect to take into account in biobleaching process, since cellulases could damage the fibers, resulting in loss of strength and performance (Terrasan et al., 2013).

The data obtained revealed that the kraft pulp pre-treatment was more efficient when conducted at 50°C, in pH 6.5, for 120 min, with an enzyme dosage of 10 U/g dried pulp. Under these conditions, the kappa number was reduced to 10.12 (6.55 points), which corresponds a kappa efficiency of 39.29. The results obtained in the treatment with *T. viride* enzymes was higher than those achieved with other fungal xylanases, which usually show reductions in kappa number between 0.9 and 5.07 units (Sandrim et al., 2005; Medeiros et al., 2007; Sangui et al., 2009; Terrasan et al., 2013; Guimaraes et al., 2013).

Conclusions

T. viride J40 strain was able to produce high levels of xylanase using pineapple peel as substrate. The *T. viride* xylanase showed optimal activity at 50°C and pH 6.0-6.5 and it was able to act and exhibited stability in moderate temperatures and acid pH values. The use of pineapple peel as carbon source can decrease the costs for the enzyme production and avoids environmental problems of the inappropriate disposition of this waste. Therefore, the reduction on kappa number achieved in this present investigation revealed the application potential of *T. viride* xylanases in biobleaching process.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors acknowledge the Coordination for the Improvement of Higher Level Personnel (CAPES - Brazil) for the scholarship awarded to the first author.

REFERENCES

Ahmed S, Imdad SS, Jamil A (2012). Comparative study for the kinetics of extracellular xylanases from *Trichoderma harzianum* and *Chaetomium thermophilum.* Electron. J. Biotechnol. 15(3):1-8.

Albert S, Chauhan D, Pandya B, Padhiar A (2011).Screening of *Trichoderma* spp. as potential fungal partner in co-culturing with white rot fungi for efficient bio-pulping. Global J. Biotechnol. Biochem. 6(3):95-101.

Ali SBR, Muthuvelayudham R, Viruthagiri T (2012). Statistical optimization of nutrients for production celulase & hemicellulase from rice straw. Asian J. Biochem. Pharm. Res. 2(2):154-174.

Bailey MJ, Buchert J, Viikari L (1993). Effect of pH on production of xylanase by *Trichoderma reesei* on xylan-and cellulose-based media. Appl. Microbiol. Biotechnol. 40(2-3):224-229.

Biely P (1993). Biochemical aspects of the production of microbial hemicellulases. In: Coughlam MP, Hazlewood GP (eds.). Hemicellulose and hemicellulases. Potland press, London, pp.127-143.

Buthelezi SP, Olaniran AO, Pillay B (2011). Sawdust and digestive bran as cheap alternative substrates for xylanase production. Afr. J. Microbiol. Res. 5(7):742-752.

Chen C, Chen JL, Lin TY (1997). Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. Enzyme Microbiol. Technol. 21(2):91-96.

Collins T, Gerday C, Feller G (2005). Xylanases, xylanases families and extremophilic xylanases. FEMS Microbiol. Rev. 29(1): 3-23.

Dashtban M, Schraft H, Qin W (2009). Fungal bioconversion of lignocellulosic residues; Opportunities & perspectives. Int. J. Biol. Sci. 5(6):578-595.

Dhiman SS, Garg G, Mahajan R, Garg N, Sharma J (2009). Single lay out and mixed lay out enzymatic processes for biobleaching of kraft pulp. Bioresour. Technol. 100(20):4736-4741.

FAOSTAT (2013). Food and Agriculture Organization. Agriculture statistics. Pineapple production (2010) by country. http://faostat.fao.org. Acessed 15 fev 2014.

Folakemi OP, Pricilla JO, Ibiyemi SA (2008). Cellulase production by some fungi cultured on pineapple waste. Nat. Sci. 6(2):64-79.

Franco PF, Ferreira HM, Filho EX (2004). Production and characterization of hemicellulase activities from *Trichoderma harzianum* strain T4. Biotechnol. Appl. Biochem. 40:255-259.

Garg G, Mahajan R, Kaur A, Sharma J (2011). Xylanase production using agro-residue in solid-state fermentation from *Bacillus pumilus* ASH for biodelignification of wheat straw pulp. Biodegradation 22(6):1143-1154.

Goswami GK, Pathak RR (2013). Microbial xylanases and their biomedical applications: a review. Int. J. Basic & Clin. Pharmacol. 2(3):237-246.

Goyal M, Kalra KL, Sarren VK, Soni G (2008). Xylanase production with xylan rich lignocellulosic wastes by a local soil isolate of *Trichoderma viride*. Braz. J. Microbiol. 39(3): 535-541.

Grigorevski-Lima AL, Oliveira MM, Nascimento RP, Bon EP, Coelho RR. Production and partial characterization of cellulases and xylanases from *Trichoderma atroviride* 676 using lignocellulosic residual biomass. Appl. Biochem. Biotechnol. 169(4):1373-1385.

Guimaraes NCA, Sorgatto M, Peixoto-Nogueira SC, Betini JHA, Zanoelo FF, Marques MR, Polizeli MLTM, Giannesi GC (2013).

Bioprocess and biotechnology: effect of xylanase from *Aspergillus niger* and *Aspergillus flavus* on pulp biobleaching and enzyme production using agro-industrial residues as substract. SpringerPlus 2:380-387.
Gupta U, Kar R (2009). Xylanase production by a thermo-tolerant *Bacillus* species under solid-state and submerged fermentation. Braz. Arch. Biol. Technol. 52(6):1363-1371.
Gupta VK, Gaur R, Gautam N, Kumar P, Yadav IJ, Darmwal NS (2009). Optimization of xylanase production from Fusarium solani F7. Am. J. Food Technol. 4(1):20-29.
Huang X, Ge J, Fan J, Chen X, Xu X, Li J, Zhang Y, Zhou D (2013). Characterization and optimization of xylanase and endoglucanase production by *Trichoderma viride* HG 623 using response surface methodology (RSM). Afr. J. Microbiol. Res. 7(36):4521-4532.
Irfan M, Syed Q (2012). Partial purification and characterization of xylanase from *Trichoderma viride* produced under SSF. Int. J. Appl. Res. Nat. Prod. 5(1):7-11.
Seyis I, Aksoz N (2005). Xylanase production from *Trichoderma harzianum* 1073 D3 with alternative carbon and nitrogen sources. Food Technol. Biotechnol. 43:37-40.
Jing-Min C, Wu Z, Ruipen R (1998). Production, properties and application of xylanase from *Aspergillus niger*. Az. Ann. N.V. Acad. Sci. 864:214-218.
Kar S, Mandal A, Mohapatra KP, Mondal CK, Pati RB (2006). Production of cellulase-free xylanase by *Trichoderma reesei* SAF3. Braz. J. Microbiol. 37(4):462-464.
Kar S, Sona GS, Das A, Jana A, Maity C, Mandal A, Das MPK, Pati BR, Mondal KC (2013). Process optimization of xylanase production using cheap solid substrate by *Trichoderma Reesei* SAF3 and study on the alteration of behavioral properties of enzyme obtained from SSF and SmF. Bioproc. Biosyst. Engin. 36(1):57-68.
Khandeparker R, Numan MT (2008). Bifunctional xylanases and their potential use in biotechnology. J. Ind. Microbiol. Biotechnol. 35(7):635-644.
Kiddinamoorthy J, Anceno AJ, Haki GD, Rakshit SK (2008). Production, purification and characterization of *Bacillus* sp. GRE7 xylanase and its application in eucalyptus kraft pulp biobleaching. World. J. Microbiol. Biotechnol. 24(5):605-612.
Ko CH, Tsai CH, Tu J, Yang BY, Hsieh DL, Jane WN, Shih TL (2011). Identification of *Paenibacillus* sp. 2S-6 and application of its xylanase on biobleaching. Int. Biodeter. Biodegr. 65(2):334-339.
Kulkarni N, Shendye A, Rao M (1999). Molecular and biotechnological aspects of xylanases. FEMS Microbiol. Rev. 23:411-456.
Kumar KS, Manimaran A, Permaul K, Singh S (2009). Production of β-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. J. Biosci. Bioeng. 107(5):494-498.
Laxmi GS, Sathish T, Rao CHS, Brahmaiah P, Hymavathi M, Prakasham RS (2008). Palm fiber as novel substrate for enhanced xylanase production by isolated *Aspergillus sp*. RSP-6. Curr. Trends Biotechnol. Pharm. 2(3):447-455.
Li S, Yang X, Yang S, Zhu M, Wang X (2012). Technology prospecting on enzymes: application, marketing and engineering. Computat. Struct. Biotechnol. J. 2(3):1-11.
Lopes AM, Valeri D, Pessoa-Júnior AP (2013). Comparison of kinetic characteristics of xylanases from *Aspergillus niger* and *Trichoderma* sp. with pH and temperature baking process parameters. Afr. J. Biotechnol. 12(19):2640-2645.
Madlala AM, Bissoon S, Singh S, Christov L (2001). Xylanase induced reduction of chlorine dioxide consumption during elemental chlorine-free bleaching of different pulp types. Biotechnol. Lett. 23:345-351.
Medeiros RG, Hanada R, Ferreira-Filho EX (2003). Production of xylan-degrading enzymes from Amazon forest fungal species. Int. Bioterior. Biodegrad. 52(2):97-100.
Medeiros RG, Silva FGS, Báo SN, Hanada R, Ferreira-Filho EX (2007). Application of xylanases from Amazon Forest fungal species in bleaching of *Eucalyptus* kraft pulps. Braz. Arch. Biol. Technol. 50(2):231-238.
Meenakshi G, Kalra KL, Sareen VK, Soni G (2008). Xylanase production with xylan rich lignocellulosic wastes by a local soil isolate of *Trichoderma viride*. Braz. J. Microbiol. 39(3):535-541.
Miller GL (1959). Use of dinitrosalicylic acid reagent for the determination of reducing sugars. Anal. Chem. 31(3):426-429.
Mohan G, Guhankumarand P, Balakrishnan V (2011). Isolation of xylan degrading enzyme from *Trichoderma* spp. Res. Plant Biol. 1(3):15-20.
Mojsov K (2010). Application of solid-state fermentation for cellulase enzyme production using *Trichoderma viride*. Perspect. Innov. Econom. Bus. 2(5):108-110.
Nagar S, Jain RK, Thakur VV, Gupta VK (2013). Biobleaching application of cellulase poor and alkali stable xylanase from *Bacillus pumilus*. 3 Biotech 3:277-285.
Norazlina I, Meenalosani N, Ku Halim KH (2013). Production of xylanase by *Trichoderma* sp. via solid state culture using sugarcane bagasse. Int. J. En. Sci. 3(2):99-105.
Paengkoum S, Wanapat M, Paengkoum P (2013). Effects of pineapple peel and rice straw ratios as basal roughage in dairy cow. World Acad. Sci. Engin. Technol. 7(1):7-8.
Pang PK, Darah I, Poppe L, Szakacs G, Ibrahim CO (2006). Xylanase production by a local isolate, *Trichoderma* spp. FETL c3-2 via solid state fermentation using agricultural wastes as substrates. Malaysian J. Microbiol. 2(1):7-14.
Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005). Xylanases from fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 67(5):577-591.
Rani DS, Nand K (2004). Ensilage of pineapple processing waste for methane generation. Waste Manag. 24(5):5523-5528.
Reena A, Anitha M, Aysha OS, Valli S, Nirmala P, Vinothkumar P (2013). Antagonistic activity of *Trichoderma viride* isolate on soil borne plant pathogenic fungi. Int. J. Bioassays 2(1):294-297.
Romanowska I, Polak J, Bielecki S (2006). Isolation and properties of *Aspergillus niger* IBT-90 xylanase for bakery. Appl. Microbiol. Biotechnol. 69(6):665-671.
Sandrim VC, Rizzatti ACS, Terenzi HF, Jorge JA, Milagres AMF, Polizeli MLTM (2005). Purification and biochemical characterization of two xylanases produced by *Aspergillus caespitosus* and their potential for kraft pulp bleaching. Process Biochem. 40(5):1823-1828.
Sanghvi GV, Koyani RD, Rajput KS (2010). Thermostable xylanase production and partial purification by solid-state fermentation using agricultural waste wheat straw. Mycology 1(2):106-112.
Sangui A, Garg N, Sharma J, Kuhar K, Kuhad RC, Gupta VK (2009). Enhanced production of cellulase-free xylanase by alkalophilic *Bacillus subtilis* ASH and its application in biobleaching of kraft pulp. World J. Microbiol. Biotechnol. 4(3):1109-1129.
Saravanan P, Muthuvelayudham R, Viruthagiri T (2013). Enhanced production of cellulase from pineapple waste by response surface methodology. J. Engin. doi: 10.1155/2013/979547.
Sathiyavathi M, Parvatham R (2013). Industrial application of xylanase in the crude enzyme extract from *Trichoderma* sp. MS 2010. Asian J. Pharm. Clin. Res. 6(2):90-96.
Savitha S, Sadhasivam S, Swaminathan K (2009). Modification of paper properties by the pretreatment of wastepaper pulp with *Graphium putredinis*, *Trichoderma harzianum* and fusant xylanases. Bioresource Technol. 100(2):883-889.
Sedmak JJ, Grossberg SE (1977). A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. Anal. Biochem. 79(1-2):544-552.
Sharma M, Kumar A (2013). Xylanases: an overview. Brit. Biotechnol. J. 3(1):1-28.
Shindu I, Chhibber S, Caplash N, Sharma P (2006). Production of cellulase free xylanase from *Bacillus megaterium* by solid state fermentation for biobleaching of pulp. Curr. Microbiol. 53(2):167-172.
Simões MLG, Tauk-Tornisielo MS, Tapia TMD (2009). Screening of culture condition for xylanase production by filamentous fungi. Afr. J. Biotechnol. 8(22):6317-6326.
Soroor, MAM, Ghazy, AM, Mandy EMS, El-Badry MO, Shousha WG, El-Khonezy MI (2013). Purification and characterization of cellulase-poor xylanases from *Trichoderma reesei* F418 grown on rice straw by solid-state fermentation. J. Appl. Sci. Res. 9(3):1702-1713.
Tran AV (2006). Chemical analysis and pulping study of pineapple crown leaves. Ind. Crops Prod. 24(1):66-74.
Terrasan CRF, Temer B, Sarto C, Silva Júnior FG, Carmona EC (2013). Xylanase and β-xylosidase from *Penicillium janczewskii*: production, physico-chemical properties and application of the crude extract to pulp biobleaching. Bioresour. 8(1):1292-1305.

Vieille C, Zeikus GJ (2001). Hyperthermophilic enzymes: sources, uses and molecular mechanisms for thermostability. Microbiol. Mol. Biol. Rev. 65(1):1-43.

Vogel HJA (1956). A convenient growth medium for *Neurospora* (Medium N). Microb. Genet. Bull 13:42-43.

Wakiyama M, Tanaka H, Yoshihara K, Hayashi S, Ohta K (2008). Purification and properties of family-10 endo-1,4-β-xylanase from *Penicillium citrinum* and structural organization of encoding gene. J. Biosci. Bioengin. 105(4):367-374.

Widjaja A, Lestari E, Tanjung A, Alfian W, Ogino H (2009). Optimized production of xylanase from fungal strains and its purification strategies. J. Appl. Sci. Environ. Sanit. 4(3):219-232.

Xiong H, von Weymarn N, Leisola M, Turunen O (2004). Influence of pH on the production of xylanases by *Trichoderma reesei* Rut C-30. Process Biochem. 39(6):731-736.

Zhang GM, Huang J, Huang GR, Lx MA, Zhang XE (2007). Molecular cloning and expression of a new xylanases gene from *Plectosphaerella cucmerina*. Appl. Microbiol. Biotechnol. 74(2): 339-346.

academicJournals

Vol. 13(22), pp. 2260-2264, 28 May, 2014
DOI: 10.5897/AJB2013.13192
Article Number: 7E30DDA44906
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Analysis and identification of oils from seed extract of *Anthonotha macrophylla* using gas chromatography-mass spectrometry (GC-MS)

Ugoeze, K. C.[1], Ehianeta, T.[2], Alaribe, C.[2] and Anyakora, C.[2]*

[1]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Port Harcourt, Nigeria.
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, University of Lagos, Lagos, Nigeria.

Received 23 August, 2013; Accepted 2 May, 2014

The volatile components obtained from the seed extract of *Anthonotha Macrophylla* were analyzed using gas chromatography-mass spectrometry (GC-MS). Prior to GC-MS analysis, Soxhelt extraction was carried out from the seeds of *A. Macrophylla.* Agilent GC-MS system comprising 6890GC model coupled with 5973 n mass selective detector was used for analysis. The GC is equipped with Agilent 7673 autosampler and a 30 m 0.25 id DB-1 MS dimethylpolysiolaxane capillary column. The MS source temperature was set at 230C and electron energy at 70V. The ionisation mode was electron ionization and the mass range was 50 to 550 while the scan time was 1 scan/min. The different compounds were identified by matching their mass spectra with the MS spectra in the NIST library. Various compounds were separated and identified but eight of these were at an elevated level. These include: n-hexadecanoic acid, n-octadecadienoic acid; cis-vaccenic acid; octadecanoic acid; hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester; campesterol; stigmasterol and gamma-sitosterol.

Key words: Gas chromatography-mass spectrometry, determination, evaluation, composition, *Anthonotha macrophylla*, seed oil.

INTRODUCTION

More than 80% of the world's population rely on Traditional medicine for their primary healthcare needs (Pierangeli, 2009). This is particularly so for the African continent (Addae-Mensah, 1992; Cunningham, 1993). Unfortunately, most of these herbal remedies have not been scientifically explored and exploited. In Africa, natural products have proven to be great sources of essential oils which combine the function of healthcare and nutrition. Essential oils have other uses such as in foods, drinks, cosmetics and medicine especially with aromatherapy becoming increasingly popular (Reische et al., 1998; Lis-Balchin et al., 1999; Ghelardini et al., 1999).

Nigeria and Africa at large are endowed with seed-bearing plants, which over the years have served various purposes and yet quite a number of them remain untapped. One of such underutilised plant is *Anthonotha*

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

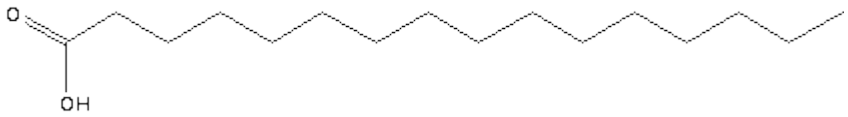


Figure 1. Chemical structure of n-hexadecanoic acid

macrophylla (AM) P. Beauv; a member of the family Leguminosae - Caesalpinioideae. AM is a small tree which is common throughout the rain forests, a medium-sized tree up to 20 m high, tree trunk seldom reaching 50 cm diameter, often multi-stemmed and low-forked, bearing a wide-spreading crown, common in the under storey of the rain-forest from Guinea to Western Cameroons and Fernando Po, and extending to Zaïre. It abounds in Nigeria with common names: (Yoruba: abata; Igbo: ububa - ikpa). Several ethnomedicinal claims have been attributed to various parts of this plant. The bark has been claimed to be useful in the treatment of venereal diseases and as vermifuges. The roots are used for intestinal related discomfort. Gums extractable from the bark have analgesic properties while the leaves could be useful as anti-diarrhoea, dysentery, skin infections, as antidotes in venomous stings and bites (Keay, 1989; Burkill, 1985). The seeds are also useful as general food. Previous studies reveal that the oil extracted from the milled dry seed gave iodine value of 1.013 ± 0.01%, free fatty acid 2.334 ± 0.04%, saponification value 5.394 ± 0.23%, un-saponifiable matter 8.33 ± 0.01% and refractive index of 1.472 ± 0.07% (Durunna, 2006).

So far, to the best of our knowledge, no data has been recorded on the profile of the oils contained in the seeds of *A. macrophylla*. The present work is aimed at identifying the fatty acids in the oil of *A. macrophylla* using gas chromatography-mass-spectrometry (GC-MS) method thereby laying a good foundation for future studies on the medicinal uses of this plant.

EXPERIMENTALS

Plant material

The matured seeds of *A. macrophylla* used for this study were collected from the forest in Akatta, Oru-East Local Government Area, Imo State of Nigeria.

Extraction

The seeds of *A. macrophylla* were shade-dried until constant weight at room temperature and milled in a hammer Mill (Thomas Willey, model 4, USA). Extraction was carried out using a standard Soxhlet apparatus (Haake FK, Germany). The Soxhlet thimble was charged with 500 g milled seeds of mean particle size (0.3 to 0.5 mm), and extracted with 1000 ml petroleum spirit (40 to 60°C) during each complete extraction step. The oil was extracted from the distillate using hexane and then dried over anhydrous sodium sulphate. After filtration, the solvent was removed by distillation under reduced pressure in a rotary evaporator at 35°C and the pure oil kept in sealed glass at 4°C in the dark, until the moment of analysis.

Gas chromatography-mass spectrometry analysis (GC/MS) conditions

The extracted oil was diluted with n-heptane in the ratio (1:50) and subjected to GC-MS. The analysis was carried out on Agilent GC-MS system comprising 6890 GC model coupled with 5973 n MSD. The GC is equipped with agilent 7673 autosampler and a DB-1 MS dimethylpolysiolaxane capillary column (30 m × 0.32 mm; film thickness 1.00 µm). The instrument operating conditions were: Carrier gas was helium at flow rate of 1.0 ml/min and at a constant pressure mode of 2.56 psi. Injector temperature was 250°C; injector volume was 1 ul in the split mode. The initial temperature was 120°C for 5 min and ramped up at 3°C/min with Nitrogen as make-up gas. The final temperature, 350°C was held for 5 min. The run time was 62 min. The MS source temperature was 230°C; electron energy was 70 V. The ionisation mode was electron ionization and the mass range of *m/z* 50 to 550 while the scan time was 1 scan/min.

Identification of phytocompounds

The interpretation of GC-MS spectra was done using the database of the National Institute of Standards and Technology (NIST). The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The nomenclatures, molecular weights and structures of the component compounds in the oil of *Anthonotha macrophylla* were ascertained. The different compounds were identified by matching their mass spectra with the MS spectra in the NIST02 library.

RESULTS AND DISCUSSION

With the help of MS spectra in the NIST library, some of the fatty acids separated in the seed extract of *A. macrophylla* were identified. Various compounds were separated and identified but eight of these were present at elevated levels and future work will focus on these compounds. Figures 1 to 8 give the chemical structures of these compounds. They include: n-hexadecanoic acid, n-octadecadienoic acid, cis-vaccenic acid, octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, campesterol, stigmasterol and γ-sitosterol. No attempt was made at quantifying the fatty acids in the oil. The relative abundance of these compounds were: 9,12-Octadecadienoic acid (33.38%); n-hexadecanoic acid (17.33%); γ-sitosterol (14.84%); 9Z- Octadec-9-enoic acid (12.85%); Campesterol (4.32%); Stigmasterol (3.63%) and Octadecanoic acid (2.97%) (Table 1). Very

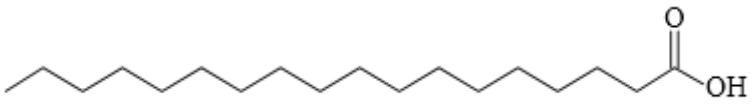


Figure 2. Chemical structure of octadecanoic acid (stearic acid)

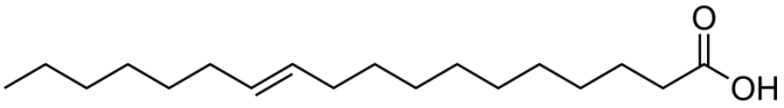


Figure 3. Chemical structure of cis-veccenic acid

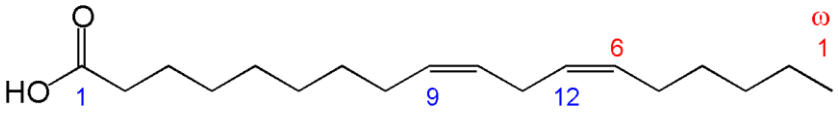


Figure 4. Chemical structure of n-octadecadienoic acid (linoleic acid)

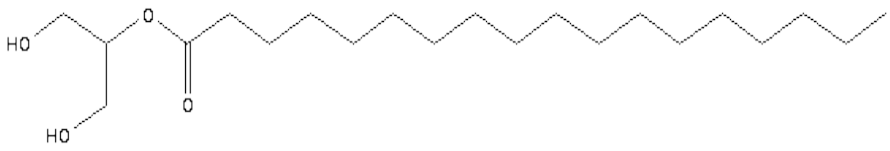


Figure 5. Chemical structure of octadecanoic acid

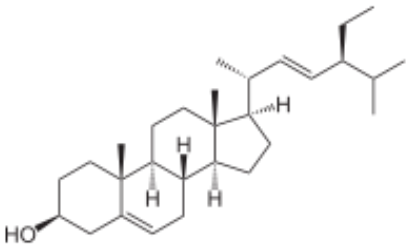


Figure 6. Chemical structure of stigmasterol

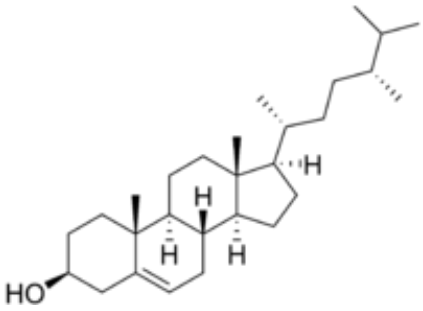


Figure 8. Chemical structure of Compesterol

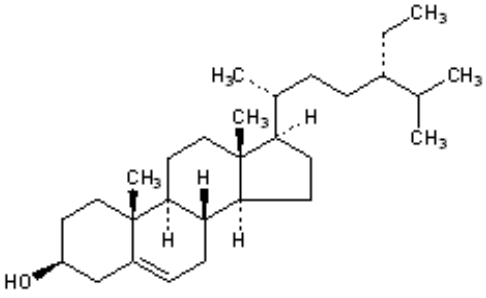


Figure 7. Chemical structure of sitosterol

many other compounds were present in the sample.

Anthonotha macrophylla oil contains n-Hexadecanoic acid (palmitic acid), a fatty acid reported to show anti-inflammatory properties by Aparna et al., (2012). From structural and kinetic studies they concluded that n-hexadecanoic acid is a potent inhibitor of phospholipase A2, an inflammatory compound. However, a downside of this phytocompound is that reported by the WHO (2003) technical report, of the convincing evidence that consumption of palmitic acid increases the risk of developing

Table 1. Phytoconstituents identified from the methanolic extract of *Anthonotha macrophylla*.

Retention time	Phytocompound	Relative Percentage (%) composition
10.04	n-Hexadecanoic acid	17.33
16.03	Octadecanoic acid (stearic acid)	2.97
	Cis-Vaccenic acid	12.85
	hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	0.61
47.59	Campesterol	4.32
	n-Octadecadienoic acid	33.38
48.61	Stigmasterol	3.63
50.42	γ - Sitosterol	14.84

cardiovascular diseases, placing it in the same evidence category as trans fatty acids, myristic acid, high sodium intake, overweight and high alcohol intake. Also, present is the common monoenoic fatty acid of bacterial lipids, cis-Vaccenic acid (cis-11-octadecenoic acid). It occurs as a minor component of most plant and animal tissues. Previous research have shown that cis-Vaccenic acid, within the range of the concentrations used, produces a proportionate inhibition of growth and respiration of *Bacillus subtilis*, thus suggesting its antibiotic effect with the cis- isomer having about twice the activity of the trans-isomer. A recent study (Djoussé et al., 2013) suggested that higher plasma levels of phospholipid cis-vaccenic acid are associated with reduced odds of heart failure with antecedent coronary heart disease.

The cholesterol-lowering potential of dietary plant sterols has been known for over 50 years (Pollack, 1953). Despite the daily consumption of plant sterols in food, the amounts are usually not high enough to have significant blood cholesterol-lowering effect. The major plant sterols are sitosterol (approx. 80%), campesterol and stigmasterol (Rao and Janezic, 1992; Ikeda et al., 2006; Phuruengrat and Phaisansuthichol, 2006). Our study showed *A. macrophylla* to contain all 3 major phytosterols in the oil.

Plant sterols are similar in structure to cholesterol (cholest-5-en-3beta-ol). The structural similarity of plant sterols to cholesterol enables them to compete with cholesterol for incorporation into the micelles, the particles that transport lipids and cholesterol into the intestinal mucosa. This competition reduces the absorption of dietary and biliary cholesterol in the gastrointestinal tract (Clifton, 2002; Lichtenstein, 2002). Phytosterols and phytostanols both inhibit the uptake of dietary and biliary cholesterol, and thus reduce the levels of low density lipoproteins and total serum cholesterol. Because the structure of β-sitosterol is similar to that of cholesterol, β-sitosterol takes the place of dietary and biliary cholesterol in micelles produced in the intestinal lumen (Moreau et al., 2002). The structural difference is the presence and positioning of methyl or ethyl moieties in their side chains.

Phytosterols possess biological functions, such as anticarcinogenic (Li et al., 2001), anti-inflammatory, antibacterial and antifungal activities (Padmaja et al., 1993) and anti-angiogenic activities (Jung-Min et al., 2007). In the mid 20th century, studies have shown plant sterols to be beneficial in lowering low density lipo-proteins and cholesterol (Farquhar and Sokolow, 1958). Since then, numerous studies have also reported the beneficial effects of the dietary intake of phytosterols, including campesterol. It is thought that the campesterol molecules compete with cholesterol and thus reduces the absorption of cholesterol in the human intestine (Heggen et al., 2010). There is rising evidence that campesterol exhibits chemopreventive effects against many cancers, including prostate (McCann et al., 2005), lung (Schabath et al., 2005) and breast (Awad et al., 2000) cancers. Our study also showed *A. macrophylla* oil to contain gamma-sitosterol and stigmasterol. Stigmasterol is reported to inhibit cholesterol biosynthesis via inhibition of sterol Delta (24)-reductase in human Caco-2 and HL-60 cell lines. A study (Batta et al., 2006) carried out on the effect of feeding 0.5% stigmasterol on plasma and liver sterols and intestinal cholesterol and sitosterol absorption in 12 wild-type Kyoto (WKY) and 12 Wistar rats showed that stigmasterol lowered plasma cholesterol levels, inhibited intestinal cholesterol and plant sterol absorption, and suppresses hepatic cholesterol and classic bile acid synthesis in Wistar as well as WKY rats. Thus, stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the animal study.

The most extensively studied members of the phytosterols, beta and gamma sitosterols, are stereoisomers and differ only in the spatial configuration of the C-17 side chain. The structural similarity of Stigmasterol and β-sitosterol strengthens the hypocholes-terolemic potential of sitosterol. Matsuoka et al. (2008) in their work reported that β-Sitosterol inhibits cholesterol absorption in the intestine. The absorbed sterol in the intestine is transported by lipoproteins and incorporated into the cellular membrane (Awad and Fink, 2000). The overall consequence being reduced cholesterol, lowered

low density lipoproteins (LDL) and fewer incidences of artherosclerosis. Sitosterol (though the β-isoform) has been reported to be beneficial to urinary and overall prostate health (Wilt et al., 1999). The GC-MS analysis of *A. macrophylla* oil of study reveals the presence of these phytosterols, suggesting that the plant will definitely find place in the scheme of drug development programmes as a useful lead.

Conclusion

With the aid of GC/MS, eight major chemical constituents have been identified from the extract of the seed oil of *A. macrophylla.* The presence of these bioactive compounds justifies the ethnomedicinal use of the plant for various ailments by traditional practitioners. This study has opened up various research opportunities on this plant and its plant parts with the aim of maximizing its potential as a possible lead in drug discovery.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Aparna V, Dileep KV, Mandal PK, Karthe P, Sadasivan C, Haridas M (2012). Anti-inflammatory property of n-hexadecanoic acid: structural evidence and kinetic assessment. Chem. Biol. Drug Des. 80(3):434-9.

Awad AB, Down AC, Fink CS (2000). Inhibition of growth and stimulation of apoptosis by β-sitosterol treatment of MDAMB-231 human breast cancer cells in culture. Int. J. Mol. Med. 5:541-546.

Awad AB, Fink CS (2000). Phytosterols as Anticancer Dietary Components: Evidence and Mechanism of Action. J. Nutr. 130(9):2127-2130.

Batta AK, Xu G, Honda A, Miyazaki T, Salen G (2006). Stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat. Metabolism 55(3):292-9.

Burkill HM (1985). A review of Dalziel's. The useful plants of west tropical Africa. Royal Botanical Garden Kew. Vol. 3.

Clifton P (2002). Plant sterols and stanols-comparison and contrasts. Sterols versus stanols in cholesterol-lowering: is there a difference? Atheroscler Suppl. 3(3):5-9.

Djousse L, Matsumoto C, Hanson NQ, Weir NL, Tsai MY, Gaziano MJ (2013). Plasma cis-vaccenic acid and risk of heart failure with antecedent coronary heart disease in male physicians. Clin. Nutr. 33(3):478-82.

Durunna CS (2006). Aspects of the bio-chemical composition of *Anthonotha macrophylla* seeds. AniM. Prod. Res. Adv. 2(4):217-220.

Farquhar JW, Sokolow M (1958). Response of serum lipids and lipoproteins of man to beta-sitosterol and safflower oil. Circulation 17(5):890-899.

Heggen E, Granlund L, Pedersen JI, Holme I, Ceglarek U, Thiery J, Kirkhus B, Tonstad S (2010). Plant sterols from rapeseed and tall oils: Effects on lipids, fat-soluble vitamins and plant sterol concentrations. Nutr. Metabol. Cardiovasc. Dis. 20(4):258-65.

Ikeda I, Konno R, Shimizu T (2006). Campest-5-en-3-one, an oxidized derivative of campesterol, activates PPAR, promotes energy consumption and reduces visceral fat deposition in rats. Biochim. Biophys. Acta. 1760:800-807.

Jung-Min Choi, Eun-Ok Lee, Hyo-Jung Lee, Kwan-Hyun Kim, Kyoo-Seok Ahn, Bum-Sang Shim, Nam-Il Kim, Myoung-Chong Song, Nam-In Baek, Sung-Hoon Kim (2007). Identification of Campesterol from *Chrysanthemum coronarium* L. and its Antiangiogenic Activities. Phytother. Res. 21:954-959.

Keay RWJ (1989). Trees of Nigeria: A revised version of Nigerian Trees (1960, 1964) by R.W.J. Keay, C.F.A. Onochie and D.P. Stanfield), Clarendon Press, Oxford. p. 223.

Li JH, Awad AB, Fink CS, Wu YW, Trevisan M, Muti P (2001). Measurement variability of plasma beta-sitosterol and campesterol, two new biomarkers for cancer prevention. Eur. J. Cancer Prev. 10:245-249.

Lichtenstein AH (2002). Plant sterols and blood lipid levels. Curr. Opin. Clin. Nutr. Metab. Care 5:147-152.

Matsuoka K, Nakazawa T, Nakamura A, Honda C, Endo K, Tsukada M (2008). Study of Thermodynamic Parameters for Solubilization of Plant Sterol and Stanol in Bile Salt Micelles. Chem. Phys. Lipids 154(2):87-93.

McCann SE, Ambrosone CB, Moysich KB et al. (2005). Intakes of selected nutrients, foods, and phytochemicals and prostate cancer risk in western New York. Nutr. Cancer 53(1): 33-41.

Moreau RA, Whitaker BD, Hicks KB (2002). Phytosterols, Phytostanols, and Their Conjugates in Foods: Structural Diversity, Quantitative Analysis, and Health-Promoting Uses. Prog. Lipid Res. 41(6):457-500.

Padmaja V, Thankamany V, Hisham A (1993). Antibacterial, antifungal and anthelmintic activities of root barks of Uvaria hookeri and Uvaria narum. J. Enthnopharmacol. 40(3):181-186.

Phuruengrat A, Phaisansuthichol S (2006). Preliminary study of steroids in *Sericocalyx schomburgkii* (Craib) Bremek by GC-MS Songklanakarin. J. Sci. Technol. 28:39-44.

Pollack OJ (1953). Successful prevention of experimental hypercholesterolemia and cholesterol atherosclerosis in rabbit. Circulation 2:696-701.

Schabath MB, Hernandez LM, Wu X, Pillow PC, Spitz MR (2005). Dietary phytoestrogens and lung cancer risk. JAMA 294: 1493-1504.

WHO (2003). Diet, Nutrition and the Prevention of Chronic Diseases, WHO Technical Report Series 916, Report of a Joint WHO/FAO Expert Consultation, World Health Organization, Geneva, 2003. p. 88 (Table 10).

Wilt TJ, MacDonald R, Ishani A (1999). Beta-sitosterol for the treatment of benign prostatic hyperplasia: a systematic review. BJU Int. 83:976-983.

academicJournals

Vol. 13(22), pp. 2265-2272, 28 May, 2014
DOI: 10.5897/AJB2014.13753
Article Number: 7B511C244907
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Ethanol extracts of *Newbouldia laevis* stem and leaves modulate serum liver marker enzymes and antioxidant enzymes activities in diabetic rats

Anaduaka, Emeka G[1]*, Ogugua, Victor N[1], Agu, Chidozie V[1,2] and Okonkwo, Chukwudi C[1,2]

[1]Department of Biochemistry, University of Nigeria Nsukka, Enugu State, Nigeria.
[2]Biotechnology and fermentation Group, The Ohio State University and Ohio Agricultural Research and Development Center, Wooster, Ohio, United States.

Received 24 February, 2014; Accepted 8 May, 2014

Diabetes is known to involve oxidative stress and changes in antioxidant enzymes. Many plants metabolites have been shown to possess antioxidant activities, improving the effects of oxidative stress complications in diabetic conditions. This study evaluated the effects of extracts from *Newbouldia laevis* leaves and stem on liver marker enzymes and antioxidant enzymes in rat model. The results confirmed that the untreated diabetic rats were subjected to oxidative stress as indicated by significantly abnormal activities of their scavenging enzymes (low superoxide dismutase, catalase and glutathione activities) to the extent of liver enzymes leakage from the hepatocytes when compared with apparently healthy rats. The ethanol extracts of *N. laevis* leaves and stem possessed antioxidant activity as shown by increased activities of superoxide dismutase and catalase, and glutathione levels of the diabetic rats after treatment. High levels of alkaline phosphatase (ALP), and alanine aminotransaminase (ALT), which are typical of oxidative stress condition were differentially ameliorated after treatment with the ethanol extracts of *N. laevis* leaves and stem in a dose dependent manner.

Key words: Diabetes mellitus, oxidative stress, *Newbouldia laevis,* liver enzymes, antioxidant enzymes.

INTRODUCTION

Oxidative stress is a term used to refer to the shift towards the pro-oxidants in the pro-oxidants/antioxidants balance that can occur as a result of an increase in oxidative metabolism (Manda et al., 2009). Reactive oxygen species (ROS) reactions with biomolecules such as lipid, protein and DNA, produce different types of secondary radicals like lipid radicals, sugar and base derived radicals, amino acid radicals depending on the nature of the ROS (Niki et al., 2005).These radicals in the presence of oxygen are converted to peroxyl radicals. Peroxyl radicals are critical in biological systems, as they often induce chain reactions. These reactions exert oxidative stress on the cells, tissues and organs of the body. The biological implications of such reactions depend on several factors like site of generation, nature of the substrate, activation of repair mechanisms, and

*Corresponding author. E-mail: anaduakaemeka@yahoo.com. Tel: +2348064212224.

redox status among many others (Koppeno, 1993; Goldstein et al., 1993). Oxidative stress is increased in diabetes mellitus owing to an increase in the production of oxygen free radicals and insufficiency in antioxidant defense mechanisms (Lawrence et al., 2008; Soliman, 2008). Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas (that is beta cells) when administered to rodents and many other animal species (Szkudelski, 2001). This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to type 1 diabetes in humans. Alloxan is selectively toxic to insulin-producing pancreatic beta cells because it preferentially accumulates in beta cells through uptake via the GLUT2 (glucose transporter 2) (Szkudelski et al., 1998). Alloxan, in the presence of intracellular thiols, generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid (Stanley and Venugopal, 2001). The beta cell toxic action of alloxan is initiated by free radicals formed in this redox reaction (Szkudelski, 2001).

Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function (Mittler, 2002). Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. It is when the availability of antioxidants is limited that this damage can become cumulative and debilitating (Cheeseman and Slater, 1993; Sies, 1997). Antioxidants are our first line of defense against oxidative damage, and are critical for maintaining optimum health and well-being. Oxidative stress is increased in diabetes mellitus owing to an increase in the production of oxygen free radicals, such as super oxide ($O^{2\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxide ($OH^{\bullet-}$) radicals which overwhelm the natural antioxidant defence mechanisms (Soliman, 2008). Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being (Traber and Atkinson, 2007). To protect the cells, organ and systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system (Vertuani et al., 2004).

Newbouldia laevis is widely used in African folk medicine for the treatment of malaria and fever, stomach-ache, coughs, sexually transmitted diseases, tooth ache, breast cancer, and constipation (Iwu, 1983). In south eastern and part of the Midwestern Nigeria, the plant is used for the treatment of septic wounds and eye problems (Akerele et al., 2011). In Nigeria, the bark is chewed and swallowed for stomach pains, diarrhea and toothache (Iwu, 2000). It has also been found useful for children's convulsion (Akunyili, 2000). The antimicrobial potential of methanol extract of the leaf (Kutete et al., 2007; Usman and Osuji, 2007; Ejele et al., 2012) have been reported in literature while the anti-inflammatory (Usman et al., 2008) and anti-malarial (Gbeassor et al., 2006) activities of the root extract have been documented. Sedative effects of the methanol leaf extract of *N. laevis* in mice and rats have also been studied and reported (Amos et al., 2002). *N. laevis* is one of such medicinal plants whose medicinal values have stood the test of time. Base on these data, the present study aims to trace how ethanol extracts of *N. laevis* stem and leaves modulate serum liver marker enzymes and antioxidant enzymes activities in alloxan induced oxidative stressed rats.

MATERIALS AND METHODS

Collection and identification of plant materials

The leaves and stem of *N. laevis* were used for this study. They were collected within University of Nigeria, Nsukka and were identified by Mr. Alfred Ozioko of Bioresources Development and Conservation Programme (BDCP) Research Centre, Nsukka, Enugu State. The fresh leaves and stem of *N. laevis* were first washed with distilled water and subsequently, normal saline to remove dirt and possible mycotoxins. The samples were dried under shade for several days and then pulverized into fine powder.

Extraction of plant materials

A quantity, 500 g of each ground sample was macerated in 1.5 L of ethanol for 48 h. The solution was filtered with Whatman no. 4 filter paper and the filtrate was concentrated to a semi-solid residue using a rotary evaporator.

Animals

Forty two (42) adult albino rats of both sexes were used for this study. All the animals used were obtained from the Animal house of the Department of Zoology, University of Nigeria Nsukka. The rats were fed with standard grower's mash rat pellets (Grand Cereals LTD, Enugu) and water.

Experimental design and analysis

Forty-two (42) Wistar albino rats of both sexes weighing between 102 and 240 g were used for the study. They were acclimatized for fourteen (14) days with free access to feed and water. After acclimatization, they were evenly distributed into seven (7) groups of six rats each. The route of administration was by oral intubation for a period of twenty one (21) days. The groups and doses administered are summarised below:

Group 1: Control (Normal rats)
Group 2: Positive control (Diabetic untreated rats)
Group 3: Alloxan induced diabetic rats + 2.5 mg/kg body weight of Glibenclamide
Group 4: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol leaves extract
Group 5: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol leaves extract
Group 6: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol stem extract
Group 7: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol stem extract

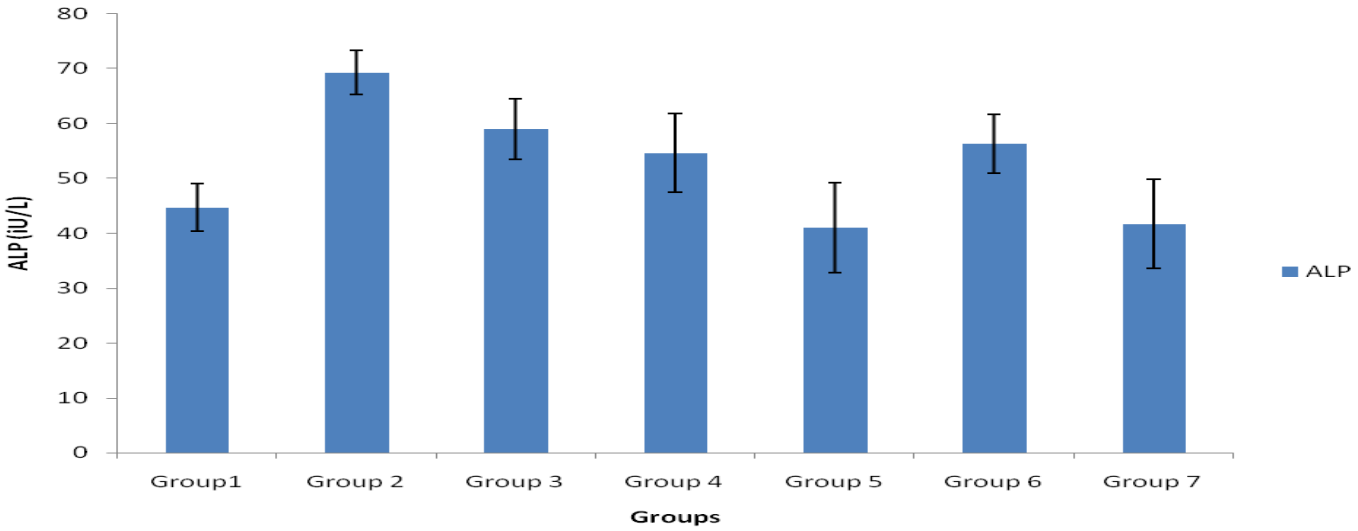


Figure 1. Effect of ethanol extracts of *Newbouldia laevis* leaves and stems on serum alkaline phosphatase (ALP). Group 1: Control (Normal rats), Group 2: Positive control (Diabetic untreated rats), Group 3: Alloxan induced diabetic rats + 2.5 mg/kg body weight of Glibenclamide, Group 4: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol leaves extract, Group 5: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol leaves extract, Group 6: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol stem extract, and Group 7: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol stem extract.

At the end of the experimental period the rats were starved for 12 h and then sacrificed under ether anaesthetized. Blood samples were received into clean dry centrifuge tube and left to clot at room temperature, then centrifuged for 10 min at 3000 r.p.m to separate serum. Serum was carefully separated into dry clean Wassermann tubes, using a Pasteur pipette and kept frozen at (-20°C) until estimation of some biochemical parameters.

Statistical analysis

Data were presented as mean of three replicates ± SD. Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 19. One way analysis of variance was adopted for comparison, and the results were subject to post hoc test using least square deviation (LSD). The data were expressed as mean ± standard deviation. P< 0.05 was considered significant.

RESULTS

Effect of ethanol extracts of *Newbouldia laevis* leaves and stems on serum alkaline phosphatase (ALP).

Figure 1 shows that the diabetic untreated group (group 2) had the highest activity of ALP but the administration of the different doses of the extracts resulted in significant ($p<0.05$) decrease in ALP activity. The 200 mg/kg body weight of the extracts (groups 4 and 6) in a similar manner as the standard drug (glibenclamide) significantly and dose dependently reduced the activity of ALP when compared with the values obtained from the diabetic untreated group (group 2). However, the 400 mg/kg body weight of the extracts caused greater reduction in the activity of ALP.

Effect of ethanol extracts of *Newbouldia laevis* leaves and stems on serum alanine aminotransaminase (ALT)

Figure 2 shows that the diabetic untreated group (group 2) had the highest activity of ALT but the administration of the different doses of the extracts resulted in significant ($p<0.05$) decrease in ALT activity. The 400 mg/kg body weight of the extracts (group 5 and 7) in a similar manner as the standard drug (glibenclamide) significantly and dose dependently reduced the activity of ALT when compared with the values obtained from the diabetic untreated group (group 2). However, the 200 mg/kg body weight of the extracts caused greater reduction in the activity of ALP even more than the standard drug.

Effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum superoxide dismutase (SOD) activity

Figure 3 shows that the diabetic untreated group (group 2) had reduced SOD activity but the administration of the different doses of the extracts resulted in significant ($p<0.05$) increase in SOD activity. The extracts stimulated SOD activity in diabetic rats treated with the leaves and stem. A significant increase ($p<0.05$) was observed in the SOD activity of rats in the test groups when compared with the positive control. Significant increases ($p<0.05$) were observed in groups 6 and 7 (stem extract treated) when compared with groups 4 and 5 (leave extract treated).

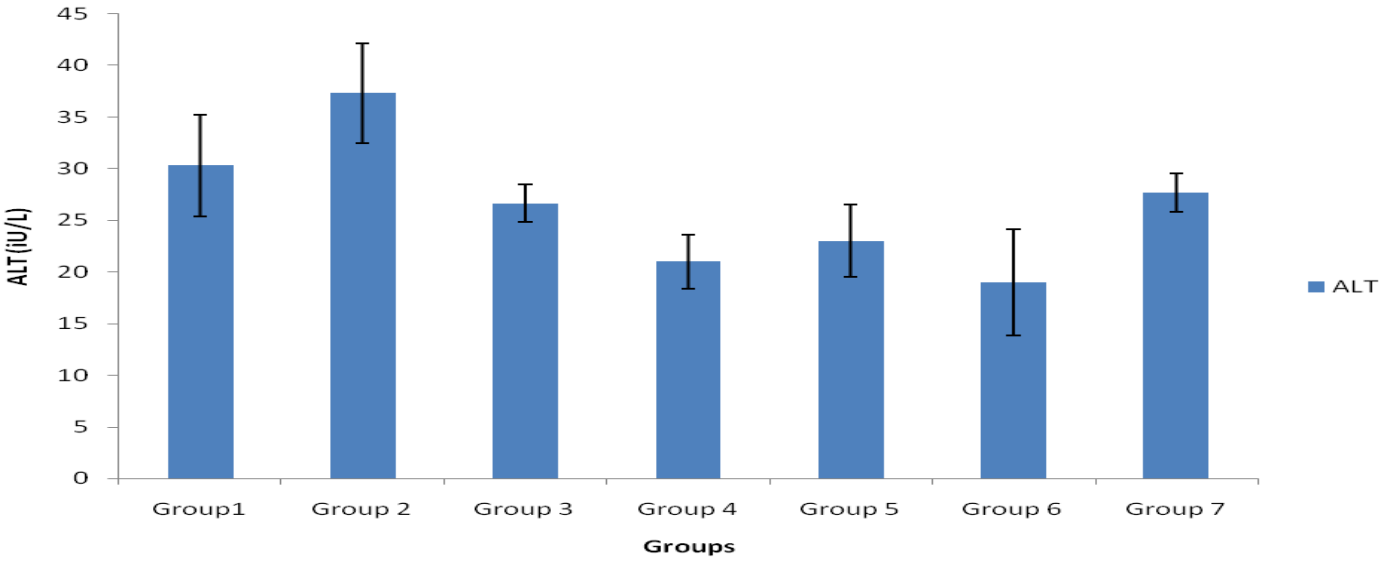


Figure 2. Effect of ethanol extracts of *Newbouldia laevis* leaves and stems on serum alanine aminotransaminase (ALT). Group 1: Control (Normal rats), Group 2: Positive control (Diabetic untreated rats), Group 3: Alloxan induced diabetic rats + 2.5 mg/kg body weight of Glibenclamide, Group 4: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol leaves extract, Group 5: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol leaves extract, Group 6: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol stem extract, and Group 7: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol stem extract.

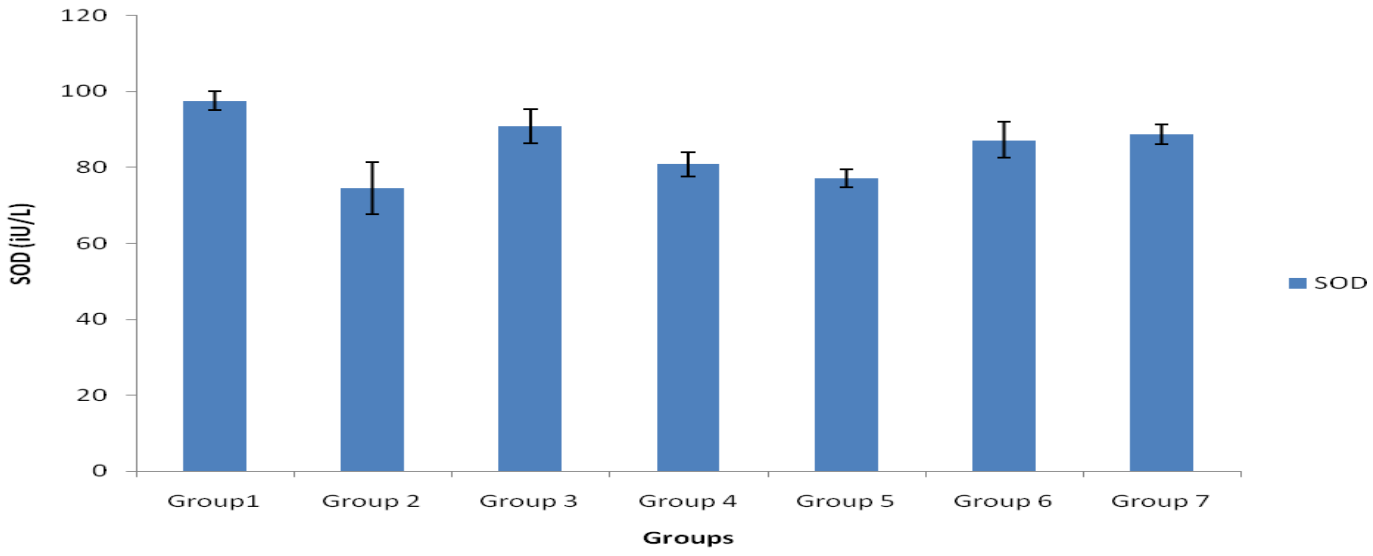


Figure 3. Effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum superoxide dismutase (SOD) activity of diabetic rats. Group 1: Control (Normal rats), Group 2: Positive control (Diabetic untreated rats), Group 3: Alloxan induced diabetic rats + 2.5 mg/kg body weight of Glibenclamide, Group 4: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol leaves extract, Group 5: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol leaves extract, Group 6: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol stem extract, and Group 7: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol stem extract.

Effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum catalase activity

Figure 4 shows that the diabetic untreated group (group 2) had reduced catalase activity but the administration of the different doses of the extracts resulted in significant ($p<0.05$) increase in catalase activity of diabetic rats treated with the leaves and stem extracts of *N. laevis*. A

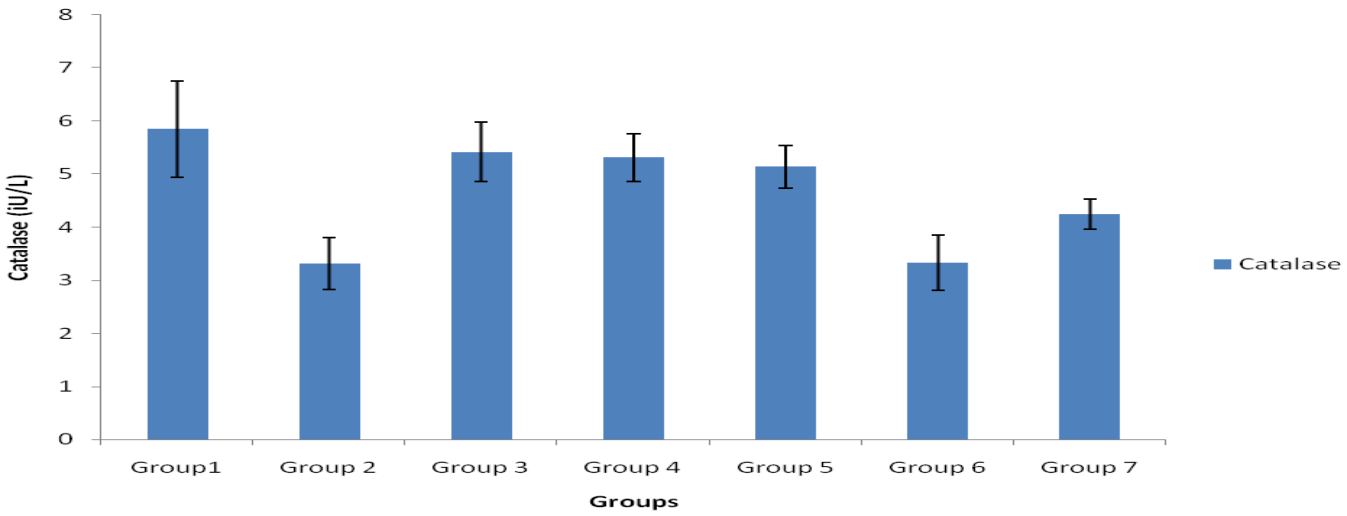


Figure 4. Effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum catalase activity. Group 1: Control (Normal rats), Group 2: Positive control (Diabetic untreated rats), Group 3: Alloxan induced diabetic rats + 2.5 mg/kg body weight of Glibenclamide, Group 4: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol leaves extract, Group 5: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol leaves extract, Group 6: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol stem extract, and Group 7: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol stem extract.

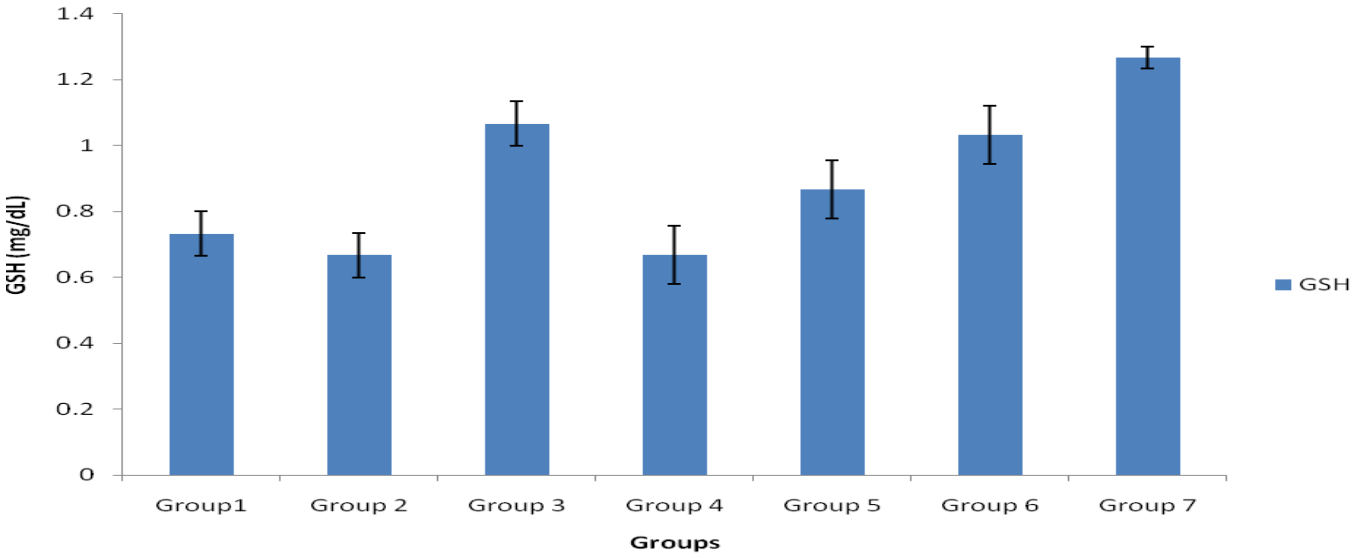


Figure 5. Effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum glutathione. Group 1: Control (Normal rats), Group 2: Positive control (Diabetic untreated rats), Group 3: Alloxan induced diabetic rats + 2.5 mg/kg body weight of Glibenclamide, Group 4: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol leaves extract, Group 5: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol leaves extract, Group 6: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol stem extract, and Group 7: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol stem extract.

significant increase ($p<0.05$) was observed in the catalase activity of rats in the test groups when compared with the control. A significant increases ($p<0.05$) in the serum catalase activity were observed in groups 4 and 5 (treated with the leave extract) when compared with groups 6 and 7 (treated with the stem extract).

Effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum glutathione

Figure 5 shows that the diabetic untreated group (group 2) had decreased glutathione but the administration of the different doses of the extracts resulted in significant

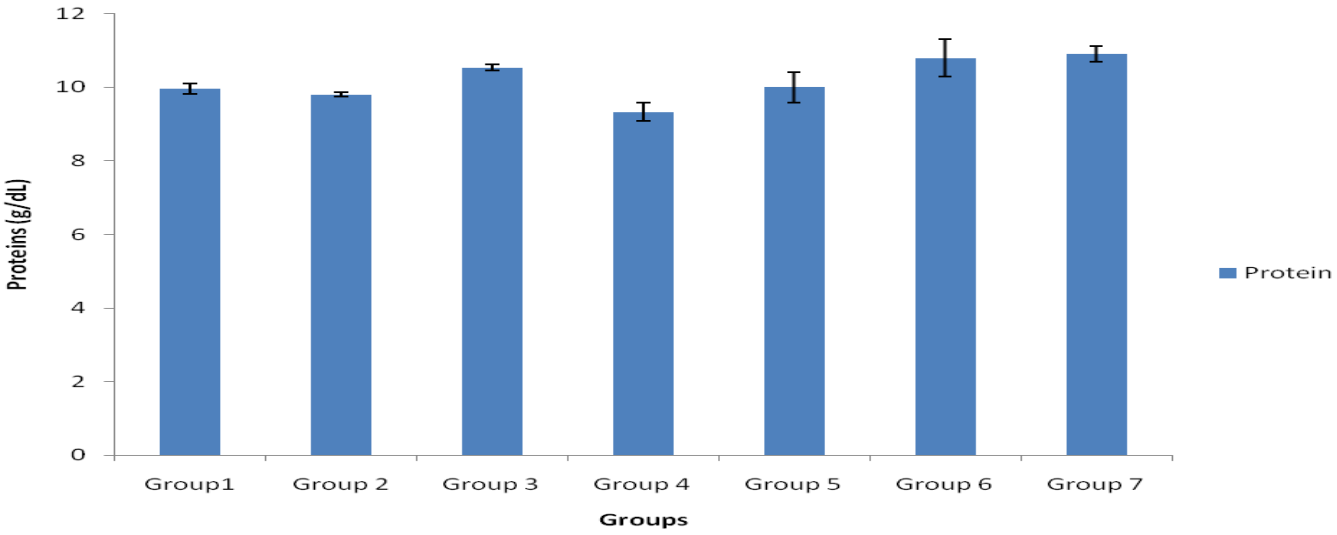


Figure 6. The effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum protein. Group 1. Control (Normal rats), Group 2. Positive control (Diabetic untreated rats), Group 3: Alloxan induced diabetic rats + 2.5 mg/kg body weight of Glibenclamide, Group 4. Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol leaves extract, Group 5: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol leaves extract, Group 6: Alloxan induced diabetic rats + 200 mg/kg body weight of ethanol stem extract, and Group 7: Alloxan induced diabetic rats + 400 mg/kg body weight of ethanol stem extract.

($p<0.05$) increase in glutathione (GSH) level in the treatment groups when compared with the positive control (group 2). The GSH helps to mop up oxidative stress in the test groups as a result of lipid peroxidation. A significant increase ($p<0.05$) was observed in the GSH of rats in the test groups, however, no significant difference ($p>0.05$) was observed in the GSH of rats in group 4 when compared with the positive control. The increase was dose dependent even as the rats treated with the stem extracts (groups 6 and 7) expressed higher level of GSH at both doses tested when compared with groups 4 and 5 (leave extract treated).

Effect of ethanol extracts of *Newbouldia laevis* leaves and stem on serum protein

Figure 6 reveals that increase in protein levels in the treatment groups as against the controls appears to be dose dependent, with group 6 and 7 expressing higher increases in protein levels.

DISCUSSION

Anti-diabetic properties of *N. laevis* leaf and stem have earlier been reported (Anaduaka et al., 2013). Increase in lipid peroxidation during diabetes may be due to the inefficient or overwhelmed antioxidant system due to free radical generation prevalent in diabetic conditions. The status of lipid peroxidation as well as altered levels of certain endogenous radical scavenger is taken as direct evidence for oxidative stress (Goldstein et al., 1993). The liver is an important insulin dependent tissue which plays a pivotal role in glucose and lipid homeostasis and is severely affected during diabetes (Seifter and England, 1982). In this study, alloxan administration to experimental rats caused a marked elevation in the levels of serum ALP and ALT which is indicative of hepatocellular damage. This might possibly be due to the release of these enzymes from the cytoplasm into the blood circulation rapidly after rupture of the plasma membrane and cellular damage.

Several studies have reported similar elevation in the activities of serum ALP and ALT during alloxan administration (Etuk and Muhammed, 2010; Gometi et al., 2014). The notable reduction in serum ALP activity recorded is suggestive of cellular membrane/-hepatocellular membrane protective effects of the extracts. ALP functions as a biochemical marker enzyme for maintaining membrane integrity. Increase in its plasma activity indicates peroxidation of cell membrane integrity. Treatment with *N. laevis* leaves and stem extracts significantly reduced the activities of these liver marker enzymes in alloxan induced oxidative stress as presented in this finding. This indicates that the extracts tend to prevent liver damage by maintaining the integrity of the plasma membrane thereby suppressing the leakage of the enzymes through the membrane, exhibiting hepatoprotective activity. A number of scientific reports indicate that certain flavonoids, terpenoids and steroids have protective effect on liver due to their antioxidant properties (Jeruto et al., 2011). Flavonoids have been reported to possess antioxidant activity (Jeruto et

al., 2011) and thus, are capable of protecting cell membranes from peroxidative actions of free radicals.

Free radical scavenging enzymes like superoxide dismutase (SOD) and catalase protect the biological system from oxidative stress (Conn, 1995; Switala and Loewen, 2002: Del-Rio et al., 2005; Waggiallah and Alzohairy, 2011). The decrease in the activity of the enzymes in the present study could be attributed to the excessive utilization of these enzymes in attenuating the free radicals generated during the metabolism of alloxan. Similar reports have shown an elevation in the status of lipid peroxidation in the liver after alloxan induction (Szkudelski et al., 1998; Gometi et al., 2013) and our findings are in accordance with these reports. Restoration in the levels of the antioxidant enzymes as shown in the above results after 21 days of treatment could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane lipids. Therefore, the antioxidant properties of the extracts may have resulted in the recoupment in the activities of the enzymic antioxidants (SOD and catalase).

Non-enzymic antioxi-dants acts synergistically to scavenge the free radicals formed in the biological system. After 21 days of treatment, the extracts effectively restored the depleted level of this non-enzymic antioxidant caused by alloxan induction.

Increase in GSH level in turn contributes to the recycling of other antioxidants such as vitamin C and E (Exner et al., 2000). This may suggest that the extracts maintain the level of antioxidant vitamins by maintaining GSH homeostasis thereby protecting the cells from further oxidative damage.

There was no significant difference ($p>0.05$) in serum protein as shown in Figure 6 when compared to the controls (group 1 and 2). This might be as a result of some proteins forming intra-chains or inter-chains disulfide bridges between cysteine residues.

The cross-links in this way help to protect the native conformation of the protein molecule from the intensity of oxidative stress (David and Cox, 2005; Biswas et al., 2006).

Conclusion

The experiment evidence obtained in the present laboratory animal study indicates that the ethanol extracts of the leaves and stem of *N. laevis* possess hepatoprotective properties for curbing oxidative stress complications. The efficacy of the extracts can be attributed to the presence of biologically active components which may be worth further investigation and elucidation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Akerele JO, Ayinde BA, Ngiagah J (2011). Phytochemical and antibacterial evaluations of the stem bark of *Newbouldia laevis* against isolates from infected wounds and eyes. Trop. J. Pharm. Res. 10(2): 211-218.

Akunyili DN (2000). Anticonvulsant activity of the ethanol extract of *Newbouldia laevis*. 2nd NAAP Scientific Conference. 155-158.

Amos S, Binda L, Vongtau H, Chindo B, Abbah J, Sambo N, Odin EM, Okwute SR, Arah P, Wambebe C, Gamaniel K (2002). Sedative effects of the methanolic leaf extract of *Newbouldia laevis in* nice and rats. Boll. Chim. Farm. 141(6): 471 -475.

Anaduaka EG, Ogugua VN, Egba S, Apeh VO (2013). Comparative anti-diabetic effects of ethanol extracts of *Newbouldia laevis* leaves and stem on serum lipid profile and lipid peroxidation status in alloxan induced diabetic rats. World J. Pharm. Pharma. Sci. 2(3): 833-845.

Biswas S, Chida AS, Rahman I (2006). Redox modifications of protein-thiols: emerging roles in cell signaling. Biochem. Pharmacol. 71: 551-564.

Cheeseman KH, Slater TF (1993). An introduction to free radical biochemistry. Br. Med. Bull. 49: 481-493.

Conn E (1995). Lipid peroxidation and cellular damage in toxic liver injury. Lab. Invest. 53(6): 599-623.

David LN, Cox MM (2005). Lehinger Principles of Biochemistry (4th Edn). W.H Freeman and Company, New York. p. 1065.

Del Rio D, Stewart AJ, Pellegrini N (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr. Metab. Cardiovasc. Dis. 15(4): 316 - 328.

Ejele AE, Duru IA, Ogukwe CE, Iwu IC (2012). Phytochemistry and antimicrobial potential of basic metabolities of *Piper umbellatum, Piper guineense, Ocimum gratissimium* and *Newbouldia laevis* extracts. J. Emerg. Trends Eng. Appl. Sci. 3(2): 309 -314.

Etuk EU, Muhammed BJ (2010). Evidence based of chemical method of induction of diabetes mellitus in experimental animals. Asian J. Exp. Biol. Sci. 1(2): 331-336.

Exner R, Wessner B, Manhart N, Roth E (2000). Therapeutic potential of glutathione. Wien. Klin. Wochenschr. 112: 610-616.

Gbeassor M, Kedjagni AY, Koumagba K, De Souza C, Agbo K, Aklikokou K, Amego KA (2006). In vitro antimalarial activity of six medicinal plants. Phytother. Res. 4(3): 115 -117.

Goldstein S, Meyerstein D, Czapski G (1993). The Fenton reagents. Free Rad. Biol. Med. 15: 435-445.

Gometi SA, Ogugua VN, Odo CE, Joshua PE (2014). Effects of some anti-diabetic plants on hepatic marker enzymes of diabetic rats. Afr. J. Biotechnol. 13(7): 905-909.

Gometi SA, Ogugua VN, Odo CE, Joshua PE, Nduka OF (2013). Synergic effects of some medicinal plants on antioxidant status and lipid peroxidation in diabetic rats. Afr. J. Pharm. Pharmacol. 7(47): 3011-3018.

Iwu MM (1983). Traditional Igbo Medicine." Institute of African Studies, University of Nigeria, Nsukka. pp. 122-144.

Iwu MM (2000). Handbook of Africa Medicinal Plants CRC Press, Inc. London. p. 19.

Jeruto P, Mutai C, Catherine L, Ouma G (2011). Phytochemical constituents of some medicinal plants used by the Nandis of South Nandi district, Kenya. J. Anim. Plant Sci. 9(3): 1201- 1210.

Koppeno WH (1993). The centennial of the fenton. Free Rad. Biol. Med. 15: 645 -651.

Kutete V, Eyong KO, Folefoc GN, Beng VP. Hussaian, H., Krohn, K, Nkengfack AE (2007). Antimicrobial activity of the methanolic extract and of the chemical constitutents isolated from *Newbouldia laevis.* Pharmazie 62(7): 552 -556.

Lawrence JM, Contraras R, Chen W, Sacks DA (2008). Trends in the prevalence of pre-existing diabetes and gestational diabetes mellitus among a racially / ethnically diverse population of pregnant women, 1999 - 2005. Diabetes Care 31(5): 899 - 904.

Manda G, Nechifor MT, Neagu TM (2009). Reactive oxygen species, cancer, and anti-cancer therapies. Curr. Chem. Biol. 3: 342 -366.

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7(9): 405-410.

Niki E, Yoshida Y, Saito Y, Noguchi N (2005). Lipid peroxidation: Mechanisms, inhibition and biological effects. Biochem. Biophys.

Res. Comm. 338(1):668-676.
Seifter S, England S (1982). Energy Metabolism, In: Arias, I., Popper H. and Schacter, D. (Eds.) The Liver: Biology and Pathology, New York Raven Press. 219-249.
Sies H (1997). Oxidative stress: oxidants and antioxidants. Exp. Physiol. 82(2): 291-295.
Soliman GZA (2008). Blood lipid peroxidation (superoxide dismutase, malondialdehyde, glutathione levels in Egyptian type 2 diabetic patients. Singapore Med. J. 49(2):129-136.
Stanley MP, Venugopal MP (2001). Antioxidant action of *Tinospora cordifolia* root extract in alloxan induced diabetic rats. Phytother. Res. 15: 213-218.
Switala J, Loewen PC (2002). Diversity of properties among catalases. Arch. Biochem. Biophys. 401(2):145-54.
Szkudelski T (2001). The mechanism of alloxan and streptozocin action in S-cell of the rats pancreas. Physiol. Res. 50:536-546.
Szkudelski T, Kandulska K, Okulicz M (1998). Alloxan *in vivo* does not only exert deleterious effects on pancreatic B cells. Physiol. Res. 47(5):343-346.
Traber MG, Atkinson J (2007). Vitamine E, antioxidant and nothing more. Free Rad. Biol. Med. 43(1):4-15.
Usman H, Yaro, AH, Garba MM (2008). Anagesic and anti-inflammatory screening of *Newbouldia laevis* flower in rodents. Trends Med. Res. 3:10-15.
Usman H, Osuji JC (2007). Phytochemical and in vitro antimicrobial assay of the leaf extract of *Newbouldia laevis*. Afr. J. Tradit. Complement. Altern. Med. 4(4): 476-480.
Vertuani S, Angusti A, Manfredini S (2004). The antioxidants and pro-antioxidants network: An overview. Curr. Pharm. Des. 10(14): 1677-1694.
Waggiallah H, Alzohairy M (2011). The effect of oxidative stress on human red cells glutathione peroxidase, glutathione reductase level, and prevalence of anemia among diabetics. N. Am. J. Med. Sci. 3: 344-347.

academicJournals

Vol. 13(22), pp. 2273-2281, 28 May, 2014
DOI: 10.5897/AJB2014.13667
Article Number: 14656F744909
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effects of extract of leaves of *Newbouldia laevis* on the activities of some enzymes of hepatic glucose metabolism in diabetic rats

Oyetunji Timothy Kolawole[1]* and Musbau Adewumi Akanji[2]

[1]Department of Pharmacology and Therapeutics, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.
[2]Department of Biochemistry, University of Ilorin, Ilorin, Nigeria.

Received 27 January, 2014; Accepted 16 May, 2014

In diabetes mellitus and insulin resistance, the activity of the enzymes involved in hepatic glucose homeostasis is disturbed. Promoting the physiologic functions of these enzymes and restoring homeostatic control by medicinal plants could be beneficial in the management of diabetes. This study investigates the effects of extract of leaves of *Newbouldia laevis* on some key enzymes of hepatic glucose metabolism in streptozotocin-induced diabetic rats. Diabetes was induced in rats by intravenous injection of streptozotocin. Diabetic rats were treated orally with *N. laevis* extract for four weeks. At the end of the treatment, the activities of hepatic glucokinase and glucose 6-phosphatase were evaluated. Effect of *N. laevis* extract on glucose 6-phosphatase activity was also assessed *in vitro* using enzyme obtained from rabbit liver. The levels of hepatic glycogen, pancreatic insulin and serum insulin were also determined. Treatment of diabetic rats with *N. laevis* extract resulted in a significant increase ($P < 0.05$) in the activity of hepatic glucokinase when compared with diabetic control. Extract of *N. laevis* showed significant inhibitory effect against the activity of glucose 6-phosphatase in both *in vivo* and *in vitro* studies. The level of hepatic glycogen in treated rats significantly increased ($P < 0.05$) compared to untreated diabetic rats. Although there was a slight increase in serum and pancreatic insulin levels of treated diabetic rats, the difference was not significant ($P > 0.05$) when compared to diabetic control. Based on the results of this study, we conclude that *N. laevis* leaf extract stimulates the activity of hepatic glucokinase and inhibits the activity of glucose 6-phosphatase in streptozotocin-induced diabetic rats. It could serve as a good alternative remedy in the management of diabetes mellitus.

Key words: Liver, glucokinase, glucose 6-phosphatase, diabetes mellitus, *Newbouldia laevis*, glucose.

INTRODUCTION

Liver, in synergy with peripheral tissues, plays an important role in the regulation of blood glucose levels. It is estimated that liver absorbs one third of the postprandial glucose and effectively convert glucose into glycogen for storage through the process of glycogenesis (Moore et al., 2012). Liver maintains stable blood glucose levels in the fasting state by producing glucose through the processes of glycogenolysis and gluconeogenesis.

*Corresponding author. E-mail: tymkol@yahoo.co.uk.

The key enzymes that play prominent roles in hepatic glucose homeostasis include glucokinase and glycogen synthase which catalyze the process of glycogenesis; and the major enzymes responsible for the regulation of gluconeogenesis are glucose 6-phosphatase, pyruvate carboxylase, phosphoenolpyruvate carboxykinase and fructose 1, 6-bisphosphatase (Eid et al., 2006). Hepatic glucose homeostatic control is also influenced at the hormonal level. The key hormones involved in glucose homeostasis are insulin and glucagon. When there is an increase in blood glucose concentration, insulin release is increased and glucagon release is suppressed. As a result, glucokinase is stimulated, glycogen synthase is activated and glycogen phosphorylase is inhibited leading to enhanced glycogen synthesis. On the other hand, glucose 6-phosphatase catalyzes the dephosphorylation of glucose 6-phosphate to glucose as the terminal step in gluconeogenesis and glycogenolysis (Dentin et al., 2007). In type 2 diabetes and insulin resistance, the control of hepatic glucose metabolism is disturbed and this leads to increased hepatic glucose output and hyperglycemia. Therefore targeting the pathways of these hepatic enzymes could lead to significant reduction of blood glucose level in type 2 diabetic patients (Tahrani et al., 2011; Agius, 2007). One way through which this could be achieved is to investigate the effects of medicinal plants on these pathways.

For centuries, medicinal plants have been employed in the management of diabetes mellitus and are well recognized as important source of new drugs. Therefore, in the search for novel anti-diabetic drugs, the potentials of medicinal plants should be diligently explored (Newman and Cragg, 2007). Such efforts have yielded positive results in the past. For instance, metformin is an anti-diabetic drug derived from *Galega officinalis*, a plant prescribed in medieval times to relieve the symptoms of diabetes (Andrade-Cetto, 2012). Metformin lowers blood glucose levels without causing overt hypoglycemia or stimulating insulin secretion. It reduces fasting plasma glucose by 25% by suppressing hepatic gluconeogenesis from substrates such as pyruvate, lactate, glycerol and amino acids. It also modulates mitochondrial respiration by increasing intramitochondrial level of calcium ions (Jaya et al., 2010). There are other plants that are used in the treatment of diabetes which may contain principles that have similar or even better therapeutic effects than metformin. Many of these plants, including *Newbouldia laevis*, have not been subjected to proper scientific investigation. *N. laevis* is an angiosperm which belongs to the Bignoniaceae family. Its common names are ‘Fertility tree’ and ‘African border tree’. The extract of the leaves has been reported to lower blood glucose level in diabetic rats (Owolabi et al., 2011). It was also shown to attenuate glycation of hemoglobin and lipid peroxidation in diabetic rats (Kolawole et al., 2013a). In the present study, we investigated the effects of extract of the leaves of *N. laevis* on insulin secretion and the activities of glucokinase and glucose 6-phosphatase in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Preparation of plant extract

Leaves of *N. laevis* were collected from the premises of Government House Annex, Osogbo, Nigeria. The leaves were identified and authenticated by Mr. Odewo of Forest Research Institute of Nigeria (FRIN) and a voucher specimen was deposited in the herbarium of the institute (voucher specimen no: FHI 107753). After the leaves have been thoroughly washed with clean distilled water, they were air-dried under shade in the laboratory for 5 days and then pulverized using an electric grinding machine. The powder sample (400 g) was extracted with 80% ethanol at 70°C by continuous hot percolation using a Soxhlet apparatus. The extraction was carried out for 24 h and the resulting ethanol extract (NLet) was concentrated at 40°C in a rotary evaporator. The solid sample obtained weighed 40.2 g (yield = 10 %). The crude ethanol extract was kept in an air-tight container and stored in a refrigerator at 4°C until the time of use.

Experimental animals

Male Wistar rats weighing 150 to 180 g were obtained from the Animal Holding Unit of the Department of Pharmacology and Therapeutics, Ladoke Akintola University of Technology (LAUTECH), Nigeria. The animals were housed in polypropylene cages in a well-ventilated area in the laboratory complex. The animals were maintained under standard laboratory conditions of temperature (22 ± 2°C), relative humidity (55-65%) and 12 h light/dark cycle. They were allowed to acclimatize for two weeks before the experiment. During the experimental period, animals were fed with a standard balanced commercial pellet diet (Ladokun Feeds Ltd. Ibadan, Nigeria) and potable tap water *ad libitum*.

Ethical consideration

All experimental procedures were conducted in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH, 1985) as well as Ethical Guidelines for the Use of Laboratory Animals in LAUTECH, Nigeria.

Induction of diabetes mellitus

Experimental diabetes was induced in rats which had fasted for 12 h by a single intravenous injection of a freshly prepared solution of streptozotocin (STZ) (60 mg/kg body weight) dissolved in 0.1 M cold citrate buffer, pH 4.5 (Chen et al., 2005). The rats were allowed to drink 5% glucose solution overnight to overcome drug-induced hypoglycemia. Estimation of fasting blood glucose (FBG) was done 72 h after injection of STZ to confirm induction of diabetes and then on the 7th day to ensure a stable diabetic condition. Fasting blood glucose was estimated by One Touch® glucometer (Lifescan, Inc. 1995 Milpas, California, USA). Blood sample for the FBG determination was obtained from the tail vein of the rats and those with blood glucose value ≥ 200 mg/dl were selected for the study.

Treatment of experimental animals

Rats were divided into a group of non-diabetic and three groups of STZ - diabetic rats. Each of the four groups consisted of six rats. The

grouping was as follows: Group I = non-diabetic control; Group II = diabetic control; Group III = diabetic rats treated with NLet (500 mg/kg); Group IV = diabetic rats treated with metformin (200 mg/kg) or glibenclamide (5 mg/kg). The drugs were administered orally everyday for 28 days using a sterile syringe fitted with a sterile cannula. Rats in groups I and II were treated orally with distilled water for the four weeks. On Day 29, the rats were euthanized under chloroform vapor. The jugular vein was exposed and cut with a sterile scalpel blade, and the rats were bled into specimen bottles. Blood samples were transferred to sterilized centrifuge tubes and allowed to clot at room temperature. The blood samples were centrifuged for 10 min at 1500 rpm. The serum obtained was used for serum insulin assay.

Tissue preparation

After 28 day treatment with the plant extract (NLet) and the reference drugs, the rats were sacrificed by cervical dislocation. Segments of the liver and pancreas tissues were excised separately from rats in all the experimental groups. The tissues were washed with phosphate buffered saline (pH 7.4) containing 0.16 mg/ml of heparin to remove any red blood cells (erythrocytes) and clots (Prasad et al., 1992). Liver and pancreas homogenates were prepared and stored at -20°C for the assays. Liver homogenate was used for glucokinase, *in vivo* glucose 6-phosphatase and hepatic glycogen assays while pancreas homogenate was used for the pancreatic insulin assay.

Glucokinase assay

Glucokinase activity was measured as previously described (Zhang et al., 2009; Davidson and Arion, 1987). About 100 mg liver tissue was homogenized in 1 ml ice-cold homogenization buffer containing 100 mM KCl, 25 mM HEPES, 7.5 mM $MgCl_2$, 4 mM dithiothreitol (pH 7.4), and then lysed overnight at 4°C. Supernatant of the tissue extract was obtained after centrifugation at 3000 rpm for 10 min at 4°C, and then supplemented with 1 mM NAD, 4 mM ATP, and 100 or 0.5 mM glucose at pH 7.4. The enzymatic reaction was started by the addition of 0.2 unit of glucose 6-phosphate dehydrogenase and incubated for 10 min at 30°C. NADH generated by glucokinase was measured by a spectrophotometer at 340 nm. The enzymatic activity of glucokinase was calculated as the difference between activities calculated at 100 and 0.5 mM glucose. Hexokinase activities were calculated as mU/mg protein. Protein concentration in liver extract was measured by Lowry method (Lowry et al., 1951).

***In vivo* glucose 6-phosphatase assay**

Glucose 6-phosphatase (G6Pase) activity was assayed according to the method of Baginsky et al. (1974) by estimation of inorganic phosphate (P_i) liberated from glucose 6-phosphate (G6P). The ultracentrifugation method of Pari and Satheesh (2006) was used to prepare liver microsomal fractions. For this assay, 1 g of frozen liver tissue was homogenized in ice-cold sucrose solution with a Polytron homogenizer. The homogenate was centrifuged sequentially at 11,000 g for 30 min, then at 105,000 g for 1 h using an ultracentrifuge (Beckman Inc., CA, USA). The solid pellet was re-suspended in ice cold sucrose/EDTA solution and used as the source of the enzyme. Tubes were divided into samples, blanks and standard. To each were added 0.1 mL of sucrose/EDTA buffer (0.25 mM, pH 7.0), 0.1 mL of G6P (100 mM), and cacodylate buffer solution. This was followed by the addition of 0.1 mL of sample to the sample tube, 0.1 mL of sucrose/EDTA solution to the blank and 0.1 mL of different concentration of K_2HPO_4 (0.5, 1, 1.5 and 2 mM) to the standard tube. All tubes were incubated at 37°C for 15 min and the enzyme activity was then terminated by adding 2 mL TCA/ascorbate (10%/2%). The tubes were centrifuged at 3000 g for 10 min. To 1.0 mL of this clean supernatant were added 0.5 mL ammonium molybdate (1%) and 1 mL of Na-arsenite/Na-citrate (2%/2%). The tubes were then allowed to stand for 15 min at room temperature and absorbance was read at 840 nm. The amount of inorganic phosphate liberated by the enzyme was calculated by comparing with absorbance values of the standard. G6Pase activity was expressed as units/mg of protein. One unit of glucose-6-phosphatase activity is defined as the amount of P_i liberated/min at 37°C under the assay conditions.

***In vitro* glucose 6-phosphatase assay**

The effect of NLet on glucose 6-phosphatase activity was performed using glucose 6- phosphatase from rabbit liver (Sigma, USA). The method of Baginsky et al. (1974) was adapted. Fifty microlitres (50 µL) of each concentration of the plant extract, sodium orthovanadate or metformin were transferred into separate test tubes followed by 100 µL of enzyme solution (glucose 6-phosphatase in 0. 25 M sodium acetate buffer, pH 6.7). The mixture was then pre-incubated in a water bath at 37°C for 20 min Thereafter 100 µL of the substrate solution (glucose 6-phosphate in 0. 25 M sodium acetate buffer pH 6. 7) was added into each of the test tube to start the reaction. The mixture was again incubated at 37°C for 15 min When the reaction was complete, 500 µL of Molybdate reagent (2.5 g of $(NH_4)_6Mo_7.4H_2O$ in 2. 45 M H_2SO_4 w/v) and 500 µL of reducing agent (1.0% Elon (p-methyl aminophenol sulphate) in 3.0% sodium bisulphite ($NaHSO_3$), w/v) were added. The intensity of the blue solution was measured with a spectrophotometer at 660 nm. Glucose 6-phosphatase inhibitory activity was expressed as percentage inhibition. The graph of percentage inhibition was plotted against extract concentration.

Hepatic glycogen assay

Hepatic glycogen content was measured according to the method of Postle and Bloxham (1980). Frozen tissue (100 mg) was placed in chilled citrate buffer (0.1 M, pH 4.5) and extracted with 1.5 mL of 6% (w/v) perchloric acid ($HClO_4$). This was then centrifuged at 2000 g for 15 min and the supernatant (0.5 mL) was neutralized with 10% (w/v) KOH. The glycogen in the supernatant was hydrolyzed by α-amyloglucosidase (50 U/mL; Sigma, USA) in sodium acetate buffer (50 mM/L; pH 4.8) overnight at room temperature. Glucose released from glycogen was estimated by glucose assay kit (Sigma, USA). The glycogen content of the liver samples was calculated as the difference between the glucose level with and without amyloglucosidase incubation. Glycogen content was expressed as mg/g wet tissue.

Insulin assay

Serum insulin level was measured by an enzyme-linked immunosorbent assay (ELISA) procedure using rat insulin ELISA kit (Mercodia, USA). Briefly, the solid phase two-site enzyme immunoassay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. After washing three times, unbound enzyme labeled antibody was removed. The bound conjugated insulin was detected by reacting with 3, 3', 5, 5'-tetramethylbenzidine. The reaction was stopped by adding acid to give a colorimetric end-point and optical density was measured with a microplate autoreader at a wavelength of 450 nm.

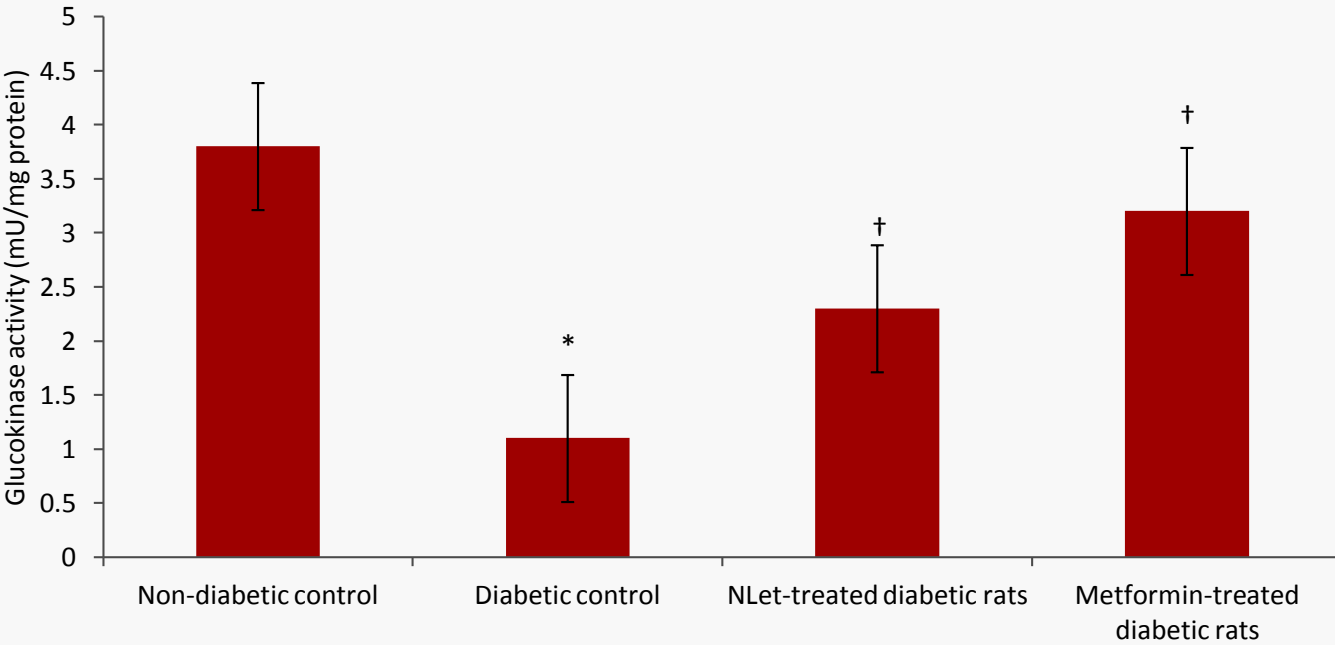


Figure 1. Effect of *N. laevis* extract on hepatic glucokinase activity in diabetic rats. Values are mean ± SEM (n = 6). **P* < 0.05 compared with non-diabetic control rats; †*P* < 0.05 compared with diabetic control.

The serum insulin was expressed as µg/L. The same procedure was followed for pancreatic insulin assay using supernatant of pancreas homogenate as sample. The results were expressed as ng/g wet weight of tissue.

Phytochemical analysis

Tests were carried out on the extract using standard procedures to identify its phytoconstituents as described by Trease and Evans (2002), Harborne (1984), and Sofowora (1993). The extract was tested for alkaloids, flavonoids, tannins, terpenoids, saponin, phenolic compounds and cardiac glycosides.

Statistical analysis

Data obtained from the experiments are expressed as mean ± standard error of mean (SEM). For statistical analysis, data were subjected to one-way analysis of variance (ANOVA) followed by Student's t- test. A level of $P < 0.05$ was taken as significant. GraphPad Prism version 5.0 for windows was used for these statistical analyses (GraphPad software, San Diego California USA).

RESULTS

Hepatic enzymes

The activity of glucokinase was significantly reduced in STZ-diabetic rats compared to nondiabetic control. Treatment of diabetic rats with NLet and metformin resulted in a significant increase ($P < 0.05$) in the activity of hepatic glucokinase when compared with diabetic control as shown in Figure 1. *In vivo*, G6Pase activity of STZ-diabetic rats was significantly increased ($P < 0.05$) compared to that of non-diabetic control. Treatment of diabetic rats with NLet and metformin for 28 days caused significant decrease ($P < 0.05$) in the activity of G6Pase compared to diabetic control (Figure 2). *In vitro* study also demonstrated that NLet has inhibitory effect against the activity of glucose 6-phosphatase in rabbit liver (Figure 3). The inhibitory activity of NLet (IC_{50} = 752.8 µg/ml) was about half that of sodium orthovanadate (IC_{50} = 355.4 µg/ml). Metformin failed to inhibit rabbit glucose 6-phosphatase *in vitro*.

Hepatic glycogen content

Hepatic glycogen level was significantly decreased ($P < 0.05$) in diabetic rats compared to non-diabetic control. After treating the animals with NLet (500 mg/kg) and metformin (200 mg/kg) for 28 days, the level of hepatic glycogen in both NLet-treated and metformin-treated groups significantly increased ($P < 0.05$) compared to diabetic control (Figure 4). However there was a significant difference ($P < 0.05$) between NLet-treated and metformin-treated groups.

Serum and pancreatic insulin levels

Serum and pancreatic insulin levels were significantly decreased ($P < 0.05$) in diabetic control rats compared to the non-diabetic group. Although there was a slight

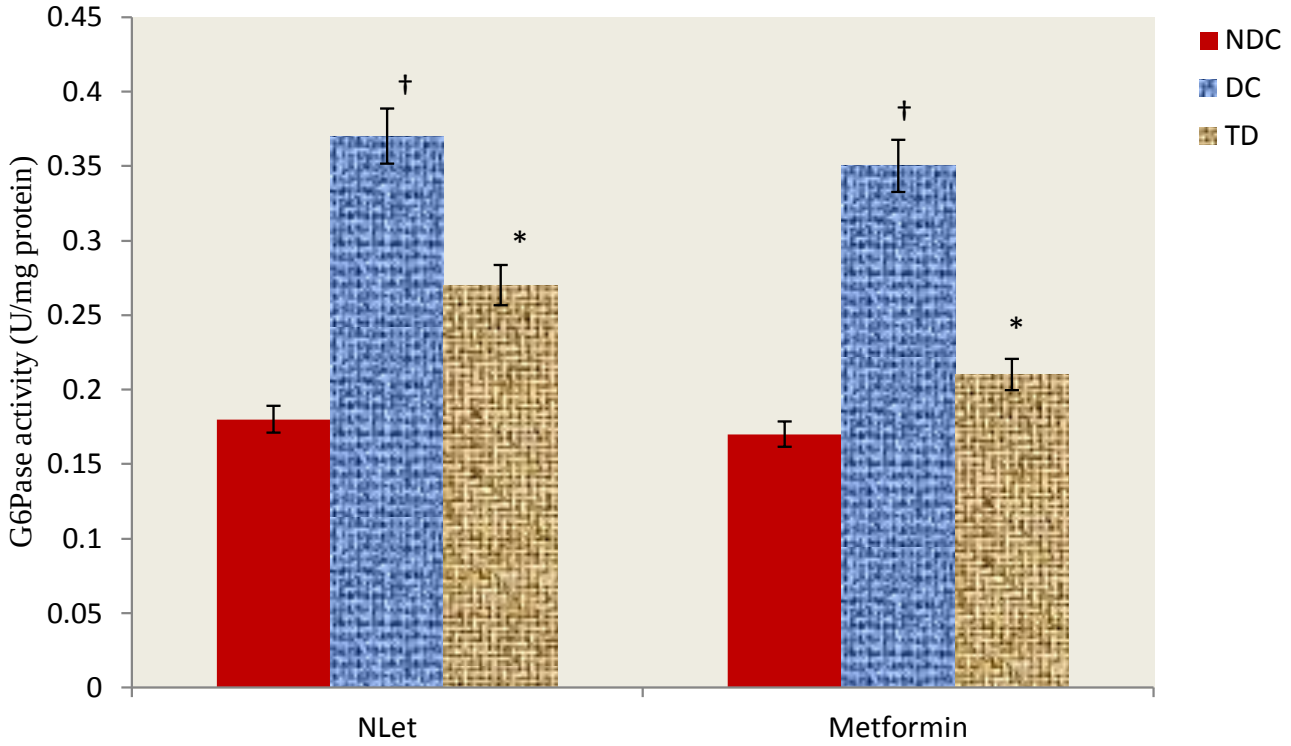


Figure 2. *In vivo* effect of *N. laevis* extract on rat liver Glucose 6-phosphatase activity. Values are mean ± SEM (n = 6). *P < 0.05 compared with diabetic control rats; †P < 0.05 compared with non-diabetic control. NDC = Non-diabetic control; DC = Diabetic control; TD = Treated diabetic rats

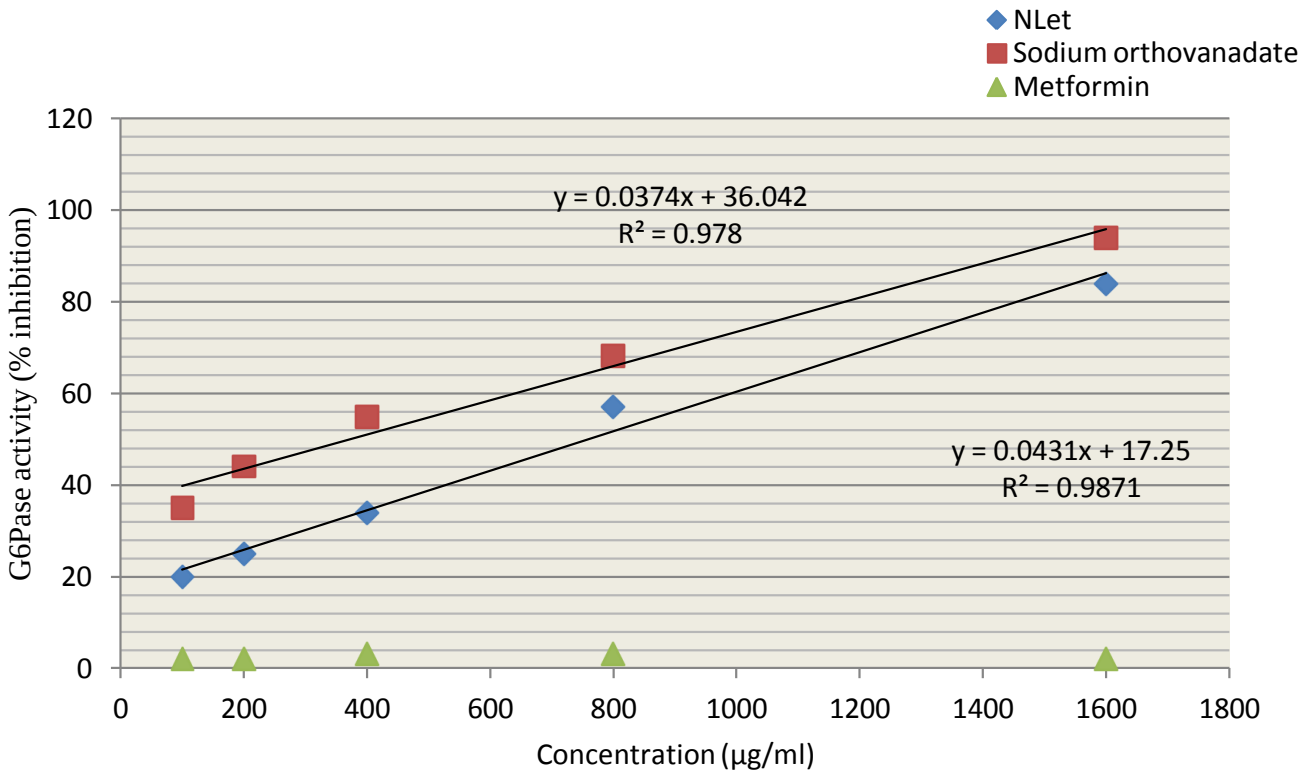


Figure 3. *In vitro* effect of *N. laevis* extract on rabbit liver glucose 6-phosphatase activity. Values represent mean ± SEM of three replicates.

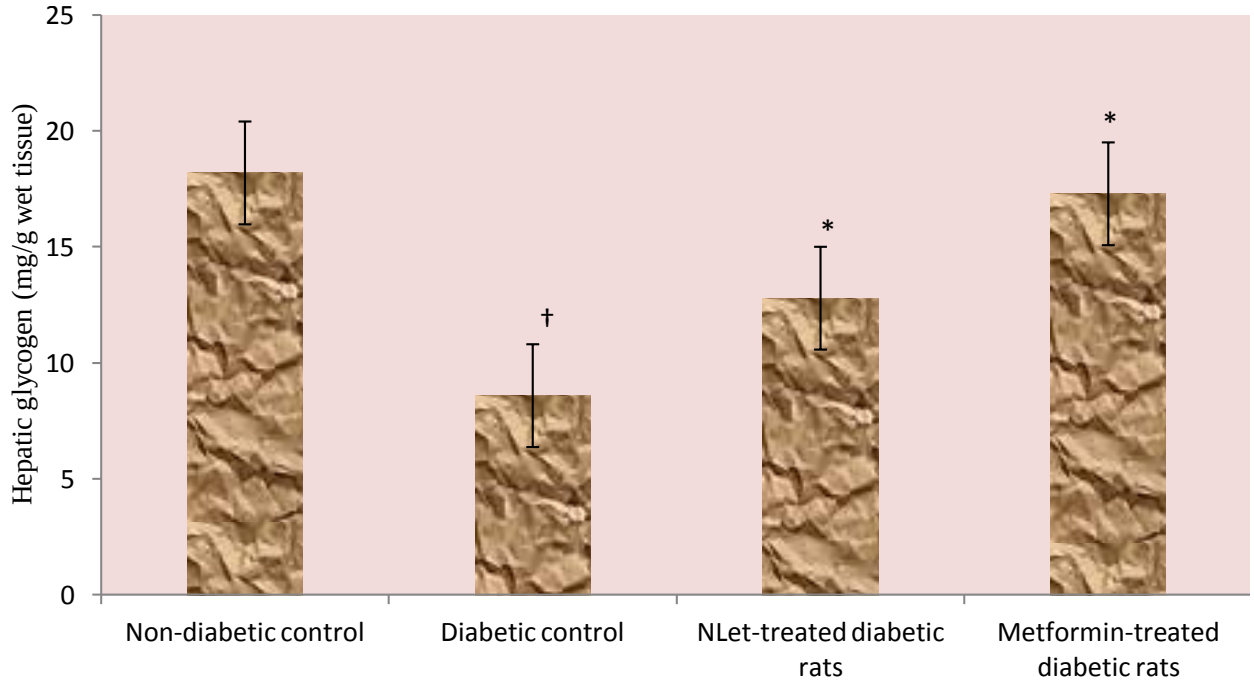


Figure 4. Effect of *N. laevis* extract on hepatic glycogen of diabetic rats. Values represent mean ± SEM (n = 6); $^{\dagger}P < 0.05$ compared with non-diabetic control, $^{*}P < 0.05$ compared with diabetic control.

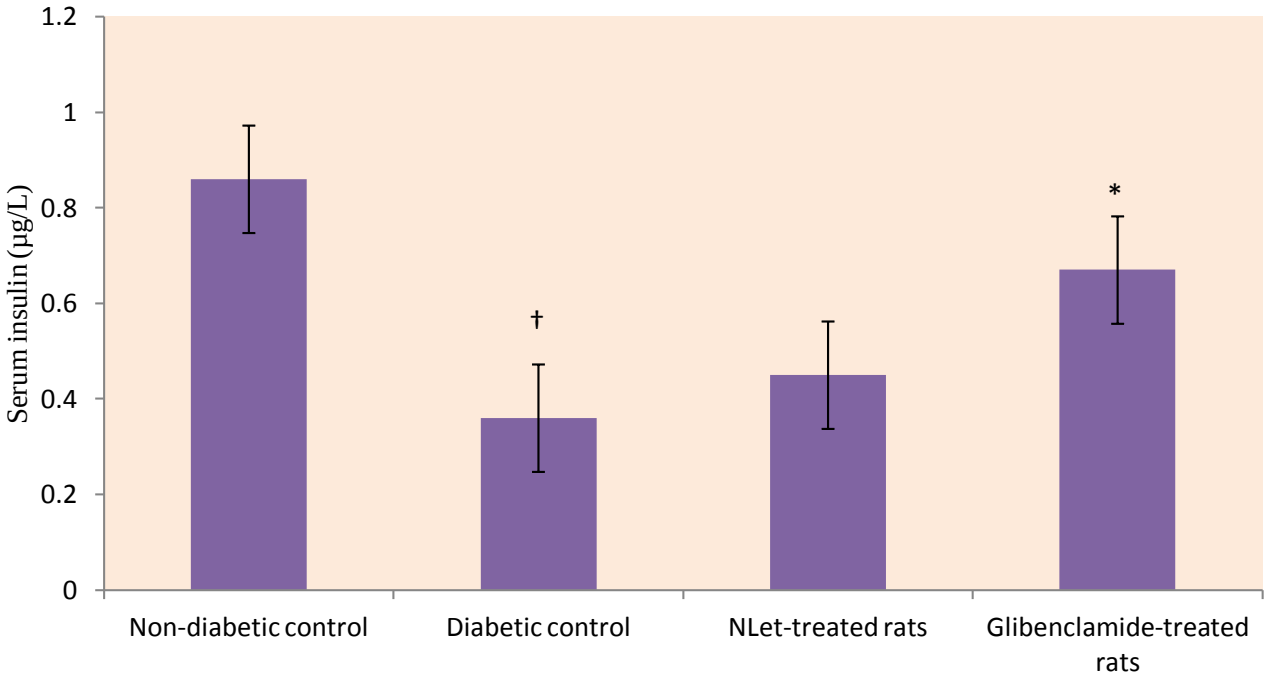


Figure 5. Effect of *N. laevis* leaf extract on serum insulin of diabetic rats. Values represent mean ± SEM (n = 6); $^{\dagger}P < 0.05$ compared with non-diabetic control, $^{*}P < 0.05$ compared with diabetic control.

increase in both serum and pancreatic insulin levels of NLet –treated diabetic rats, the difference was not significant ($P > 0.05$) when compared to diabetic control as shown in Figures 5 and 6, respectively.

Phytochemical screening

The results of the analysis show that the crude ethanolic extract of *N. laevis* leaves contain tannins, saponin,

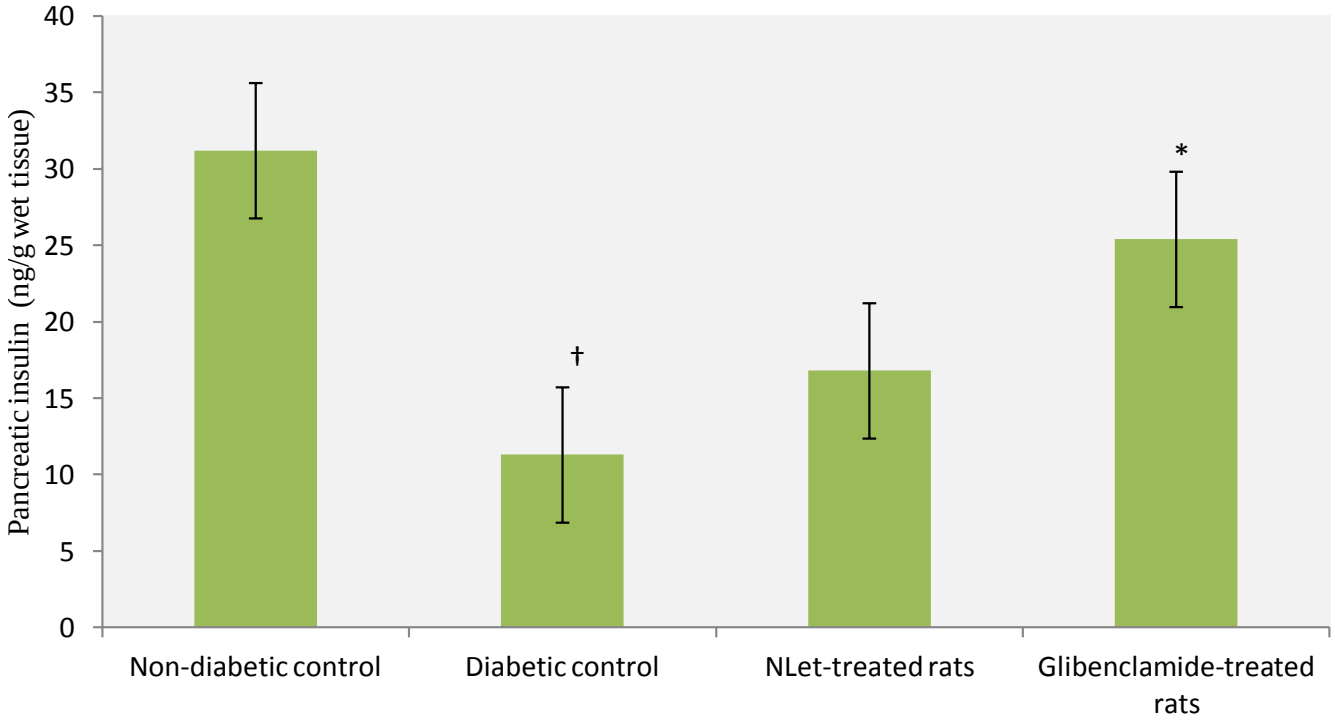


Figure 6. Effect of *N. laevis* leaf extract on pancreatic insulin of diabetic rats. Values represent mean ± SEM (n = 6); $^{\dagger}P < 0.05$ compared with non-diabetic control, $P < 0.05$ compared with diabetic control.

Table 1. Phytoconstituents of the leaf extract of *Newbouldia laevis*.

Compound tested	Test	Observation	Inference
	Dragendorff	(-)	Absent
Alkaloids	Mayer	(-)	Absent
	Wagner	(-)	Absent
	Shinoda	(+)	Present
Flavonoids	Lead acetate	(+)	Present
	Ammonia	(+)	Present
Saponin	Frothing	(+)	Present
Tannins	Prussian blue	(+)	Present
Phenolic compounds	Ferric chloride	(+)	Present
Cardiac glycosides	Keller-Killani	(-)	Absent
Terpenoids	Salkowski's	(+)	Present

(+) = positive; (-) = negative.

flavonoids, terpenoids and phenolic compounds. Cardiac glycosides and alkaloids were not detected. The results of the analysis are presented below (Table 1).

DISCUSSION

Glucokinase is one the enzymes that play key role in hepatic glucose homeostasis. It catalyzes the rate limiting step of glycolysis by phosphorylating glucose leading to the synthesis of glucose -6-phosphate (Postic et al., 2004). In pancreatic β-cells, glucokinase acts as a glucose sensor, ensuring that insulin release is appropriate to the plasma glucose concentration. In the liver, glucokinase is essential for this tissue to carry out its unique metabolic functions (Agius, 2008). Under basal glucose concentrations (< 5.5 mM), hepatic glucokinase is bound to the glucokinase regulatory protein (GKRP) in

the nucleus (Zimmet et al., 2005). However, when subjected to high glucose concentration (10-30 mM), glucokinase is released from the GKRP into the cytoplasm where it exists in the unbound active form (Alberti et al., 2005). Impairment of glucokinase activity plays a major role in precipitating glucose intolerance and the development of diabetes mellitus. Glucokinase gene mutation has been associated with a form of diabetes known as maturity onset diabetes of the young (Zhang et al., 2009).

In the present study, the activity of glucokinase significantly decreased in STZ-diabetic rats. In the NLet-treated diabetic rats, there was an increase in the activity of this enzyme which was significant ($P < 0.05$) when compared with the diabetic control. This is probably a reflection of the slight increase in plasma insulin concentration following treatment with NLet. Glucokinase activity is impaired in pancreas and liver following the damage caused by free radicals that are generated by streptozotocin in diabetic rats (Zhang et al., 2009; Srinivasan and Ramarao, 2007). Therefore the observed effects of the extract could also be due to direct activation of glucokinase. Activators of glucokinase have been reported to enhance the activity of the enzyme in drug-induced diabetic animals (Priyadarsini et al., 2012).

Glucose 6-phosphatase (G6Pase) catalyzes the final step in both glycogenolytic and gluconeogenic pathways, cleaving phosphate from glucose 6-phosphate to liberate free glucose into the circulation (Smith et al, 2005). It is thus uniquely situated to regulate both circulating concentrations of glucose and the storage of excess glucose as glycogen. In diabetic condition, glucose 6-phosphatase activity is elevated and the symptoms associated with hyperglycemia are exacerbated. In this study, *in vivo* activity of G6Pase in the rat liver was significantly reduced ($P < 0.05$) in both NLet- and metformin-treated groups when compared to diabetic control group. The hypoglycemic activity of NLet reported by Owolabi et al. (2011) could be due to the suppression of G6Pase activity as reported for other hypoglycemic agents such as vanadate compounds (Mosseri et al., 2000). Normalization of serum blood glucose concentration in diabetes with insulin usually results in decreased G6pase gene expression and activity (Massillon et al., 1996). However, the same report also indicated that correction of hyperglycemia in diabetic rats leads to normalization of hepatic gene expression of G6Pase regardless of the circulating insulin concentration. This indicates that *in vivo* gene expression of G6Pase in the diabetic liver is regulated by glucose independent of insulin. Therefore the reduction in G6Pase activity in rat liver observed in this study after treatment with NLet may be attributed to the hypoglycemic effect due to insulin or other mechanisms independent of insulin. *In vitro* study also confirmed the inhibitory activity of NLet against G6Pase. The inhibitory activity of NLet was about half that of sodium orthovanadate. This result is promising especially when one takes into consideration the toxicity of sodium orthovanadate. It was earlier reported that NLet has low toxicity profile with LD_{50} > 5g/kg body weight in mice (Kolawole et al., 2013b). Metformin is a well known inhibitor of G6pase *in vivo* (Jung et al., 2006). However the results of this study showed that metformin failed to inhibit the activity of hepatic G6Pase *in vitro*. Enzyme inhibitors are known to act via different mechanisms (Lieberman and Marks, 2009). Some inhibitors induce conformational changes in the structure of an enzyme; such inhibitors could be expected to inhibit enzymes *in vitro.* Other enzyme inhibitors influence the activity of enzymes by suppressing the synthesis of enzymes at the genetic level, and as such will show no inhibitory effect *in vitro*. The result of this study suggests that metformin belongs to the latter group of inhibitors.

The results of this study indicate that hepatic glycogen level was significantly decreased in diabetic rats compared to non-diabetic control. After the 28-day treatment with NLet and metformin, the level of hepatic glycogen in both NLet-treated and metformin-treated groups significantly increased compared to diabetic control. A significant difference was also observed between NLet-treated and metformin-treated groups. This may be a reflection of insulin level in each experimental group. *Newbouldia laevis* extract appears to have very little insulinotropic effect and the stimulation of glycogen synthase activity in the NLet-treated group was not as pronounced as in the group treated with metformin. Metformin on the other hand appears to have stimulated the activities of glycogen synthase and glucokinase better than NLet. The slight increase in serum and pancreatic insulin levels observed in diabetic rats following treatment with NLet could be due to the protection of the functional β-cells from further deterioration so that they remain active and produce insulin. Studies have shown that the presence of phytochemicals such as flavonoids, phenols, saponins, tannins and terpenoids in plants are responsible for their anti-diabetic properties (Narender et al., 2011; Momoh et al., 2011). Phytochemical analysis of NLet revealed the presence of tannins, flavonoids, saponins, terpenoids and phenolic compounds, while cardiac glycosides and alkaloids were not detected. The active ingredients responsible for antidiabetic activities of NLet probably reside in one or more of these phytochemicals.

Conclusion

Our data indicate that extract of the leaves of *N. laevis* promotes good hepatic glucose homeostasis in streptozotocin-induced diabetic rats by enhancing the activity of hepatic glucokinase and inhibiting the activity of glucose 6-phosphatase. It could serve as a good alternative medicine in the management of diabetes mellitus.

Structural elucidation of the active components of the plant could also provide a lead molecule for the development of novel antidiabetic drug.

Conflict of Interests

The author(s) have not declared any conflict of interests

REFERENCES

Agius L (2007). New hepatic targets for glycaemic control in diabetes. Best Pract Res Clin Endocrinol. Metab. 21(4):587- 605.

Agius L (2008). Glucokinase and molecular aspects of liver glycogen metabolism. Biochem. J. 414(1):1-18.

Alberti GK, Zimmet P, Shaw J (2005). The metabolic syndrome - a new worldwide definition. Lancet 366:1059-1062

Andrade-Cetto A (2012). Effects of medicinal plant extracts on gluconeogenesis. Botanics: Targets and Therapy. 2:1- 6.

Baginsky ES, Foa PP, Zak B (1974). Glucose 6-phosphatase. In: Bergymeyer, H.U. (ed.), Methods of Enzymatic Analysis, Vol. 2 (2nd edition), Academic Press, New York, pp. 788-792.

Chen H, Brahmbhatt S, Gupta A, Sharma AC (2005). Duration of streptozotocin-induced diabetes differentially affects p38-Mitogen-Activated Protein Kinase (MAPK) phosphorylation in renal and vascular dysfunction. Cardiovascular Diabetol. DOI:10.1186/1475-2840-4-3

Davidson A, Arion WJ (1987). Factors underlying significant underestimation of glucokinase activity in crude liver extracts: physiological implications of higher cellular activity. Arch Biochem. Biophys. 253:156 -167.

Dentin R, Liu Y, Koo SH, Hedrick S, Vargas T, Heredia J, Yates J III, Montminy M (2007). Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. Nature 449:366-369.

Eid A, Bodin S, Ferrier B, Delage H, Boghossian M, Martin M, Baverel G, Conjard A (2006). Intrinsic gluconeogenesis is enhanced in renal proximal tubules of zucker diabetic fatty rats. J. Am. Soc. Nephrol. 17: 398-405.

Harborne JB (1984). Phytochemical Methods. 2nd ed. Chapman and Hall, London, p. 279

Jaya A, Shanthi P, Sachdanandam P (2010). Modulation of oxidative/nitrosative stress and mitochondrial protective effect of *Semecarpus anacardium* in diabetic rats. J. Pharm. Pharmacol. 62:507-513.

Jung M, Park M, Lee HC, Kang YH, Kang ES, Kim SK (2006). Antidiabetic agents from medicinal plants. Curr. Med. Chem. 13:1203-1218.

Kolawole OT, Akanji MA, Akiibinu MO (2013b). Toxicological assessment of ethanolic extract of the leaves of *Newbouldia laevis* (P. Beauv). Am. J. Med. Medic. Sci. 3(4):74 - 80 DOI:10.5923/j.ajmms.20130304.04

Kolawole OT, Akanji MA, Awe EO, Akiibinu MO (2013a). Ethanolic extract of leaves of *Newbouldia laevis* attenuates glycosylation of hemoglobin and lipid peroxidation in diabetic rats. Am. J. Pharmacol. Toxicol. 8(4):179-186. DOI:10.3844/ajptsp.2013.179.186

Lieberman M, Marks AD (2009). Marks' Basic Medical Biochemistry: A Clinical Approach. 3rd Ed. Lippincott Williams and Wilkins, Philadelphia. ISBN- 10: 078177022X, pp.139-155.

Lowry OH, Rosenbrough NJ, Farr AL, Randall JL (1951). Protein measurement with the Folin Phenol reagent. J. Biol. Chem.193:265-275.

Massillon D, Barzilai N, Chen W, Hu M, Rossetti L (1996). Glucose regulates *in vivo* glucose-6- phosphatase gene expression in the liver of diabetic rats. J. Biol. Chem. 271:9871-9874.

Momoh S, Yusuf OW, Adamu MM, Agwu COC, Atanu FO (2011). Evaluation of the phytochemical composition and hypoglycemic activity of methanolic leaves extract of *Costus afer* in albino rats. British J. Pharmaceut. Res. 1(1):1-8.

Moore MC, Coate KC, Winnick JJ, An Z, Cherrington AD (2012). Regulation of hepatic glucose uptake and storage in vivo. Adv. Nutr. 3:286-294. DOI: 10.3945/an.112.002089

Mosseri R, Waner T, Shefi M, Shafrir E, Meyerovitch J (2000). Gluconeogenesis in non-obese diabetic (NOD) mice: in vivo effects of vanadate treatment on hepatic glucose-6-phoshatase and phosphoenolpyruvate carboxykinase. Metabol. 49:321-325.

Narender T, Khaliq T, Madhur G (2011). Naturally occurring anti-hyperglycemic and antidyslipidemic agents. Oportunity, Challenge and Scope of Natural Products in Medicinal Chemistry pp.155-185.

Newman DJ, Cragg GM (2007). Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70:461- 477.

NIH (1985). Principles of Laboratory Animal Care. NIH Publication No. 85-23 revised.

Owolabi OJ, Amaechina FC, Okoro M (2011). Effect of ethanol leaf extract of *Newboulda laevis* on blood glucose levels of diabetic rats. Trop. J. Pharmaceut. Res. 10:249-254.

Pari L, Satheesh MA (2006). Effects of pterostilbene on hepatic key enzymes of glucose metabolism in streptozotocin and nicotinamide induced diabetic rats. Life Sci. 79(7):641-645.

Postic C, Dentin R, Girard J (2004). Role of the liver in the control of carbohydrate and lipid homeostasis. Diabetes Metab. 30:389-408.

Postle AD, Bloxham DP (1980). The use of tritiated water to measure absolute rates of hepatic glycogen synthesis. Biochem J. 192:65-73.

Prasad K, Lee P, Mantha SV, Kalra J, Prasad M, Gupta JB (1992). Detection of ischemia- reperfusion cardiac injury by cardiac muscle chemiluminescence. Mol. Cell Biochem. 115:49-58.

Priyadarsini RL, Namratha JR, Reddy DRS (2012). Glucokinase activators: a glucose sensor role in pancreatic islets and hepatocytes. Int. J. Pharm. Pharmaceut. Sci. 4(2):81-87.

Smith C, Marks AD, Lieberman M (2005). Mark's Basic Medical Biochemistry: A Clinical Approach (2nd edition), Lipincott Williams and Wilkins, Baltimore, Maryland, pp. 120-136.

Sofowora A (1993). Medicinal plants and traditional medicine in Africa. Spectrum Books Ltd., Nigeria, pp. 142-157.

Srinivasan K, Ramarao P (2007). Animal models in type 2 diabetes research: an overview. Indian J. Med. Res. 125: 451-472.

Tahrani AA, Bailey CJ, Del Prato S, Barnett AH (2011). Management of type 2 diabetes: new and future developments in treatment. Lancet 378(9786):182-197.

Trease GE, Evans WC (2002). Pharmacognosy. 15th ed. Saunders Publishers, London, pp. 42-44.

Zhang X, Liang W, Mao Y, Li H, Yang Y, Tan H (2009). Hepatic glucokinase activity is the primary defect in alloxan-induced diabetes of mice. Biomed. Pharmacotherap. 63:180-186.

Zimmet PZ, Alberti GK, Shaw JE (2005). Mainstreaming the metabolic syndrome: a definitive definition. This new definition should assist both researchers and clinicians. Med. J. Aust. 183:175-176.

academicJournals

Vol. 13(22), pp. 2282-2287, 28 May, 2014
DOI: 10.5897/AJB2013.13342
Article Number: 804249444912
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Detection of dentin antigenic fractions by salivary immunoglobulin G in patients undergoing orthodontic treatment

Tânia Maris Pedrini Soares da Costa[1], Solange de Paula Ramos[2], Mirian Marubayashi Hidalgo[3], Alberto Consolaro[4], Shahzad Akbar Khan[1] and Eiko Nakagawa Itano[1]*

[1]Department of Pathological Sciences, Universidade Estadual de Londrina, Paraná, Brazil.
[2]Department of Histology, Universidade Estadual de Londrina, Paraná, Brazil.
[3]Department of Dentistry, Universidade Estadual de Maringá, Maringá, Paraná, Brazil.
[4]Department of Oral Pathology, Bauru College of Odontology, Universidade de São Paulo, Bauru, São Paulo, Brazil.

Received 2 October, 2013; Accepted 8 May, 2014

This study aimed to partially characterize the antigenic fractions of human dentin extract, and to evaluate the anti-dentin antibodies levels in patients undergoing orthodontic treatment. Two dentin extract fractions (FI and FIIa) were used to analyze the saliva samples from three groups of subjects. One group had 13 subjects who presented radiographic signs of mild to moderate root resorption 12 months after starting orthodontic treatment (T12); saliva samples taken prior to treatment served as controls (T0). The other groups had 10 subjects with radiographic signs of mild to severe root resorption up to 48 months after orthodontic therapy (PT); 10 individuals not undergoing orthodontic treatment were selected as controls (ST). Western blot analysis revealed the presence of dentinal fractions of approximately 35 to 70 kDa in (T12) and (PT) but not in the control samples. In immunoenzymatic assays, the anti-FI salivary IgG levels were significantly higher in T12 and PT groups than in the controls (P< 0.05). Our results demonstrate that dentinal fractions may be presented to the immune system and detected by salivary IgG. The levels of anti-dentin antibodies may remain elevated even years after finishing of orthodontic therapy.

Key words: Root resorption, dentinal antigens, humoral response, enzyme-linked immunosorbent assay (ELISA), Western blot.

INTRODUCTION

External apical root resorption (EARR) is an immunopathological manifestation of inflammatory origin that presents high prevalence (39 to 99%) in patients undergoing orthodontic treatment (Apajalahti and Peltola, 2007; Dudic et al., 2008). This process has consequence the irreversible shortening from apex root. Although in most cases the root resorption is mild and minor clinical importance, moderate (lesser than 2 mm) to severe

*Corresponding author. E-mail: itanoeiko@hotmail.com.

(greater than 2 mm) root resorption has been reported to occur at a frequency of 10 to 20% (Brin et al., 2003; Marques et al., 2010), representing a serious problem for orthodontists. The combination of several mechanical and biological risk factors, reported in the literature as: extensive tooth movement, movement type, orthodontic force magnitude, duration and type of force, individual susceptibility, anatomical factors, genetic influence, systemic factors (Sameshima and Sinclair, 2001; Al-Qawasmi et al., 2003; Segal et al., 2004; Artun et al., 2009), may predispose EARR. However, the destruction of the cementoblastic layer, that protect the tooth root, is the local factor required for the initiation of (Consolaro, 2005). Although the mechanisms of induction and regulation of this pathology are not yet completely understood, some results have indicated the potential of dentin to induce inflammatory and immune response in the root resorption process. Dentin contains numerous non-collagenous proteins (NCP) and signaling molecules usually hidden, trapped in their mineralized matrix. These dentin constituents, that seem to be multifunctional, can be exposed and released in consequence of local injury as from orthodontic movement (Butler et al, 1998; Silva et al., 2004).

The exposure of the dentin *in vivo* is, fre-quently, accompanied by inflammatory cell infiltration on periodontal tissues and by signals and cytokines production, for activation and differentiation of clasts, responsible by resorption of root surface (Bassaran et al., 2006). It is possible that clasts dissolve the mineralized matrix, endocytose, transport and continuously release dentin molecules in the tooth/periodontium microen-vironment during the resorption process (Nesbitt 1997).

These components may act as chemotactic factor (Ogata et al., 1997) and activation factor of inflammatory cells (Lara et al., 2003) and resorptive cells (Nakagawa et al., 2000). Additionally, previous studies already have established the immunogenic potential of dentin and confirmed the presence of anti-dentin antibodies (autoantibodies), by using a model of root resorption in dogs and mice (King and Courts, 1989; NG et al., 1990; Wheeler and Stroup, 1993). Subsequent studies investigated the cellular response in root resorption process (Lara et al., 2003) and the presence of serum IgG and IgM levels was shown in patients with apical root resorption resulting in dental trauma (Hidalgo et al., 2005). And more recently, Ramos et al. (2011), used saliva samples to determine the levels of secretory IgA anti-human dentin extract. Because there are few studies in literature about the antigenic components of the dentin in EARR, the present research, described here, aimed to partially characterize human dentin antigenic fractions detected through the saliva and additionally to evaluate the anti-dentin antibodies profile in patients undergoing orthodontic treatment. The subjects in this study had mild to severe root resorption and were evaluated during or post-treatment. This analysis could help in further studies to obtain early diagnosis, development of preventive methods and understanding of the immunopathological mechanisms involved in root resorption.

METHODOLOGY

All procedures involving human were performed after consent given by the subjects or by a parent/legal guardian and were approved by the Human Ethics Committee at State University of Londrina.

Subjects' selection

This study had three different groups (A, B, C). The A study group had 13 subjects (mean age 16 ± 4 years old) with nine females and four males. This group was analyzed over two phases: 12 months following the beginning of the fixed appliance therapy, at which time the patients presented with radiographic signs of mild to moderate root resorption (T12); these subjects taken prior to treatment served as controls (T0). The B study group had 10 subjects (mean age 21 ± 2 years old) with five females and five males. This group was analyzed approximately 48 months following orthodontic treatment, at which time they presented with radiographic signs of mild to severe root resorption (PT). The C study group had ten volunteers matched by gender and age without treatment and no radiographic evidence of root resorption were used as controls (ST). Inclusion criteria were: no previous orthodontic treatment and no radiographic evidence of root resorption. Exclusion criteria were: trauma to the primary or permanent dentition, autoimmune disease, chronic inflammatory disease, periodontal disease, periapical lesions, asthma, active caries or oral mucosa lesions, use steroidal or non-steroidal anti-inflammatory drugs for at least one month before sampling occurred.

Saliva samples

Saliva samples were collected at T0, T12, ST and PT. These samples were obtained between 10:00 and 16:00 to avoid the effect of cardian circle in salivary IgG secretion into saliva. Unstimulated whole saliva samples (2 ml) were collected by expectoration into sterilized vials after the subjects had rinsed their mouth twice with water. Saliva samples were centrifuged at 12000 rpm for 10 min, and the supernatants were then stored at -20°C until use.

Antigen preparation

Extract of human dentin containing NCP from the dentin matrix was used as the antigen. Powder dentin was obtained using a modified technique described by Wheeler and Stroup (1993); the third molars were donated by patients for whom extraction was indicated. The dentin was removed using a drill with a high-speed bit. The powder obtained was diluted at demineralizing solution (guanidine-HCl 5 M, 10%, ethylenediamine tetraacetic acid pH 5.0 and 1 µM phenylmethylsulfonylfluoride) for 14 days at 4°C and then centrifuged two times at 3.218 rpm for 20 min. The supernatant was collected. The protein content was assessed by the Lowry method (1951). Dentin extract was stored at -80°C until use.

Radiographs

Periapical radiographs were obtained from subjects at T0, T12, ST and PT (70 kV, 10 mA, exposure time 0.7 s). The radiographs of the upper central incisors were taken by using the long cone paralleling technique. Kappa values for the intra-examiner variation ranged from 0.85 to 0.9. The most resorbed incisor was considered

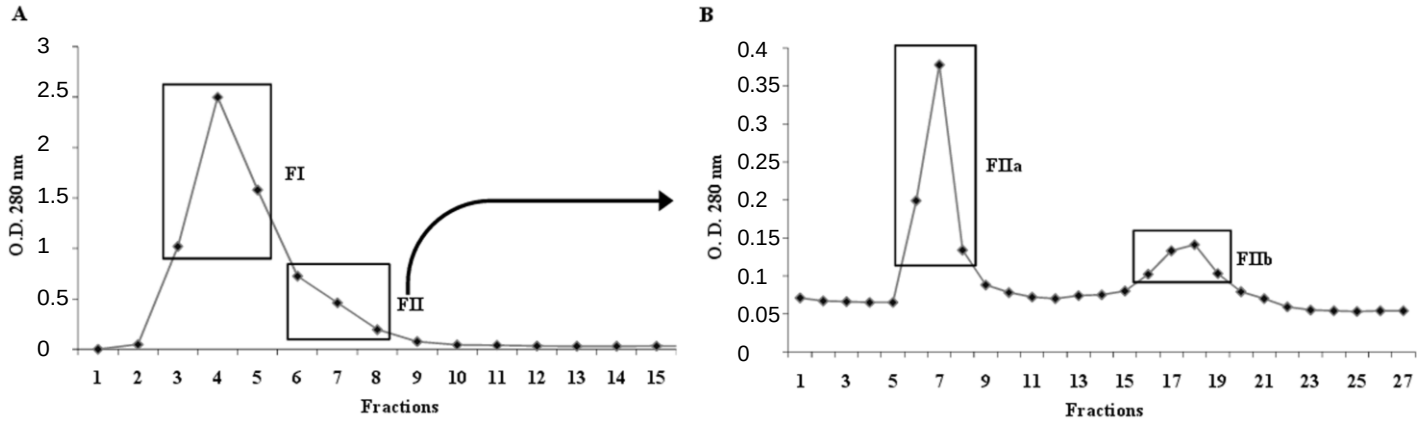


Figure 1. Chart of Gel filtration chromatography - Human dentin extract. (A) Fraction FI (tubes 3, 4 and 5) and fraction FII (tubes 6, 7 and 8) were fractioned in a Sephadex G-25 column. (B) Fraction FII was refractionated in a Sephadex G-100 column, yielding fraction FIIa (tubes 6, 7 and 8) and fraction FIIb (tubes 16, 17, 18 and 19). Only fractions FI and FIIa were used in these experiments.

for analysis. Tooth length was measured from the incisal edge to the apex. The measurements were made with a pachymeter (0.02 mm precision; Mitutoyo Sul Americana, São Paulo, Brasil) placed parallel to the pulp canal. Image distortion was determined by comparing the image length of a radiopaque object (metal clips) placed on the film. Image distortion between T0 and T12 radiographs was determined by comparing crown length. The maximum acceptable distortion was 5%. For analysis of degree of resorption, upper central incisor was selected, in subjects after treatment with orthodontic appliances. The degree of root resorption was classified by using criteria described by Levander and Malmgren (1988): mild resorption or degree I- with irregular apical root contour; moderate resorption or degree II- lesser 2 mm, severe resorption or degree III- 2 mm to one-third of the original root length. The patients presented Class I or Class II malocclusion.

Gel filtration chromatography on Sephadex G-25 and Sephadex G-100

To desalinate the extract, crude dentin extract was fractionated in a Sephadex G-25 gel filtration column (Sigma, St. Louis, USA). The automatic fraction collector (FC 203B; Gilson, Middleton, USA) was maintained at 4°C. Only one fraction was concentrated and refractionated on a Sephadex G-100 column (Sigma, St. Louis, USA). The eluted fractions in phosphate buffered saline (PBS) were read in a spectrophotometer at 280 nm (Pharmacia Biotech, Sweden). The total protein content of each fraction was assessed by the Lowry method (1951).

ELISA for the detection of anti-dentin salivary IgG

ELISA immunoplates (Techno Plastic Products, Zurich, Switzerland) were sensitized with 30 µg/mL FI or FIIa fractions in carbonate-bicarbonate buffer (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, distilled water, qsp 1000 ml, pH 9.6), incubated for 1 h at 37ºC and overnight at 4°C. The plate was washed four times with phosphate-buffered saline pH 7.2 (PBS), containing 0.05% Tween 20, and 0.5% skimmed milk. Then, the plates were blocked with PBS containing 0.5% Tween 20 and 5% skimmed milk for 1 h at room temperature. After washing, undiluted saliva samples were incubated at 37°C for 90 min. After washing, the conjugated goat-anti-human IgG-peroxidase (A8775; Sigma–Aldrich, St. Louis, USA) was added at 1:4000 dilution, and incubated for 90 min at 37°C. It was added after washing the O-phenylenediamine substrate solution. The reaction was stopped after 15 min using 50 µL/well of $4NH_2SO_4$. Absorbances were read at 492 nm in a Multiskan EX reader (Lab Systems, Helsinki, Finland). Antibody levels were expressed as absorbance in optical density units (O.D.).

SDS-PAGE electrophoresis and Western blot

FI and FIIa were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5-15% gradient acrylamide gels and were transferred to nitrocellulose membranes (NCM). The NCM was blocked with PBS containing 0.5% Tween 20 and 5% skimmed milk for 60 min h at room temperature. The NCM were cut into individual strips containing fractions F or FIIa and a molecular weight standard. Each strip was incubated with pools of undiluted saliva samples (T0, T12, ST or PT) with 10 samples from each group, followed by the addition of 1: 1000 conjugated goat-anti-human IgG-peroxidase (A8775; Sigma-Aldrich, St. Louis, USA) 90 min at 37°C and overnight at 4°C. After washing tetramethyl benzidine (00-2019; Invitrogen, San Francisco, USA) was added. The reaction was stopped with distilled water. The controls were saliva samples from the T0 and ST subjects.

Data analysis

The following non-parametric tests were used: the Wilcoxon test for paired groups (T0 and T12) and the Mann-Whitney test for unpaired group (ST and PT). These tests were used to detect differences in the ELISA absorbances (antibody levels expressed as O.D.). The results were considered statistically significant when $P<0.05$. The Lilliefors and Shapiro-Wilks normality tests were used.

RESULTS

Gel filtration chromatography

Chromatography of dentin extract on a Sephadex G-25 column resulted in one main peak of light absorption at 280 nm. From this peak, two fractions were obtained: FI and FII (chart demonstrated in Figure 1A). The FII

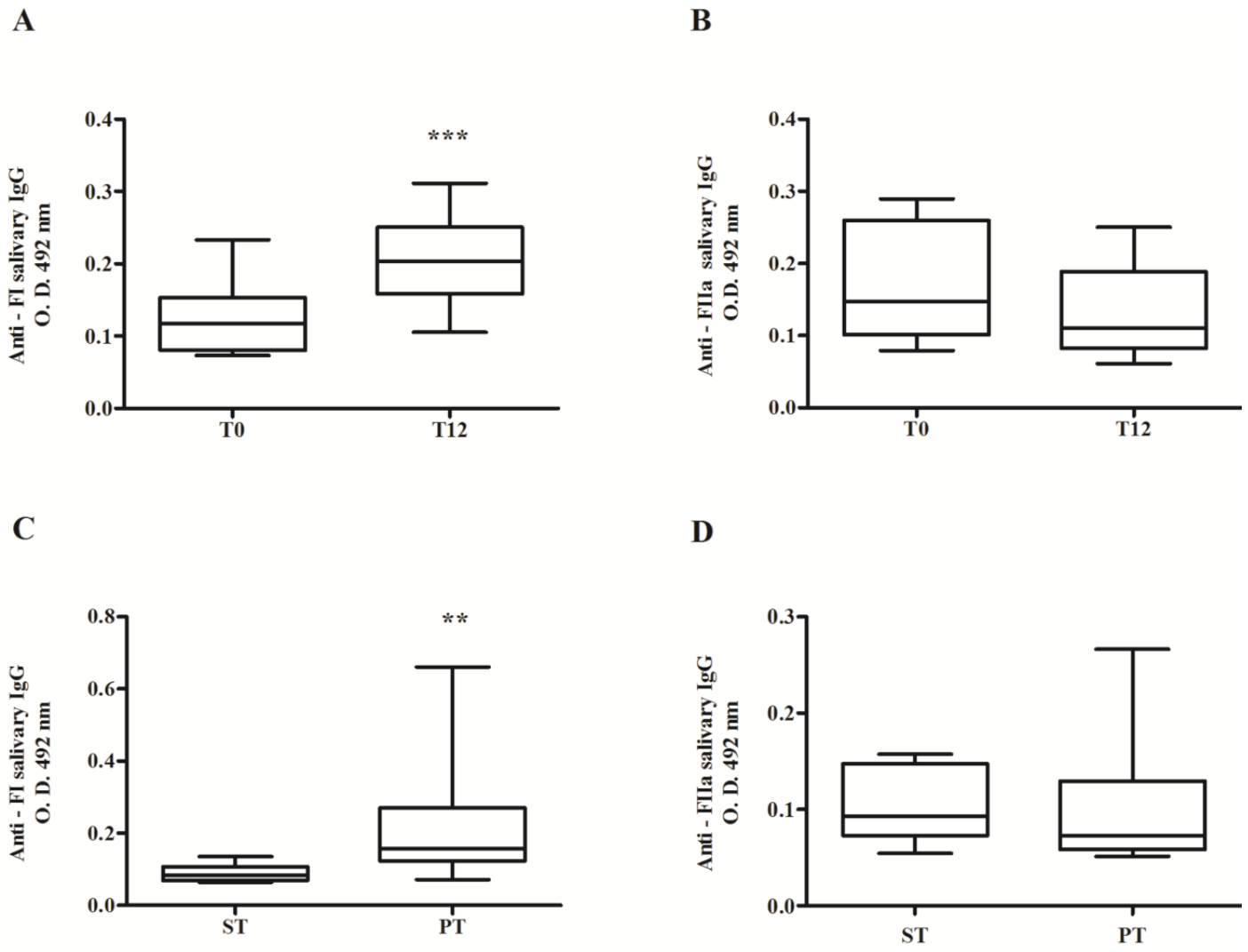


Figure 2. Analysis of the levels of salivary IgG against FI or FIIa fractions of human dentin extract using ELISA assays. Statistically significant differences (P < 0.05) are marked with asterisks. (A) Salivary IgG against FI fraction of human dentin extract in T0 or T12. (B) Salivary IgG against FIIa fraction of human dentin extract in T0 or T12. (C) Salivary IgG against FI fraction of human dentin extract in patients PT or ST. (D) Salivary IgG against FIIa fraction of human dentin extract in patients in PT or ST.

fraction was concentrated by lyophilization and was refractionated on a Sephadex G-100 column, resulting in a 280 nm spectrophotometric profile containing two peaks. Fractions corresponding to each peak were mixed, resulting in pool FIIa and FIIb (chart demonstrated in Figure 1B). Only fractions FI and FIIa were used in the following experiments.

Anti-FI and anti-FIIa salivary IgG levels detected by ELISA pre-treatment (T0) and after 12 months of orthodontic treatment (T12)

The anti-FI salivary IgG levels was higher at T12 than at T0 (P <0.05, Wilcoxon test), as is shown in Figure 2A. No statistically significant differences were observed in anti-FIIa salivary IgG levels between T0 and T12 (P>0.05, (Wilcoxon test), as is demonstrated in Figure 2B.

Anti-FI and anti-FIIa salivary IgG levels detected by ELISA in control donors (ST) and in patients approximately 48 months after orthodontic treatment (PT)

The anti-FI salivary IgG levels were higher in the PT group than in the ST group (P <0.05, Mann-Whitney test; Figure 2C), but no statistically significant differences in the anti-FIIa IgG levels were observed between these groups (P >0.05, Mann-Whitney test), as shown in Figure 2D.

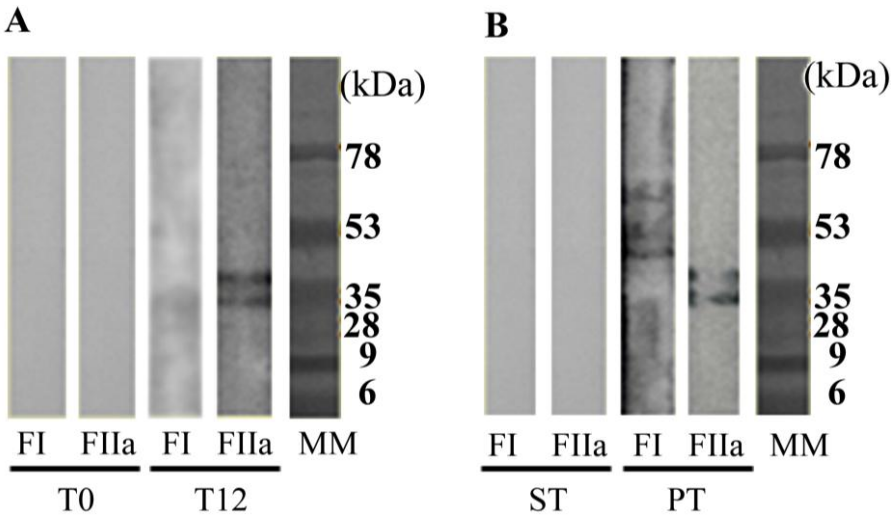


Figure 3. Western blot results of fractions FI or FIIa using pooled saliva samples from patients. (A) T0 or T12, (B) PT or ST.

FI and FIIa fraction analysis by Western blot

Western blot results show that the salivary IgG of the T12 or PT groups (but not the T0 or St groups) detected dentin antigens in the FI fraction (approximately 50 to 70kDa) and the FIIa fraction (approximately 35 kDa and 40 kDa). These data are shown in Figure 3A and 3B.

DISCUSSION

Based on the evidence that anti-dentin antibodies can be detected in animal experimental models and in patients with root resorption (King and Courts, 1989; Ng et al., 1990; Wheeler and Stroup, 1993; Hidalgo et al., 2005; Ramos et al., 2011), it has been hypothesized that pathological root resorption may be associated with autoimmune responses against dentinal components. The development of autoimune disorders could be bound to the presence of autoreactive T lymphocytes in the thymus that have not been deleted (Weiner et al., 1995). An effective mechanism to prevent self-reactivity is the compartmentalization of certain self-antigen under normal conditions (Kindt et al., 2008). And the exposure of some hidden antigens, released after injury, makes them accessible to specific receptors of antigen-presenting cells (APCs) and adaptive immune cells, which can result in the production of autoantibodies (Janeway, 2007). The presence of anti-dentin autoantibodies in the saliva of control groups could be explained by the indexes of dental resorption described in the general population (Massler and Malone, 1954). However, the high level of specific autoantibodies observed in this study in T12 may be due to the release of dentinal antigens as consequence of orthodontic movement, compared to control, without treatment. When dentin antigen-IgG complexes are internalized by APCs (macrophages, dendritic cells) and presented to autoreactive effector T cells, an inflammatory response will occur. Then, the antigen is eliminated, the ratio of IgG to antigen increases, decreasing immune responses and reducing tissue damage, with a slope of the response towards tolerance (De Groot et al., 2008). Natural regulatory T cell (nTreg) $CD4^{+}CD25_{Hi}FoxP3^{+}$, is an important mechanism of effector T cell regulation, and may represent one of the critical forms of autoregulatory response to self-antigens. On the other hand, the course of resorption will depend on a complex interaction of secreted molecules, inflammatory and bone cells within the tooth surrounding tissues (Ne et al., 1999).

The results shown, also indicated a significantly higher level of anti-FI fraction salivary IgG in (PT) than subjects (ST). This fact could be due to the persistence of the stimulus and the high half-life of the memory cells that produce antibodies anti-dentinal components (Schittek and Rajewsky, 1990; Slifka, 1998). Thus, the induction of inflammatory events by dentin molecules released into microenviroment may contribute to the maintenance of this process (Silva et al., 2004). Western blot analysis of the FI fraction also detected another high molecular weight band that occurred in all the samples, including the control groups (data not shown). No significant difference in the FIIa fraction was observed between the groups and its control using an ELISA. However, western blot analysis detected dentinal antigens of approximately 35 and 40 kDa by saliva of the T12 and PT groups but not in the control groups. The concentration of FIIa fraction by lyophilization was necessary to detection in western blot, by salivary IgG. The results often contradictory in the literature about *in vivo* and *in vitro* dentin studies can be explained by the use of different extraction methods, source of dentin and methods of cell

treatment. Based on data of this study, we hypothesized that not all dentinal proteins may be immunogenic and some of them may be tolerogenic. It is possible that in the case of EARR, specially, mild or moderate, anti-dentin natural autoantibodies have a homeostatic function in mediating removal of dentinal components released within periodontal ligament microenvironment (HANS et al., 2007). In conclusion, our results suggest that antigenic fractions of approximately 35 to 70 kDa may be released from dentin, presented to the immune system and detected by salivary IgG in orthodontic patients with mild to severe root resorption. The high levels of salivary IgG to fraction of human dentin following orthodontic treatment may remain elevated even years after completion of therapy. The use of saliva samples provide great advantages of adherence by patients and may be a route of choice for the development of preventive methods, early diagnosis and understanding of the immunopathological mechanisms involved in the root resorption process. Further studies, with a larger number of patients, should be performed to confirm these results.

Conflict of Interests

The author(s) have not declared any conflict of interests

REFERENCES

Al-Qawasmi RA, Hartsfield JK Jr, Everett ET, Flury L, Liu L, Foroud TM, Macri JV, Roberts WE (2003).Genetic predisposition to external apical root resorption in orthodontic patients: linkage of chromosome-18 marker. J. Dent. Res. 82:356-360.

Apajalahti S, Peltola J (2007). Apical root resorption after orthodontic treatment-a retrospective Study. Eur. J. Orthod. 29: 408-412.

Artun J, Van't Hullenaar R, Doppel D, Kuijpers-Jagtman AM (2009). Identification of orthodontic patients at risk of severe apical root resorption. Am. J. Orthod. Dent. Orthop. 135:448-455.

Bassaran G, Ozer T, Kaya FA, Hamamci O (2006). Interleukins 2, 6, and 8 levels in human gingival sulcus during orthodontic treatment. Am. J. Orthod. Dent. Orthop. 130:7e1-7e6.

Brin I, Tulloch JF, Koroluk L, Philips C (2003). External apical root resorption in Class II malocclusion: a retrospective review of 1-versus 2-phase treatment. Am. J. Orthod. Dent. Orthop. 124:151-156.

Butler WT (1998). Dentin matrix proteins. Eur. J. Oral Sci. 106: 204-210.

Consolaro A (2005). Reabsorções dentárias. Dental Press Maringá.

De Groot AS, Moise L, McMurry JA, Wambre E, Van Overtvelt L, Moingeon P, Scott DW, Martin WX (2008). Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes." Bl. 8: 3303-3311.

Dudic A, Giannopoulou C, Martinez M, Montet X, Kiliaridis S (2008). Diagnostic accuracy of digitized periapical radiographs validated against micro-computed tomography scanning in evaluating orthodontically induced apical root resorption. Eur. J. Oral Sci. 116:467-472.

Hans UL (2007). Homeostatic roles of naturally occurring antibodies: An overview. J. Autoimm. 29: 287-294.

Hidalgo MM, Itano EN, Consolaro A (2005). Humoral immune response of patients with dental trauma and consequent replacement resorption. Dent. Traumatol. 21:218-221.

Janeway CA, Travers P, Walport M (2007). Imunobiologia. Porto Alegre: Artemed.

Kindt, TJ, Goldsby, RA, Osborne, BA (2008). Tolerância e Autoimunidade In: Imunologia de Kuby. Artemed. Porto Alegre.

King G, Courts F (1989). Humoral immune response to active root resorption. In: Norton LA, Burstone CJ, edtors. The Biology of Tooth. Boca Raton: Movement CRC 276-285.

Lara VS, Figueiredo F, da Silva TA, Cunha FQ (2003). Dentin-induced *in vivo* inflammatory response and *in vitro* activation of murine macrophages. J. Dent. Res. 6:460-465.

Levander E, Malmgren O (1988). Evaluation of the risk of root resorption during orthodontic treatment: a study of upper incisors. Eur. J. Orthod. 10:30-38.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Marques LS, Ramos-Jorge ML, Rey AC, Armond MC, Ruellas AC (2010). Severe root resorption in orthodontic patients treated with the edgewise method: prevalence and predictive factors. Am. J. Orthod. Dent. Orthop. 384-388.

Massler M, Malone AJ (1954). Root resorption in human permanent teeth. Am. J. Orthod.40: 619-633.

Nakagawa M, Kaneda T, Morita S, Sato T, Yomada T, Hanada K, Kumegawa M, Hakeda Y (2000). Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. FEBS. Let. 473:161-164.

Ne RF, Whiterspoon DE, Gutmann JL (1999). Tooth resorption. Quint. 30:9-25. Nesbitt SA, Horton

MA (1997). Trafficking of matrix collagens through bone-resorbing osteoclasts. Sci. 276:266-269.

Ng KT, King GJ, Courts FJ (1990). Humoral immune response to active root resorption with a murine model. Am. J. Orthod. Dent. Orthop. 98:456-462.

Ogata Y, Niisato N, Moriwaki K, Yokota Y, Furuyama S, Sugiya H (1997). Cementum, root dentin and bone extracts stimulate chemotatic behavior in cells from periodontal tissue. Comp. Biochem. Physiol. 116: 359-365.

Ramos SP, Ortolan GO, Dos Santos LM, Tobouti PL, Hidalgo MM, Consolaro A, Itano EN (2011). Anti-dentine antibodies with root resorption during orthodontic treatment. Eur. J. Orthod. 33: 584-591.

Sameshima GT, Sinclair PM (2001). Predicting and preventing root resorption: Part II Treatment factors. Am. J. Orthod. Dent. Orthop.119:511-515.

Schittek B and Rajewsky K (1990). Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. Nat. 346:749-751.

Segal GR, Schiffman PH, Tuncay OC (2004). Meta analysis of the treatment-related factors of external apical root resorption. Orthod. Cran. 7: 71-78.

Silva TA, Rosa AL, Lara VS (2004). Dentin matrix proteins and soluble factors: intrinsic regulatory signals for healing and resorption of dental and periodontal tissues? Oral Dis. 10: 63-74.

Slifka MK, Antia R, Whitmire JK, Ahmed R (1998). Humoral immunity due to long-lived plasma cells. Imm. 8:363-372.

Wheeler TT, Stroup SE (1993). Traumatic root resorption in dentine-immunized mice. Am. J. Orthod. Dent. Orthop. 103: 352- 357.

Wheiner HL, Miller A, Khoury SJ et al. (1995). In: J. Mestecky et al. Advances in Mucosal Immunology. Plenum Press New York 1243-1244.

academicJournals

Vol. 13(22), pp. 2288-2294, 28 May, 2014
DOI: 10.5897/AJB2013.13242
Article Number: DC3889644923
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Preliminary study on the dye removal efficacy of immobilized marine and freshwater microalgal beads from textile wastewater

S. Dinesh Kumar, P. Santhanam*, R. Nandakumar, S. Ananth, B. Balaji Prasath, A. Shenbaga Devi, S. Jeyanthi, T. Jayalakshmi and P. Ananthi

Marine Planktonology and Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli-620 024, Tamil Nadu, India.

Received 6 September, 2013; Accepted 16 May, 2014

Discharge of textile wastewater containing toxic dyes can adversely affect aquatic organisms and human health. The objective of the study was to investigate the potential of immobilized marine microalgae (*Chlorella marina, Isochrysis galbana, Tetraselmis* sp. *Dunaliella salina* and *Nannochloropsis* sp.) and freshwater microalga (*Chlorella* sp.) in removing dye from textile wastewater (TW). The present study incorporated the use of 2% sodium alginate matrixes for decoloration. Among the algal species tested, the highest colour removal was noticed in *Isochrysis galbana* (55%) followed by freshwater *Chlorella* sp. (43%). The present method is easy to use, cost effective and devoid of technical problems.

Key words: Marine microalga, immobilization, textile wastewater, *Chlorella marina, Isochrysis galbana, Dunaliella salina,* biosorption, bioremediation.

INTRODUCTION

Biosorption is one of the most innovative technologies to remove contaminants from the aqueous solution and wastewaters. Textile industrial wastewaters are characterized with high amount of biochemical oxygen demand (BOD), total suspended solid (TSS), chemical oxygen demand (COD), alkalinity and total dissolved solids (Kaushik and Malik, 2009). Therefore, degradation of wastes from these industrial discharges becomes difficult (Fewson, 1998). These dyes cause problems to human health, because they have toxic, carcinogenic and even mutagenic compounds that pose a serious hazard to aquatic organisms (Ozer et al., 2005). Dyes can be segregated as anionic (direct, acid and reactive dyes); cationic (basic dyes); and nonionic (disperse dyes). The chromophores in anionic and nonionic dyes mostly consist of azo groups or anthraquinone types.

Anthraquinone based dyes are more resistant to degradation due to their fused aromatic structures. The

*Corresponding author. E-mail: sanplankton@yahoo.co.in. Tel: +9198942-23482. Fax: +91431-2407045.

metal complex dyes are mostly based on chromium. Dye wastewater is usually treated by physical or chemical treatment processes. These include flocculation combined with flotation, electro flocculation, membrane filtration, electro kinetic coagulation, electrochemical destruction, ion-exchange, irradiation, precipitation, ozonation, and katox treatment method involving the use of activated carbon and air mixtures. However, these technologies are generally ineffective in color removal, expensive and less adaptable to a wide range of dye wastewaters (Banat et al., 1996). Adsorption, especially immobilization has been observed to be an effective process for color removal from dye wastewater because, immobilization of the biomass overcomes many of these problems. Amongst the various immobilization methods, encapsulation (whereby the biomasses are enclosed within a polymeric matrix) is one of the most commonly used methods. In addition to that, the immobilization matrix is suitable for practical use in biosorption; it must be mechanically strong and chemically stable to withstand actual process conditions. Mass transfer considerations are also of paramount importance: the matrix must be sufficiently porous to enable diffusion of the sorbate to the sorbent surface. The ability of the gel matrix to facilitate diffusion of sorbate is also influenced by the size and shape of the immobilization gels (Jen et al., 1996). Microalgae have been shown to be capable of removing colour from various dyes through mechanisms such as biosorption, bioconversion and bioagulation (Khalaf, 2008), microalgae can be a better choice for bioremediation compared to other microorganisms owing to their photosynthetic capabilities, thereby absorbing CO_2 from atmosphere and converting solar energy into useful biomasses and incorporating nutrients and other pollutants.

Several workers have reported dye removal potential of freshwater algae (Acuner and Dilek, 2004; Dhaneshwar et al., 2007; Khalaf, 2008). However, very few attempts have been made by some workers using marine algae for dye removal (Mubarak et al., 2011; Soumya, 2012; Henciya et al., 2013). The objective of the present attempt was to investigate the efficiency of some marine (*Chlorella marina*, *Isochrysis galbana*, *Tetraselmis* sp., *Nannochloropsis* sp. and *Dunaliella salina*) and freshwater microalgal cells (*Chlorella* sp.) in dye removal from the textile effluent. This is the first report for dye removal by these marine microalgal cells.

MATERIALS AND METHODS

Microalgal culture

Marine microalgae *C. marina, I. galbana, Tetraselmis* sp., *D. salina*, and *Nannochloropsis* sp. strains were obtained from the Central Institute of Brackishwater Aquaculture, Chennai, India. Freshwater microalgae *Chlorella* sp. was isolated from pond located at Bharathidasan University campus, using agar plating technique. Indoor algal stock culture was maintained according to Perumal et al. (2012).

Immobilization of microalgae

The microalgal beads were prepared by the method described by Santos et al. (2002) with minor modifications in respect to alginate and cation solution concentrations. To prepare 100 ml of alginate solution with the required alginate concentration, the alginate was first carefully dissolved by slow stirring in 70 ml of distilled water. A 1.3% (w/v) solution of sodium alginate (Himedia, MB114-100G, Mumbai, India) was prepared with warm (room temperature) distilled water, autoclaved (for 15 min at 120°C), cooled to room temperature, and mixed in a magnetic stirrer until the sodium alginate was completely dissolved. In the remaining 30 ml of distilled water, 3.5 g sodium chloride (Himedia, RM853-500G) was dissolved to obtain 35 g L^{-1} salinity. Cation solutions were prepared in nanopure water. Beads were formed by adding the alginate solution drop wise by means of a 20 ml syringe (0.8 × 40 mm needle; Braun, Melsungen, Germany), into the cation solution, from a height of approximately 15 cm and at a rate of approximately one drop per second. Beads were kept stirring in the cation solution for 45 min to allow complete hardening of the alginate, and washed several times with filtered (0.45 m) natural seawater to eliminate the remaining cation.

Characteristics of dye wastewater

The textile wastewater was collected from local dyeing industry located in Karur, Tamil Nadu, India. To understand the TW before treatment, physico-chemical parameters were determined (Table 1) using standard methods (Jenkins and Medsken, 1964; Strickland and Parsons, 1979; APHA, 1998) prior to experiment.

Spectrophotometer analysis

Scanning was performed between 300 and 1000 nm by using UV-vis spectrophotometer (1800 Shimadzu UV) to determine the maximum absorbance (λ max) wavelength of the diluted (1:10) untreated textile effluent (Khalaf, 2008). This dye wastewater showed (λ max) maximum absorbance at 350 nm (Figure 1). The absorbance (350 nm) was used for further analyses of dye.

Experimental setup

The first set of experiment deliberated on the effect of sample conditions (Shaking and Stable). Each conical flask (250 ml round bottom erlenmeyer flasks) was inoculated with 50 numbers of microalgal beads. The alginate beads (without microalgae) were used as control. The flasks with algal beads were kept at 37°C in metabolic shakers (RIVOTEK, SELEC RC5100, India) at 200 rpm for shaking condition and without shaking (static condition). The samples were withdrawn at defined time intervals (12 and 24 h) and suspended particles from the sample were removed by centrifugation at 7,000 rpm for 20 min using centrifuge (REMI, R8C, G-Force value- 8232 RCF). Decolorization was monitored by measuring the absorbance (according to λ max) of the supernatant at 350 nm using UV- Spectrophotometer. Second set of experiment were studied to the effect of different algal species for dye removal. The sodium alginate beads were prepared using five marine microalgal species (*C. marina, I. galbana, Tetraselmis* sp*., D. salina* and *Nannochloropsis* sp.) and one fresh water microalgal species (*Chlorella* sp.) with same number of beads added to conical flasks. The samples were collected at 12 and 24 h time interval and stopped when maximum decolorization was achieved. The

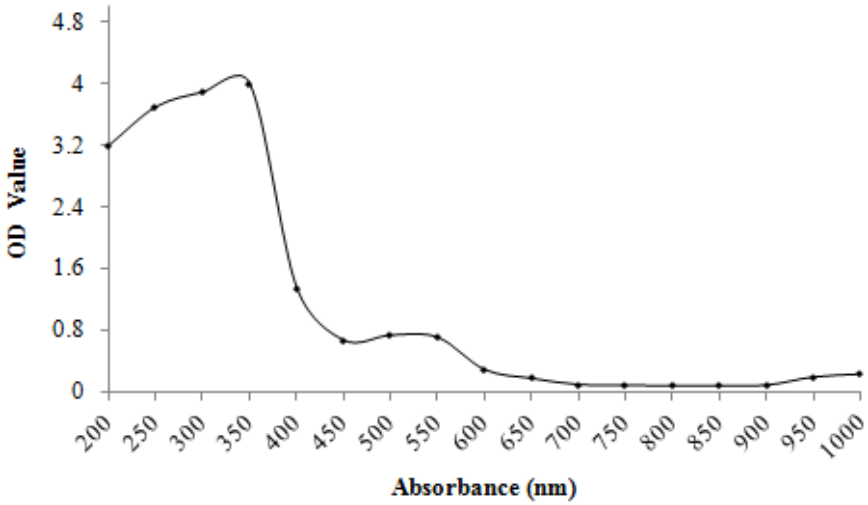


Figure 1. Spectrophotometer analyses for untreated textile wastewater.

Table 1. The physico-chemical characteristics of untreated textile wastewater

Parameter	Value
pH	10.6
Colour	Pink
Conductivity (µS)	2,228
Salinity (ppt)	8
Dissolved oxygen (mg L^{-1})	5.9
Temperature (°C)	23.5
Total suspended solid (mg L^{-1})	0.109
COD (mg L^{-1})	30.15
Phosphate (µmol/l)	7.88
Nitrate (µmol/l)	2.94
Ammonia (µmol/l)	17.42
Silicate (µmol/l)	42.27
Nitrite (µmol/l)	17.89

absorbance was noted according to Telke et al. (2010). The decolorization rate was calculated as follows:

$$\text{Decolorization (\%)} = \frac{\text{(Initial absorbance)} - \text{(Observed absorbance)}}{\text{Initial absorbance}} \times 100$$

RESULTS AND DISCUSSION

Characteristics of untreated textile wastewater

The textile wastewater temperature was 23.5°C, was highly colored and had alkaline condition with strong and objectionable odour that presents significant disposal or treatment problem. Physico-chemical characteristics of wastewater are given in Table 1.

Effect of treatment method

Two set of triplicates samples were studied for the discoloration of wastewater (one set at shaker + one set at static condition). The maximum colour reduction (13.66 ± 0.2%) noticed in shaker condition was recorded at 120 min in incubation, whereas at the static condition it was recorded as 21.77 ± 0.4% at 120 min. The results show that the decolorization of dyes was increased with time up to 120 min. However, the rate of dye decolorization was quite slow after 120 min which may be probably due to products inhibition. This observation suggested that initial two hour was significant for dyes decolorization and

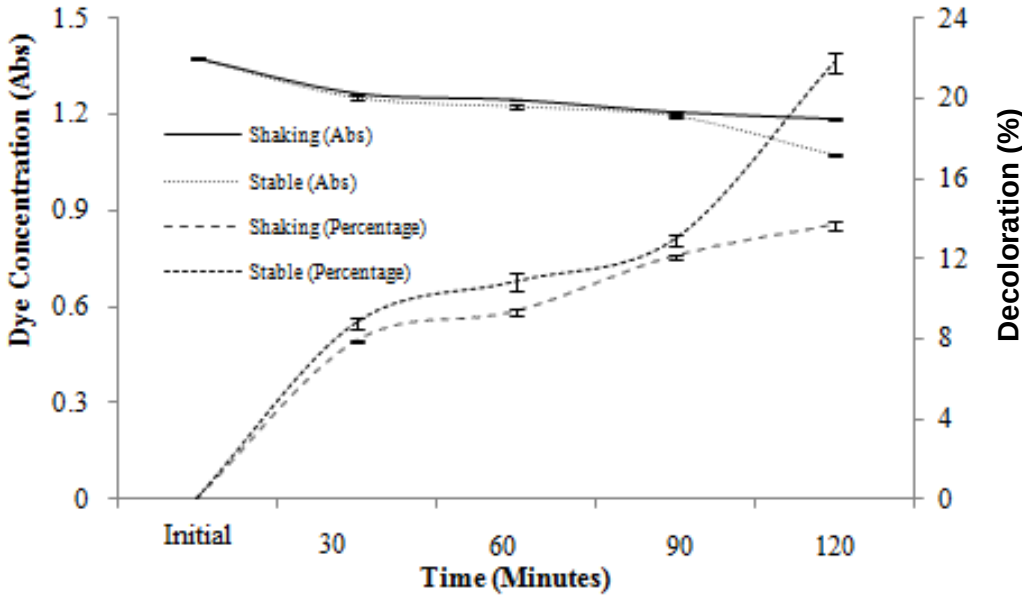


Figure 2. Decoloration of dye wastewater using immobilized microalgal beads with reference to treatment methods.

results were agreed by earlier workers (Khan and Husain, 2007; Arabaci and Usluoglu, 2014). In both (Static and Shaking) condition, experiments were performed until 120 min beyond which the removal rate of the microalgae decreased. The beads used in static condition proved to be more efficient on colour removal (Figure 2). Earlier studies also revealed the high colour removal from textile wastewater in more static condition than that shaking condition using various biological biomasses like fungi, bacteria, enzymes etc (Sugiura et al., 1999; Jang et al., 2007; Dhanve et al., 2009). The microaerophilic (Static) conditions which resulted in higher colour removal may be due to the presence of oxygen that would normally inhibit the activity of decolorization, resulting in less efficiency of color removal capacity in aerophilic condition by the same organisms (Meiying et al., 2007).

Effect different algal species

Algae have been found to be potential biosorbents because of their availability in both fresh and saltwater. The biosorption capacity of algae is attributed to their relatively high surface area and high binding affinity (Donmez and Aksu, 2002). Cell wall properties of algae play a major role in biosorption; electrostatic attraction and complexation are known to take part during algal biosorption (Satiroglu et al., 2002). Previous reports have suggested that level of discoloration of dye wastewater using algae can change based on their growing water salinity (Liu et al., 2013). The time dependent experiment showed that the colour removal increased with increasing time as agreed by previous worker (Saraswathi and Balakumar, 2009). They stated that the maximum time is (not exceeding seven days) an ideal way to reduce the colour from the dye wastewater. The percentage of colour reduction using immobilized beads were in the order of *I. galbana* (55.75%), fresh water C*hlorella* sp. (43.77%), *Tetraselmis* sp. *(*41.93%*), C. marina (*36.75%*), Nannochloropsis* sp. (32.87%) and *D. salina* (29.54%*)*, respectively (Figure 3). The present results have proved that *I. galbana* posesses efficient colour removal ability as agreed by Ang (2008). He proved that the *I. galbana* being suitable for bioremediation and removing pollutants from various effluents compared to *Chaetoceros* sp and *Tetraselmis* sp. Higher decolourization capacity of *I. galbana* can be attributed to size of the cells (Kishore et al*.,* 2006). Wolfe et al. (1998) described that the *I. galbana* play a role in the fate of dispersed oil, it is important to understand how dispersants may influence the bioavailability of pollutants like metals, dye compositions in primary tropic levels of marine food chains. The decolorization of present study (*I. galbana*; 55.75%) were quietly high compared to other workers (Henciya et al., 2013) has been dealt with marine species (*Lyngbya* sp.; 46.34%).

The colour removal capacity, especially by using algae, may be attributed to the accumulation of dye ions on the surface of algal biopolymers and further to the diffusion of the dye molecules from aqueous phase onto the solid phase of the biopolymer (Ozer et al., 2006). Extracellular polymers consist of surface functional groups, which enhance sorption of the dye molecules onto the surface of the polymer (floc) during dye removal process. The

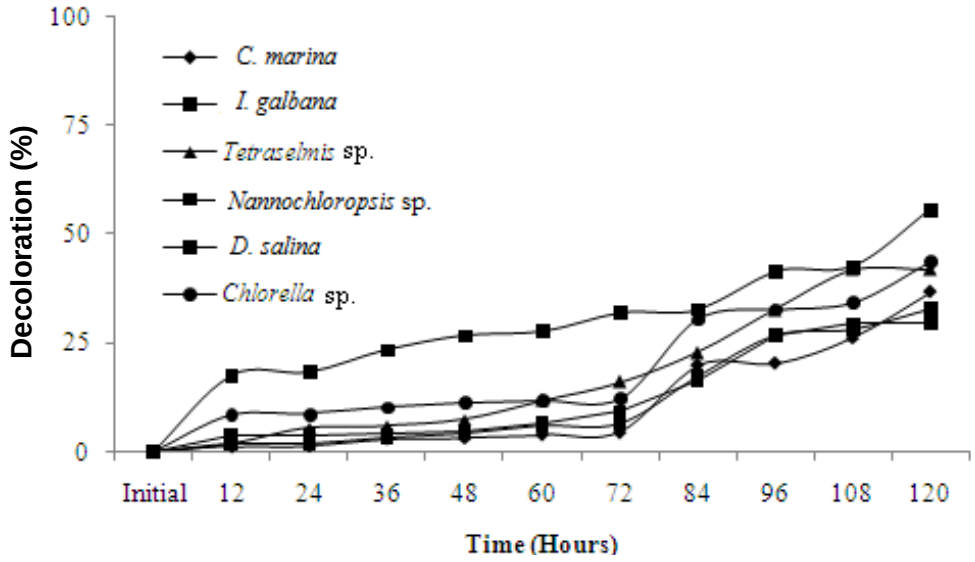


Figure 3. Effect of incubation time on dye removal efficiency (%) of six microalgal species.

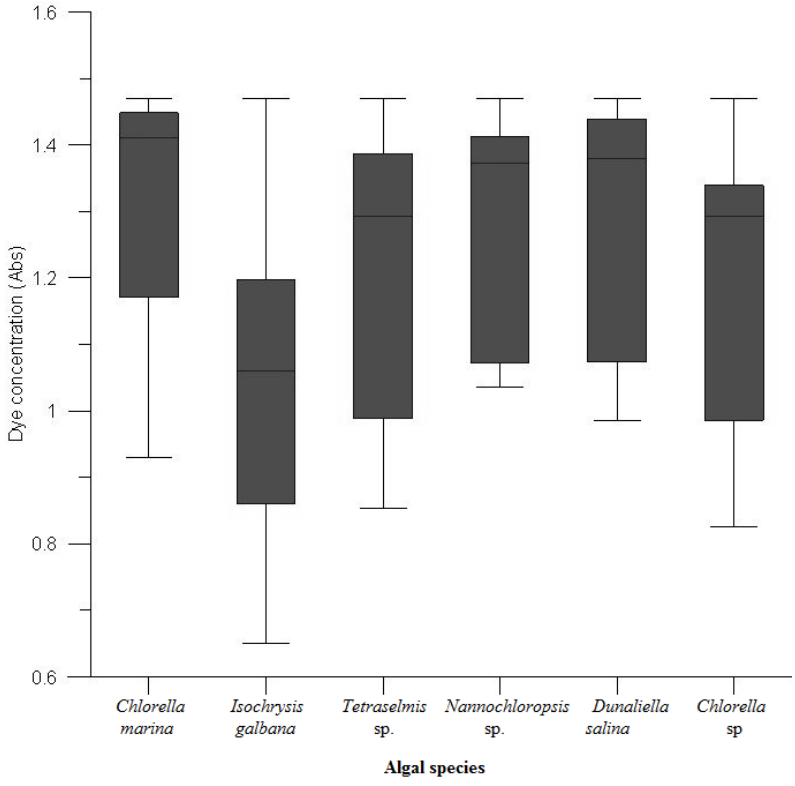


Figure 4. Box-Whisker plot shows the decoloration of dye wastewater using different immobilized microalgal beads.

released metabolic intermediates (long chain biopolymers) which have excellent coagulation capacity along with the dye remaining in the aqueous phase tend to adsorb onto the surface of the polymers and settle (biocoagulation) (Mohan et al., 2002).

Many authors reported that microalgae is a common and effective species for the immobilization and adsorption purposes (Tam et al., 1994; Lau et al., 1998; Abdel Hameed, 2002; Dinesh et al., 2013) (Figure 4). Alginate is the most frequent polymer used for algal immobilization.

Studies have adequately verified cell viability in the alginate matrix (Vilchez et al., 2001). In a freely suspended algal treatment system, the removal efficiency is often directly related to the cell mass. Increasing the algal biomass would improve the removal efficiency and shorten the retention time (Lau et al., 1995). On the contrary, the super-concentrated cell stocking in the beads, posed a serious leakage problem (Lau et al., 1997) and affects the treatment efficiency by the number of beads in dye wastewater (algal bead concentration).

Conclusion

Microalgae *I. galbana* can be considered as an important candidate among the six (five marine and one fresh water) microalgae studied, applicable to efficient removal of synthetic dyes from textile effluents. Similar studies on physiology and biochemical aspects of microalgal farming and fresh methods of immobilization together with co-immobilization of various capable species are necessary to develop a good decolorization method. Immobilized microalgae in alginate will be useful for final polishing of DW after undergoing crucial treatment before discharge. Cartridges of immobilized algae will be the optional system for such purpose as this will take up less land space compared to suspension cultures in raceway ponds. In addition, the effects of pH, contact time and temperature need to be optimized before commer-cialization.

Conflict of Interests

The author(s) have not declared any conflict of interests

ACKNOWLEDGEMENT

Authors thank the Head, Department of Marine Science and authorities of Bharathidasan University for the facilities provided. Authors acknowledge Dr. A. R. Thirunavukkarasu, Principal Scientist, Fish Culture Division, Central Institute of Brackishwater Aquaculture, Chennai, for providing algal culture for our experiments.

REFERENCES

Ang MY (2008). Use marine micro algae in bioremediation of balm oil mill effluent, Ph. D Thesis, University Malaysia Sabah, Malasiya. P. 85.

APHA (1998). Standard methods for the examination of water and wastewater. American Public Health Association, American Water Works Association, Water Pollution Control Federation, 20th ed. Washington DC, USA. P. 133.

Arabaci G, Usluoglu A (2014). The enzymatic decolorization of textile dyes by the immobilized polyphenol oxidase from quince leaves. Sci. World J. 1-5.

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

Dhaneshwar N, Ayazloo M, Khataee AR, Pourhassan M (2007). Biological decolorization of dye solution containing Malachite Green by microalgae *Cosmarium* sp. Bioresour. Technol. 98:1176-1182.

Dhanve RS, Kalyani DC, Phugare SS, Jadhav JP (2009). Coordinate action of exiguobacterial oxidoreductive enzymes in biodegradation of reactive yellow 84A dye. Biodegradation 20:245-255.

Dinesh KS, Santhanam P, Jayalakshmi T, Nandakumar R, Ananth S, Shenbaga DA, Balaji PB (2013). Optimization of pH and retention time on the removal of nutrients and heavy metal (zinc) using immobilized marine microalga *Chlorella marina*. J. Biolog. Sci. 13:400-405.

Donmez G, Aksu Z (2002). Removal of chromium (VI) from saline wastewaters by *Dunaliella* species. Process Biochem. 38: 751-762.

Fewson CA (1998). Biodegradation of xenobiotic and other persistent compounds: the causes of recalcitrance. Trends Biotechnol. 6:148-153.

Henciya S, Murali SA, Malliga P (2013). Decolorization of Textile dye effluent by Marine cyanobacterium *Lyngbya* sp. BDU 9001 with coir pith. Int. J. Environ. Sci. 3:1909-1918.

Jen AC, Wake C, Mikos AG (1996). Review: Hydrogels for cell immobilization, Biotechnol. Bioeng. 50:357-364.

Jenkins D, Medsken L (1964). A Brucine method for the determination of nitrate in ocean, estuarine and fresh waters. Anal. Chem. 36: 61.

Kaushik P, Malik A (2009). Fungal dye decolorization: Recent advances and future potential. Environ. Int. 35:127-141.

Khalaf MA (2008). Biosorption of reactive dye from textile wastewater by nonviable biomass of *Aspergillus niger* and *Spirogyra* sp. Bioresour. Technol. 99: 6631-6634.

Khan AA, Husain Q (2007). Decolorization and removal of textile and non-textile dyes from polluted wastewater and dyeing effluent by using potato (*Solanum tuberosum*) soluble and immobilized polyphenol oxidase. Biores. Technol. 98:1012-1019.

Kishore KK, Krishna Prasad M, Sarma GVS, Murthy VR (2006). Biosorption studies for removal of chromium using immobilized marine alga *Isochrysis galbana.* Indian J. Mar. Sci. 35:263-267.

Lau PS, Tam NFY, Wong YS (1995). Effect of algal density on nutrient removal from primary settled wastewater. Environ. Pollut. 89: 59-66.

Lau PS, Tam NFY, Wong YS (1997). Wastewater Nutrients (N and P) removal by carrageenan and alginate immobilized *Chlorella vulgaris*. Environ. Technol. 18:945-951.

Lau PS, Tam NFY, Wong YS (1998). Operational optimization of batch wise nutrient removal from wastewater by carrageenan immobilized *Chlorella vulgaris*. Water Sci. Technol. 38:185-192.

Liu G, Zhou J, Meng X, Fu SQ, Wang J, Jin R, Lv H (2013). Decolorization of azo dyes by marine *Shewanella* strains under saline conditions. Appl. Microbiol. Biotechnol. 97: 4187-4197.

Meiying X, Jun G, Goupig S (2007). Biodegradation of textile azo dye by *Shewanella* decoloration is S12 under microaerophilic conditions. Appl. Microbiol. Biotechnol. 76:719-726

Mohan SV, Rao NC, Prasad K, Karthikeyan J (2002). Treatment of simulated reactive yellow 22 (azo) dye effluents using *Spirogyra* species. Waste Manage. 22: 575-582.

Mubarak AD, Suresh A, Praveen Kumar R, Gunasekaran M, Thajuddin N (2011). Efficiency of textile dye decolorization by marine Cyanobacterium, *Oscillatoria formosa* NTDM02. Afr. J. Basic Appl. Sci. 3:09-13.

Ozer A, Akkaya G, Turabik M (2005). Biosorption of Acid Red 274 on *Enteromorpha prolifera* in a batch system. J. Hazard. Mater. 126:119-127.

Ozer A, Akkaya G, Turabik M (2006). The removal of Acid Red 274 from wastewater: combined biosorption and biocoagulation with *Spirogyra rhizopus*. Dyes Pigments 71: 83-89.

Perumal P, Balaji PB, Santhanam P, Ananth S, Shenbaga DA, Dinesh Kumar S (2012). Isolation and Culture of Microalgae. Manual on Advances in Aquaculture Technology(ed P. Santhanam), pp.166-181.

Santos MM, Moreno-Garrido I, Goncalves F, Soares AMVM, Ribeiro R (2002). An in situ bioassay for estuarine environments using the microalga *Phaeodactylum tricornutum*. Environ. Toxicol. Chem. 21:567-574.

Saraswathi K, Balakumar S (2009). Biodecolourization of azodye

(pigmented red 208) using *Bacillus firmus* and *Bacillus laterosporus*. J. Biosci. Tech. 1: 1-7.
Satiroglu N, Yalcinkaya Y, Denizli A, Arica MY, Bektas S, Genc O (2002). Application of NaOH treated *Polyporus versicolor* for removal of divalent ions of group IIB elements from synthetic wastewater. Process Biochem. 38:65-72.
Soumya G (2012). An investigation on nutrients and textile dye removal efficacy of seagrass *Cymodocea rotundata* immobilized beads, M. Phil., Thesis, Bharathidasan University, India. p. 86.
Strickland SC, Parsons TR (1979). A practical handbook of seawater analyses. Ottawa. Bulletin of Fisheries Research Board of Canada.
Sugiura W, Miyashita T, Yokoyama T, Ara M (1999). Isolation of Azo-dye-degrading microorganisms and their application to white discharge printing of fabric. J. Biosci. Bioeng. 88:577-581.
Tam NFY, Lau PS, Wong YS (1994). Wastewater inorganic N and P removal by immobilized *Chlorella vulgaris*. Water Sci. Tech. 30: 369-374.
Telke AA, Joshi SM, Jadhav SU, Tamboli DP, Govindwar SP (2010). Decolorization and detoxification of Congo red and textile industry effluent by an isolated bacterium *Pseudomonas* sp. SU-EBT. Biodegradation 21:283-296.
Vilchez C, Garabayo I, Marckvichea E, Galvan F, Leon R (2001). Studies on the suitability of alginate-entrapped *Chlamydomonas reinhardtii* cells for sustaining nitrate consumption processes. Biores. Technol. 78:55-61.
Wolfe MF, Schwartz GJB, Singaram S, Mielbrecht EE, Tjeerdema RS, Sowby ML (1998). Effects of salinity and temperature on the bioavailability of dispersed petroleum hydrocarbons to the golden-brown algae *Isochrysis galbana.* Arch. Environ. Contam. Toxicol. 35:268-273.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *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 Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals