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Full Length Research Paper

Diclofenac inhibits virulence of *Proteus mirabilis* isolated from diabetic foot ulcer

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Diclofenac is an analgesic and anti-inflammatory drug, used to relief 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

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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 10^6 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 swarming 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 swarming 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 swarming 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 swarmer cells, sections of agar from swarming 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 swarmer 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 immersions lens.

For swimming 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 (Liaw et al., 2004). Briefly, bacterial suspension (50 µl) was mixed with a 2% erythrocyte suspension (450 µl) in 0.85% NaCl and 20 mM $CaCl_2$ 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 × 10^6 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 × 10^6 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.

Tested agent	MIC (µg/ml)	MBIC(µg/ml)	Ratio MBIC/MIC
Ciprofloxacin	2	128	64
Amoxicillin/Clavulinic acid	265	2048	8
Cefoperazone	64	1024	16
Gentamycin	16	512	32
Tetracycline	64	2048	32
Chloramphenicol	64	2048	32
Imipenem	4	8	2
Diclofenac	2	4	2

Then, ethanol was added and optical densities were measured at 590 nm. The assay was repeated triplicate and the means and slandered 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 5×10^5 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 (½ 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) inhabited 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.

Percentage of reduction	Diclofenac (½ MIC, %)	Diclofenac (¼ MIC, %)
Swarming	100	35
Swimming	84	63
Hemolysin production	23.5	6.8
Protease production	38.8	12
Adhesion	21.3	0
Biofilm formation	89.7	40
Urease	92.5	42.6

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

Antibiotic	MIC	MIC_{Dec}	FIC	MBIC	$MBIC_{Dec}$	FIC
Ciprofloxacin	2	1	0.5	128	32	0.25
Amoxicillin/Clavulinic acid	265	64	0.25	2048	256	0.25
Cefoperazone	64	32	0.5	1024	128	0.125
Gentamycin	16	4	0.25	512	64	0.125
Tetracycline	64	32	0.5	2048	256	0.125
Chloramphenicol	64	16	0.25	2048	512	0.25
Imipenem	4	4	1	8	4	0.5

*MIC and MBIC were determined in µg/ml. *MIC_{Dec} and $MBIC_{Dec}$, are the minimum inhibitory concentration and minimum biofilm inhibitory concentration of tested antibiotics respectivitely, 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).

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 dicolfenac (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 ½MIC concentrations of dicolfenac (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. Dicolfenac (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 dicolfenac (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

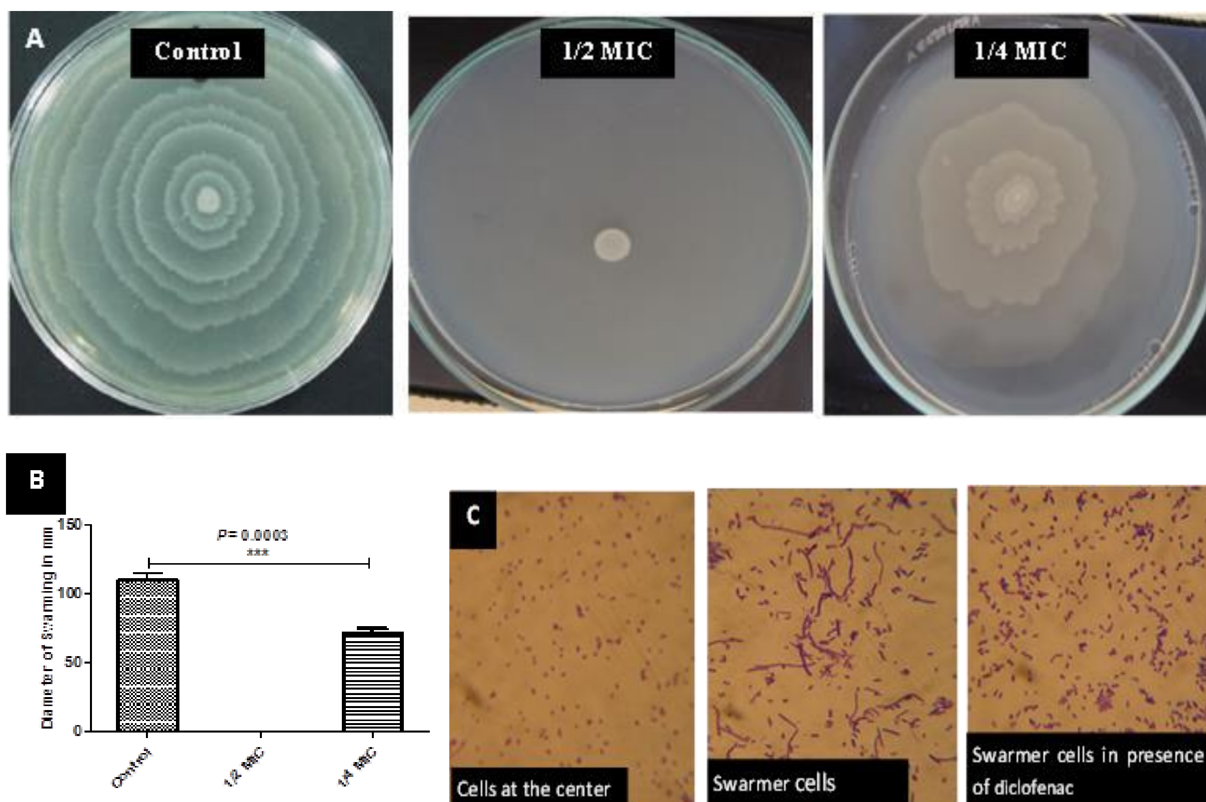

Figure 1. The inhibitory effect of sub MIC (½ MIC or ¼ MIC) of diclofenac on swarming of isolated *P. mirabilis* from diabetic foot. A) Sub MIC of dicolfenac showed inhibition of swarming of isolated *P. mirabilis* on LB swarming agar (1.5% agar); ½ MIC of diclofenac showed complete inhibition while concentration of ¼ MIC showed partial inhibition of the swarming. B) Dicolfenac (½ MIC) inhibited the swarming of *P. mirabilis*, moreover, the concentration of ¼ MIC was significantly enough to inhibit the swarming of *P. mirabilis* (P= 0.0003). C) 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 cell.

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 forassessment 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

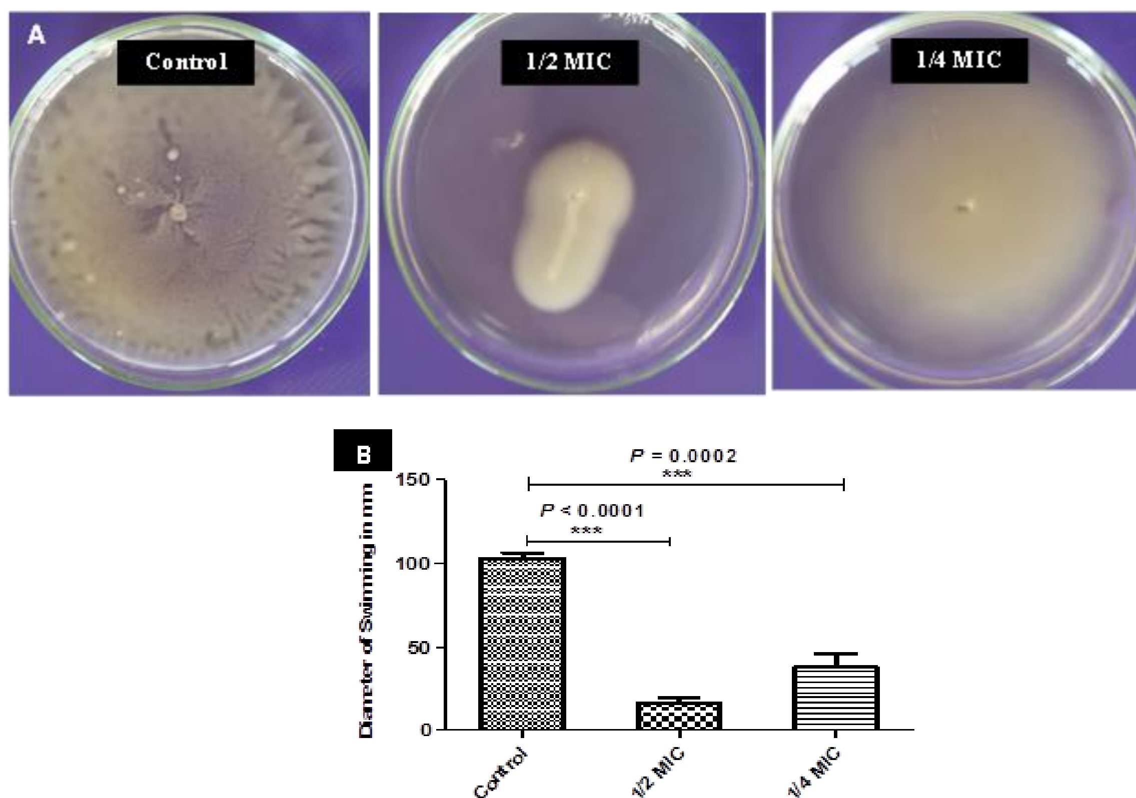

Figure 2. The inhibitory effect of sub MIC (½ MIC or ¼ MIC) of diclofenac on swimming of isolated *P. mirabilis* from diabetic foot. A) Sub MIC of dicolfenac showed inhibition of swimming of isolated *P. mirabilis* on LB swimming agar (0.4% agar). B) Sub MIC of dicolfenac (½ MIC or ¼ MIC) inhibited the 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, dicolfenac did not show a much significant inhibition of adhesion as shown in biofilm inhibition. Diclofenc 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;

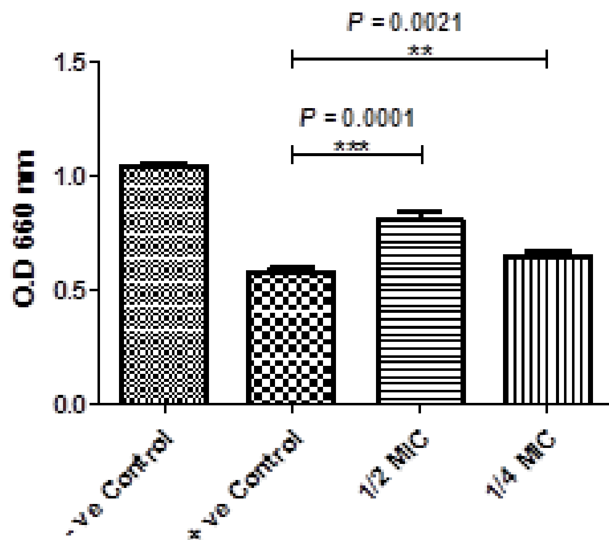

Figure 3. Protease assay. Extracellular protease activity was measured in the presence or absence of sub MIC diclofenac (½ MIC or ¼ MIC) using a casein substrate, the optical densities were measured at 660 nm. Sub MIC of dicolfenac (½ MIC or ¼ MIC) decreased the protease activity of *P. mirabilis* significantly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

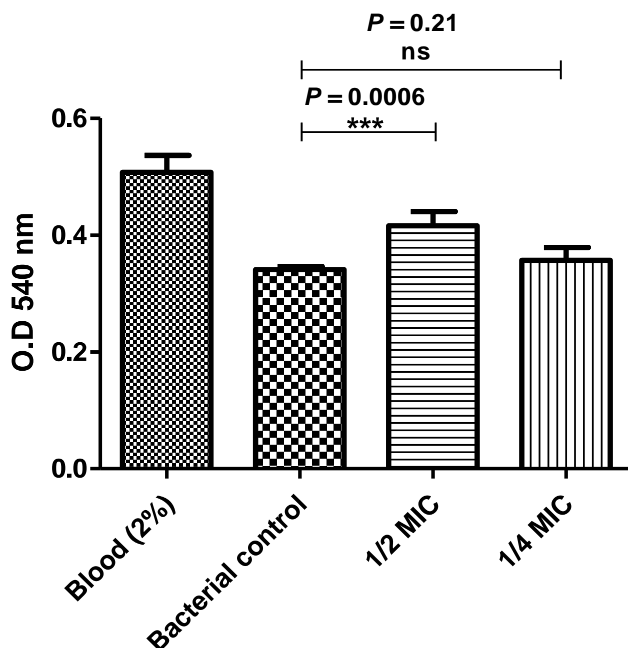

Figure 4. Haemolysin assay. Extracellular haemolysin activity was measured in the presence or absence of sub MIC diclofenac (½ MIC or ¼ MIC), the optical densities were measured at 540 nm. Sub MIC of dicolfenac (½ MIC) decreased the haemolysin activity significantly (*P* = 0.0006), however lower concentration (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;

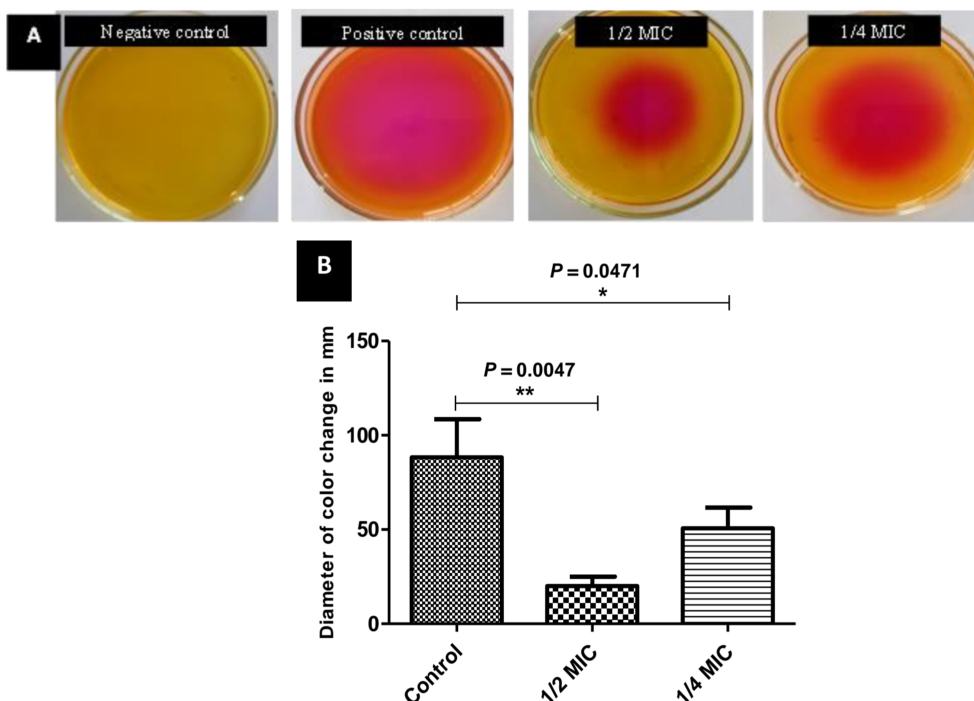

Figure 5. Urease assay. Urease activity was measured in the presence or absence of sub MIC diclofenac (½ MIC or ¼ MIC). A) Urease activity was demonstrated on Christensen's urea agar plates, change in pH indicator color due to urea release, the zones of color change were measured in mm. B) Sub MIC of dicolfenac (½ MIC or ¼ MIC) decreased the urease activity of *P. mirabilis* significantly (P = 0.0047 or P = 0.0471, respectively).

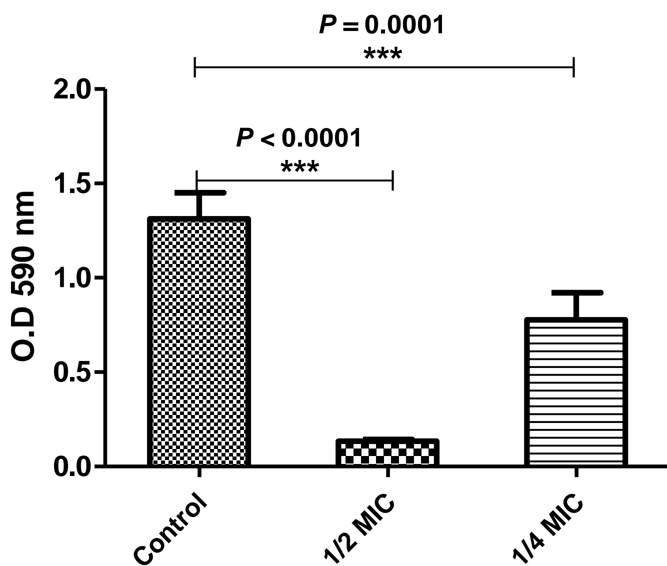

Figure 6. Inhibition of biofilm formation. *P. mirabilis* isolate was cultured with diclofenac in concentration ½ MIC or ¼ MIC in micro-titer plate, incubated at 37°C overnight, the attached cells were stained with crystal violet and their optical densities were measured at 590 nm. Sub MIC of dicolfenac (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* (Rózalski 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 (Rózalski 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

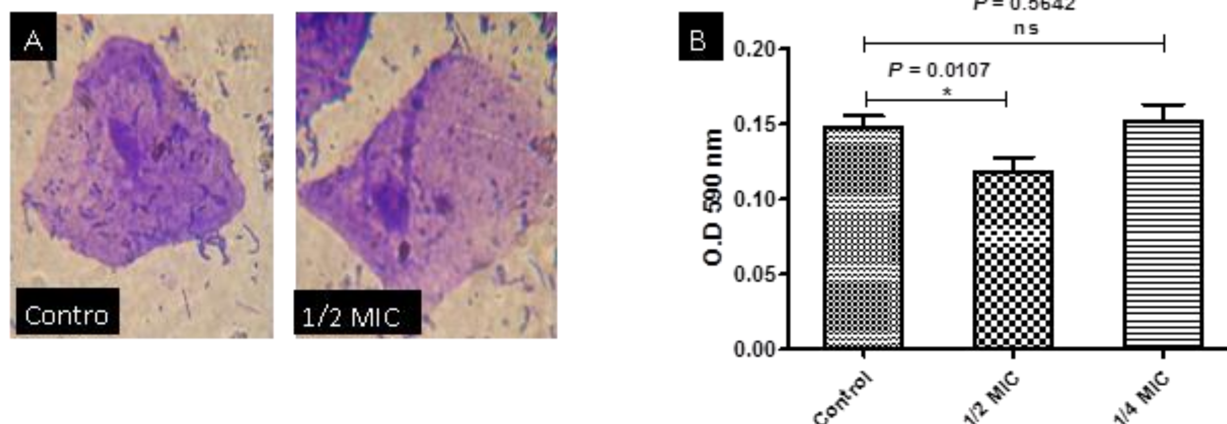

Figure 7. Adhesion assay. A) Adhesion to epithelial cells, collected from pregnant urine and co-cultured with *P. mirabilis* isolate in absence and presence of diclofenac in concentration ½ MIC or ¼ MIC. Diclofenac did not inhibit the bacterial adhesion to epithelial cells significantly. B) *P. mirabilis* isolate was cultured with diclofenac in concentration ½ MIC or ¼ MIC in micro-titer plate, incubated at 37°C for 1 h, the adhered cells were stained with crystal violet and their optical densities were measured at 590 nm. Dicolfenac at concentration of ½ MIC showed significant inhibition of adhesion (P = 0.01) while in concentration of ¼ MIC did not show significant reduction of adhesion.

inhibition on both protease and urease which may reduce bacterial virulence potentially.

Haemolysin is another virulence factor of *P. mirabilis* because of its cytotoxicity on epithelial cells (Armbruster and Mobley, 2012). It is most interesting that mutation in the gene encoding Haemolysin *hpmA* did not affect the colonization or tissue damage during infection (Alamuri et al., 2009), which may indicate that its activity is either diminished *in-vivo* or masked by contribution of other virulence factors (Armbruster and Mobley, 2012). *P. mirabilis* genome is transcribed in several distinguished rRNA operons, surprisingly the two-partner secretion system containing the hemolysin genes *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 *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 *in-vivo* evidence of diclofenac inhibitory effect on virulence, it may be more helpful to prescribe diclofenac for diabetic patients when needed.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

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

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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

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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 CIP-like 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 (HNO_3) 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 (Tauveron 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) were added to 400 µL of the polar or apolar solvent, vortexed for 1 min and settled for 15 min. The optical density of 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 $(1 - A/Ao) \times 100$, 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

Spore 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% HNO_3 w/v to absorbance values between 0.8-1. Tubes containing NaOH or HNO_3 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 *B. cereus* isolates from a pasteurized milk processing line*.

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

*Pasteurized milk was obtained from reconstituted and processed milk powder, in the investigated dairy plant. [a]*B. cereus* isolates were kindly characterized at the genotypic level at UMR 408 INRA Avignon, France in a previous work (Malek et al., 2013). [b]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. [c] 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 *B. cereus* spores by immersion in the wells of a 6-well polystyrene plate (Nunc multidish) for 3 h in a saline spore suspension (10^7-10^9 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 *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 exanimated in a 100 TM Hitachi environmental scanning electron microscope (Hitachi, Japan), at pressure in microscope chamber of 4 Torr. To ovoid 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 *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 *B. cereus* ATCC 11778, included for a comparative purpose. It is interesting to note the variability in hydrophobic/hydrophilic characters

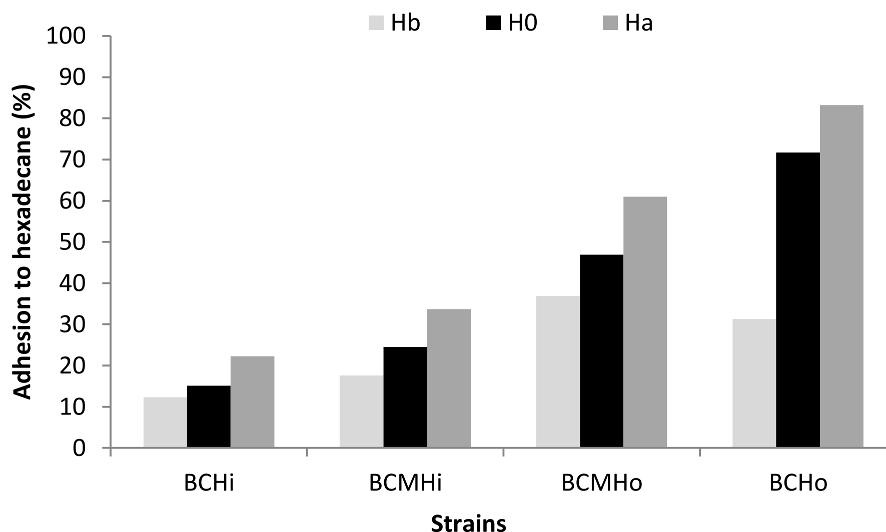

Figure 1. Variation in spore surface hydrophobicity of 13 *B. cereus* strains following alkali or acid stresses. H0: hydrophobicity of untreated spores (initial hydrophobicity), Hb: alkali-induced hydrophobicity, Ha: acid-induced hydrophobicity. H0, Hb and Ha bars represent means of spore hydrophobicity values of strains grouped based on their initial hydrophobicity. BCHi: *B. cereus* hydrophilic spores (4 strains), BCMHi: *B. cereus* moderately hydrophilic spores (3 strains), BCMHo: *B. cereus* moderately hydrophobic spores (5 strains), BCHo: *B. cereus* ATCC 11778: highly hydrophobic strain.

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-donnor 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

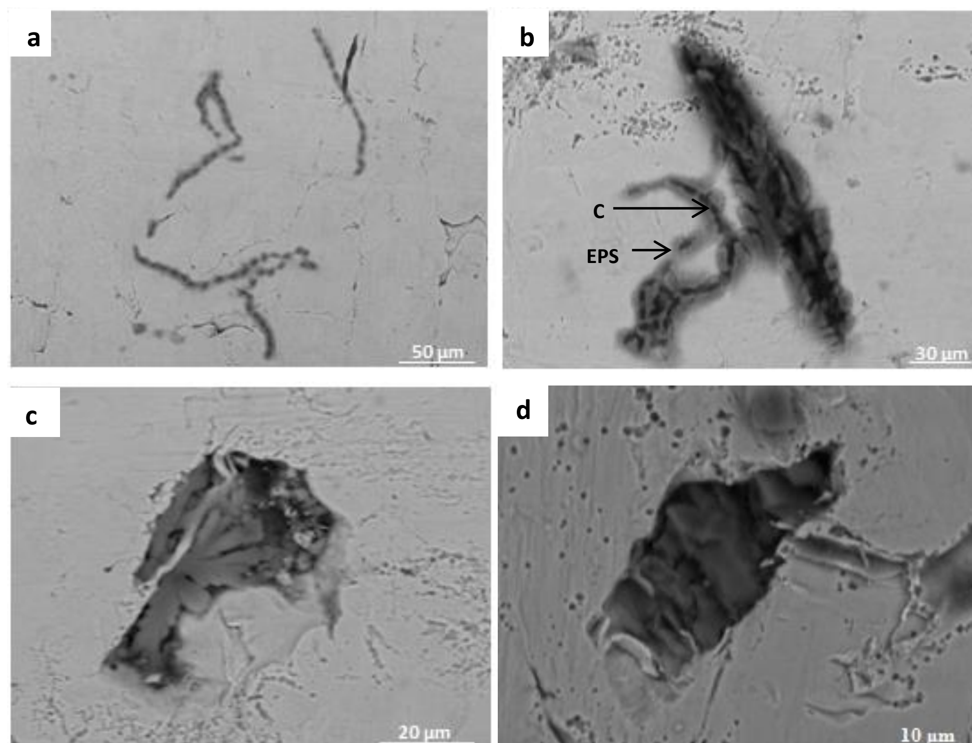

Figure 2. ESEM micrographs of 24 h old biofilms formed in diluted milk by *B. cereus* strain P53. (a) adherent cells surrounded by clear areas of EPS. (b) cells surrounded or covered by EPS material. (c and d) Young biofilms in crevices of damaged stainless steel. C Indicates cells and EPS exocellular polymeric substances.

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 harborages, 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.

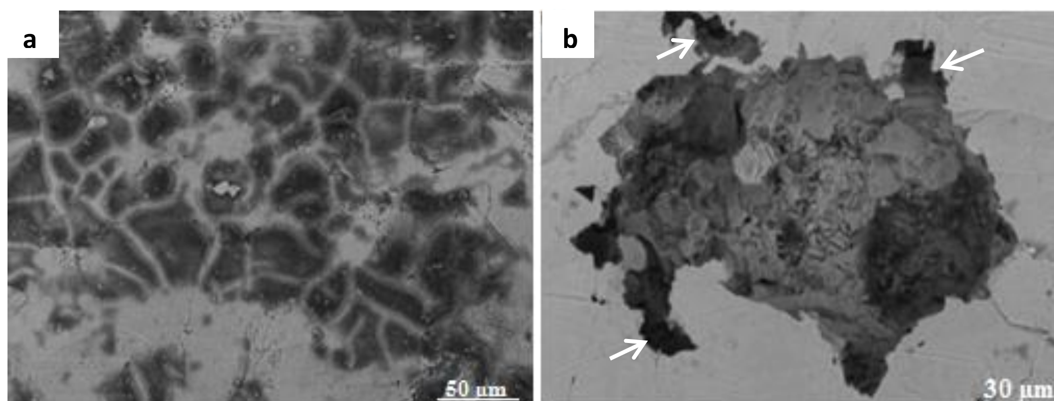

Figure 3. Comparison of 48 h old biofilms formed in nutrient broth by pH- treated (a) and untreated spores (b) of *B. cereus* strain S113. Arrows indicate detachment of small portions of the biofilm.

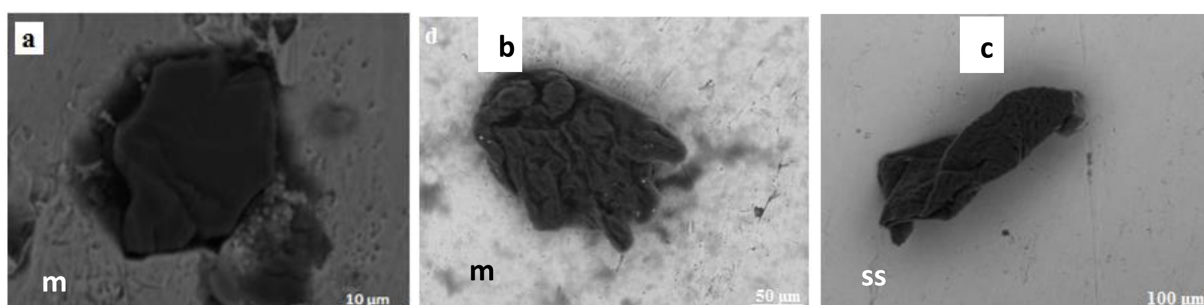

Figure 4. Smooth and wrinkled matrix surface topographies of 7 days old biofilms formed in non-diluted milk (a and b) and nutrient broth (c) by *B. cereus* strains S78 (a), A7 (b) and P56 (c). (m) Indicates milk fouling coating stainless steel surface topography and (ss) stainless steel.

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 P53 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 harborages 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.

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Full Length Research Paper

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

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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

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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 (Saliih 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). 16SrRNA forward primer (CGCACGGGTGAGTAAGGTAT) and 16SrRNA reverse primer (TAAACACATGCTCCACCGCT) 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, $MgCl_2$ 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 Exispin 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

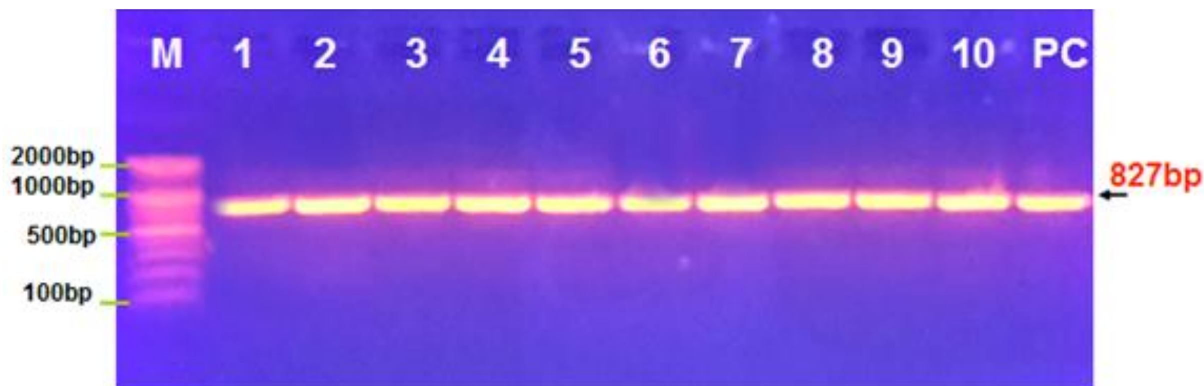

Figure 1. Agarose gel electrophoresis image that shown the PCR product of 16S rRNA gene that using in detection of *Campylobacter jejuni* Where M: Marker (1500-100bp), lane (1-3) domestic chicken, lane (4-5) pigeon, (6-10) Human were positive *Campylobacter jejuni* isolates and lane (PC) DNA Campylobacter genus positive control (Genekam, Germany) at 827bp PCR product size.

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

Host	No. of samples	*Campylobacter jejuni* positive	Percentage
Human	40	16	40
Chicken	20	3	15
Pigeon	20	2	10

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.

***Campylobacter* spp.**	**Sources/sample**	**Gen bank accession numbers**
IQDC-1	Domestic chicken/Feces	KR133485.1
IQDC-2	Domestic chicken/Feces	KR133486.1
IQDC-3	Domestic chicken/Feces	KR133487.1
IQP-1	Pigeons/Feces	KR133488.1
IQP-2	Pigeons/Feces	KR133489.1
IQH-1	Human/Stool	KR133490.1
IQH-2	Human/Stool	KR133491.1
IQH-3	Human/Stool	KR133492.1
IQH-4	Human/Stool	KR133493.1
IQH-5	Human/Stool	KR133494.1

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

***Campylobacter* spp. strains**	**Accession number**	***Campylobacter* spp. Iraq isolates of human**									
		IQ.H-1		**IQ. H -2**		**IQ. H-3**		**IQ. H -4**		**IQ. H -5**	
		Score	**Identify (%)**	**Score**	**Identify (%)**	**Score**	**Identify (%)**	**Score**	**Identify (%)**	**Score**	**Identify (%)**
C. Jejuni	Y19244.1	1495	99	1498	99	1469	99	1504	99	1495	99
C. faecalis	AJ.276874.1	1168	92	1194	93	1147	92	1170	92	1168	92
C. fetus	AJ.306569.1	1280	95	1273	95	1254	95	1184	95	1280	95
C. subantarctic	AM. 933373.1	1478	99	1482	99	1452	99	1509	99	1478	99
C. subantarctic	AM. 933374.1	1478	99	1482	99	1452	99	1509	99	1478	99
C. volucis	FM.883695.1	1439	98	1443	98	1413	98	1476	99	1439	98
C. hominis	AJ.251584.1	1114	91	1149	92	1098	92	1096	91	1114	91
C. coli	AM.042699.1	1210	99	1498	99	1258	99	1020	99	1210	99

sequences of eight strains of *Campylobacter* spp. 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

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

Campylobacter spp. strains	Accession number	*Campylobacter* spp. Iraq isolates of birds									
		IQ DC -1		IQ DC -2		IQ DC -3		IQ P-1		IQ P-2	
		Score	Identify (%)	Score	Identify (%)	Score	Identify (%)	Score	Identify (%)	Score	Identify (%)
C. Jejuni	Y19244.1	1509	99	1495	99	1506	99	1511	99	1500	99
C. faecalis	AJ.276874.1	1158	92	1168	92	1218	93	1206	93	1157	92
C. fetus	AJ.306569.1	1168	95	1280	95	1303	95	1286	95	1264	94
C. subantarctic	AM. 933373.1	1515	99	1478	99	1489	99	1495	99	1483	99
C. subantarctic	AM. 933374.1	1515	99	1478	99	1489	99	1495	99	1483	99
C. volucis	FM.883695.1	1482	99	1439	98	1450	98	1456	98	1445	98
C. hominis	AJ.251584.1	1090	91	1114	91	1168	92	1162	92	1109	91
C. coli	AM.042699.1	1003	99	1210	99	1522	99	1506	99	1194	99

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

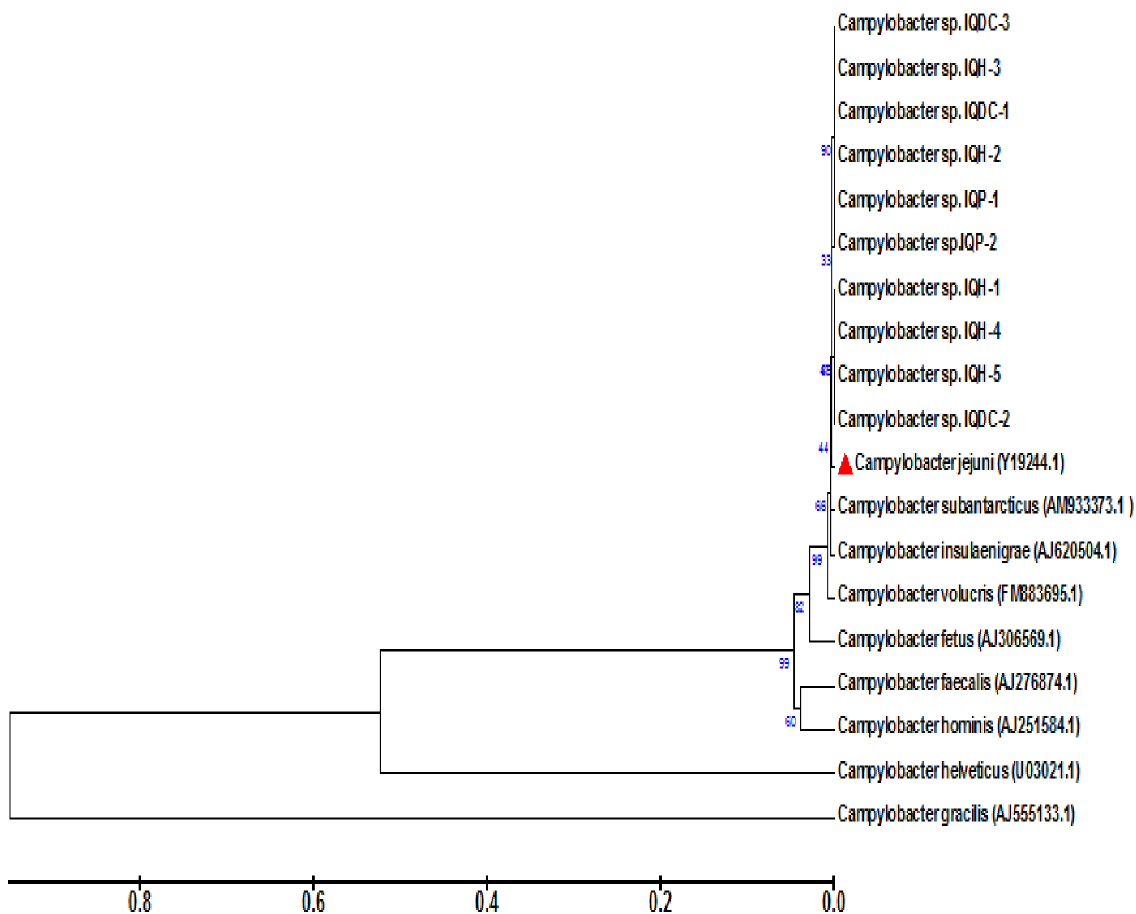

Figure 2. The phylogenetic tree analysis of *Campylobacter* spp. Iraq isolates based on partial 16SrRNA gene sequences using unweight pair group method with arithmetic mean (UPGMA tree) in (MEGA 6.0 version).

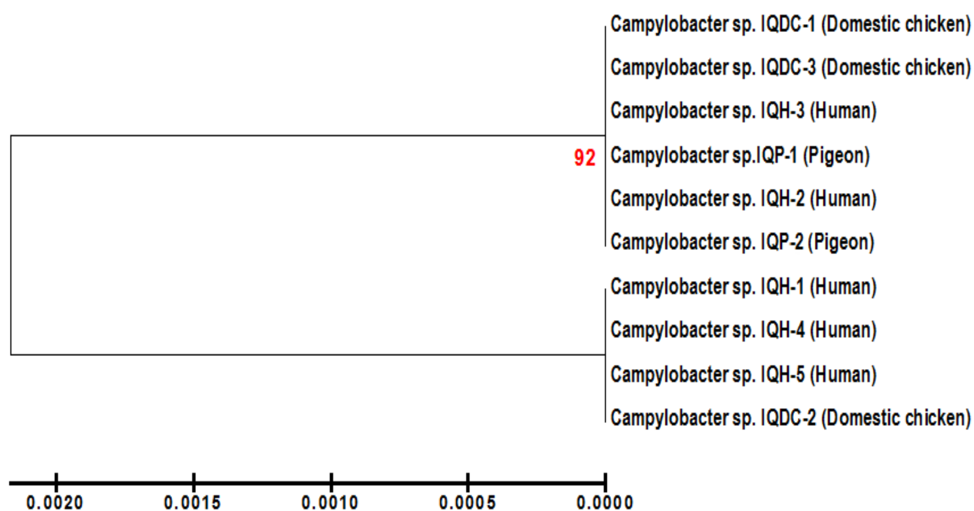

Figure 3. Phylogenetic tree analysis of *Campylobacter* spp. Iraq isolates based on the partial 16S ribosomal RNA gene sequence that used for host relationship analysis. The phylogenetic tree was constructed using Unweight Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).

Campylobacter gastroenteritis.

Conflict of Interests

The author has not declared any conflict of interests.

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Full Length Research Paper

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

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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

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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; Rebedeaux 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 antiserum 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 (2:1w/v) containing 1% 2-mercaptoethanol. The slurry was filtered in a double cheesecloth, clarified with chloroform (1/2 vol) and stirred for 20 min at 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 centrifuged at 10,000 g for 10 min and the supernatant rescued and stored. Virus yield was determined by assuming an extinction coefficient of 5.1 (Salazar et al., 1982).

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). Nonspecific 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 immuno-reactive 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 RNeasy 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'TGCTCGCATGGGTCATAGAC 3') at 70°C for 10 min. To the

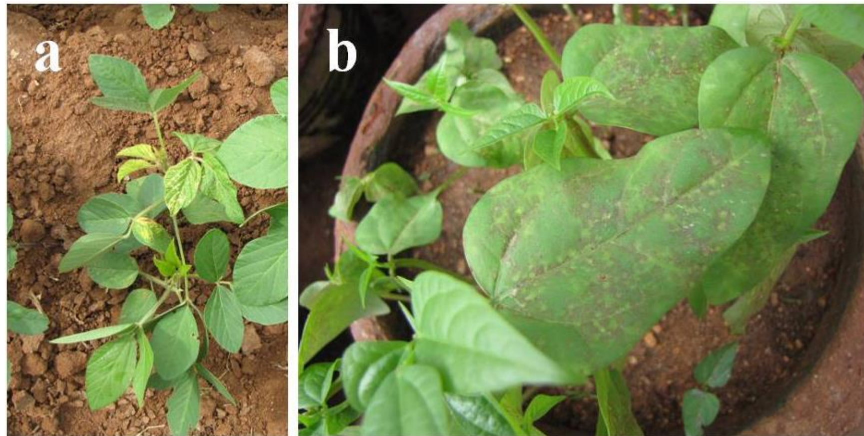

Figure 1. a. Symptoms of TSV on soybean leaves necrotic lesion under field condition; b. Development of circular necrotic lesions on the leaves of cowpea on artificial inoculation.

transcription mixture, various reaction components were added [(RNase inhibitor 1 µl (20 U); dNTPs 2 µl (10 mM); 4 µl 5× reverse transcriptase buffer containing Tris HCl 250 mM, pH 8.3 at 25°C, KCl 250 mM, $MgCl_2$ 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 $MgCl_2$ 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

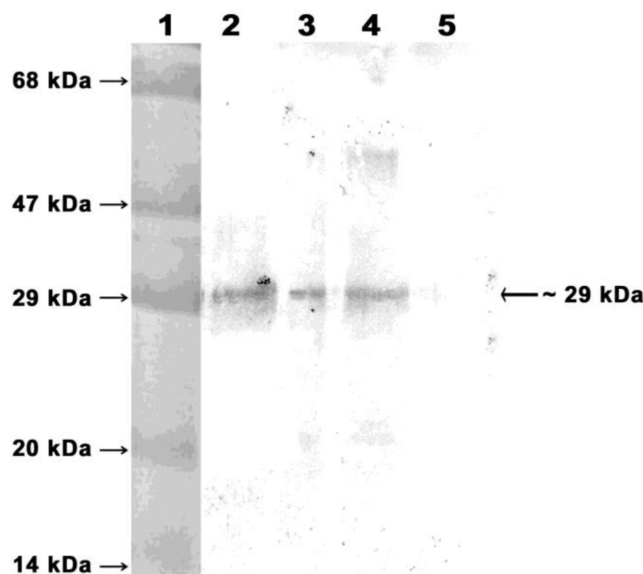

Figure 2. Western blot assay of TSV infected cowpea (primary inoculum from naturally infected soybean); Lane 2-4: TSV infected sample; Lane 5: Healthy sample (Control).

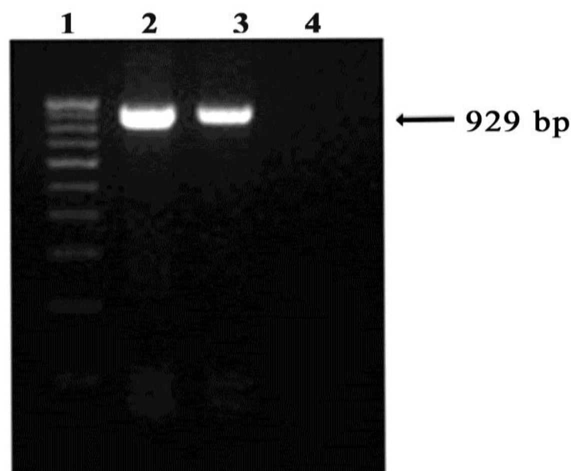

Figure 3. RT-PCR amplification of TSV coat protein gene from naturally infected leaves of soybean. Lane 1: 100 bp ladder; lanes 2-3: Amplified DNA fragment from infected samples; lane 4: Healthy samples (Control).

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 immuno assay 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.

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

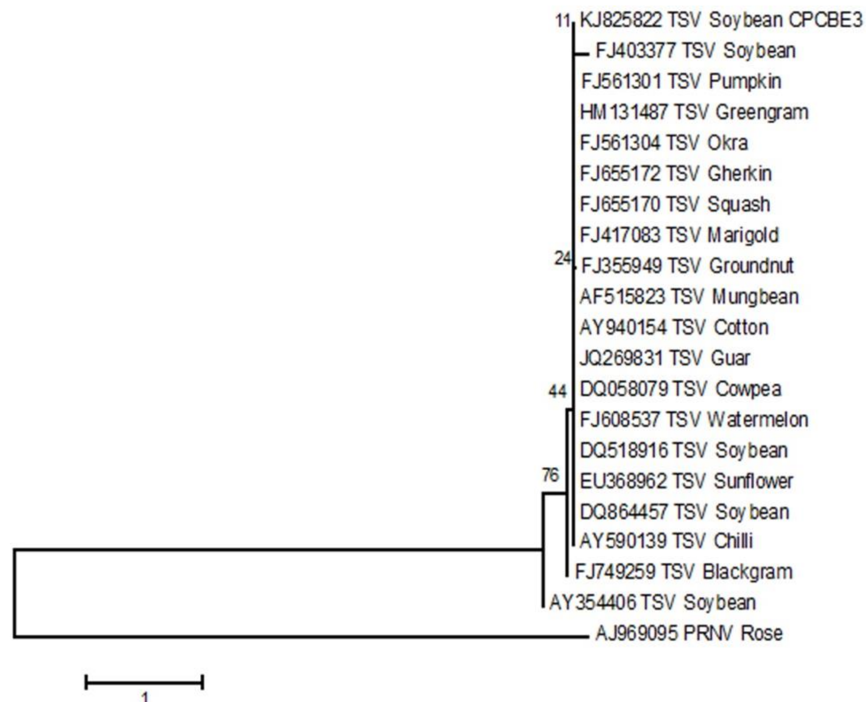

Figure 4. Neighbour-joining phylogenetic tree based on the nucleotide sequences of the coat protein gene of TSV (KJ825822) and *P. ringspot necrosis virus* is defined as an out-group.

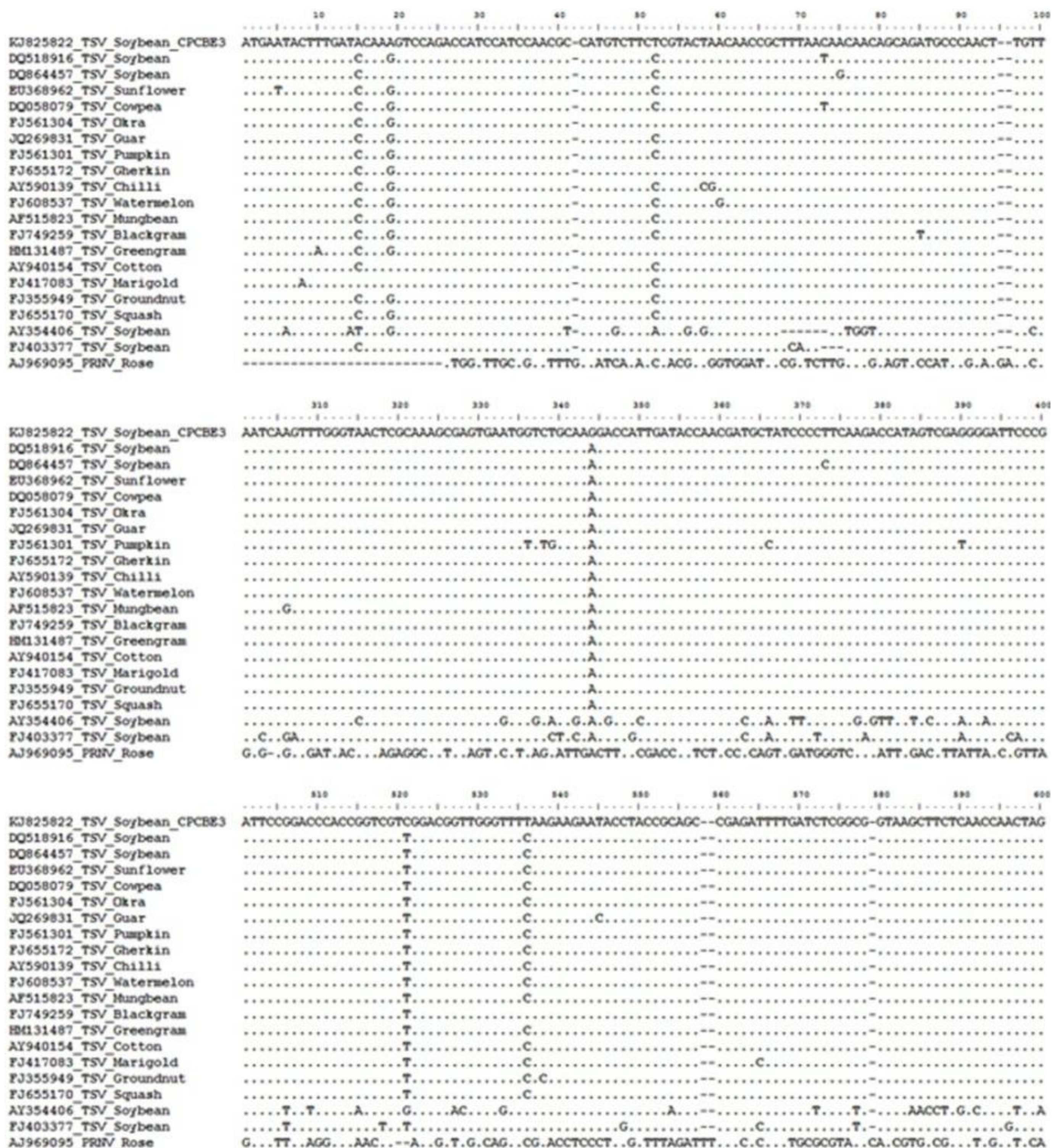

Figure 5. Multiple sequence alignment of the 717 nucleotide sequences of the coat protein gene of the soybean strain of TSV and corresponding sequences of 19 selected strains of TSV. *P. ringspot necrosis virus* is defined as an out-group.

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

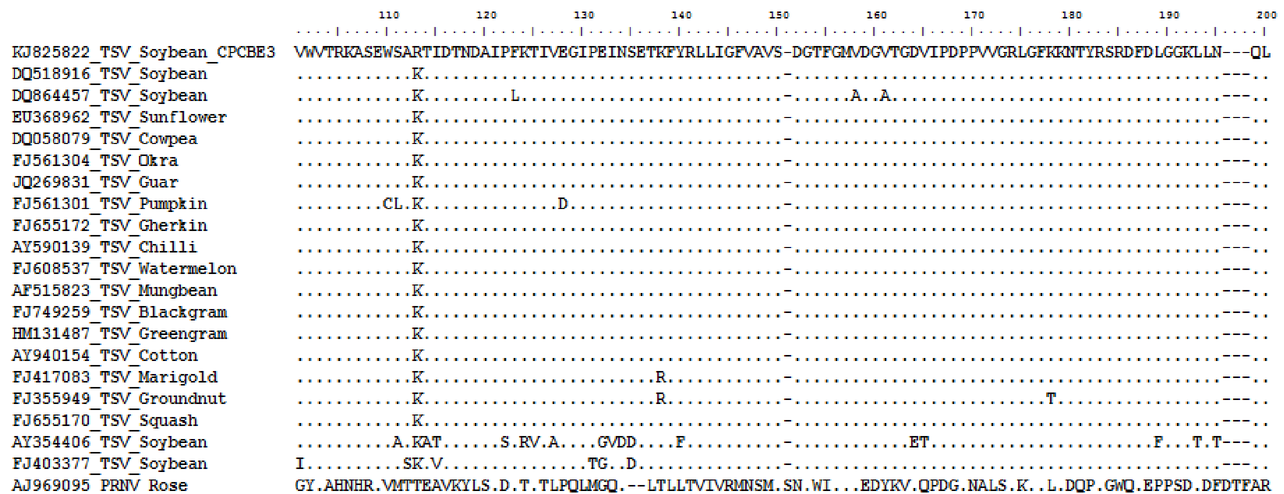

Figure 6. Multiple sequence alignment of the 239 amino acid sequences of the 3' end of the coat protein gene of the soybean strain of TSV and corresponding sequences of 19 selected strains of TSV. *P. ringspot necrosis virus* is defined as an out-group.

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

Characterization 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 veinal 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 antiserum 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'-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 *Ilarvirus* 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.

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Full Length Research Paper

Biofixation of CO_2 on a pilot scale: Scaling of the process for industrial application

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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).

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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 (Soccol, 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 microalga *S. platensis* (*Arthrospira*), as grown in modified Zarrouk culture medium (Zarrouk, 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 Zarrouk medium, 16.8 $g.L^{-1}$ $NaHCO_3$). CO_2 was injected into the medium at a rate of 10 L CO_2.h in the light phase (when the concentration of $NaHCO_3$ was 1.0 $g.L^{-1}$) and 20 L CO_2.h 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_{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_{max}) was defined as the highest productivity using Equation 1:

$$P = \left(\frac{X_t - X_0}{t_t - t_0} \right) \quad (1)$$

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 t_0 (d).

The maximum specific growth rate (μ_{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:

$$\varphi = \frac{(X_t - X_0) * V * CX * \left(\frac{M_{CO_2}}{M_C} \right)}{\left(Q * Y_{CO_2} * \frac{1}{22{,}4} * M_{CO_2} * t_I \right) * \varepsilon} \quad (2)$$

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 t_0, CX is the fraction of carbon determined in the microalgal biomass, V (L) is the volume of medium in the photobioreactor, M_{CO2} ($g.mol^{-1}$) and M_C are the molar masses of carbon dioxide and carbon present in the biomasses, respectively, Q is the injected gas flow, Y fraction CO_2 in the gas injected and Ɛ 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.

$$\phi = \frac{(X_t - X_0) * V * CX * \left(\frac{M_{CO_2}}{M_C} \right)}{\left(Q * Y_{CO_2} * \frac{1}{22{,}4} * M_{CO_2} * t_I \right)} \quad (3)$$

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

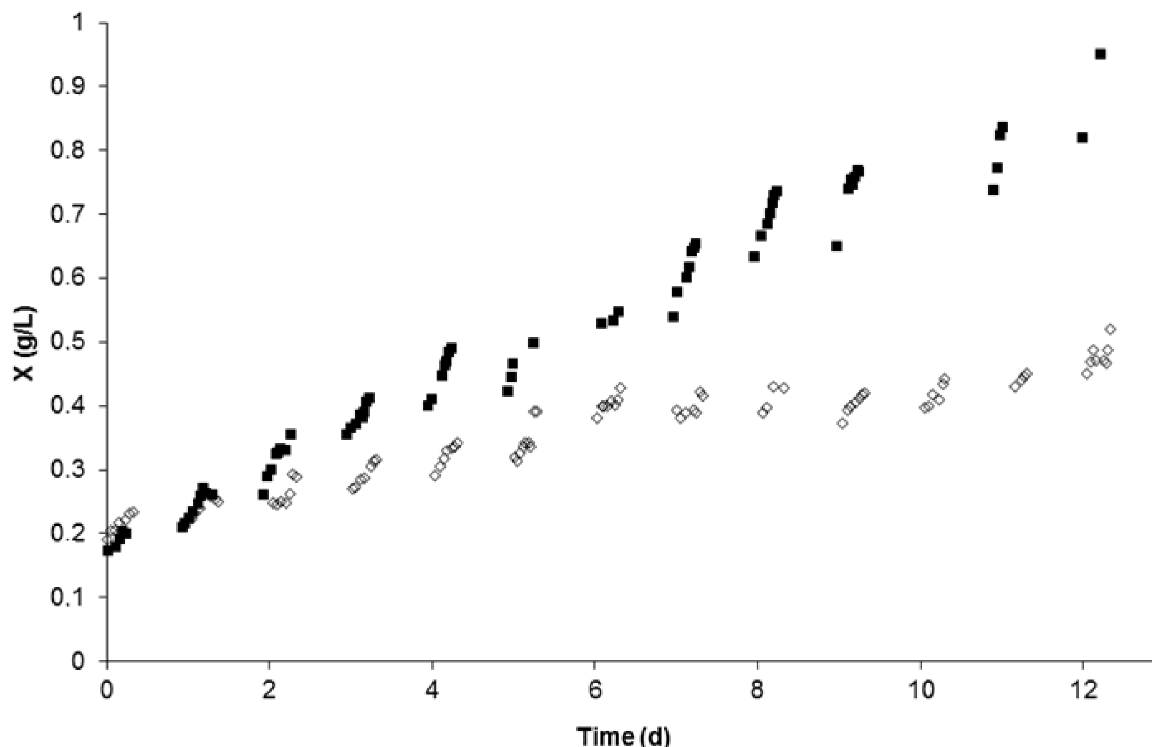

Figure 1. Growth curves of *S. platensis*: $NaHCO_3$ 1.0 g.L^{-1} (◊); $NaHCO_3$ 2.0 g.L^{-1} (■)

Table 1. Kinetic parameters obtained.

$NaHCO_3$ (g.L^{-1})	X_{max} (g.L^{-1})	P_{max} (g.L^{-1}.d^{-1})	μ_{max} (d^{-1})
1.0	0.52	0.07	0.76
2.0	1.00	0.11	0.76

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 ($HCO_3^- \xleftrightarrow{k} CO_2 + OH^-$), 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 show that, during the light phase (day time), growth occur 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

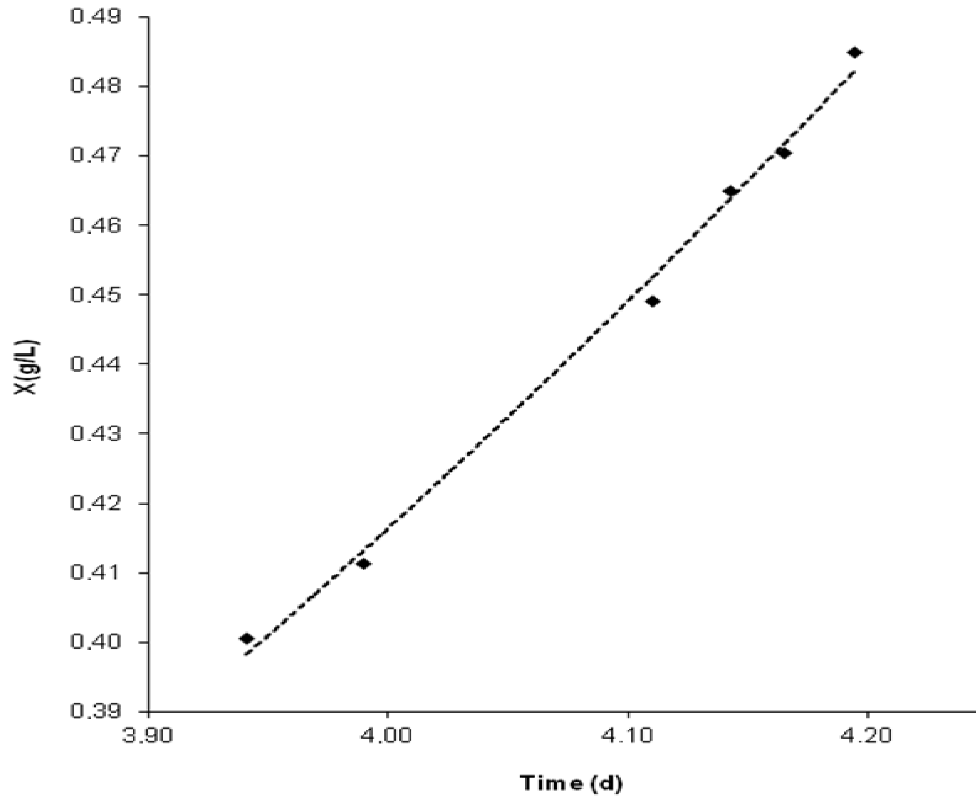

Figure 2. Growth of *S. platensis* during the light phase.

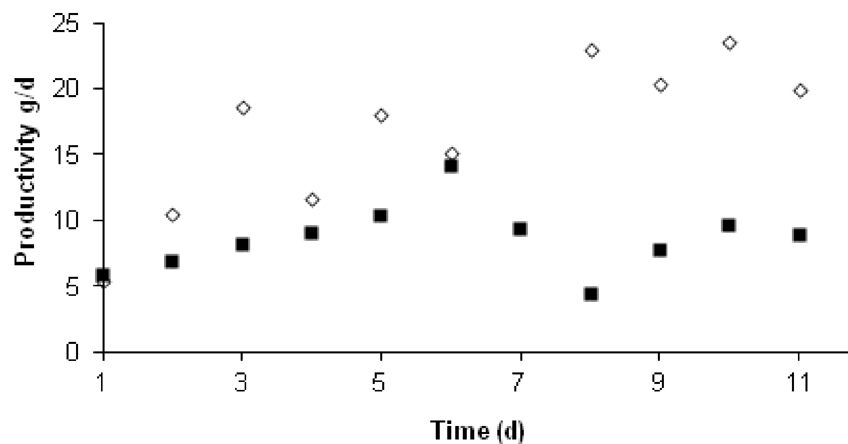

Figure 3. Productivity of cultures containing $NaHCO_3$ 1.0 $g.L^{-1}$ (■) or $NaHCO_3$ 2.0 $g.L^{-1}$ (◊)

(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

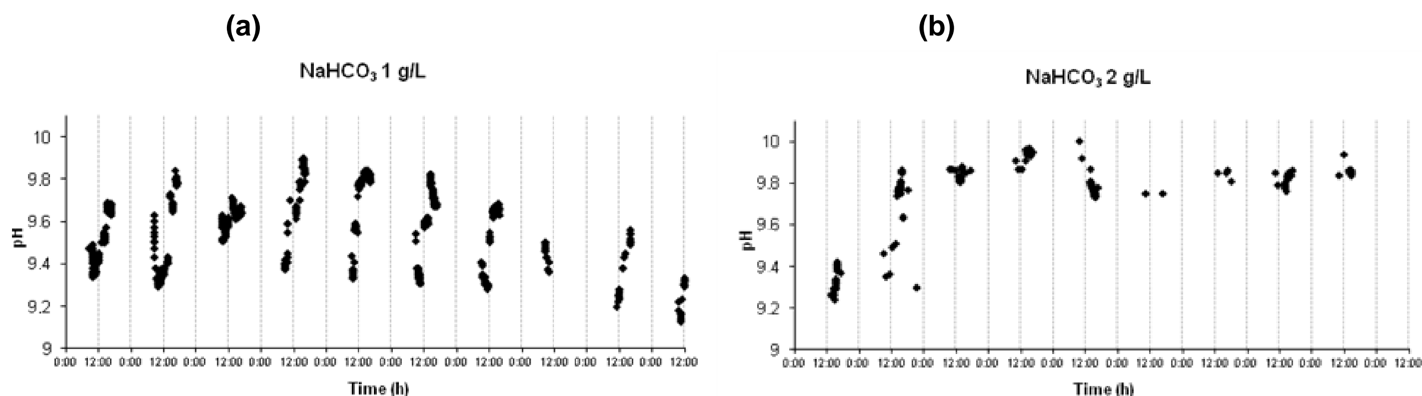

Figure 4. pH of the medium throughout the cultivations.

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

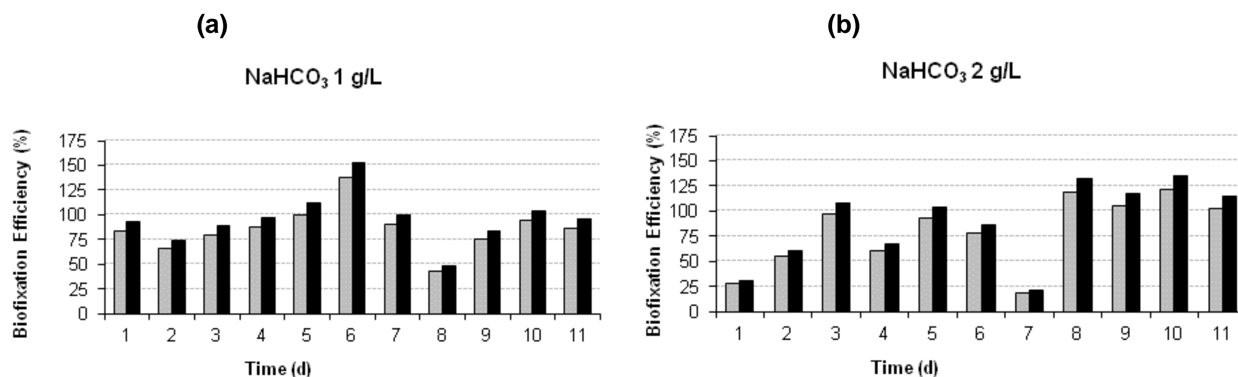

Figure 5. Biofixation efficiency– Overall ; net .

greater than the amount that could be provided by the CO_2 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 $NaHCO_3$ (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/CO_2 mixture was usually continuous. When the CO_2 supply was carried out on-demand, losses were minimized, and biofixation efficiency increased. In the raceway type of bioreactors, the maximum efficiencies of CO_2 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 CO_2 to the culture medium.

Conclusions

The maximum concentration of cells in cultures containing 1.0 $g.L^{-1}$ of $NaHCO_3$ was 520 $mg.L^{-1}$. In cultures containing 2.0 $g.L^{-1}$ of $NaHCO_3$, 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 microalgae. The maximum productivity obtained was 0.13 $g.L^{-1}.d^{-1}$ in the experiment containing 2.0 $g.L^{-1}$ of $NaHCO_3$. 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.

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