ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org
Help Desk: helpdesk@academicjournals.org
Website: http://www.academicjournals.org/journal/AJMR
Submit manuscript online http://ms.academicjournals.me/
## Editors

<table>
<thead>
<tr>
<th>Name</th>
<th>Institution and Address</th>
</tr>
</thead>
</table>
| Prof. Stefan Schmidt  | *Applied and Environmental Microbiology*  
University of KwaZulu-Natal  
Pietermaritzburg,  
South Africa. |
| Prof. Fukai Bao       | *Department of Microbiology and Immunology*  
Kunming Medical University  
Kunming,  
China. |
| Dr. Jianfeng Wu       | *Dept. of Environmental Health Sciences*  
University of Michigan  
USA. |
| Dr. Ahmet Yilmaz Coban | *OMU Medical School*  
Department of Medical Microbiology  
Samsun,  
Turkey. |
| Dr. Seyed Davar Siadat | *Pasteur Institute of Iran*  
Pasteur Square, Pasteur Avenue  
Tehran,  
Iran. |
| Dr. J. Stefan Rokem    | *The Hebrew University of Jerusalem*  
Department of Microbiology and Molecular Genetics  
Jerusalem,  
Israel. |
| Prof. Long-Liu Lin     | *National Chiayi University*  
Chiayi,  
Taiwan. |
| Dr. Thaddeus Ezeji     | *Fermentation and Biotechnology Unit*  
The Ohio State University  
USA. |
| Dr. Mamadou Gueye      | *MIRCEN/Laboratoire commun de microbiologie*  
IRD-ISRA-UCAD  
Dakar, Senegal. |
| Dr. Caroline Mary Knox | *Department of Biochemistry, Microbiology and Biotechnology*  
Rhodes University  
Grahamstown,  
South Africa. |
| Dr. Hesham Elsayed Mostafa | *Genetic Engineering and Biotechnology Research Institute (GEBRI)*  
Mubarak City For Scientific Research  
Alexandria, Egypt. |
| Dr. Wael Abbas El-Naggar | *Microbiology Department*  
Faculty of Pharmacy  
Mansoura University  
Mansoura, Egypt. |
| Dr. Barakat S.M. Mahmoud | *Food Safety/Microbiology*  
Experimental Seafood Processing Laboratory  
Mississippi State University  
Pascagoula,  
USA. |
| Prof. Mohamed Mahrous Amer | *Faculty of Veterinary Medicine*  
Department of Poultry Diseases  
Cairo university  
Giza, Egypt. |
Editors

Dr. R. Balaji Raja  
Department of Biotechnology  
School of Bioengineering  
SRM University  
Chennai, India.

Dr. Aly E Abo-Amer  
Division of Microbiology  
Botany Department  
Faculty of Science  
Sohag University  
Egypt.

Editorial Board Members

Dr. Haoyu Mao  
Department of Molecular Genetics and Microbiology  
College of Medicine  
University of Florida  
Florida, USA.

Dr. Yongxu Sun  
Department of Medicinal Chemistry and Biomacromolecules  
Qiqihar Medical University  
Heilongjiang  
P.R. China.

Dr. Ramesh Chand Kasana  
Institute of Himalayan Bioresource Technology  
Palampur, India.

Dr. Pagano Marcela Claudia  
Department of Biology,  
Federal University of Ceará - UFC  
Brazil.

Dr. Pongsak Rattanachaikunsopon  
Department of Biological Science  
Faculty of Science  
Ubon Ratchathani University  
Thailand.

Dr. Gokul Shankar Sabesan  
Microbiology Unit, Faculty of Medicine  
AIMST University  
Kedah, Malaysia.

Dr. Kamel Belhamel  
Faculty of Technology  
University of Bejaia  
Algeria.

Dr. Sladjana Jevremovic  
Institute for Biological Research  
Belgrade, Serbia.

Dr. Tamer Edirne  
Dept. of Family Medicine  
Univ. of Pamukkale  
Turkey.

Dr. Mohd Fuat ABD Razak  
Institute for Medical Research  
Malaysia.

Dr. Minglei Wang  
University of Illinois at Urbana-Champaign  
USA.

Dr. Davide Pacifico  
Istituto di Virologia Vegetale – CNR  
Italy.

Prof. N. S. Alzoreky  
Food Science & Nutrition Department  
College of Agricultural Sciences & Food  
King Faisal University  
Saudi Arabia.

Dr. Chen Ding  
College of Material Science and Engineering  
Hunan University  
China.

Dr. Sivakumar Swaminathan  
Department of Agronomy  
College of Agriculture and Life Sciences  
Iowa State University  
USA.

Dr. Alfredo J. Anceno  
School of Environment, Resources and Development (SERD)  
Asian Institute of Technology  
Thailand.

Dr. Iqbal Ahmad  
Aligarh Muslim University  
Aligrah, India.
## Editorial Board Members

<table>
<thead>
<tr>
<th>Name</th>
<th>Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Juliane Elisa Welke</td>
<td>UFRGS – Universidade Federal do Rio Grande do Sul Brasil.</td>
</tr>
<tr>
<td>Dr. Iheanyi Omezuruike Okonko</td>
<td>Department of Virology Faculty of Basic Medical Sciences University of Ibadan Ibadan, Nigeria.</td>
</tr>
<tr>
<td>Dr. Giuliana Noratto</td>
<td>Texas A&amp;M University USA.</td>
</tr>
<tr>
<td>Dr. Babak Mostafazadeh</td>
<td>Shaheed Beheshty University of Medical Sciences Iran.</td>
</tr>
<tr>
<td>Dr. Mehdi Azami</td>
<td>Parasitology &amp; Mycology Department Baghaei Lab. Isfahan, Iran.</td>
</tr>
<tr>
<td>Dr. Rafel Socias</td>
<td>CITA de Aragón Spain.</td>
</tr>
<tr>
<td>Dr. Anderson de Souza Sant’Ana</td>
<td>University of São Paulo Brazil.</td>
</tr>
<tr>
<td>Dr. Juliane Elisa Welke</td>
<td>UFRGS – Universidade Federal do Rio Grande do Sul Brazil.</td>
</tr>
<tr>
<td>Dr. Jorge Reinheimer</td>
<td>Universidad Nacional del Litoral (Santa Fe) Argentina.</td>
</tr>
<tr>
<td>Dr. Qin Liu</td>
<td>East China University of Science and Technology China.</td>
</tr>
<tr>
<td>Dr. Samuel K Ameyaw</td>
<td>Civista Medical Center USA.</td>
</tr>
<tr>
<td>Dr. Xiao-Qing Hu</td>
<td>State Key Lab of Food Science and Technology Jiangnan University China.</td>
</tr>
<tr>
<td>Prof. Branislava Kocic</td>
<td>University of Nis School of Medicine Institute for Public Health Nis, Serbia.</td>
</tr>
<tr>
<td>Prof. Kamal I. Mohamed</td>
<td>State University of New York Oswego, USA.</td>
</tr>
<tr>
<td>Dr. Adriano Cruz</td>
<td>Faculty of Food Engineering-FEA University of Campinas (UNICAMP) Brazil.</td>
</tr>
<tr>
<td>Dr. Mike Agenbag</td>
<td>Municipal Health Services, Joe Gqabi, South Africa.</td>
</tr>
<tr>
<td>Dr. D. V. L. Sarada</td>
<td>Department of Biotechnology SRM University Chennai India.</td>
</tr>
<tr>
<td>Prof. Huaizhi Wang</td>
<td>Institute of Hepatopancreatobiliary Surgery of PLA Southwest Hospital Third Military Medical University Chongqing China.</td>
</tr>
<tr>
<td>Prof. A. O. Bakhiet</td>
<td>College of Veterinary Medicine Sudan University of Science and Technology Sudan.</td>
</tr>
<tr>
<td>Dr. Saba F. Hussain</td>
<td>Community, Orthodontics and Peadiatric Dentistry Department Faculty of Dentistry Universiti Teknologi MARA Selangor, Malaysia.</td>
</tr>
</tbody>
</table>
**Editorial Board Members**

Prof. Zohair I. F. Rahemo  
*Department of Microbiology and Parasitology*  
*Clinical Center of Serbia*  
*Belgrade, Serbia.*

Dr. Afework Kassu  
*University of Gondar*  
*Ethiopia.*

Dr. How-Yee Lai  
*Taylor’s University College*  
*Malaysia.*

Dr. Nidheesh Dadheech  
*MS. University of Baroda,*  
*Vadodara, India.*

Dr. Franco Mutinelli  
*Istituto Zooprofilattico Sperimentale delle Venezie*  
*Italy.*

Dr. Chanpen Chanchao  
*Department of Biology,*  
*Faculty of Science,*  
*Chulalongkorn University*  
*Thailand.*

Dr. Tsuyoshi Kasama  
*Division of Rheumatology,*  
*Showa University*  
*Japan.*

Dr. Kuender D. Yang  
*Chang Gung Memorial Hospital*  
*Taiwan.*

Dr. Liane Raluca Stan  
*University Politehnica of Bucharest*  
*Department of Organic Chemistry*  
*Romania.*

Dr. Mohammad Feizabadi  
*Tehran University of Medical Sciences*  
*Iran.*

Prof. Ahmed H Mitwalli  
*Medical School*  
*King Saud University*  
*Riyadh,*  
*Saudi Arabia.*

Dr. Mazyar Yazdani  
*Department of Biology*  
*University of Oslo*  
*Blindern,*  
*Norway.*

Dr. Babak Khalili Hadad  
*Department of Biological Sciences*  
*Islamic Azad University*  
*Roudehen,*  
*Iran.*

Dr. Ehsan Sari  
*Department of Plant Pathology*  
*Iranian Research Institute of Plant Protection*  
*Tehran,*  
*Iran.*

Dr. Snjezana Zidovec Lepej  
*University Hospital for Infectious Diseases*  
*Zagreb,*  
*Croatia.*

Dr. Dilshad Ahmad  
*King Saud University*  
*Saudi Arabia.*

Dr. Adriano Gomes da Cruz  
*University of Campinas (UNICAMP)*  
*Brazil.*

Dr. Hsin-Mei Ku  
*Agronomy Dept.*  
*NCHU*  
*Taichung, Taiwan.*

Dr. Fereshteh Naderi  
*Islamic Azad University*  
*Iran.*

Dr. Adibe Maxwell Ogochukwu  
*Department of Clinical Pharmacy and Pharmacy Management,*  
*University of Nigeria*  
*Nsukka,*  
*Nigeria.*

Dr. William M. Shafer  
*Emory University School of Medicine*  
*USA.*

Dr. Michelle Bull  
*CSIRO Food and Nutritional Sciences*  
*Australia.*
Editorial Board Members

Prof. Márcio Garcia Ribeiro
School of Veterinary Medicine and Animal Science-UNESP,
Dept. Veterinary Hygiene and Public Health,
State of Sao Paulo
Brazil.

Prof. Sheila Nathan
National University of Malaysia (UKM)
Malaysia.

Prof. Ebiamadon Andi Brisibe
University of Calabar,
Calabar,
Nigeria.

Dr. Julie Wang
Burnet Institute
Australia.

Dr. Jean-Marc Chobert
INRA- BIA, FiPL
France.

Dr. Zhilong Yang
Laboratory of Viral Diseases
National Institute of Allergy and Infectious Diseases,
National Institutes of Health
USA.

Dr. Dele Raheem
University of Helsinki
Finland.

Dr. Biljana Miljkovic-Selimovic
School of Medicine,
University in Nis,
Serbia.

Dr. Xinan Jiao
Yangzhou University
China.

Dr. Endang Sri Lestari, MD.
Department of Clinical Microbiology,
Medical Faculty,
Diponegoro University/Dr. Kariadi Teaching Hospital,
Semarang
Indonesia.

Dr. Hojin Shin
Pusan National University Hospital
South Korea.

Dr. Yi Wang
Center for Vector Biology
Rutgers University
New Brunswick
USA.

Prof. Natasha Potgieter
University of Venda
South Africa.

Dr. Sonia Arriaga
Instituto Potosino de Investigación Científica y Tecnológica/
División de Ciencias Ambientales
Mexico.

Dr. Armando Gonzalez-Sanchez
Universidad Autonoma Metropolitana Cuajimalpa
Mexico.

Dr. Pradeep Parihar
Lovely Professional University
Punjab,
India.

Dr. William H Roldán
Department of Medical Microbiology
Faculty of Medicine
Peru.

Dr. Kanzaki, L. I. B.
Laboratory of Bioprospection
University of Brasilia
Brazil.

Prof. Philippe Dorchies
National Veterinary School of Toulouse,
France.

Dr. C. Ganesh Kumar
Indian Institute of Chemical Technology,
Hyderabad
India.

Dr. Zainab Z. Ismail
Dept. of Environmental Engineering
University of Baghdad
Iraq.

Dr. Ary Fernandes Junior
Universidade Estadual Paulista (UNESP)
Brasil.
Editorial Board Members

Dr. Fangyou Yu  
*The first Affiliated Hospital of Wenzhou Medical College*  
China.

Dr. Galba Maria de Campos Takaki  
*Catholic University of Pernambuco*  
Brazil.

Dr. Kwabena Ofori-Kwakye  
*Department of Pharmaceutics*  
*Kwame Nkrumah University of Science & Technology*  
Kumasi, Ghana.

Prof. Liesel Brenda Gende  
*Arthropods Laboratory, School of Natural and Exact Sciences, National University of Mar del Plata*  
Buenos Aires, Argentina.

Dr. Hare Krishna  
*Central Institute for Arid Horticulture*  
Rajasthan, India.

Dr. Sabiha Yusuf Essack  
*Department of Pharmaceutical Sciences*  
*University of KwaZulu-Natal*  
South Africa.

Dr. Anna Mensuali  
*Life Science*  
*Scuola Superiore Sant’Anna*  
Italy.

Dr. Ghada Sameh Hafez Hassan  
*Pharmaceutical Chemistry Department*  
*Faculty of Pharmacy*  
*Mansoura University*  
Egypt.

Dr. Kátia Flávia Fernandes  
*Department of Biochemistry and Molecular Biology*  
*Universidade Federal de Goiás*  
Brasil.

Dr. Abdel-Hady El-Gilany  
*Department of Public Health & Community Medicine*  
*Faculty of Medicine*  
*Mansoura University*  
Egypt.

Dr. Radhika Gopal  
*Cell and Molecular Biology*  
*The Scripps Research Institute*  
San Diego, CA USA.

Dr. Mutukumira Tony  
*Institute of Food Nutrition and Human Health*  
*Massey University*  
New Zealand.

Dr. Habip Gedik  
*Department of Infectious Diseases and Clinical Microbiology*  
*Ministry of Health Bakırköy Sadi Konuk Training and Research Hospital*  
*Istanbul, Turkey*.

Dr. Annalisa Serio  
*Faculty of Bioscience and Technology for Food Agriculture and Environment*  
*University of Teramo*  
Teramo, Italy.
**ARTICLES**

**Diclofenac inhibits virulence of Proteus mirabilis isolated from diabetic foot ulcer**
Wael Abdel Halim Hegazy, 733

**Hydrophobicity and specific biofilm features of Bacillus cereus spores subjected to pH stresses**
Fadila Malek 744

**Phylogenetic analysis of Campylobacter jejuni from human and birds sources in Iraq**
Huda Abdal-Hadei Ali Al-Nasrawi 752

**Molecular characterization and genetic diversity of Tobacco streak virus infecting soybean (Glycine max L.)**
Rajamanickam, S., Ganesamurthy, K. and Karthikeyan, G. 759

**Biofixation of CO2 on a pilot scale: Scaling of the process for industrial application**
Felipe Camerini, Michele da Rosa Andrade Zimmermann de Souza, Michele Greque de Morais, Bruna da Silva Vaz, Etiele Greque de Morais and Jorge Alberto Vieira Costa 768
Diclofenac inhibits virulence of *Proteus mirabilis* isolated from diabetic foot ulcer

Wael Abdel Halim Hegazy¹,²

¹Department of Microbiology and Immunology, Faculty of Pharmacy, Zagzig University, Egypt.
²Oman Pharmacy Institute, Ministry of Health, Muscat, Oman.

Received 6 April 2016; Accepted 11 May, 2016.

Diclofenac is an analgesic and anti-inflammatory drug, used to relieve the secondary complications of diabetes. Wound infections are much more serious particularly in diabetic patients. *Proteus mirabilis* are Gram negative rods, show a wide range of pathogenesis based on arsenal of diverse virulence factors. Recently, *P. mirabilis* and other Gram negative rods were isolated from diabetic foot ulcers; moreover, these isolates showed an increase resistance and more aggressive virulence behavior. The promising approaches to overcome such kind of infections include the improvement of patient's immunity and/or challenging the bacterial virulence. Diclofenac is used frequently by diabetic patients, and it showed antimicrobial activity. This study was conducted to screen the effect of diclofenac on the virulence of *P. mirabilis* isolated from diabetic foot. Interestingly, diclofenac significantly inhibited or decreased the *P. mirabilis* virulence which indicates its additional beneficial use in diabetic foot patients.

**Key words:** *Proteus mirabilis*, diabetic foot, diclofenac.

INTRODUCTION

Proteus, Homer’s Odyssey, was known by his ability to foretell the future to anyone capable of capturing him; he changed shape to evade his followers. Hauser (1885), first used the name Proteus in bacterial nomenclature to describe a shapeshifting bacterium isolated from putrefied meat. *Proteus mirabilis*, family Enterobacteriaceae, is a Gram-negative, motile, non-lactose fermenter and produce hydrogen sulphide by incubation in triple sugar iron media. *P. mirabilis* is dimorphic and has the ability to differentiate from short rods into elongated, multinucleate swarm cells that express thousands of flagella. Members of the genus *Proteus* are widely distributed in nature and can be isolated from stagnant water, sewage, soil, and the intestinal tract (Armbruster and Mobley, 2012). *P. mirabilis* can cause a wide range of pathogenesis to the
infected host that varied from pyelonephritis, urolithiasis to prostatitis. Moreover, it is one of the major causes of catheter-associated urinary tract infections (CAUTIs) as it causes approximately 3% of all nosocomial infections and up to 44% of CAUTIs in the United States (O’Hara et al., 2000; Jacobsen et al., 2008).

Diabetic foot ulcers are known to be a complicated serious problem of diabetes as it increases the risk of amputation (Grayson, 1995). *P. mirabilis* is considered one of the most common infectious agents of diabetic foot ulcers (Sekhar et al., 2014; Perim et al., 2015). The most critical that it is showing a vast resistance to several antimicrobial agents which worsen the diabetic foot ulcers and delay the treatment (Tansarli et al., 2013; Perim et al., 2015). The understanding of bacterial behavior, life style and virulence factors is a crucial determinant in developing vaccines and introducing more efficient antimicrobial treatments (Hegazy and Hensel, 2012). The virulence of *P. mirabilis* is described on genetic basis which are chromosomally integrated or extra-chromosomally imported. For instance, 94 Kb ICE PM1 mobile pathogenicity island of *P. mirabilis* is present with up to 100% sequence identity for some genes in several Proteus species, which indicate DNA transfer between these species (Flannery et al., 2009). The virulence factors of *P. mirabilis* are widely diverse, adhesive fimbria, flagella swarming motility and production of toxins as haemolysin and extracellular enzymes as protease and urease all are working cooperatively on pathogenesis enhancement (Jacobsen et al., 2008; Morgenstein et al., 2010; Armbruster and Mobley, 2012). The biofilm formation by *P. mirabilis* constitutes an additive obstacle in antibiotic treatment of infections and its prevention is considered as an aim (Jacobsen et al., 2008; Zhao and Hu, 2013).

In serious bacterial infections as in diabetic foot ulcers, in order to control the aggressive bacterial invasion, it is a mandatory not only to prevent microbial infections by antibiotics, but also to inhibit microbial virulence to guarantee effectiveness. Controlling of diabetic foot ulcers, the surgical intervention and treatments are both applied. Diabetes traditional treatment regimens include basically analgesics and anti-inflammatory which are used to mask the other complications and relief the pain (Park and Anand, 2015; Santema et al., 2016).

Diclofenac inhibits synthesis of prostaglandin by inhibition of cyclooxygenase; it is widely used as sodium salt or potassium salt as anti-inflammatory (Chakraborti et al., 2010). Moreover, it is available indifferent dosage forms which ease its use and its safety is approved. Several drugs which are available in the market and commonly used by diabetic patients for their activity against bacterial virulence were screened. In this study, the effect of direct effect of diclofenac was studied on the virulence factors of a highly resistant *P. mirabilis* isolated from ulcerated diabetic foot. The effect of diclofenac in combination with antibiotics was studied.

**MATERIALS AND METHODS**

**Bacterial strain**

Clinical isolate of *P. mirabilis* was obtained from diabetic foot ulcers from patients admitted to the Surgery Department in Zagazig University Hospitals. The isolate was identified by morphology, Gram staining and biochemical reactions (Koneman et al., 1997).

**Determination of minimum inhibitory concentration (MIC)**

MIC of antibiotics or diclofenac sodium salt (Novartis, Egypt) was determined by the broth microdilution method according to Clinical Laboratory and Standards Institute Guidelines (CLSI) (Wayne, 2006). Briefly, bacterial inoculum were prepared and standardized to have a turbidity matching that of ½ McFarland standards. Sterile saline was used to dilute the bacterial suspensions to achieve a cell density approximating 10⁶ CFU/ml. Equal volumes of antibiotics or diclofenac sodium salt and aliquots of the bacterial suspensions in Mueller-Hinton broth were added. After incubation of the plates at 37°C overnight, the MIC was calculated as the lowest concentration that showed no visible growth in the tubes. The test was repeated triplicate.

**Swarming and swimming motilities assay**

The effect of diclofenac on swimming and swarming was examined (Liaw et al., 2001, 2004). For swimming assay, overnight culture of *P. mirabilis* was prepared and 5 μl from this culture was inoculated on the center of the surface of dried LB swimming agar (1.5%) plates containing different sub-inhibitory concentrations of diclofenac (½ MIC or ¼ MIC). The plates were incubated overnight at 37°C, the swimming zones diameters were measured in mm. Control plates were also prepared and inoculated in the same way. The experiment was repeated in triplicates and the mean and standard deviation were calculated. In order to differentiate swarmers cells, sections of agar from swimming assay plates with and without inhibitor were cut under aseptic conditions. The sections were cut from the center of the colony which contains vegetative cells and from the edge of the colony with swarmers cells. After removal of the bacteria from the cut agar pieces with phosphate buffered saline, they were simple stained with crystal violet and examined under the oil immersion lens.

For swarming assay, the overnight *P. mirabilis* culture was stabbed into the center of the dried LB swimming agar (0.4%) with diclofenac (½ MIC or ¼ MIC). After overnight incubation of the plates at 37°C, the swimming zones diameters were measured in mm. Control plates were also prepared and inoculated in the same way. The experiment was repeated in triplicate and the mean and standard deviation were calculated.

**Protease assay**

The tested strain was grown overnight in LB broth with and without sub-MIC (½ MIC or ¼ MIC) of diclofenac at 37°C. The bacterial suspension was centrifuged and the supernatant was collected for Protease assay (Keay et al., 1970). Protease activity was measured in the presence and absence of diclofenac using a casein substrate; 1 ml of the culture supernatant was mixed with 1 ml 0.05
M phosphate buffer-0.1 M NaOH (pH 7.0) containing 2% casein, and incubated for 10 min at 37°C. The reaction was stopped by adding 2 ml 0.4 M trichloroacetic acid. After 30 min stand at room temperature, the precipitate was removed by centrifugation and the optical density of the assays was measured at 660 nm. The positive and negative controls were prepared in the same way. The assay was repeated in triplicate and the mean and standard deviation were calculated.

**Hemolysis assay**

The tested strain was grown overnight in LB broth with and without sub-MIC (½ MIC or ¼ MIC) of diclofenac at 37°C. The bacterial suspension was centrifuged and supernatant was collected for Haemolysin assay (Liau et al., 2004). Briefly, bacterial suspension (50 μl) was mixed with a 2% erythrocyte suspension (450 μl) in 0.85% NaCl and 20 mM CaCl₂ and incubated at 40°C for 15 min. Hemolytic activity was determined by the haemoglobin release using a 100% positive haemolysin-positive reference and the optical density of the assays was measured at 540 nm. The assay was repeated triplicate and the mean and standard deviation were calculated.

**Urease activity assay**

To examine the effect of diclofenac on urease production, a modification of Koneman et al. (1997) method was applied. An overnight culture of *P. mirabilis* was prepared and 5 μl from this culture was inoculated on the center of the surface of dried Christensen's urea agar plates containing different sub-inhibitory concentrations of diclofenac (½ MIC or ¼ MIC). The plates were incubated overnight at 37°C, the activity of urease was indicated by the pH indicator color change from yellow to pink and pink zones diameters were measured in mm. Control plates were also prepared and inoculated in the same way. The experiment was repeated in triplicate and the mean and standard deviation were calculated.

**Biofilm formation**

**Assessment of biofilm production**

Overnight cultures of *P. mirabilis* tested isolate was prepared, diluted with fresh tryptone soya broth and adjusted to a cell density of 1 × 10⁶ CFU/ml for assessment of biofilm production (Stepanovic et al., 2000). Aliquots of 200 μl of the adjusted bacterial suspension were inoculated in sterile 96-well polystyrene microplates, incubated for 24 h at 37°C. The wells were gently aspirated and washed three times with sterile phosphate buffered saline (pH 7.2). The adherent cells were fixed with 200 μl of 99% methanol for 20 min and stained with 200 μl crystal violet (1%) for 20 min. The excess dye was washed out under running distilled water, and then the plates were air dried. The crystal violet bound dye was extracted by 95% ethanol and the optical densities were measured at a wavelength of 590 nm. The test was repeated three times, and the mean optical densities were calculated. The cut-off OD (ODc) was defined as three times standard deviations above the mean OD of the negative control. The tested isolate was categorized into one of four groups: non-biofilm forming (OD ≤ ODc), weak biofilm forming (OD > ODc, but ≤ 2x ODc), moderate biofilm forming (OD >2x ODc, but ≤ 4x ODc), or strong biofilm forming (OD > 4x ODc).

**Inhibition of biofilm formation**

For evaluation of the inhibitory effect of diclofenac on biofilm formation, the same procedure described for assessment of biofilm production was followed. Aliquots of 100 μl of the prepared bacterial suspension were added to the wells of sterile 96-well polystyrene microplate containing 100 μl of ½ MIC or ¼ MIC of diclofenac. The optical densities of the stained adherent biofilms were measured in the presence and absence of diclofenac at a wavelength of 590 nm. The assay was repeated in triplicate and the means and standard deviations were calculated.

**Determination of minimum biofilm inhibitory concentration (MBIC)**

The MBICs of the antibiotics or diclofenac, the minimum concentrations which inhibit regrowth of the bacterial biofilm cells, were determined by broth dilution method in polystyrene microtiter plates (Cernohorská and Votava, 2008). Briefly, an overnight culture adjusted with TSB to achieve a turbidity equivalent to that of a ½ McFarland standard, 75 μl aliquots of the inoculated media were added to the wells of microtiter plates. The plates were incubated for 24 h at 37°C. The wells were washed three times with PBS under aseptic conditions. Volumes of 100 μl of appropriate two-fold dilutions of the respective antimicrobial agents or diclofenac in Mueller–Hinton broth were transferred into the dried wells with established biofilms. The microtiter plates were incubated for 18 to 20 h at 37°C and MBIC was determined, as the lowest concentration of antibiotic showed no visible growth in the wells. A positive control and a negative control were included in all experiments. The experiment was repeated in triplicate.

**Adhesion assay**

Overnight cultures of *P. mirabilis* tested isolate was prepared, diluted with fresh tryptone soya broth and adjusted to a cell density of 1 × 10⁶ CFU/ml for adhesion assay (Vesterlund et al., 2005).

**Adhesion to epithelial cells**

Epithelial cells were collected from pregnant urine, washed and resuspended in phosphate buffer saline (PBS). Epithelial cells were counted by methylene blue method and distributed eventually in microtitre-plate and co-cultured with bacterial strain in total volume 150 μl in absence and presence of diclofenac in concentration ½ MIC or ¼ MIC. Cells were incubated at 37°C for 1 h, washed 3 times with PBS, fixed at 60°C for 20 min, stained with equal volume of crystal violet (0.1%) for 45 min and washed 5 times with PBS. Finally, 150 μl 20 mole/L citrate buffer (PH 4.3) was used to lysis cells for 45 min and optical density was measured at 570 nm. The experiment was repeated in triplicate and the means and slandered deviations were calculated.

**Adhesion to abiotic surface**

Bacterial strain was cultured with diclofenac in concentration ½ MIC or ¼ MIC in micro-titer plate, incubated at 37°C for 1 h, washed 3 times with PBS, fixed at 60°C for 20 min, stained with equal volume of crystal violet (0.1%) for 15 min and washed 5 times with PBS.
Table 1. The MIC and MBIC of antibiotics and diclofenac against isolated *Proteus mirabilis* from diabetic foot.

<table>
<thead>
<tr>
<th>Tested agent</th>
<th>MIC (µg/ml)</th>
<th>MBIC (µg/ml)</th>
<th>Ratio MBIC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>2</td>
<td>128</td>
<td>64</td>
</tr>
<tr>
<td>Amoxicillin/Clavulinic acid</td>
<td>265</td>
<td>2048</td>
<td>8</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>64</td>
<td>1024</td>
<td>16</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>16</td>
<td>512</td>
<td>32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>64</td>
<td>2048</td>
<td>32</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>64</td>
<td>2048</td>
<td>32</td>
</tr>
<tr>
<td>Imipenem</td>
<td>4</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Then, ethanol was added and optical densities were measured at 590 nm. The assay was repeated triplicate and the means and stan-dered deviations were calculated.

Combination of antibiotics and diclofenac

To determine the effect of combining diclofenac with antimicrobial agents, the MICs of these antimicrobial agents were determined in the presence of ¼ MIC of diclofenac. The wells of microtiter plates with 50 µl of 4 fold the final concentration of each diclofenac and antibiotics were inoculated with standardized bacterial suspensions to have a final inoculum of 5x10^5 CFU/ml and incubated at 37°C overnight. The MIC was calculated as the lowest concentration of antimicrobial agent that can completely inhibit visible growth in the wells. Fractional inhibitory concentration (FIC) of antibiotic was determined according to Mackay et al. (2000). FIC of drug A = MIC drug A in combination/MIC drug A alone. The result of the combination may be synergistic (FIC ≤ 0.5), indifferent (FIC > 0.5 to 4), or antagonistic (FIC > 4).

Statistical analysis

The assays were repeated in triplicates and the data are presented as median and range unless specified. The differences between the control and diclofenac were analyzed by t-test using the Graphpad Prism 5 software. The relationship between variables was evaluated using the Pearson rank correlation test. A two-tailed P value <0.05 was considered statistically significant. The percentage of inhibition of diclofenac was calculated.

RESULTS

Identification of *P. mirabilis* isolates

*P. mirabilis* isolate was identified as Gram-negative rods. They produced lactose non-fermenting colonies on MacConkey's agar and showed swarming on nutrient agar. They produced hydrogen sulphide from triple sugar iron agar and were urease positive and indole fermentation negative.

Determination of MIC

The MIC of antibiotics or diclofenac sodium salt was determined by the broth microdilution method according to Clinical Laboratory and Standards Institute Guidelines (CLSI). The results were summarized in Table 1. The MIC for tested antibiotics was determined in the presence of diclofenac (¼ MIC) and FIC was calculated for combinations. It was shown that diclofenac synergistically decrease the MIC of tested antibiotics, and FIC ranged from 0.25 to 0.5, except combination with imipenem was indifferent (FIC = 1). The results of diclofenac combination with antibiotics were summarized in Table 3.

Inhibition of swarming and swimming motilities

An overnight culture of *P. mirabilis* was prepared and 5 µl from this culture was inoculated on the center of the surface of dried LB swarming agar (1.5%) or LB swimming agar (0.4%) plates containing different sub-inhibitory concentrations of diclofenac (¼ MIC or ¼ MIC). The plates were incubated overnight at 37°C, the diameters of swarming zones or swimming zones were measured in mm. Control plates were also prepared and inoculated in the same way (Figures 1A and 2A). In order to differentiate swarmer cells, sections of agar from swarming assay plates with and without diclofenac were cut under aseptic conditions. The sections were cut from the center of the colony which contains vegetative cells and from the edge of the colony with swarmer cells. The swarmer cells in the presence of diclofenac were shorter and more or less similar to vegetative cells (Figure 1C). The experiment was repeated in triplicate and the mean, standard deviation and significance of inhibition were calculated (Figures 1B and 2B). Sub-inhibitory concentrations of diclofenac inhibited the swarming and swimming motilities significantly. Diclofenac (½ MIC) inhibited swarming completely and swimming significantly (P < 0.0001), moreover diclofenac in ¼ MIC
Table 2. Percentage of reduction of *P. mirabilis* virulence by diclofenac in sub MIC concentrations.

<table>
<thead>
<tr>
<th>Percentage of reduction</th>
<th>Diclofenac (½ MIC, %)</th>
<th>Diclofenac (¼ MIC, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swarming</td>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>Swimming</td>
<td>84</td>
<td>63</td>
</tr>
<tr>
<td>Hemolysin production</td>
<td>23.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Protease production</td>
<td>38.8</td>
<td>12</td>
</tr>
<tr>
<td>Adhesion</td>
<td>21.3</td>
<td>0</td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>89.7</td>
<td>40</td>
</tr>
<tr>
<td>Urease</td>
<td>92.5</td>
<td>42.6</td>
</tr>
</tbody>
</table>

Table 3. Modification of the susceptibility of isolated *P. mirabilis* to antibiotics in presence of diclofenac in concentration of ¼ MIC.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC</th>
<th>MIC&lt;sub&gt;Dec&lt;/sub&gt;</th>
<th>FIC</th>
<th>MBIC</th>
<th>MBIC&lt;sub&gt;Dec&lt;/sub&gt;</th>
<th>FIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>128</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>Amoxicillin/Clavulinic acid</td>
<td>265</td>
<td>64</td>
<td>0.25</td>
<td>2048</td>
<td>256</td>
<td>0.25</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>64</td>
<td>32</td>
<td>0.5</td>
<td>1024</td>
<td>128</td>
<td>0.125</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>16</td>
<td>4</td>
<td>0.25</td>
<td>512</td>
<td>64</td>
<td>0.125</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>64</td>
<td>32</td>
<td>0.5</td>
<td>2048</td>
<td>256</td>
<td>0.125</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>64</td>
<td>16</td>
<td>0.25</td>
<td>2048</td>
<td>512</td>
<td>0.25</td>
</tr>
<tr>
<td>Imipenem</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>*MIC and MBIC were determined in µg/ml. *MIC<sub>Dec</sub> and MBIC<sub>Dec</sub>, are the minimum inhibitory concentration and minimum biofilm inhibitory concentration of tested antibiotics respectively, in presence of diclofenac in concentration of ¼ MIC. *FIC of drug A= MIC drug A in combination/MIC drug A alone. The result of the combination may be synergistic (FIC ≤ 0.5), indifferent (FIC > 0.5 to 4), or antagonistic (FIC > 4).*</sup>

showed significant inhibition for swarming and swimming (*P = 0.0003 and P = 0.0002, repetitively*).

The percentage of the inhibition of swarming was 100% as in case of ½ MIC concentrations, while decreased to 35% in concentration of ¼ MIC (Table 2). Diclofenac inhibited swimming activity of isolated *P. mirabilis* 84 and 63% in concentrations ½ MIC and ¼ MIC, repetitively (Table 2).

**Protease assay**

Protease activity was measured in the presence and absence of diclofenac using a casein substrate. Sub MIC of diclofenac (½ MIC or ¼ MIC) decreased the protease activity of *P. mirabilis* significantly (*P = 0.0001 or P = 0.0021, respectively*) (Figure 3). The percentage of inhibition of protease activity varied from 12 to about 39% in ¼ MIC and ½ MIC concentrations of diclofenac (Table 2).

**Hemolysis assay**

The tested strain was grown with and without sub-MIC (½ MIC or ¼ MIC) of diclofenac at 37°C. The bacterial suspensions were centrifuged and the supernatant was collected for Haemolysin assay. Diclofenac (½ MIC) showed a significant inhibition in haemolysin activity (*P = 0.0006) and the reduction percentage was about 24%, however, it did not show a significant inhibition in ¼ MIC concentration (*P = 0.21) (Figure 4 and Table 2).

**Urease activity assay**

An overnight culture of *P. mirabilis* was prepared and 5 µl from this culture was inoculated on the center of the surface of dried Christensen's urea agar plates containing different sub-inhibitory concentrations of diclofenac (½ MIC or ¼ MIC) (Figure 5A). The diameter of color change were measured in mm and statistically calculated. Sub MIC of diclofenac (½ MIC or ¼ MIC) showed a decrease in the urease activity of *P. mirabilis* significantly (*P = 0.0047 or P = 0.0471, respectively*) (Figure 5B). The percentages of reduction of urease activity were about 93 and 43% for ½ MIC and ¼ MIC of diclofenac (Table 2).

**Biofilm formation**

For assessment of biofilm production, the ODc and OD of
tested *P. mirabilis* were determined. ODc was 0.064 and OD was 0.344 (OD > 4x ODc), *P. mirabilis* isolate was considered strong biofilm forming according to Stepanovic et al. (2000). The minimum biofilm inhibitory concentration was performed using sterile 96-well polystyrene microplate plates against tested antibiotics and diclofenac (Table 1).

For evaluation of the inhibitory effect of diclofenac on biofilm formation, the same procedure described for assessment of biofilm production was followed in sterile 96-well polystyrene microplate containing ½ MIC or ¼ MIC of diclofenac. Diclofenac in sub MIC concentrations showed a significance inhibition in biofilm formation (P < 0.0001 and P = 0.0001 in ½ MIC and ¼ MIC) (Figure 6). The percentages of inhibition were calculated about 90 and 40% in concentration of ½ MIC or ¼ MIC of diclofenac repetitively (Table 2). Moreover, diclofenac (¼ MIC) decreased the MBIC of tested antibiotics, the FIC ranged from 0.125 to 0.5 (synergism). The summary of synergistic effect of diclofenac and antibiotics is summarized in Table 3.

### Adhesion assay

For evaluation the effect of diclofenac on adhesion of isolated *P. mirabilis*, epithelial cells were collected from pregnant urine and distributed eventually in microtitre plate and co-cultured with bacterial strain in absence and presence of diclofenac in concentration ½ MIC or ¼ MIC (Figure 7A). The cells were incubated at 37°C for 1 h, fixed at 60°C for 20 min, stained with equal volume of crystal violet and the adherent cells were lysed with citrate buffer (pH 4.3) and optical density was measured at 570 nm. No significant inhibition was observed to be considered.

Furthermore, the inhibitory effect of diclofenac on adhesion of *P. mirabilis* to abiotic surface was examined. *P. mirabilis* was cultured with diclofenac in concentration ½ MIC or ¼ MIC in micro-titer plate, incubated at 37°C for
Figure 2. The inhibitory effect of sub MIC (½ MIC or ¼ MIC) of diclofenac on swimming of isolated *P. mirabilis* from diabetic foot. A) Sub MIC of diclofenac showed inhibition of swimming of isolated *P. mirabilis* on LB swimming agar (0.4% agar). B) Sub MIC of diclofenac (½ MIC or ¼ MIC) inhibited the swimming of *P. mirabilis* significantly (*P* < 0.0001 or *P* = 0.0002, respectively).

1 h. The adherent cells were stained with crystal violet, ethanol was added and optical densities were measured at 590 nm. Compatible to findings with adhesion to epithelial cells, diclofenac did not show a much significant inhibition of adhesion as shown in biofilm inhibition. Diclofenac did not show a significant inhibition of adhesion in concentration of ¼ MIC (*P* = 0.56), while it showed inhibition in concentration of ½ MIC (*P* = 0.01) (Figure 7.B).

**DISCUSSION**

*Proteus* is a causative agent of wide range of infections; the potential of its virulence is not only due to production of several extracellular enzymes, but also due to inherent capability of pretrichrous flagellar translocation and its biofilm formation capability (Morgenstein et al., 2010; Armbruster and Mobley, 2012). In the last decades, resistance development is one of the serious emerging problems and it is owed to genotypic and/or phenotypic modifications. In general, bacterial virulence is thought to be a factor of resistance and overcoming the virulence is hypothesized to enhance the antimicrobial eradication process. Overcoming the bacterial virulence is more crucial in immunocompromised patients, as in diabetes. Inspite, the vast advances in controlling diabetes, complications usually happen. The most serious complications are those due to bacterial infections, particularly, in immunocompromised patients (Assmann et al., 2015). In this study, *P. mirabilis* was isolated from diabetic foot ulcer and its virulence behavior and resistance to common prescribed antibiotics were evaluated. Anti-inflammatory drugs and analgesics are widely prescribed for diabetic patients for several medical reasons. Diclofenac is one of these anti-inflammatory and analgesic drugs which showed some antibacterial activity. This work was to evaluate the effect of diclofenac sodium salt on inhibition or reduction of important virulence factors of *P. mirabilis*.

Swarming and swimming describe flagellum-dependent movement across a surface or through liquid or soft agar. This form of motility allows *P. mirabilis* to migrate across the infected ulcer, spreading the infection (Rather, 2005;

Figure 3. Protease assay. Extracellular protease activity was measured in the presence or absence of sub MIC diclofenac (½ MIC or ¼ MIC) using a casein substrate, the optical densities were measured at 660 nm. Sub MIC of diclofenac (½ MIC or ¼ MIC) decreased the protease activity of _P. mirabilis_ significantly (\(P = 0.0001\) or \(P = 0.0021\), respectively).

Figure 4. Haemolysin assay. Extracellular haemolysin activity was measured in the presence or absence of sub MIC diclofenac (½ MIC or ¼ MIC), the optical densities were measured at 540 nm. Sub MIC of diclofenac (½ MIC) decreased the haemolysin activity significantly (\(P = 0.0006\)), however lower concentration (¼ MIC) did not show significant decrease in activity (\(P = 0.21\)).

Jacobsen et al., 2008). Moreover, _P. mirabilis_ biofilms contain protruding swarm cells which enhance the microbial resistance (Jones et al., 2007). It showed that sub-inhibitory concentrations of diclofenac inhibited the swarming and swimming motilities significantly which may affect the infection spread which may influence the pathogenesis as a consequence. Several factors participated in the regulation of swarming motility including the up-regulator of flagellar master operon (Umo) proteins and other factors reviewed elsewhere (Morgenstein et al., 2010). Moreover, all flagellum-related genes are arranged within a single 53.3-kb locus (Pearson et al., 2008), which may indicate the significance of inhibition of the transcriptional regulator on motility. There are several approaches which described the mode of diclofenac action; one of them is its capability to down regulate Umo proteins (work in progress).

Biofilms are closely associated microbial cells embedded in dynamic communities within a hydrated extracellular polymeric substance on an air-liquid interface, or adherent to inert (abiotic) or living surfaces constituting the major proportion of bacterial biomass in nature (De Kievit et al., 2001). Biofilms are formed in a sequential manner, started by reversible attachment of free-floating cells to surfaces. A variety of direct interactions, generally associated with the onset of the production extracellular polymeric substances, are responsible for the transition to irreversible attachment which entraps bacteria and results in aggregation of cells (Gómez-Suárez et al., 2002). Diclofenac in sub-inhibitory concentration did not affect the bacterial adhesion significantly, while the formation of biofilms are significantly inhibited in the same concentrations, which indicate its action on prevention of late stages of biofilm formation. Many species of bacteria use a system of stimuli and response correlated to population density called quorum sensing (QS). Intercellular signaling regulates functions contributing to virulence of many bacterial pathogens. Thus, interference with signaling is a promising approach to improve the outcome of bacterial, and in particular, biofilm infections (Zhang and Li, 2016).

It was assumed that effect of diclofenac on biofilm formation may be due to interference with QS signaling (under investigation) which may explain its significant inhibition of biofilm versus non considerable inhibition of bacterial adhesion. Diclofenac in sub-inhibitory concentrations do not only lowered the inhibitory concentration of antibiotics in combination, but also lowered their inhibitory concentration in the presence of biofilm.

The effect of diclofenac is extended to inhibit extracellular enzymes which may contribute significantly in lowering the pathogenesis. Urease enzyme is a significant virulence factor in human and animal infections of the urinary and gastrointestinal tracts;
Figure 5. Urease assay. Urease activity was measured in the presence or absence of sub MIC diclofenac (½ MIC or ¼ MIC). A) Urease activity was demonstrated on Christensen's urea agar plates, change in pH indicator color due to urea release, the zones of color change were measured in mm. B) Sub MIC of diclofenac (½ MIC or ¼ MIC) decreased the urease activity of *P. mirabilis* significantly (P = 0.0047 or P = 0.0471, respectively).

Figure 6. Inhibition of biofilm formation. *P. mirabilis* isolate was cultured with diclofenac in concentration ½ MIC or ¼ MIC in microtiter plate, incubated at 37°C overnight, the attached cells were stained with crystal violet and their optical densities were measured at 590 nm. Sub MIC of diclofenac (½ MIC or ¼ MIC) inhibited the biofilm formation of *P. mirabilis* significantly (P < 0.0001 or P = 0.0001, respectively).

moreover, its role in recycling of nitrogenous wastes may take part in resistance to some biocides (Mobley et al., 1995). The urease activity is constitutive in most *P. mirabilis* strains and inducible in some *P. mirabilis* (Rózsalki et al., 1997). The urease of *P. mirabilis* is reported to be regulated by pH; moreover, it was shown that the expression is developmentally regulated as swarm cells have higher levels of urease and of urease transcript (Mobley et al., 1995). Protease is an extracellular proteolytic enzyme which cleaves two classes of antibodies, IgA and IgG, as well as non-Ig proteins such as gelatin, secretory component, casein, and bovine serum albumin (Loomes et al., 1990). It had been demonstrated that the differentiation of *P. mirabilis* short vegetative rods into hyper-flagellate swarmer cells is accompanied by substantial increases in the activities of virulence factors including proteases (Allison et al., 1994). The ability of *P. mirabilis* to invade human epithelial cells is basically characteristic of swarmer cells but not vegetative cells; the protease activity is hypothesized to be relevant to *P. mirabilis* pathogenesis (Rózsalki et al., 1997). Moreover, *P. mirabilis* strains synthesize urease, which degrades urea, providing alkaline optimal conditions for the action of proteases (Senior et al., 1993). Diclofenac showed a significant
inhibition on both protease and urease which may reduce bacterial virulence potentially.

Haemolysin is another virulence factor of \textit{P. mirabilis} because of its cytotoxicity on epithelial cells (Armbruster and Mobley, 2012). It is most interesting that mutation in the gene encoding Haemolysin \textit{hpmA} did not affect the colonization or tissue damage during infection (Alamuri et al., 2009), which may indicate that its activity is either diminished \textit{in-vivo} or masked by contribution of other virulence factors (Armbruster and Mobley, 2012). \textit{P. mirabilis} genome is transcribed in several distinguished rRNA operons, surprisingly the two-partner secretion system containing the hemolysin genes \textit{hpmBA} are transcribed in separate operons than those in which protease and urease are transcribed (Pearson et al., 2008). Diclofenac showed a significant inhibition of haemolysin production in higher concentrations in comparison to that needed to inhibit protease or urease, that may be due to an expected inhibitory effect of diclofenac on transcription of urease or protease (under investigation). Conclusively, diclofenac showed a significant inhibition of \textit{P. mirabilis} virulence, which may be helpful to diabetic patients who need anti-inflammatory and analgesic treatments. This study showed the effect of diclofenac phenotypically while it will be more interesting to show the molecular basis of this effect (work in progress). Diclofenac is widely used drug in different medical conditions and in different pharmaceutical formulations; inspite of the fact that our study lacks the \textit{in-vivo} evidence of diclofenac inhibitory effect on virulence, it may be more helpful to prescribe diclofenac for diabetic patients when needed.

\textbf{Conflict of Interests}

The authors have not declared any conflict of interests.

\textbf{REFERENCES}


Hydrophobicity and specific biofilm features of *Bacillus cereus* spores subjected to pH stresses

Fadila Malek

Department of Biology, SNV-STU Faculty, University of Tlemcen, Algeria.

Received 10 March 2016, Accepted 29 April, 2016.

*Bacillus cereus* is a foodborne pathogen that often persists on food processing surfaces due the formation of spores and biofilms. Spores of 12 selected *B. cereus* strains from genotypes that recurred in a pasteurized milk processing line were investigated in this study, for their surface and biofilm characteristics. The main objective was to have an insight into their persistence strategies. Spore surface hydrophobicity and acid-base properties, were assessed using the microbial adhesion to solvents (MATS) method. To determine how hydrophobicity was affected by cleaning procedures, this property was measured when spores were submitted to alkali or acidic stresses mimicking those of cleaning-in-place (CIP) procedures. Biofilms formation on stainless steel coupons by pH-treated spores was investigated in three culture media and imaged by using environmental scanning electron microscopy (ESEM). Results showed that spores were either hydrophilic or moderately hydrophobic. Alkali-stress reduced spore surface hydrophobicity, whereas acidic shock increased it. More limited hydrophobicity changes following alkaline stress suggest alkali adaptation of spores. In addition, spores submitted to pH-stresses produced specific biofilm features on stainless steel as shown by ESEM imaging. Alkali tolerance and the biofilm lifestyle are strategies that permit *B. cereus* recurrent genotypes to persist in the milk processing line. Overall, this study gives an insight into hydrophobicity and specific biofilm features of *B. cereus* spores submitted to chemical cleaning.

**Key words:** *Bacillus cereus*, biofilms, spores, hydrophobicity, CIP-like stress, dairy industry, environmental scanning electron microscopy (ESEM).

INTRODUCTION

Tolerance of bacteria to low and high-pH stresses is of major concern to the food industry. As shown by several studies (Lindsay et al., 2002; Cotter and Hill, 2003; Giotis et al., 2009; Mols and Abee, 2011), the pH stresses encountered in the food processing environments may induce acidic and/or alkaline resistance of contaminating bacteria and thus contribute to their survival and persistence in the factories. Unfortunately, this adaptive
behavior is largely reported for important foodborne pathogenic microorganisms such as *Listeria monocytogenes* and *Bacillus cereus*. As an illustration, in the dairy industry, certain *B. cereus* genotypes were shown to recur in milk processing lines for several years (Svensson et al., 2004; Shaheen et al., 2010; Malek et al., 2013). In addition, *B. cereus* forms biofilms that are responsible of spore dissemination into food environments (Wijmann et al., 2007) and resist to removal (Kumari and Sarkar, 2014).

The high adhesion potential of *B. cereus* spores is well established and related to spore surface hydrophobicity which relies on morphological structures notably exosporium and appendages (Husmark and Ronner, 1992; Faille et al., 2002; Ankolekar and Labbé, 2010). Spores of *B. cereus* have mainly been investigated for survival, adhesion and biofilm formation after CIPLike stresses (Faille et al., 2010; Salutiano et al., 2010; Shaheen et al., 2010). However, it remains unknown how the conditions encountered by spores during cleaning procedures affected spore surface hydrophobicity. CIP systems are alkaline (NaOH) and/or acidic (HNO3) washes often performed at high temperature (70 – 80°C) (Bremer et al., 2006). Similarly, little is known about the structure of the biofilms developed by *B. cereus* on dairy processing equipment after CIP procedures. That is why this study dealt with the analysis of spore surface hydrophobicity and biofilm features following pH-stresses by using *B. cereus* dairy recurrent strains, in order to understand their persistence strategies. For this purpose, spore hydrophobicity and acid-base properties of a set of 12 *B. cereus* dairy isolates and a comparative reference strain, *B. cereus* ATCC 11778, were, first assessed. Hydrophobicity was further measured when spores were submitted to alkali and acid stresses that mimicked those of CIP systems. Finally, pH-treated spores were used to form biofilms on stainless steel coupons, under static conditions and observed in ESEM.

**MATERIALS AND METHODS**

**Bacterial strains**

*B. cereus* strains were previously isolated from a pasteurized milk processing line (Malek et al., 2013). These strains were fingerprinted by M13 PCR, and clustered into three distinct M13-PCR groups: one major group (genotype A), which included 17 out of 20 strains and two minor groups (genotypes B and C). Genotypes A and B which recurred in this processing line for more than four years (Table 1), were affiliated to the mesophilic *B. cereus* group III while the last genotype was affiliated with the mesophilic *B. cereus* group IV, according to the phylogenetic classification of Guinebretière et al. (2008).

**Spore surface properties**

Different solvents were used to evaluate the hydrophobic/hydrophilic spore surface properties of *B. cereus* and their Lewis acid–base characteristics. Both apolar solvents hexadecane and hexane were used to estimate the hydrophobicity properties of spore surfaces while the two monopolar solvents, chloroform and diethyl ether, were selected for the estimation of the Lewis acid/base (that is, electron donor/acceptor) character, according to the microbial adhesion to solvents (MATS) partitioning assay (Bellon-Fontaine et al., 1996). Hydrophobicity is expressed as the percentage (P) of adhesion to hexadecane. Spores are very hydrophilic (P < 20%), hydrophilic (20 > P < 40%), moderately hydrophobic (40 > P < 60%) and highly hydrophobic (P > 60%). The acid-base interactions can be assessed based on the comparison between the microbial cell affinity to chloroform, an acidic solvent (electron acceptor) and the apolar solvent hexadecane as well as between spore affinity to diethyl ether, a basic solvent (electron donor) and the apolar solvent, hexane (Bellon-Fontaine et al., 1996). Results are expressed as percentages of adhesion to each solvent. Spores had an electron donor character when their affinity to chloroform is higher than with hexadecane and an electron acceptor character when their affinity to diethyl ether is higher than to hexane. Spore suspensions were prepared as previously described (Simmonds et al., 2003), and prior to use, they were washed one time and suspended in saline (0.15 M NaCl) at pH 7. Hydrophobic and acid-base properties of spore surfaces were measured using the MATS method (Bellon-Fontaine et al., 1996) with modifications based on observations from other reports (Tauferon et al., 2006). In short, saline spore suspensions were adjusted to an absorbance of 0.6 to 1 at 595 nm. Spore suspension (2 mL) was added to 400 µL of the polar or apolar solvent, vortexed for 1 min and settled for 15 min. The optical density of the water phase was measured using a spectrophotometer at 595 nm. As described by Bellon-Fontaine et al. (1996), the percentage of spores bound to a given solvent was expressed as (1 – A/A0) x 100, where A0 is the absorbance measured at 595 nm of the bacterial suspension before mixing and A is the absorbance after mixing. The mean and standard error were calculated from five measurements. Chemical products (Hexadecane, chloroform, hexane and diethyl ether) were obtained from Aldrich chemical, Co., Inc. USA.

**Hydrophobicity of pH-treated spores**

Spores were investigated for their surface hydrophobicity following mixing with sodium hydroxide (pH 12.7) at 80°C and into nitric acid (pH 1.2) at 70°C, to mimic CIP conditions as applied at the investigated dairy plant. Spore suspensions were pH-treated using the protocol of Faille et al. (2010), with minor modification. One volume of the stock suspensions was added to 1 volume of aqueous 2% w/v NaOH or 1% HNO3 w/v to absorbance values between 0.8–1. Tubes containing NaOH or HNO3 spore mixtures were respectively incubated at 80 and 70°C for 10 min. After each treatment, spores were rapidly cooled, harvested as previously described (Faille et al., 2010) and re-suspended in saline to absorbance values between 0.8–1. Hydrophobicity of the pH-treated spores was assessed as described above. Experiments were performed with repetitions. The obtained data were submitted to variance analysis and correlation tests using Matlab 7.0 France software.

**Adhesion of pH-treated spores to stainless steel coupons**

The pH-treated spores were used to adhere on stainless steel coupons (AISI 304 L, 10 x 10 mm), cleaned according to the protocol described by Peng et al. (2001). For the adhesion assay
Table 1. Spore surface hydrophobicity and acid-base properties of \textit{B. cereus} isolates from a pasteurized milk processing line*.

<table>
<thead>
<tr>
<th>Strains\textsuperscript{ab}</th>
<th>Hexadecane  ((% \pm SE))</th>
<th>Chloroform  ((% \pm SE))</th>
<th>Hexane  ((% \pm SE))</th>
<th>Ether  ((% \pm SE))</th>
<th>Character\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13PCR group A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S78</td>
<td>12.5 ± 6.5</td>
<td>15.7 ± 6</td>
<td>14.9 ± 5.4</td>
<td>31.5 ± 7</td>
<td>D and A</td>
</tr>
<tr>
<td>S19</td>
<td>14.2 ± 3.2</td>
<td>17.6 ± 4.7</td>
<td>17.5 ± 6.2</td>
<td>20.1 ± 6.8</td>
<td>D and A</td>
</tr>
<tr>
<td>P56</td>
<td>18.4 ± 4.2</td>
<td>23.9 ± 5.3</td>
<td>21.1 ± 3.7</td>
<td>27.3 ± 3.9</td>
<td>D and A</td>
</tr>
<tr>
<td>P53</td>
<td>21.5 ± 4.5</td>
<td>34.4 ± 4.1</td>
<td>23.4 ± 7.7</td>
<td>27.4 ± 6.7</td>
<td>D and A</td>
</tr>
<tr>
<td>A9</td>
<td>28.4 ± 6.2</td>
<td>42.8 ± 7.8</td>
<td>34.2 ± 4.8</td>
<td>28.4 ± 1.9</td>
<td>D</td>
</tr>
<tr>
<td>P52</td>
<td>42.3 ± 1.6</td>
<td>37.3 ± 3.9</td>
<td>45.4 ± 7.2</td>
<td>19.9 ± 6.1</td>
<td>ND. NA</td>
</tr>
<tr>
<td>S66</td>
<td>43.6 ± 3.5</td>
<td>37.3 ± 3.2</td>
<td>45.2 ± 6.1</td>
<td>19.9 ± 5.8</td>
<td>ND. NA</td>
</tr>
<tr>
<td>S113</td>
<td>49.3 ± 5.2</td>
<td>21.1 ± 4.3</td>
<td>53.6 ± 5.6</td>
<td>8.4 ± 5.8</td>
<td>ND. NA</td>
</tr>
<tr>
<td>S79</td>
<td>51.6 ± 2.4</td>
<td>38.9 ± 2.7</td>
<td>53.3 ± 3.1</td>
<td>15.2 ± 4.2</td>
<td>ND. NA</td>
</tr>
<tr>
<td>M13PCR group B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S35</td>
<td>23.6 ± 3.7</td>
<td>33.8 ± 3.6</td>
<td>25.8 ± 3.8</td>
<td>27.6 ± 3.8</td>
<td>D and A</td>
</tr>
<tr>
<td>A7</td>
<td>15.2 ± 2</td>
<td>29.1 ± 6.1</td>
<td>18.2 ± 2.8</td>
<td>21.1 ± 5.6</td>
<td>D and A</td>
</tr>
<tr>
<td>M13PCR group C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S116</td>
<td>46.7 ± 2.8</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ungrouped strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC ATCC 11778</td>
<td>71.7 ± 7.1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*Pasteurized milk was obtained from reconstituted and processed milk powder, in the investigated dairy plant. *\textit{B. cereus} isolates were kindly characterized at the genotypic level at UMR 408 INRA Avignon, France in a previous work (Malek et al., 2013). \textsuperscript{c}Strains are coded as follow: Letters indicate isolation origin and period. P: milk powder in 2010, S: milk processing equipment in 2010, A: milk processing equipment in 2006. \textsuperscript{d}D: electron donor, A: electrons acceptor, ND. NA: non-electron donor and non-electron acceptors. Hydrophobicity is expressed as percentage of adhesion to hexadecane.

coupons were fouled with \textit{B. cereus} spores by immersion in the wells of a 6-well polystyrene plate (Nunc multdish) for 3 h in a saline spore suspension \((10^7\cdot10^9\) spores mL\(^{-1}\)) and quickly immersed in sterile water to remove weakly attached spores.

**Biofilm formation by pH-treated spores**

To establish laboratory conditions that mimic the industrial setting being studied, especially soiling conditions, the formation of biofilms by \textit{B. cereus} spores was investigated in three culture media: non-diluted milk, 100 fold diluted milk and nutrient broth, at various incubation times. Milk culture medium consisted of 10% (w/v) medium heat milk powder (Germany), diluted in distilled water. The coupons with adhered spores were placed into wells containing culture media and incubated under static conditions for 24, 48 or 168 h (7 days) at 30°C. The media were refreshed every 2 days with the same fresh sterile medium and prior to that, the coupons were water rinsed to remove loosely attached cells.

**Microscopy**

ESEM imaging does not require any sample preparation or specific method. After the incubation time, all the above biofilm carrying stainless steel coupons were washed thrice with distilled water, air dried and examined in a 100 TM Hitachi environmental scanning electron microscope (Hitachi, Japan), at pressure in microscope chamber of 4 Torr. To avoid biofilm dehydration, the samples must be rapidly observed.

**RESULTS**

**Hydrophobicity of untreated spores**

Spore surface hydrophobicity measured by MATS method was expressed as percentage of adhesion to hexadecane (Table 1). Results showed that hydrophobicity of spores varied among the analyzed \textit{B. cereus} dairy isolates which displayed either a hydrophilic or hydrophobic character. Seven out of 12 strains were markedly (< 20%) or moderately hydrophilic (< 40%), adherence to hexadecane range between 12.5 and 28.4% respectively. Remaining strains shared moderately hydrophobic character, with hydrophobicity values spanning a narrow range from 42.1 to 51.6%. The unique highly hydrophobic strain (71.7%) was \textit{B. cereus} ATCC 11778, included for a comparative purpose. It is interesting to note the variability in hydrophobic/hydrophilic characters...
among closely related strains of M13 PCR genotype A, and the dominance of hydrophilic spores among *B. cereus* dairy isolates.

**Lewis acid-base properties of untreated spores**

The affinity of spores to the polar and apolar solvents, according to the MATS method, is presented in Table 1. The affinity of hydrophilic *B. cereus* spores of dairy origin to the polar solvents (chloroform/diethyl ether) were higher than to alkanes (hexadecane/decane), indicating their electron-donorr and electron-acceptor properties. However, their affinity for the different solvents did not exceed 40% and confirmed their hydrophilic nature. Whereas moderately hydrophobic spores sharing low affinity to both chloroform (< 40%) and diethyl ether (< 20%) did not express any acid-base characters. Conversely and regardless of the solvent used, the affinity of spores from the reference strain *B. cereus* ATCC 11778, was high (> 70%), indicating its hydrophobic nature.

The moderately hydrophilic spores produced by *B. cereus* strain A9 showed higher affinity for the electron acceptor solvent (chloroform) than hexadecane indicating an electron donor character. The electron acceptor property expressed by a higher affinity to diethyl ether (basic solvent) than to hexane was not observed for all spore surfaces.

**Hydrophobicity of pH-treated spores**

The variation in the hydrophobic/hydrophilic character of spores when mixed with 1% v/v sodium hydroxide (pH 12.7) at 80°C or 0.5% v/v nitric acid (pH 1.2) at 70°C is presented in Figure 1. Results showed that high alkaline stress led to a decrease in spore surface hydrophobicity while high acid stress increased it. The analysis of variance indicated that the variability in the hydrophobicity values was explained by the initial hydrophobicity and not by the strain effect. Nevertheless, in contrast to acid-induced hydrophobicity, the alkali-induced changes were significantly correlated with the initial hydrophobicity of spores (Pearson coefficient $r = 0.579 \ [P < 0.05]$). Accordingly, lower spore hydrophobicity values, resulted in lower alkali-induced hydrophobicity values indicating that hydrophilic spores were the least affected by alkaline shock.

**Structures of biofilms formed by pH- treated spores**

A selection of representative ESEM pictures is shown in Figures 2 to 4. ESEM images were captured at different incubation times. Biofilms incubated both in nutrient broth and non-diluted milk or diluted milk for 24 or 48 h, were little developed structures. The 48 h old biofilm shown in Figure 3a is a less elaborated monolayer structure which presents dehydration signs. In comparison, native spores
of the same strain formed a substantial thick mature biofilm, at the detachment stage (Figure 3b). It is also interesting to note that, after 24 h of cultivation, the biofilms formed in 1/100 diluted milk were still at the adhesion stage (Figure 2a) or just starting to be covered with the EPS-matrix (Figure 2b). In contrast, in the biofilms formed in crevices (Figure 2c and d), cells are completely hidden in the extracellular matrix. It appears that in these harborage, the production of the biofilm-matrix was enhanced, enabling cells to form a compact matrix structure devoid of obvious pores or channels. Older biofilms (7 days) formed in non-diluted milk or nutrient broth, were also compact shapes characterized by smooth or wrinkled surface topography (Figure 4). However, these biofilms are most likely devoid of living cells.

**DISCUSSION**

In this study, the authors attempted to phenotypically characterize spores from a collection of *B. cereus* strains that recurred in a pasteurized milk processing line, in order to understand their persistence strategies. Interesting findings were the variability in spore surface hydrophobicity recorded among closely related *B. cereus* genotypes and the predominance of hydrophilic spores. Spores of *B. cereus* are generally recognized to be hydrophobic or highly hydrophobic (Simmonds et al., 2003; Tauveron et al., 2006, Ankelokar and Labbé, 2010). Hydrophilic spores have already been reported among strains of *B. cereus* isolated in the dairy environment (Bernardes et al., 2010; Salustiano et al., 2010), and strains of thermophilic bacilli isolated from milk powder (Seale et al., 2008). Accordingly, the dairy environment appeared to be a source of hydrophilic spores of both mesophilic and thermophilic bacilli.

Based on the results of the MATS method, Lewis acid-base properties exhibited by *B. cereus* spores were consistent with data of the literature. Electron donor and electron acceptor characters were found for very hydrophilic strains belonging to other bacterial species (Faille et al., 2002; Hamadi et al., 2004; Djeribi et al., 2013). Similarly, the lack of any electron donor electron acceptor properties was described in hydrophobic *B. cereus* spores (Faille et al., 2002). Both characteristics were associated with high adhesion potential to inert surfaces.
The authors also attempted to explain the variability recorded in spore surface hydrophobicity among closely related *B. cereus* strains (belonging to genotype A). Hydrophobicity of *B. cereus* has been already reported to be strain-associated and not related to the ecological niche (Tauveron et al., 2006). Nevertheless, adaptation of dairy-associated *B. cereus* to alkaline pH was previously reported (Lindsay et al., 2002). Hydrophilic strains of this bacterium were isolated in the dairy industry from CIP solutions (Salustiano et al., 2010) or the filling machine (Bernardes et al., 2010). Since CIP systems, are mainly alkaline and/or acidic washes often performed at high temperature (Bremer et al., 2006), the existence of a relationship between the hydrophilic character of *B. cereus* spore surfaces and alkali adaptation is believed. To give more insight into this issue, hydrophobicity was assessed when spores were submitted to hot alkali stress or hot acidic stress mimicking those of CIP procedures. Based on the results of the percentage of adhesion to hexadecane, high-pH stress results in a decrease of hydrophobicity values while low-pH stress increases them. These results are consistent with data from previous works concerning *B. cereus* (Lindsay et al., 2000) or other bacteria (Giotis et al., 2009; Moorman et al. 2008), after exposure to mild pH-stresses. However, to the best of the authors' knowledge, this property has not been investigated when cells were submitted to more severe pH-stresses, like those encountered during cleaning procedures. Faille et al. (2010) have already shown exosporium glycoproteins to be seriously damaged by spore treatments using severe alkaline stress (2% NaOH at 80°C for 20 min) and this should result in a decrease in spore hydrophobicity as shown in the current study. On another hand, hydrophilic spores displayed more limited hydrophobicity changes as compared to highly hydrophobic spores, so that the highest percentage of hydrophobicity change (40.5%) was recorded for the most hydrophobic strain, BC ATCC 11778. More limited hydrophobicity reduction following alkali treatment has been related to cell alkali adaptation of bacteria (Giotis et al., 2009). Consequently, hydrophilic spores behave as alkali adapted cells, whilst the
reference strain should be considered as non-alkali adapted strain. This result constitutes one possible explanation for the occurrence of markedly hydrophilic strains, among these B. cereus dairy isolates. At the dairies, cleaning procedures may select some spores with specific surface chemistry, and it is likely that hydrophilic spores are part of such category. In good agreement with this finding, the surface chemistry of Bacillus spores has been described to significantly influence the efficiency of cleaning procedures (Faille et al., 2013).

The biofilms formed by pH-treated spores, in all culture media were not well developed structures, in terms of tridimensional architecture, or net-like patterns. This should be ascribed to the loss of the viability of most of the pH-treated spores or the loss of their ability to adhere due to damaged structures, as previously demonstrated (Faille et al., 2010; Shaheen et al., 2010). As an illustration, the 48 h old biofilm formed in nutrient broth was little elaborated structure which consists of a two-dimensional net-like attachment pattern, previously described for biofilms formed in poorly nutritional conditions (Marsh et al., 2003).

Likewise, in 100 fold diluted milk, the biofilm formation process is also seriously affected, since after 24 h incubation at 30°C, bacteria were still at early biofilm formation stages, namely the adhesion step. Based on the ESEM pictures, it is clear that only small numbers of spores survived the pH-stresses, and adhered to stainless steel. In good agreement, Faille et al. (2010) demonstrated that, a small percentage of adherent B. cereus spores were able to resist the conditions found during CIP procedures and the spores detached during the CIP procedure would re-adhere along the CIP rig. In the current study, pH-treated spores of B. cereus strain PS3 were able to adhere on stainless steel surfaces but had lower propensity to develop a mature biofilm in 100 fold diluted milk, within 24 h. This may probably be due to the incapacity of adhered spores to rapidly germinate in diluted milk. Consistent with results of Shaheen et al. (2010), spores of some B. cereus strains were not capable of germinating in 10 fold diluted milk. However, ESEM pictures showed that, when protected in harborsages of impaired material, spores were able to develop young biofilms, once a minimum initial bacterial load is necessary for bacteria to persist in a harborage site (Carpentier and Cerf, 2011).

Similarly, older biofilms (7 days) are associated with low numbers of living cells when they are first formed, but may be devoid of cells or contain only few spores, once the structures mature. Nevertheless, the observed old wrinkled structures were already described in B. subtilis biofilms and shown to be highly resistant to penetration of gas and liquid and thus to withstand biocide effects (Epstein et al., 2011). This is of crucial concern to the efficiency of cleaning procedures against biofilms exhibiting such recalcitrant structures. These results are interesting cues with regard to the persistence of foodborne pathogens in industrial settings.

Conclusion

Spores and biofilms are considered the most important reservoir of B. cereus in milking pipelines and on surfaces of equipment, and have to be deeply characterized. This study showed how cleaning procedure may affect spore surface hydrophobicity in B. cereus. Hydrophilic spores which seem to be common among dairy-associated B. cereus strains should be selected in the dairy environment by CIP-like procedures. Hydrophilic spores selected by cleaning systems were best able to withstand chemical cleaning, and to form specific biofilm features on stainless steel. This should constitute possible strategies whereby B. cereus recurrent genotypes persist in the dairy processing line. Improved knowledge on spore surface characteristics and a better understanding of B. cereus biofilm formation, through comparison of worldwide gathered data, may help develop efficient strategies for their control.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

ESEM imaging was performed at the University of Tlemcen, Algeria. Pr. Tabti Boufeldja is gratefully acknowledged for the facilities offered during the microscopic analysis and Cherifa Belabassi for assistance in the ESEM observations.

REFERENCES


Phylogenetic analysis of *Campylobacter jejuni* from human and birds sources in Iraq

Huda Abdal-Hadei Ali Al-Nasrawi

College of Veterinary Medicine, University of Al-Qadissiya, Iraq.

Received 19 February, 2016; Accepted 8 April, 2016

The present study was designed for a phylogenetic tree analysis of *Campylobacter* species as molecular method for genetic identification of *Campylobacter jejuni* from human and birds sources and birds and amplified by polymerase chain reaction assay using specific primers for 16S rRNA gene of *Campylobacter jejuni* (GenBank: EF136575.1). In this study, the multiple sequence alignment analysis and neighbor joining phylogenetic tree analysis was performed by using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in MEGA 6.0 version that analyzes 827 bp for ribosomal 16S rRNA gene. *C. jejuni* was detected in 40% (16/40) of stool samples collected from patients suffering from gastroenteritis, while detection rates of *C. jejuni* were 15% (3 /20) and 10% (2/20) of fecal samples of domestic chicken and pigeon respectively by PCR assay. The phylogenetic analysis results revealed that all local isolates of *Campylobacter* spp. were closed related to NCBI-Blast *C. jejuni* strain No.Y19244.1, whereas other NCBI-Blast *Campylobacter* spp. were out of tree and more different to ten *Campylobacter* spp. Iraq isolates and also found the relationships between the local isolates of *Campylobacter* spp. (Human, Domestic chicken, and Pigeon). This study represents the first report on the use of molecular phylogeny to *Campylobacter* spp. obtained in Iraq and confirmed the zoonotic potential of *C. jejuni*.

**Key words:** Phylogenetic tree, *Campylobacter* species, 16S ribosomal gene, human, birds, Iraq.

INTRODUCTION

Campylobacteriosis is a common zoonotic disease that affect human and cause gastrointestinal disturbances (Barakat et al., 2013). *Campylobacter jejuni* is responsible for 90% of *Campylobacter* species human infections and they occur in sporadic way (Schielke et al., 2014). *Campylobacter* is one of the most frequently occurring bacterial agents of gastroenteritis in human (WHO, 2012). Most bird flocks are colonized within several days and still so until slaughter. The handling and ingesting of contaminated meat with *Campylobacter*, especially poultry meat is considered an important source of food-borne gastroenteritis in human (Hermans et al., 2011). Today, attention has turned to nucleic acid technology; the polymerase chain reaction (PCR) and related techniques are rapid, specific and sensitive as compared to other tests used in detection of...
Campylobacter spp. (Englen and Kelley, 2000). C. jejuni is the Campylobacter spp. predominantly found in infected humans and colonized broilers. Sequence analysis of the 16S rRNA gene is very useful for identification of bacteria to genus and species level (Hansson et al., 2008). The potential application of the 16S rRNA gene for determining phylogenetic relationships among all living organisms had attracted much interest and would play a major role in extensive rearrangement of Campylobacter taxonomy (Woese, 1987). Phylogenetic analysis may be used as a molecular tool in future studies in the surveillance of Campylobacter-like organisms (Nayak et al., 2014). C. jejuni is isolated from stool samples of diarrheic children and confirmed phenotypically on the basis of biochemical tests in many provinces in Iraq (Salih and Al-Saad, 1994; Mohammad et al., 2004; Al-Ani et al., 2008). C. jejuni is identified by conventional PCR assay in human and domestic chicken in Al-Qadissiya province, Iraq (Al-Hisnaway, 2008). Abd (2014) proved that the detection rate of C. jejuni in human was 55.2% by Real-Time PCR Assay in Al-Muthanaa province, Iraq. The present study aimed at examining and analyzing the partial 16S ribosomal RNA gene sequence for construction of phylogenetic trees analysis of Campylobacter spp. Iraq isolates from infected humans, domestic chicken and pigeons in comparison to those of other NCBI-Blast Campylobacter spp.

MATERIALS AND METHODS

Samples collections

Human stool samples

A total of 40 stool samples of patients suffering from enteritis with ages ranging from 1 to 50 years were collected from general hospital in Al-Qadissiya province, Iraq during a period 6 months from October 2014 to March 2015 and after clinician consultation (included diarrhea, symptoms comprising vomiting, abdominal pain, fever) and microscopically examination in the hospitals where many samples contain motile bacteria, pus and few contain mucous and blood.

Bird samples

Fresh fecal samples were collected randomly from 20 flocks of domestic chicken from different farms, as well as 20 fecal samples of pigeon were collected from the same farms in Al-Qadissiya province, Iraq. The samples were collected during a period 6 months from October 2014 to March 2015.

All samples were placed in test tube containing 3 ml of peptone water in sterile condition and were immediately transported to the laboratory during 3 to 6 h in a cooler with ice packs. All the samples were frozen at -20°C for DNA extraction.

Genomic DNA extraction

Genomic DNA was extracted from stool samples by using AccuPrep® Stool DNA Extraction Kit, Bioneer, Korea. The extraction was done according to company instructions. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, store in -20°C in refrigerator until perform PCR.

Polymerase chain reaction (PCR)

PCR assay was carried out by using specific primer which was designed in this study from highly conserved regions of 16S ribosomal of C. jejuni (GenBank: EF136575.1). 16S rRNA forward primer (CGCACGGGTGAGTAAGGTAT) and 16SrRNA reverse primer (TAACACATGCTTCCACCGT) were provided by Bioneer company, Korea and using DNA C. jejuni as positive control and it was provided by Genekam, Germany. PCR master mix was prepared by using AccuPower® PCR PreMix kit Bioneer, Korea. The PCR premix tube contains freeze-dried pellet of Taq DNA polymerase 1U, dNTPs 250 µM, Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, MgCl₂ 1.5 mM, stabilizer, and tracking dye and the PCR master mix reaction was prepared according to kit instructions in 20 µl total volume by adding 5 µl of purified genomic DNA and 1.5 µl of 10 pmole of forward primer and 1.5 µl of 10 pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20 µl and briefly mixed by Exisip vortex centrifuge (Bioneer, Korea). The reaction was performed in a thermocycler (Mygene Bioneer, Korea) by set up following thermocycler conditions; initial denaturation temperature of 95°C for 5 min; followed by 30 cycles at denaturation 95°C for 30 s, annealing 58°C for 1 min, and extension 72°C for 1 min and then final extension at 72°C for 10 min. The 827 bp PCR products were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under UV trans-illuminator.

DNA sequencing method

The 827 bp PCR product was purified from agarose gel by using EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada. The purified 16S rRNA gene PCR product samples were sent to Bioneer Company in Korea to perform the DNA sequencing using 16SrRNA forward primer by AB DNA sequencing system. DNA sequencing method was performed for confirmative Phylogenetic tree relationship analysis of Campylobacter spp. based on 16S ribosomal RNA gene by Phylogenetic tree analysis using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in MEGA 6.0 version.

RESULTS AND DISCUSSION

Identification of C. jejuni in human and birds by PCR assay

Campylobacter is considered as human pathogen despite of it commensal organisms in domestic poultry and livestock. The present study describes a molecular method for detection C. jejuni from human and bird sources by using specific primer of 16S ribosomal of C. jejuni (Figure 1). Polymerase chain reaction (PCR) analysis using Campylobacter genus-specific partial 16S rRNA primers revealed the presence of Campylobacter spp. DNA in the faces (Turowski et al., 2014), where conventional PCR is rapid as nearly 2 times and sensitive method to determine Campylobacter spp. in comparison with culturing and this enhance its application.
Figure 1. Agarose gel electrophoresis image that shown the PCR product of 16S rRNA gene that using in detection of Campylobacter jejuni. Where M: Marker (1500-100bp), lane (1-3) domestic chicken, lane (4-5) pigeon, (6-10) Human were positive Campylobacter jejuni isolates and lane (PC) DNA Campylobacter genus positive control (Genekam, Germany) at 827bp PCR product size.

Table 1. The detection rates of Campylobacter jejuni in human and birds.

<table>
<thead>
<tr>
<th>Host</th>
<th>No. of samples</th>
<th>Campylobacter jejuni positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>40</td>
<td>16</td>
<td>40</td>
</tr>
<tr>
<td>Chicken</td>
<td>20</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Pigeon</td>
<td>20</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

as timesaving method of Campylobacter spp. by using 16SrRNA gene primer (Stoyanchev, 2004). Zhang et al. (2013) proved that PCR assay was sensitive (100%) in comparison with (49%) sensitivity of direct bacterial culture.

In present study, detection rate of C. jejuni in human was 16 (40%) out of (40) stool samples collected from infected patients which suffered from diarrhea and some of them suffered other symptoms such as fever, colic and vomiting. This result shows that detection rate of C. jejuni in human was relatively low, compared with the results of other studies reported in Iraq describing Campylobacter in human, which reported that the prevalence of campylobacteriosis was 55.2 and 66.7% (Abd, 2014; Al-Amri et al., 2007), while this result was higher than that record by Al-Hisnaway (2008) who found C. jejuni in 33.3% of stool samples of human by PCR assay in Al-Qadissiya province in Iraq. The different detection rate of the present study in comparison with other studies may influence many factors such as age, season, geography and immune state of human.

In this study, 3/20 (15%) fecal samples of domestic chicken were identified as C. jejuni by using specific primers of 16S ribosomal of C. jejuni by PCR assay and this result agree with Al-Hisnaway (2008) who detected C. jejuni with 17.6% from chicken fecal samples by conventional PCR assay, where C. jejuni has been reported to be the most frequent species recovered from poultry and poultry carcasses (Jorgensen et al., 2002).

The occurrence of C. jejuni in pigeon feces has been studied in several countries worldwide. In the present study, the detection rate of C. jejuni in pigeons fecal samples was 10% (2/20) (Table 1), this result was lower than that record by Casanovas et al. (1995) who found Campylobacter spp. in 26.2% of fecal pigeon samples and all of Campylobacter species isolated from pigeon fecal samples was C. jejuni (100%).

Sequencing analysis of 16S rRNA genes of Campylobacter spp. Iraqi isolates

The partial sequences for 16S ribosomal RNA genes of ten Iraq isolates Campylobacter spp. can be found under the accession numbers at NCBI-Gen Bank submission and they are shown in Table 2. Sequence analysis of ten samples positive for Campylobacter spp. was performed to confirm the PCR results in this study, the DNA sequencing analysis of 16S rRNA gene 827 bp PCR product by multiple sequence alignment Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in MEGA 6.0 version showed specific detection of C. jejuni. These studies agreed with Dewhirst et al. (2005) who identified representing either C. jejuni or Campylobacter coli by 16S rRNA sequence analysis.

The nucleotide sequences of the 16S rRNA genes of ten Campylobacter spp. Iraq local isolates of human and birds were determined and compared with 16S rRNA
Table 2. Gen bank accession numbers of 16S ribosomal RNA gene, partial sequence for Campylobacter spp. Iraq isolates from Human and bird sources.

<table>
<thead>
<tr>
<th>Campylobacter spp.</th>
<th>Sources/sample</th>
<th>Gen bank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQDC-1</td>
<td>Domestic chicken/Feces</td>
<td>KR133485.1</td>
</tr>
<tr>
<td>IQDC-2</td>
<td>Domestic chicken/Feces</td>
<td>KR133486.1</td>
</tr>
<tr>
<td>IQDC-3</td>
<td>Domestic chicken/Feces</td>
<td>KR133487.1</td>
</tr>
<tr>
<td>IQP-1</td>
<td>Pigeons/Feces</td>
<td>KR133488.1</td>
</tr>
<tr>
<td>IQP-2</td>
<td>Pigeons/Feces</td>
<td>KR133489.1</td>
</tr>
<tr>
<td>IQH-1</td>
<td>Human/Stool</td>
<td>KR133490.1</td>
</tr>
<tr>
<td>IQH-2</td>
<td>Human/Stool</td>
<td>KR133491.1</td>
</tr>
<tr>
<td>IQH-3</td>
<td>Human/Stool</td>
<td>KR133492.1</td>
</tr>
<tr>
<td>IQH-4</td>
<td>Human/Stool</td>
<td>KR133493.1</td>
</tr>
<tr>
<td>IQH-5</td>
<td>Human/Stool</td>
<td>KR133494.1</td>
</tr>
</tbody>
</table>

Table 3. The sequence identity for Campylobacter spp. Iraq isolates from human.

<table>
<thead>
<tr>
<th>Campylobacter spp. strains</th>
<th>Accession number</th>
<th>Campylobacter spp. Iraq isolates of human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IQ.H-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Score</td>
</tr>
<tr>
<td>C. Jejuni</td>
<td>Y19244.1</td>
<td>1495</td>
</tr>
<tr>
<td>C. faecalis</td>
<td>AJ.276874.1</td>
<td>1168</td>
</tr>
<tr>
<td>C. fetus</td>
<td>AJ.306569.1</td>
<td>1280</td>
</tr>
<tr>
<td>C. subantarctic</td>
<td>AM. 933373.1</td>
<td>1478</td>
</tr>
<tr>
<td>C. subantarctic</td>
<td>AM. 933374.1</td>
<td>1478</td>
</tr>
<tr>
<td>C. volucis</td>
<td>FM.883695.1</td>
<td>1439</td>
</tr>
<tr>
<td>C. hominis</td>
<td>AJ.251584.1</td>
<td>1114</td>
</tr>
<tr>
<td>C. coli</td>
<td>AM.042699.1</td>
<td>1210</td>
</tr>
</tbody>
</table>

The results showed that the sequence identity was 99% between ten Campylobacter spp. Iraq local isolates and C. Jejuni (Y19244.1), Campylobacter subantarctic (AM. 933373.1), C. subantarctic (AM. 933374.1) and C. coli (AM.042699.1) (Tables 3 and 4). The bacteria with relatively small genomes, such as C. jejuni may undergo genetic variation to increase their potential to adapt to new environments; such genotypic variation could result in phenotypic changes. These variations are probably important in the transmission route from broiler to man, where Campylobacter spp. must survive several hostile environments (Hansson et al., 2008).

Phylogenetic analysis

Phylogenetic tree analysis based on the clone 16S rRNA gene, partial sequence used for confirmative detection of Campylobacter spp. Iraq
isolates that included this study where phylogenetic analysis of 16S rRNA gene sequences has become the primary method for determining prokaryotic phylogeny. Therefore, the validity of 16S rRNA gene based phylogenetic analyses is of fundamental importance for prokaryotic systematics (Dewhirst et al., 2005). However, studies have suggested that multiple strains should be investigated to evaluate the degree of sequence diversity within and between species (Clayton et al., 1995). In the present study, the phylogenetic tree was constructed based on the ten *Campylobacter* spp. Iraq isolates included (n=5) human, (n=3) chicken and (n=2) pigeons and nine strains of NCBI-Blast *Campylobacter* spp. The ten *Campylobacter* spp. Iraq isolates showed close relationship with NCBI-Blast *C. jejuni* (Y19244.1) compared to other strains of NCBI-Blast *Campylobacter* spp. (Figure 2). These results agreed with Weis et al. (2014) who used phylogenetic analyses of 16S rRNA sequence data to distinguish *C. jejuni* from other species and to map strains found in crows with strains previously isolated from humans, livestock, and poultry. Nayak et al. (2014) referred to phylogenetic analysis providing a rapid, accurate and effective method for identification of species within the *Campylobacter*.

### Host relationship analysis of *Campylobacter* spp. Iraq strains

In present study, we have investigated putative specificity of the host using phylogenetic analysis of genetically closely related *Campylobacter* spp. from different sources where recent studies have suggested a potential role for birds in zoonotic transmission of *Campylobacter* spp., the leading cause of gastroenteritis in humans worldwide (Petersen et al., 2001; Broman et al., 2004; Weis et al., 2014). The results showed *Campylobacter* spp. IQH-2 (KR133491.1) and *Campylobacter* spp. IQH-3 (KR133492.1) isolates of human were more close relationship with *Campylobacter* spp. IQP-1 (KR133488.1) and *Campylobacter* spp. IQP-2 (KR133489.1) isolates of pigeons, as well as with *Campylobacter* spp. IQDC-1 (KR133485.1) and *Campylobacter* spp. IQDC-3 (KR133487.1) isolates of domestic chickens. *Campylobacter* spp. IQH-1 (KR133490.1), *Campylobacter* spp. IQH-4 (KR133493.1) and *Campylobacter* spp. IQH-5 (KR133494.1) of human were close related with *Campylobacter* spp. IQDC-2 (KR133486.1) of domestic chicken (Figure 3). These results agreed with Schouls et al. (2003) who record about 75% of the human strains were found to be most closely related to the patterns of the other human strains, and the patterns of 20% of the human strains were more similar to the patterns of the strains isolated from poultry.

### Conclusion

This study suggested that phylogenetic tree analysis is based on 16S ribosomal RNA gene, partial sequence can be used for confirmative detection of *Campylobacter* spp. isolates and determine the close relationship between *Campylobacter* spp. isolated from human, domestic chicken and pigeon. These results highlighted the importance of domestic chicken and pigeon as a potential source of human

---

**Table 4.** The sequence identity for *Campylobacter* spp. Iraq isolates from domestic chicken and pigeon.

<table>
<thead>
<tr>
<th><em>Campylobacter</em> spp. strains</th>
<th>Accession number</th>
<th><em>Campylobacter</em> spp. Iraq isolates of birds</th>
<th>Score</th>
<th>Identify (%)</th>
<th>Score</th>
<th>Identify (%)</th>
<th>Score</th>
<th>Identify (%)</th>
<th>Score</th>
<th>Identify (%)</th>
<th>Score</th>
<th>Identify (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>Y19244.1</td>
<td>IQ DC -1</td>
<td>1509</td>
<td>99</td>
<td>1495</td>
<td>99</td>
<td>1506</td>
<td>99</td>
<td>1511</td>
<td>99</td>
<td>1500</td>
<td>99</td>
</tr>
<tr>
<td><em>C. faecalis</em></td>
<td>AJ.276874.1</td>
<td>IQ DC -2</td>
<td>1158</td>
<td>92</td>
<td>1168</td>
<td>92</td>
<td>1218</td>
<td>93</td>
<td>1206</td>
<td>93</td>
<td>1157</td>
<td>92</td>
</tr>
<tr>
<td><em>C. fetus</em></td>
<td>AJ.306569.1</td>
<td>IQ DC -3</td>
<td>1168</td>
<td>95</td>
<td>1280</td>
<td>95</td>
<td>1303</td>
<td>95</td>
<td>1286</td>
<td>95</td>
<td>1264</td>
<td>94</td>
</tr>
<tr>
<td><em>C. subantarctic</em></td>
<td>AM. 933373.1</td>
<td></td>
<td>1515</td>
<td>99</td>
<td>1478</td>
<td>99</td>
<td>1489</td>
<td>99</td>
<td>1495</td>
<td>99</td>
<td>1483</td>
<td>99</td>
</tr>
<tr>
<td><em>C. subantarctic</em></td>
<td>AM. 933374.1</td>
<td></td>
<td>1515</td>
<td>99</td>
<td>1478</td>
<td>99</td>
<td>1489</td>
<td>99</td>
<td>1495</td>
<td>99</td>
<td>1483</td>
<td>99</td>
</tr>
<tr>
<td><em>C. volucis</em></td>
<td>FM.883695.1</td>
<td></td>
<td>1482</td>
<td>99</td>
<td>1439</td>
<td>98</td>
<td>1450</td>
<td>98</td>
<td>1456</td>
<td>98</td>
<td>1445</td>
<td>98</td>
</tr>
<tr>
<td><em>C. hominis</em></td>
<td>AJ.251584.1</td>
<td></td>
<td>1090</td>
<td>91</td>
<td>1114</td>
<td>91</td>
<td>1168</td>
<td>92</td>
<td>1162</td>
<td>92</td>
<td>1109</td>
<td>91</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>AM.042699.1</td>
<td></td>
<td>1003</td>
<td>99</td>
<td>1210</td>
<td>99</td>
<td>1522</td>
<td>99</td>
<td>1506</td>
<td>99</td>
<td>1194</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure 2. The phylogenetic tree analysis of *Campylobacter* spp. Iraq isolates based on partial 16SrRNA gene sequences using unweight pair group method with arithmetic mean (UPGMA tree) in (MEGA 6.0 version).

Figure 3. Phylogenetic tree analysis of *Campylobacter* spp. Iraq isolates based on the partial 16S ribosomal RNA gene sequence that used for host relationship analysis. The phylogenetic tree was constructed using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).
Campylobacter gastroenteritis.

Conflict of Interests

The author has not declared any conflict of interests.

REFERENCES

Molecular characterization and genetic diversity of Tobacco streak virus infecting soybean (Glycine max L.)

Rajamanickam, S.1*, Ganesamurthy, K.2 and Karthikeyan, G.1

1Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore - 641 003, India.
2Department of Oil Seeds, Tamil Nadu Agricultural University, Coimbatore - 641 003, India.

Received 22 November, 2015; Accepted 5 May, 2016

Soybean (Glycine max L.), the most popular oil seed crop, grown in the experimental fields of Tamil Nadu Agricultural University and adjoining areas exhibited symptoms of necrosis and stunting. The symptoms were similar to bud blight of soybean caused by the Tobacco streak virus (TSV) and the disease is most prevalent throughout the country. To study the nature of infection, sap inoculation of the soybean strain induced local as well systemic infection on cowpea plants cv. C 152 and resulted in the production of circular necrotic lesions and death of plants. The samples were also serologically positive in DAC-ELISA and it has also yielded a protein band of approximately 29 kDa corresponding to coat protein of TSV in Western blot assay. For the characterization of virus, RT-PCR was carried out with a newly designed coat protein gene specific primer, which resulted in amplification of the expected 929 bp size. Sequence analysis of the CP gene had nucleotide similarity of 80.6 to 99.3% with known isolates of TSV. The multiple sequence alignment revealed that CP gene showed single unique variation and some of deletion and addition mutation was found in nucleotide and amino acid sequences against the isolates of other soybean Brazil and USA isolates and produced single unique variation at position 344 where adenine was substituted with guanine. There was no deletion and addition between nucleotide sequences in the group of Indian isolates, further confirms the placement of the soybean isolate of TSV in a single subgroup.

Key words: Soybean, Tobacco streak virus, coat protein gene, diversity analysis.

INTRODUCTION

Soybean (Glycine max L.) is known as ‘Golden bean’ and is a native of North China, Asia belongs to family Fabaceae. It is a versatile and fascinating crop with innumerable possibilities of not only improving agriculture but also supporting industries. It is a rich source of lysine (6.4%) in addition to other essential amino acids, vitamins.

*Corresponding author. E-mail: rajamanickam.path@gmail.com. Tel: +91 9786293446.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
and minerals. Like other economically important crops soybean is susceptible to different diseases caused by viruses. Yellow mosaic virus (YMV), Soybean mosaic virus (SMV) and Groundnut bud necrosis virus (GBNV) are the major viral diseases of soybean in India, which causes considerable reduction in yield up to 80% under severe conditions (Thakur et al., 1998; Rebadeaux et al., 2004; Lal et al., 2005). In addition to above, a recent outbreak of soybean bud blight caused by Tobacco streak virus (TSV) being reported to be an emerging virus and considered as a major constraint in soybean (Arun Kumar et al., 2008). This may be an indication of the occurrence of a virus disease which also reduces yield and in some varieties 100% yield losses have been observed. The characteristic symptoms caused by the TSV in soybean under field conditions are stunting of plant and necrotic patches on the leaves of growing tip of plants. Johnson (1936) reported infection of TSV in tobacco and it is a member of the genus Ilarvirus under the family Bromoviridae. In India, the TSV infects several other crops in addition to soybean (Jain et al., 2005; Sivaprasad et al., 2010; Bhaskara Reddy et al., 2012). TSV can be transmitted mechanically, but the transmission of TSV commonly occurs through different species of thrips viz., Megalurothrips usitatus, Frankliniella schultzei, Scirtothrips dorsalis, Thrips palmi and Thrips tabaci under field conditions (Jagtap et al., 2012). Alternative host plants have been suspected to harbour TSV which have contributed in its transmission. The virus causes asymptomatic infections in several common weed species, including Parthenium hysterophorus, Ageratum conyzoides and Corchorus trilocularis, whose pollen is a major source of TSV and these plants, also harbour thrips (Prasada Rao et al., 2003; Shukla et al., 2005). Though the occurrence of TSV has been reported from many hosts in India, only limited reports are available on the biological and molecular characterization of these isolates and their exact identification remains unaddressed in soybean. In this study, we report the natural occurrence of TSV on soybean and its molecular properties and phylogenetic relationship with other TSV isolates.

MATERIALS AND METHODS

Virus isolates and maintenance of inoculum

Soybean (Glycine max L.) plants showing characteristic symptoms of TSV were collected from naturally infected field at Coimbatore (Tamil Nadu) and used as inoculum of virus. The infected plants were identified by the presence of veinal necrosis on the growing leaves, necrotic spots on the leaves and stunting of plants (Figure 1a). The TSV infected samples collected from field were subjected to direct antigen coating ELISA (DAC-ELISA) as per the procedure described by Hobbs et al. (1987) with the polyclonal antisera specific to TSV (kindly provided by the ICRISAT, Hyderabad). The cowpea plants cv. C 152 was used for propagating the virus. The cowpea C 152 plants were raised in the glasshouse under insect proof conditions. The virus extract was prepared by macerating TSV infected leaf samples with 0.1 M sodium phosphate buffer pH 7.0 using ice tray and inoculated mechanically in cowpea cv. C125 cotyledonary leaves of six day old plants previously dusted with 600 mesh carborundum powder. The inoculated plants were kept under observation for 4 to 5 days for the expression of symptoms (Subramanian and Narayanasamy, 1973).

Purification of Tobacco streak virus

Virus infected leaves from cowpea C 152 were ground in 0.01 M potassium phosphate buffer, pH 8.0 (2:1 w/v) containing 1% 2-mercaptoethanol. The slurry was filtered in a double cheese cloth, clarified with chloroform (1/2 vol) and stirred for 20 min inc 4°C. The extract was centrifuged at 10,000 g for 10 min at 4°C and the supernatant was mixed with 6% PEG 8,000 at 4°C for 2 h. After low speed centrifugation (10,000 g for 10 min) the pellet was dissolved overnight at 4°C. After another low speed centrifugation the supernatant was centrifuged at 180,000 g for 2 h in a swinging bucket rotor using 25% sucrose frozen gradient (Baxter-Gabbard, 1972; Davis and Pearson, 1978). The virus was fractionated using an ISCO density gradient fractionator and UV analyzer. Fractions were diluted in 0.01 M potassium phosphate buffer, pH 8.0 and centrifuged at 100,000 g for 90 min. The pellet was dissolved and the condition for the expression of symptoms was observed.

Western blot analysis

A mixture of an equal amount of virus preparation and dissociation buffer (0.125 M Tris-HCl, pH 6.7, 3% SDS, 20% glycerol and 10% 2-mercaptoethanol) was boiled for 5 min. Electrophoresis was carried out in 12.5% (separating) polyacrylamide gels according to Laemmli’s method (1970). The pre-stained molecular weight markers (Fermentas Life Sciences) along with the samples were loaded (20 μl) into the slot of the gel. The protein was electrophoretically transferred to nitrocellulose blotting membrane (NC, Sartorius, 0.2 μm pore size) in Tris-glycine buffer containing 20% (v/v) methanol overnight at 25 V at 4°C. The membrane was washed and blocked for 1 h with 1% (w/v) hemoglobin in phosphate-buffered saline (PBS) and then separately incubated for 2 h with polyclonal antiserum specific to TSV (1:1,000) respectively in PBS-Tween (PBST). Non-specific antibodies were removed by 10 min washing (4 times) with PBST. Goat-anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) was used as the secondary antibody (1:3,000 in PBST). The NC membrane was incubated for 1.5 h at room temperature, excess antibody was removed and immunoreactive proteins were visualized after subjecting the NC membrane to the reaction mixture containing 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

RNA isolation and cDNA synthesis

The inoculum of TSV soybean isolate was maintained on cowpea cv. C152 and total RNA was extracted from 100 mg leaves of soybean using RNAeasy plant extraction kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturers' protocol and resuspended in 50 μl nuclease free water. For cDNA synthesis of TSV, 1 μg total isolated RNA (200 ng/μl) was annealed with 0.3 μM downstream primers (GKTSV CPR-5’TGCTCGCATGGTCATAGAC 3’) at 70°C for 10 min. To the
transcription mixture, various reaction components were added ([RNase inhibitor 1 µl (20 U); dNTPs 2 µl (10 mM); 4 µl 5X reverse transcriptase buffer containing Tris HCl 250 mM, pH 8.3 at 25°C, KCl 250 mM, MgCl₂ 20 mM, 1 µl DTT 50 mM]). The reaction mixture was incubated at 37°C for 10 min, 40 U M-MuLV reverse transcriptase was added and the mixture re-incubated at 37°C for 60 min. The reaction was stopped by heating the mixture at 70°C for 10 min.

Reverse transcription polymerase chain reaction (RT-PCR)

TSV:cDNA product (5 µl) was added to 50 µl of PCR reaction mixture containing 0.20 mM each of dNTPs, 0.25 µM of each primer (GKTSV CPF: 5’AGATAAGTCGCTTCTCGGAC 3’ and GKTSV CPR: 5’TGCTCGCATGGGTCATAGAC 3’), 5 µl 10X Taq polymerase buffer, 2.0 mM MgCl₂ and 2 U Taq DNA polymerase. The RT-PCR was performed in Eppendorf Mastercycler Gradient ES with the following thermal programme: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 1 min and final extension of 72°C for 10 min.

Molecular cloning and sequencing

The amplicon of coat protein and replicase genes were purified using QIAGEN gel extraction kit (Qiagen Inc., Chatsworth, CA, USA) and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer’s instructions and transformed into Escherichia coli DH5α by following standard molecular biology procedures (Sambrook et al., 1989). Plasmid DNA was isolated from the potential recombinant clones using Wizard plus DNA purification kit (Promega, Madison, WI) according to the manufacturer’s protocol and the positive clones were identified by restriction digestion analysis using EcoRI enzyme. The three independent clones were sequenced at Chromos Biotech Pvt. Ltd., Bangalore from both orientations for each fragment separately. The sequences were then edited using the BIOEDIT Software (Hall, 1999). Sequence similarity search of the GenBank database was done using the Basic Local Alignment Search Tool (BLAST) program.

Sequence diversity and phylogenetic analysis

The amino acid sequences of the TSV coat protein gene was translated from the consensus nucleotide sequences using the EMBOSS Transeq program (Rice et al., 2000). Both the nucleotide and amino acid sequences were then aligned with selected sequences of TSV strains using the CLUSTAL W program (Larkin et al., 2007). Phylogenetic analysis was done on MEGA 5.1 (Tamura et al., 2011) and trees were created using the neighbour-joining method (Saitou and Nei, 1987). The robustness of the trees was determined by bootstrap using 1,000 replicates. Prunus ringspot necrotic virus (PRNV) was used as a reference out group member of the genus Ilarvirus for rooting the phylogenetic tree.

RESULTS

Isolation of virus and serodiagnosis

The soybean plant samples showing characteristic symptoms of TSV were collected and inoculated separately on cowpea cv. C152 plants through mechanical sap inoculation. The assay host cowpea cv. C152 expressed distinct local lesions on three to four days after inoculation. The inoculated cowpea cotyledonary leaves developed necrotic lesions and then the systemic veinal necrosis occurred. The veinal necrosis resulted in severe stem necrosis and lead to the collapse of the entire inoculated plants (Figure 1b). The results of DAC-ELISA revealed that, the samples exhibited characteristic symptoms of TSV showed strong positive reaction with approximately five fold increase in absorbance values than the apparently healthy samples.

Western blot analysis

The molecular weight of coat protein was determined by
Comparing its mobility with the marker proteins on a 12% PAGE under denaturing conditions. The isolated virus particles revealed the presence of a single band corresponding to ~29 kDa coat protein subunit. Western blot immunoassay was performed by using 1:1000 dilution of primary antibody specific to TSV (ICRISAT, Hyderabad). A 1:1500 dilution of IgG conjugated to alkaline phosphatase (secondary antibody) produced a positive reaction with the ~29 kDa band obtained with SDS PAGE. The resulted band was of viral coat protein and confirming the virus isolate under study was TSV (Figure 2).

Cloning and sequencing

Total RNA extracted from cowpea samples infected with TSV inoculum from soybean was analyzed by RT-PCR with specific primers corresponding to coat protein gene. The result revealed the infected samples resulted in the amplification of 929 bp corresponding to CP gene (Figure 3). The amplified DNA fragment of coat protein gene was excised, cloned into pGEM-T easy vector and sequence determined. The gene sequence was edited using BIOEDIT software and obtained full length nucleotide sequence. The nucleotide sequence analysis using NCBI BLAST confirmed the association of TSV.

Coat protein gene sequence analysis

The amplified fragment corresponding to CP gene contains single ORF consist of 717 bases encoded the protein with 239 amino acids. The coat protein gene sequence of soybean strain of TSV was submitted in NCBI Genbank database (Accession No. KJ825822). The sequence (KJ825822) was compared with corresponding genes from known TSV isolates at the nucleotide and amino acid sequence levels. The sequence had 98.8 and 98.4%, nucleotide homology with other soybean TSV strains of Indian isolates (DQ518916 and DQ864457), 80.6 and 87.9 % nucleotide homology with other TSV strains from soybean of Brazil and USA respectively (AY354406 and FJ403377) (Table 1). Multiple nucleotide sequence alignment and phylogenetic analysis revealed very high homologies between the TSV strains and confirmed the formation of single subgroup (Figure 4). Multiple sequence alignment further revealed a near perfect homology between the nucleotide sequence of the soybean strain and the nucleotide sequences of other strains except for a single unique variation at position 344 where adenine was substituted with guanine. Also strain produced variation at the position 15 where cytosine was substituted with adenine (DQ518916 and DQ864457). Similarly strain had variation at position 52 where cytosine was substituted with thiamine, position 521 where thiamine was substituted with cytosine and position 536 where cytosine was substituted with thiamine with other Indian isolates (DQ518916 and DQ864457) (Figure 5). The results revealed that nucleotide sequences in the group, further confirms the placement of the soybean strain of TSV as a single subgroup. Analysis of the 239 deduced amino acid sequence of the 3’ end of the coat protein gene of RNA 3 revealed that the soybean strain of our TSV had 79.5 to 99.5% homology with other strains of the same virus (AY354406 and AY940154). The amino
Table 1. Nucleotide (nt) and amino acid (aa) identities of the coat protein gene of *Tobacco streak virus* (TSV) soybean strain (KJ825822) with corresponding sequences of selected isolates of TSV.

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Strain/host</th>
<th>Country</th>
<th>% identity</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ518916</td>
<td>Soybean</td>
<td>India</td>
<td>98.8</td>
<td>99.1</td>
</tr>
<tr>
<td>DQ864457</td>
<td>Soybean</td>
<td>India</td>
<td>98.4</td>
<td>97.4</td>
</tr>
<tr>
<td>EU368962</td>
<td>sunflower</td>
<td>India</td>
<td>98.8</td>
<td>98.7</td>
</tr>
<tr>
<td>DQ058079</td>
<td>Cowpea</td>
<td>India</td>
<td>98.8</td>
<td>99.1</td>
</tr>
<tr>
<td>FJ561304</td>
<td>Okra</td>
<td>India</td>
<td>99.1</td>
<td>98.7</td>
</tr>
<tr>
<td>JQ269831</td>
<td>Guar</td>
<td>India</td>
<td>98.8</td>
<td>99.1</td>
</tr>
<tr>
<td>FJ561301</td>
<td>Pumpkin</td>
<td>India</td>
<td>98.4</td>
<td>97.8</td>
</tr>
<tr>
<td>FJ655172</td>
<td>Gherkin</td>
<td>India</td>
<td>99.0</td>
<td>98.7</td>
</tr>
<tr>
<td>AY590139</td>
<td>Chilli</td>
<td>India</td>
<td>98.0</td>
<td>97.8</td>
</tr>
<tr>
<td>FJ608537</td>
<td>Watermelon</td>
<td>India</td>
<td>98.8</td>
<td>98.3</td>
</tr>
<tr>
<td>AF515823</td>
<td>Mungbean</td>
<td>India</td>
<td>98.7</td>
<td>98.7</td>
</tr>
<tr>
<td>FJ749259</td>
<td>Blackgram</td>
<td>India</td>
<td>98.4</td>
<td>97.4</td>
</tr>
<tr>
<td>HM131487</td>
<td>Greengram</td>
<td>India</td>
<td>98.8</td>
<td>98.7</td>
</tr>
<tr>
<td>AY940154</td>
<td>Cotton</td>
<td>India</td>
<td>99.3</td>
<td>99.5</td>
</tr>
<tr>
<td>FJ417083</td>
<td>Marigold</td>
<td>India</td>
<td>98.8</td>
<td>98.7</td>
</tr>
<tr>
<td>FJ355949</td>
<td>Groundnut</td>
<td>India</td>
<td>97.9</td>
<td>97.4</td>
</tr>
<tr>
<td>FJ655170</td>
<td>Squash</td>
<td>India</td>
<td>99.0</td>
<td>99.1</td>
</tr>
<tr>
<td>AY354406</td>
<td>Soybean</td>
<td>Brazil</td>
<td>80.6</td>
<td>79.5</td>
</tr>
<tr>
<td>FJ403377</td>
<td>Soybean</td>
<td>USA</td>
<td>87.9</td>
<td>90.0</td>
</tr>
<tr>
<td>AJ969095</td>
<td>Rose - PRNV</td>
<td>India</td>
<td>37.3</td>
<td>18.2</td>
</tr>
</tbody>
</table>

Figure 4. Neighbour-joining phylogenetic tree based on the nucleotide sequences of the coat protein gene of TSV (KJ825822) and *P. ringspot necrosis virus* is defined as an out-group.
Figure 5. Multiple sequence alignment of the 717 nucleotide sequences of the coat protein gene of the soybean strain of TSV and corresponding sequences of 19 selected strains of TSV. *P. ringspot necrosis virus* is defined as an out-group.

The amino acid sequence had 99.1 and 97.4% homology with other soybean TSV strains of Indian isolates (DQ518916 and DQ864457), 79.5 and 90.0% amino acid homology with other TSV strains from soybean of Brazil and USA.
disorder. P. ringspot necrosis virus is defined as an outgroup.

Respectively (AY354406 and FJ403377). The soybean strain had single unique amino acid variation at position 113 where Lysine substituted with arginine (Figure 6). Also amino acid sequence has deletion mutation from the position 43 and 44 against the TSV soybean strains from Brazil and USA respectively (AY354406 and FJ403377).

DISCUSSION

Charaterization of Tobacco streak virus infecting soybean (Glycine max L.) in Tamil Nadu provides knowledge of better understanding the genetic composition, variation caused by mutation and recombination and correct taxonomic position. TSV had wider host range and extending its host range day by day. Due to different environmental constraints on the evolution of new strains, it is important to study the phylogenetic relationship of the viruses locally. TSV on soybean showed symptoms viz., stunting and necrotic patches on leaves of growing tip of plants under field conditions. The soybean exhibiting typical symptoms of TSV were inoculated on cowpea cv. C 152 plants, resulted in production of typical necrotic lesions on inoculated primary leaves, systemic veinial necrosis and death of plants under glasshouse conditions. The circular necrotic lesions on cowpea are the characteristic symptoms of TSV by mechanical inoculation (Ramiah et al., 2001; Ladhalakshmi et al., 2006; Arun Kumar et al., 2008). Serological or immunological assays have been developed and successfully used for a number of years for the detection of plant viruses. TSV infected soybean collected from field were found to be positive for TSV specific polyclonal antibody. This type of results was supported by Bhaskara Reddy et al. (2012) who raised the polyclonal antibody against the TSV and showed positive reaction for sample collected from natural infection of Hibiscus cannabinus in DAC-ELISA. Prasad Rao et al. (2003) also proved the detection of a new strain SB-10 of TSV from potato through DAC-ELISA. In our study virus preparation has reacted with the antisem specific to TSV, which was produced approximately 29 kDa protein in western blot assay. This results was supported by Almeida et al. (2005) reported coat protein gene of TSV from infected soybean in Brazil had a molecular mass of 29.880 kDa. This suggested that necrosis disease of soybean was caused by the TSV.

PCR has been shown effective in rapid and sensitive detection of many plant viruses (Candresse et al., 1998). To detect TSV infection in soybean plants, RT-PCR technique was used to amplify CP gene with self-designed primers. Approximately 929 bp including UTR corresponding to CP gene was amplified using specific primer, while no such band was observed when total RNA extracted from healthy tissue. The products were cloned and its nucleotide sequences were determined. The CP gene of TSV isolate was compared with corresponding gene from known TSV isolates at the nucleotide and amino acid sequence levels. Phylogenetic analysis revealed soybean strain has very high homologies between the TSV strains of other crops and confirmed the formation of single subgroup (Figure 4). Multiple sequence alignment revealed a near perfect homology between the nucleotide sequence of the soybean strain and the nucleotide sequences of other strains. The CP gene showed single unique variation and some of deletion and addition mutation was found in
nucleotide sequences against the strains from soybean Brazil (AY354406) and USA (FJ403377) isolates. Rajamanickam and Karthikeyan (2014) characterized the CP gene of TSV okra stain and reported sequence had two unique variations at the position 15 where cytosine was substituted with adenine and it produced unique variation at the position 526 where cytosine was substituted with thiamine. There was no deletion and addition between nucleotide sequences in the group. Cornelissen et al. (1984) cloned and sequenced TSV RNA3 genome reveals that, complete sequence of 2,205 nucleotides of TSV RNA 3, confirming 140 bp 3’s-terminal residues. Two long open reading frames starting with a methionine codon are revealed by this sequence. Similarly, Bhat et al. (2002) conducted serology and characterization of coat protein studies for the sunflower Ilarivirus from India and they reported that it should be regarded as a strain of TSV belonging to subgroup I, designated as TSV-SF, which shared 90% amino acid sequence identity with TSV (strain WC). Almeida et al. (2005) amplified coat protein gene of TSV with a size of 717 nucleotides along with 287 nucleotides at 3’ untranslated region using RT-PCR and the results revealed that nucleotides and amino acids showed 96 to 98% similarity to other TSV isolates. They also reported TSV isolate causing soybean bud blight disease in Brazil was reported to be a distinct strain of TSV (TSV-BR), which shared 81.3 and 80.7% nucleotide sequence homology with the CP gene of TSV-WC and TSV-MB (mungbean isolate from India). In conclusion, the CP gene of TSV soybean strain revealed, single unique variation and some of deletion and addition mutation was found in nucleotide and amino acid sequences of CP gene of TSV. But, there were no amino acid changes except, three positions compared to other Indian isolates used in the study. Such studies would help in the development of strategies for the control of viral diseases.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge the Department of Science and Technology (SERB), Government of India, New Delhi for the financial support of the project and Tamil Nadu Agricultural University, Coimbatore - 641 003

REFERENCES


Bhat AI, Jain RK, Kumar A, Ramiah M, Varma A (2002). Serological and coat protein sequence studies suggest that necrosis disease on sunflower in India is caused by a strain of Tobacco streak ilarivirus. Arch. Virol. 147:651-658


Biofixation of CO$_2$ on a pilot scale: Scaling of the process for industrial application

Felipe Camerini, Michele da Rosa Andrade Zimmermann de Souza, Michele Greque de Morais, Bruna da Silva Vaz, Etiele Greque de Morais and Jorge Alberto Vieira Costa*

Laboratory of Biochemical Engineering, College of Chemical and Food Engineering, Federal University of Rio Grande, Rio Grande, RS, Brazil.

Received 23 June, 2015; Accepted 18 February, 2016

The use of *Spirulina* in CO$_2$ biofixation, aside from its contribution to reducing the greenhouse effect, enables the use of the biomass to obtain biocompounds. In this work, *Spirulina platensis* was used for CO$_2$ biofixation under different conditions of inorganic carbon. *S. platensis* was inoculated into 200 L bioreactors containing modified Zarrouk’s medium (concentration of the carbon was from 1.0 to 2.0 g.L$^{-1}$). CO$_2$ (12% v/v) was injected into the culture medium intermittently to maintain the inorganic carbon concentration and pH levels favorable for growth. The values of the maximum specific growth rate obtained for both conditions were the same (0.76 day$^{-1}$). However, the culture in which a concentration of 2.0 g.L$^{-1}$ of NaHCO$_3$ was maintained allowed higher concentrations of biomass (1.0 g.L$^{-1}$) and higher productivity (0.11 g.L$^{-1}$.d$^{-1}$) to be obtained as compared to the same parameters obtained in cultures containing 1.0 g.L$^{-1}$ of NaHCO$_3$.

Key words: Bioreactor, cyanobacteria, microalgae, *Spirulina*.

**INTRODUCTION**

The risk of irreversible effects on global climate caused by greenhouse gases has stimulated scientific research on reduced carbon dioxide emissions (Binaghi et al., 2003). Alternatives such as reforestation intensification, fertilizing the ocean with iron and fertilization using major or trace elements (Stewart and Hessami, 2005) are among the technologies studied with the aim of fixing atmospheric carbon in terrestrial or aquatic organisms.

The photoautotrophic growth of microalgae requires supply of CO$_2$ as source of carbon. At the same time, the supply of CO$_2$ helps to control the pH of the culture (Radmann et al., 2011). Chemical analyses of the biomass have shown that the microalgal biomass contains 40 to 50% of carbon, suggesting that approximately 1.83 ton of carbon dioxide are required to produce 1.0 ton of biomass (Ho et al., 2011).

Microalgal cultivation technologies have been studied in recent decades in the context of mitigating the emissions of greenhouse gases. The biological fixation of carbon dioxide by microalgae growing in environmental conditions is considered the best way to fix CO$_2$, because the algae’s use of solar energy is much higher than that of terrestrial plants, the maximum photosynthetic capacity of which only lasts for a short period (Rosa et al., 2011).

*Corresponding author. E-mail: jorgealbertovc@terra.com.br. Tel: (+55)(53) 3233-6908.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
Some of the most noteworthy microorganisms used in the studies of carbon dioxide biofixation are photoautotrophic cyanobacteria, such as *Spirulina platensis*, that fixates CO$_2$ at a faster rate than eukaryotic organisms. *S. platensis* is a filamentous cyanobacterium capable of forming colonies in tropical or sub-tropical shallow water containing high levels of carbonates and bicarbonates (Lourenço, 2006).

The biomass generated in the CO$_2$ fixation process has physicochemical characteristics that have a high potential for applicability and can be used in human food or animal feed, for extracting biocomposts (Morais et al., 2015) and obtaining biofuels (Pandey, 2014). *Spirulina* is the most studied microalga and has a proven ability to fix inorganic carbon. This microalga is distinguished for having a generally recognized as safe (GRAS) certificate granted by the Food and Drug Administration (FDA), which ensures its use in food and medicine. Its biomass has a high protein content (64-74%), polyunsaturated fatty acids, pigments and vitamins (Socclo, 2013).

The aim of this study was to use *S. platensis* in a process of carbon dioxide biofixation with different concentrations of inorganic carbon using an open raceway type bioreactor equipped with a carbon dioxide fuel injection system under environmental light conditions.

**MATERIALS AND METHODS**

**Microorganism and culture conditions**

This study used the microalgae *S. platensis* (*Arthospira*), as grown in modified Zarrour culture medium (Zarrour, 1966). The carbon source (NaHCO$_3$) was added in two concentrations (1.0 and 2.0 g.L$^{-1}$). The cultivations were carried out in 200 L open “raceway” type reactors at 30°C under natural lighting. The inoculum was pre-filtered and rinsed to remove NaHCO$_3$ originating from its propagation (carried out in standard Zarrour medium, 16.8 g.L$^{-1}$ NaHCO$_3$). CO$_2$ was injected into the medium at a rate of 10 L CO$_2$.h$^{-1}$ in the light phase (when the concentration of NaHCO$_3$ was 1.0 g.L$^{-1}$) and 20 L CO$_2$.h$^{-1}$ in the light phase (when the concentration of NaHCO$_3$ was 2.0 g.L$^{-1}$). The light phase was defined as the daily period between 8 and 17 h (9 h per day).

**Analytical determinations**

The biomass concentration was measured by reading the optical density at 670 nm in a spectrophotometer (Femto 700-Plus, Brazil), with a calibration curve that related optical density to the dry biomass weight; the pH was measured using a digital pH meter (QUIMIS Q400H, Brazil). Ambient and culture medium temperatures were measured with a mercury thermometer.

**Assessed kinetic parameters**

The maximum concentration of the *S. platensis* biomass ($X_{\text{max}}$, g.L$^{-1}$) was measured, and productivity ($P$, g.L$^{-1}$d$^{-1}$) was calculated using Equation 1 (Bailey and Ollis, 1986). The maximum yield ($P_{\text{max}}$) was defined as the highest productivity using Equation 1:

$$P = \frac{X_t - X_0}{t_t - t_0}$$

Where $X_t$ was the cell concentration (g.L$^{-1}$) at time t (d) and $X_0$ was the cell concentration (g.L$^{-1}$) at time to (d).

The maximum specific growth rate ($\mu_{\text{max}}$, d$^{-1}$) was calculated by the exponential regression of the logarithmic phase of the growth curve for each daily cycle (Bailey and Ollis, 1986).

**Calculation of the net efficiency of CO$_2$ biofixation**

The liquid efficiency of biofixation is the ratio between CO$_2$ fixed in the form of microalgal biomass and the CO$_2$ transferred to the culture medium, which is given by CO$_2$ supplied to the system, considering the transfer efficiency of the injection conditions employed. The net efficiency of biofixation is determined by Equation 2:

$$\phi = \left( \frac{Q \cdot Y_{\text{CO}_2} \cdot \frac{1}{22.4} \cdot M_{\text{CO}_2} \cdot t_i}{M_a \cdot V \cdot C_{\text{biomass}}} \right) \cdot \Phi$$

where $X_t$ (g.L$^{-1}$) is the biomass concentration at time t (d), $X_0$ (g.L$^{-1}$) is the biomass concentration at time to, CX is the fraction of carbon determined in the microalgal biomass, V (L) is the volume of medium in the photobioreactor, $M_{\text{CO}_2}$ (g.mol$^{-1}$) and $M_a$ are the molar masses of carbon dioxide and carbon present in the biomass, respectively, Q is the injected gas flow, Y fraction CO$_2$ in the gas injected and $\Phi$ is the transfer efficiency. The percentage of carbon present in biomass (CX) is considered to be 50%, according to Benemann (1997).

**Calculating the overall efficiency of the CO$_2$ biofixation**

The overall efficiency of biofixation is the ratio between CO$_2$ fixed in the form of microalgal biomass and the total CO$_2$ supplied to the system. The overall efficiency of biofixation was determined using Equation 3.

$$\left( \frac{X_t - X_0} {M_{\text{bio}} \cdot M_{\text{biomass}}} \right) \cdot \frac{1}{22.4} \cdot M_{\text{CO}_2} \cdot t_i$$

**RESULTS AND DISCUSSION**

Figure 1 shows the growth of the cyanobacterium *S. platensis* for the two conditions assessed (1 and 2 g.L$^{-1}$ of NaHCO$_3$). In the experiment, the concentration of NaHCO$_3$ of 2.0 g.L$^{-1}$ resulted in the greatest cell concentrations (1.0 g.L$^{-1}$). In cultures carried out with 1.0 g.L$^{-1}$ of NaHCO$_3$, the production rates (via photosynthesis) and consumption of biomasses (via respiration) were equal after the sixth day. This phenomenon is known as the compensation point and occurs when the photosynthetic activity carried out during
the day equals the heterotrophic activity that occurs during the night. In this assay, the compensation point is associated with the limitation of growth due to lack of nutrients (in this case, the carbon).

When the NaHCO$_3$ concentration was 2.0 g.L$^{-1}$, the rate grew continuously throughout the cultivation period, and there was no compensation point in this condition. The maximum cell concentration obtained in these experiments was twice as high as that obtained when 1.0 g.L$^{-1}$ of NaHCO$_3$ was added, showing that there is a direct relationship between the concentration of dissolved inorganic carbon with the cellular concentration maintained in the cultures (Table 1).

The bicarbonate concentration in the medium determines the rate of formation of carbon dioxide ($HCO_3^{} \leftrightarrow k CO_2 + OH^-$), where k is the kinetic constant of the reaction. The growth rate and cell concentration determine the culture’s carbon dioxide demand; thus, the bicarbonate concentration maintained in the medium depends on these parameters. Table 1 presents the kinetic parameters obtained during the experiments. The same maximum specific growth was obtained for both experiments (0.76 day$^{-1}$), demonstrating that growth is not impaired under any of the conditions. However, the parameter that must be followed in this case is the cell concentration to be maintained, as this metric will determine whether the carbon is excessive (which would cause the loss of carbon into the atmosphere) or in short supply (thus limiting the growth).

Figure 1 shows that, during the light phase (day time), growth occurs at higher rates than those observed when evaluated over several days of cultivation. This is due to the consumption of the biomass resulting from the activation of the heterotrophic metabolism during the dark phase (night). Under environmental conditions, the cultures are not illuminated during the night. Vonshak and Richmond (1988) reported that the loss of biomass in *Spirulina* cultures caused by respiration during the night can represent 35% of the biomass produced during the day. Figure 2 shows the growth curve determined over the light phase on the fourth day with the medium containing 2.0 g.L$^{-1}$ of NaHCO$_3$. One alternative to significantly increase growth rates would be to keep the cultures illuminated at night, thereby avoiding the consumption of biomass. Another option to avoid the nocturnal loss of carbon is the use of organic carbon sources, such as glucose or acetate, during the night (Ogbonna and Tanaka, 1996). Such measures would ensure biomass growth, even when the heterotrophic

### Table 1. Kinetic parameters obtained.

<table>
<thead>
<tr>
<th>NaHCO$_3$ (g.L$^{-1}$)</th>
<th>$X_{max}$ (g.L$^{-1}$)</th>
<th>$P_{max}$ (g.L$^{-1}$.d$^{-1}$)</th>
<th>$\mu_{max}$ (d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.52</td>
<td>0.07</td>
<td>0.76</td>
</tr>
<tr>
<td>2.0</td>
<td>1.00</td>
<td>0.11</td>
<td>0.76</td>
</tr>
</tbody>
</table>
Camerini et al.          771

Figure 2. Growth of *S. platensis* during the light phase.

Figure 3. Productivity of cultures containing NaHCO$_3$ 1.0 g.L$^{-1}$ (■) or NaHCO$_3$ 2.0 g.L$^{-1}$ (◊) (Ogbonna and Tanaka, 1996). Such measures would ensure biomass growth, even when the heterotrophic metabolism is activated, although the consumption of carbon dioxide would be zero during the entire period in which the microalga stopped photosynthesizing (Andrade and Costa, 2007).

The productivity of the culture (Figure 3) increased for the test containing 2.0 g.L$^{-1}$ of NaHCO$_3$ during the study period. However, when 1.0 g.L$^{-1}$ of NaHCO$_3$ was added, the productivity fell after the sixth day of cultivation. On the first day of cultivation, when the cellular concentration was low, the difference in the concentrations of inorganic carbon did not influence the productivity of the cultures. However, when the cell concentration increased, the
growth was limited by the carbon source.

In the study by Pelizer et al. (2003), the best results with related to specific growth rate were obtained when the cell concentration used in the inoculum was 50 mg.L$^{-1}$. However, according to Vonshak et al. (1982), the density of the *Spirulina* population must be 400-500 mg.L$^{-1}$. These authors showed that the maximum daily production rates of *Spirulina* occurred in this range, with a significant reduction in concentrations above these values.

Radmann et al. (2007) evaluated the blend concentration that must be maintained and the Zarrouk medium dilution in semi-continuous cultivations of *S. platensis*. According to the results obtained in this study, the highest specific growth rates were seen when the blend concentration was 400 mg.L$^{-1}$ in Zarrouk medium containing 20% v/v of the original formulation, which produced a NaHCO$_3$ concentration of 3.36 g.L$^{-1}$.

Figure 4a shows the behavior of the pH of the medium as a function of time of the cultivation for the experiments with 1.0 g.L$^{-1}$ of NaHCO$_3$. During the light phase, the pH increases, whereas during the dark phase, the pH falls, returning to its initial value. This cycle is due to two phenomena that occur during the daily cycle. During the light phase, the autotrophic metabolism consumes CO$_2$ and increases the medium's pH; during the dark phase, the heterotrophic metabolism releases CO$_2$ in the medium, thereby reducing the pH.

Shiraiwa et al. (1993) reported an increase in the pH from 6-9 in the medium of *Chlorella* cultures after just a few minutes in the presence of inorganic carbon (in the form of HCO$_3^-$) and light. This pH increase has been linked with the carbon consumption and the production of O$_2$ during photosynthesis. The conversion of HCO$_3^-$ into CO$_2$ and OH$^-$ was the main cause of the change in the medium's pH. The medium pH is a function of biological activity; thus, during the light phase when carbon dioxide is being consumed, the pH increases. During the night, when the respiration rate causes the release of carbon dioxide, the pH drops.

In the assay containing 2.0 g.L$^{-1}$ of NaHCO$_3$ (Figure 4b), the pH did not follow the same patterns as in the assay with 1.0 g.L$^{-1}$ of NaHCO$_3$; instead, the pH increased during the first days of cultivation although normally it would drop overnight. In this case, the high concentration of inorganic carbon present under these conditions led to a transfer of CO$_2$ to the external medium, raising the pH. In the experiment during which 2.0 g.L$^{-1}$ of NaHCO$_3$ was initially added, the CO$_2$ concentration in the medium during the first days of cultivation exceeded the demand for the cultivation. This made the pH to rise, even at night when the photosynthetic activity ceased and when the consumption of biomass would usually decrease the medium's pH.

Richmond and Grobbelaar (1986) studied the relationship between the medium's pH and the purity of the monoalgal culture. They found that, under high pH conditions (above 10.0), *Spirulina* cultures presented a reduction in contamination by other microorganisms. However, during this study, it was found that productivity is maintained at its maximum value between pH 9.5 and 10.5. Above pH 10.5, a sharp decrease in culture productivity can be seen.

The pH controls the growth of *Spirulina*. In fact, at a pH above 10.2 to 10.4, a clear decrease in productivity was noted (Richmond and Grobbelaar, 1986; Vonshak et al. 1982). Experiments conducted on a laboratory scale by Jiménez et al. (2003) showed that the growth rate of *Spirulina* was significantly reduced (15-20%) at a pH above 9.5.

In order to determine the culture conditions that provide the highest efficiency of carbon dioxide fixation, the fixation efficiencies were measured on each day of cultivation. Figure 5 shows the fixation efficiency.

In the experiment containing 1.0 g.L$^{-1}$ of NaHCO$_3$ (Figure 5a), the initial biofixation efficiency was high, because the amount of carbon supplied is relatively low as compared to the amount in the experiment containing 2.0 g.L$^{-1}$ of NaHCO$_3$. After this point, values above 100% can be seen, indicating that the carbon consumption was

![Figure 4. pH of the medium throughout the cultivations.](image-url)
greater than the amount that could be provided by the CO₂ from the injected gas alone. In this case, growth takes place under limited carbon conditions, and the growth rate falls. In the experiment with 2.0 g.L⁻¹ of NaHCO₃ (Figure 5b), the initial efficiency was low due to the high carbon dioxide concentration, and the best values were achieved after 3 days when the cell concentration reached 0.4 g.L⁻¹. After the seventh day of cultivation, the efficiency exceeded 100% (cell concentration of 0.7 g.L⁻¹).

Morais and Costa (2007) and Watanabe and Hall (1996) both reported efficiencies of 53.29 and 54% in cultures of S. platensis in the tubular photobioreactors. However, in this type of bioreactor, the injection of the air/CO₂ mixture was usually continuous. When the CO₂ supply was carried out on-demand, losses were minimized, and biofixation efficiency increased. In the raceway type of bioreactors, the maximum efficiencies of CO₂ conversion into biomass obtained were approximately 80% (Vonshak and Richmond, 1988). However, these authors showed that high efficiencies are rarely found in large systems, due to the means used to transfer CO₂ to the culture medium.

Conclusions

The maximum concentration of cells in cultures containing 1.0 g.L⁻¹ of NaHCO₃ was 520 mg.L⁻¹. In cultures containing 2.0 g.L⁻¹ of NaHCO₃, the cell concentrations can be maintained between 800 and 900 mg.L⁻¹ with no reduction in productivity, showing the importance of the carbon source for the photosynthetic growth of microalga. The maximum productivity obtained was 0.13 g.L⁻¹.d⁻¹ in the experiment containing 2.0 g.L⁻¹ of NaHCO₃. Regarding environmental issues, the use of carbon dioxide from gas streams as combustion gases represents an alternative for reducing the emissions of greenhouse gases into the atmosphere. Economically, the use of flue gas as a source of inorganic carbon markedly reduces the costs of the processes required to obtain Spirulina biomass.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the Brazilian Electric Power Company (ELETROBRAS) and the Thermal Generation of Electric Energy Company (CGTEE) for their financial support of this study.

REFERENCES

Radmann EM, Camerin PV, Santos TD, Costa JAV (2011). Isolation and application of SOX and NOX resistant microalgae in biofixation of CO₂ from thermoelectricity plants. Energy Convers. Manag. 52:


African Journal of Microbiology Research

Related Journals Published by Academic Journals

- African Journal of Biotechnology
- African Journal of Biochemistry Research
- Journal of Bacteriology Research
- Journal of Evolutionary Biology Research
- Journal of Yeast and Fungal Research
- Journal of Brewing and Distilling