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Full Length Research Paper

Urinogenital trichomoniasis in women in relation to candidiasis and gonorrhoea in University of Port-Harcourt Teaching Hospital

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This study was conducted to identify and estimate the proportion of patients who had trichomoniasis in relation to gonorrhea and candidiasis in two hundred and twenty (220) women aged between 18 and 55 years from the month of February to June 2005. High vaginal swab (HVS) samples from symptomatic and asymptomatic women in University of Port-Harcourt Teaching Hospital were examined in antenatal, gynecology, family planning and sexually transmitted diseases clinics. Statistical analysis of the data revealed a significant differences ($p<0.05$) with lower prevalence of gonorrhea co-infected with trichomoniasis among the categories of women examined. Out of two hundred and twenty (220) women examined, 10 (4.5%) were found to be positive for trichomoniasis. The latter percentage was also positive for candidiasis and gonorrhea. From these 10 (4.5%) women, 3 (1.4%) were for gonorrhea in addition to trichomoniasis while 7 (3.2%) out of the 10 (4.5%) examined were positive for both trichomoniasis and candidiasis. Observation showed the most infected women were between ages of eighteen and thirty. In conclusion, this study has shown that co-infection with trichomoniasis and candidiasis is more prevalent in the University of Port Harcourt Teaching Hospital (UPTH).

Key words: Prevalence, *Trichomonas vaginalis, Neisseria gonorrhea, Candida albicans,* sexually transmitted infection (STI), women.

INTRODUCTION

Trichomonas vaginalis is a sexually transmitted parasitic protozoa known to be responsible for an estimated 180 million new infections per year, making it the most prevalent nonviral sexually transmitted pathogen worldwide (Abdurehman et al., 2013). *T. vaginalis* is a flagellate which belongs to the order trichomonadida, having three to five flagellae and recurrent flagellum which may be attached to the body to form an undulating

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membrane (Ferrer, 2000; Smyth, 1994). All five flagella arise from basal bodies grouped at the anterior end, just in front of the single nucleus. With the aid of these flagella the organism can swim actively (Kulda et al., 1978; Smyth 1994; Honigbera, 1983; Honigbera, 1978; Kannel and Levine, 2003).

T. vaginalis is a worldwide pathogen of men and women (WHO, 2001). It is a sexually anaerobic parasite, which frequently co-exists with gonorrhea, the World Health Organisation (WHO) estimates the world wide prevalence of trichomoniasis to be 174 million and to account for 10 to 25% of vaginal infections (WHO, 2001; Rosemarie, 1985). This species is a relatively common pathogenic specie of the female and male urinogenital tract.

It is an important complication in pregnancy as it has been related to prematurity and low birth weight (Kannel and Levine, 2003). It has been supported by the hypothesis that *T. vaginalis* may be an important co-factor in promoting the spread of HIV in Africa-America communities (Adeoye et al., 2009; Sorvillo et al., 2001). The organism causes trichomoniasis, resulting in vaginitis in women and urethritis in men; 200 million people annually are probably infected (Quinn and Holmes, 1984).They live in vaginal of woman and urethral of men, hence causes vaginal discharges, annoying but not serious disease in woman, while it causes urethral discharge in men (Monica, 2002; Kreier and Baker, 1987).

T. vaginalis is rather more commonly responsible for mild vaginal inflammation associated with a copious, foul-smelling discharge. It has been suggested that the parasite's pathogenicity is associated with endocrinal or other charges resulting in variation in the normal bacterial flora of the vaginal, leading to a reduction in the acidity of its contents from the usual pH 4-5 to 5.5. The organism can survive at neutrality (pH 7) (ACOG 1996).

Evans (1976) and Sobel (1997) stated in their investigations that *T. vaginalis* does not invade tissue, but causes frothy, foul smelling greenish grey vaginal discharge. It causes Puntate hemorrhages and granular appearance of vaginal and cervix yielding classic strawberry appearance of trichomonal cervicitis, post coital bleeding might occur.

From the work of Kreier and Baker (1987) it was deduced that the infection rate of trichomoniasis caused by *T. vaginalis* among those with vaginal upsets was higher, up to seventy percent. Fifty to seventy percent of infected men appear to be asymptomatic and the infection may be self-limited. Juliano et al. (1986) stated that the clinical manifestation range from asymptomatic carriage to severe vaginitis, there is usually intense inflammation with itching and copious discharge called leucorrhea.

Rosemarie (1985) emphasized the etiology and prevalence of vulvovaginitis and stated that prevalence ranges from as low as 5% of private gynaecologic patients to as high as 50 to 75% of prostitutes. *Neisseria gonorrhea* causes the sexually transmitted disease gonorrhea, the most frequently reported communicable diseases in the United State of America. Barnes and Holmes (1984) and Benenson, (1985) worked on candi-diasis, which is caused by *Candida albicans* saprophytic in the gastrointestinal tracts in 25 to 50% of the population and in the vaginal of 25 to 50% asymptomatic women. The clinical presentation in the vulva is usually erythematous and edematous (Tanb, 1976). Vaginal discharge may be thick and white, resembling cottage cheese. Occassionally, discharge is thin and watery satellite lesions may spread to the groin. Many women have no symptoms; sexual partners may develop balantidies or cutaneous lesion on penis.

This work is thus undertaken to identify and estimate the proportion of patients suffering from trichomoniasis in relation to gonorrhea and candidosis. This is to enable us know the incidence and prevalence rates and thereby enhance proper treatment given by health care providers to the patients.

MATERIALS AND METHODS

Sampling method

High vaginal swabs (HVS) were taken from women attending University of Port-Harcourt Teaching Hospital from antenatal, gynecology, family planning and sexually transmitted disease clinics from February to June, 2002. The samples were randomly sampled every 3rd day from February to June 2002. A week each was used for each department in a month which means 1 week was used for antenatal patients, the 2nd week for gynecology department, the 3rd week for family planning section, and the 4th week for sexually transmitted disease department in the month of February 2002. The same process was performed in the months-March, April and June 2002. The swabs were examined for *T. vaginalis,* cultural method for *C. albicans and N. gonorrhea.* A total of 220 women examined.

Test materials and reagents

The following equipment were used- Microscope, microscopy slides, cover slips, cotton wool, sterile swab sticks. Reagents used include physiological saline, Giensa's stain, absolute method and distilled water.

Preparation of films

Clean grease-free slides soaked in ethanol to reduced contamination to minimum were used and wiped with sterile cotton wool.

Wet preparation

Some drops of sterile physiological saline were added to the swab container, mixing and making a smear with the swab onto a ready for use slide and covered with a cover slip. Some minutes of incubation or warming on a heated microscope stage then followed, especially for samples that have stayed some time outside the

Table 1. Overall prevalence concomitant infections with trichomoniasis, co-incident with candidiasis and gonorrhea

Affected patients	Number	Percentage (%)
Patients with *Trichomonas vaginalis* only	0	0
Patients with *Trichomonas vaginalis* and *Candida albicans*	7	70
Patients with *Trichomonas vaginalis* and *Neisseria gonorrhea*	3	30

body, then examined immediately.

Stained preparation

A smear from the HVS sample was made onto a ready for use slide, care was taken not to make the smear too thick. Drying of the film was effected by flaying in the air or keeping on the table to dry horizontally. Giensa's thin film technique was used, using already prepared Giensa's stain, method and distilled water. *N. gonorrhea* and *C. albicans* were cultivated using blood agar medium and Sarbouroud dextrose agar, respectively. They were prepared according to the manufacturer's instructions.

Stain procedure

Already prepared Giensa's stain was diluted 1 in 30 before use. One milliliter of Giensa's stain was added to twenty-nine milliliter of distilled water in that ratio for use. Using a staining rack, the samples were first fixed in methanol for two minutes, covered with dilute Giensa's stain on each slide. The film was left to stain for 30 min. The back of the slides was wiped dry. The film was allowed to air dry and examined under oil immersion (100 x).

Microbial examination

Wet preparation

Using 10 and 40x objectives (with the condenser iris closed to give good contrast), *T. vaginalis* was seen very motile, round to oval in shape and a little larger than pus cells in positive samples. They were seen around pus cells. The stained film preparation was used to study the morphological features of *T.s vaginalis.* These features include the nucleus and anterior flagella.

RESULTS

Two hundred and twenty two (220) high vaginal swab (HVS) samples were successfully studied using wet preparations and the positive cases stained by Giensa's to confirm *T. vaginalis*. Ten (10) samples of the total number were positive for *T. vaginalis*, giving a prevalence of 4.5%. Three (3) of the positive 10 samples were also positive for *N. gonorrhea* while the remaining seven (7) samples were positive for both *C. albicans* and *T. vaginalis*.

Table 1 shows the overall prevalence concomitant infections with trichomoniasis, co-incident with candidosis and gonorrhea at the University of Port-Harcourt Teaching Hospital.

DISCUSSION

In this study, the occurrence of trichomoniasis in University of Port Harcourt Teaching Hospital in Port Harcourt is low (4.5%) when compared with the work of Abdurehman et al. (2013), in Southwest Ethiopia (4.98%), Ogbona et al. (1991), 37.6% and WHO (2001) 32%, but high in comparison with the work done in Sagamu by Anate (1991), (0%) and Perazzi et al. (2010), in Argentina (4%). It was discovered that none of the ten (10) women had *T. vaginalis* as a single infection but were co-infected with *C. albicans* and *N. gonorrhea*. Observations also showed that no woman was infected by all three organisms at the same time in University of Port Harcourt Teaching Hospital (UPTH). The prevalence of trichomoniasis co-infected with candidiasis is higher (70%) when compared with trichomoniasis co-infected with gonorrhea (30%). The observed difference in the rate of infection could be due to variation in age distribution, personal hygiene practice, climatic conditions, socio-economic and literacy status of the study women. The co-existence of these diseases in their host body could attribute to the basic origin of the causative agents which is mainly of sexually transmitted origin. Certain hormonal changes could also initiate their co-infection, most especially trichomoniasis and candidiasis according to the findings of Matini et al. (2012), Al-Saeed et al. (2011), Fule et al. (2012) and Rosemarie (1985).

The percentage of trichomoniasis in this study could be attributed to many multiple factors such as awareness of people towards the spread of human immuno-deficiency virus (HIV) due to public campaign by World Health Organization (WHO), National Agency for Food Drug Administration Control (NAFDAC) and some other agencies in Nigeria and abroad. This has led to a change in attitude of people towards unsafe sex and increase in the use of condoms. The use of condoms helps reduce the rate of sexually transmitted diseases and since the organisms especially *T. vaginalis* infects the vaginal mucosa in women asymptomatically and self-limiting in men, this could have attributed to the lower percentage (4.5%) inferred in this study. This is in concordance with the findings of Evans et al. (1995).

Port Harcourt dwellers are medically enlightened; hence they seek medical attention very seriously and practice good hygiene. The women with trichomoniasis that also had candidiasis, could be due to poor hygiene

especially with the use of general toilet facilities. Some women prefer using betel leaf for douching, whilest others use water after sexual contact. This is in concordance with findings of Joesoef el al. (1996) which stated that some women prefer douching as a cleaning method and after sexual contact. Douching especially with water helps reduce and protect against trichomniasis, candidosis and gonorrhea, hence contributed to the low rate of 4.5% of trichomoniasis in women as observed in this work.

Bro-Jorgensen and Jensen, (1971) reported that gonorrhea is associated with trichomoniasis, because both organisms are sexually transmitted (John, 1989). Only 3 women amongst those who were positive for trichomonaisis infection were positive for gonorrhea infection.

Good personal hygiene could have contributed to reducing the occurrence of the infection, and good eating habit according to Rosemarie (1985) investigation that eating of food that does not lower the acidity within the body system helps ward off the infection, and she recommended yoghurt due to its lactobacilli content for women, this helps minimizing trichomonaisis and candidiasis since these infectious organism are associated with a lowered acidity.

In conclusion, trichomoniasis should be treated when noticed immediately to avoid tubal infertility and ectopic pregnancy (Goyal et al., 2011; Anate, 1991). Good hygiene should be practiced among women. Women should also maintain good and healthy eating habit, premarital sex should be discouraged. Use of condom is advisable. Self-medication should be avoided among women, they should seek the help of health care givers where necessary. Through this (Fernando et al., 2011) laboratory investigations are done to detect this organism and appropriate drugs would be administered. Good cleaning and disinfecting habits are advised, especially in the use of toilet and wears, example towels. 200 mg Metronidazole (Flagyl) T.D.S for 7-10 days not in early pregnancy is suggested. Likewise 5 mg pentrance or Clotrimazole is recommended.

Conflict of Interests

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

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REFERENCES

Al-Saeed WM (2011). Detection of Trichomonas vaginalis by different methods in Women from Dohok province. Eastern Mediterranean Health J. 17(9):706-709.

Abdurehman E, Zeleke M, Ahmed Z (2013). *Trichomonas vaginalis* Infection among Pregnant Women in Jimma University Speciallized Hospital, Southwest Ethiopia, Infectious Dis. 2013: 439-444.

Adeoy IM, Jok A, Umeora OJ, Okonta PI (2009). "The prevalence of *Trichomonas vaginalis and Candida alb*icans infections in the lower genital tracts of antenatal patients in Abakaliki Southerneastern Nigeria." Published In: Nepal J. Obstetrics Gynaecol. 4(1):11-14.

ACOG Technical Bulletin on "Vaginitis" (1996). Published In: American Family Physician 54(6):293-302.

Anate M (1991). "The infertility couple problem: Role of family planning choices." Published In: Nigeria Medical Practitioner. 21: 26-31.

Barnes RC, Holmes KK (1984). ''Epidemiology of Gonorrhea Current Perpectives. ''Published In: Epidemiologic Rev. 6: 1-30.

Benenson AS (1985). "Control of communicable Diseases in Man,"Published In: Am. Public Health 6:106 -111.

Bro- Jorgensen A, Jensen I (1971). "Gonorrhea tonsillar infections. "Published In: Br. Med. J. 4: 660.

Evans BA, Bond PD, Mac Rae KD (1995). "Heterosexual relationships and condom use in the spread of sexual transmitted diseases to woman." Published In: Genito - Urinary Medicine 71(5): 291-294.

Evans TN (1976). "Sexually Transmissible Diseases." Published In: Am .J Obstet Gynecol. 125:116-131.

Fernando SD, Herath S, Rodrigo C, Rajapakse R (2011). Improving diagnosis of *Trichomonas vaginalis* infections in resource limited healthcare settings in Sri lanka, J. Global Infectious Dis. 3(4): 324 328.

Ferrer J (2000). "Vaginal Candidiasis:epidemiological and etiological factors."Published In: Int. J. Gyn Obstet. 71: 521-527.

Goyal M, Hayes K, McGowan KL, Fein JA, Mollen C (2011). Prevalence of *Trichomonas vaginalis* infection in Symptomatic Adolescence Females presenting to a Pediatric emergency department. Soc. Acad Emerg. Med. 18: 763-766.

Honigberg BM (1978a). Trichomonads Parasitic in human. pp 53-54 Spring-Verlag, New York.

Honigberg BM, Mattern CFT, Warton A (1983). "Fine Structure of *Trichomonas vaginalis* in relation to that of other trichomonads. Published In: *Wiadomosci Parazytologiczne* vol. 29:5- 7.

Joesoef MR, Wiknjosastro G, Norojono W, Sumampouw H, Linma M, Hansell MJ, Hills SE, Lewis J (1996). "Co - infection with Chlamydia and Gonorrhea among pregnant woman and bacteria Vaginosis."Published In*:* Int. J. Sexual Transmitted Dis. Acquired Immuno - Deficiency Syndrome 7(1): 61 -64.

John BW (1989). Pathology of Human Disease. pp. 244 - 246, Phliadelphia. London press USA.

Juliano C, Rubino S, Ziccon D. Cappuccinelli P (1986). "An immunofluorescen study of the microtubule organization in *Trichomonas vaginalis*."Published In: J. Prot. 33:56 -59.

Kannel WB, Levine BS (2003). "Candida infection as a risk factor for HIV transmission."Published In: J. Womens Health 12(5):487-494.

Kulda J, Nohyynkova E (1978). "Flagellates of human intestine and of intestines of other species."Published In: In Parasitic Protozoa 2:132-138.

Matini M, Rezaie S, Mohebali M (2012). Prevalence of *Trichomonas vaginalis* infection in Hamadan City, Western Iran, Iran J. Parasitol. 7(9):67-72.

Ogbona CI I, Ogbona IB, Ogbona AA, Anosike JC (1991). "Study on the incidence of *Trichomonas vaginalis* among pregnant women in Jos area of Plateau State, Nigeria."Published In: Angew Parasitology 32(4):198-204.

Perazzi BE, Menghi CI, Coppolillo EF (2010). Prevalence and Comparison of diagnostic methods for *Trichomonas vaginalis* infection in pregnant women in Argentina, 'Koran J. Parasitol. 48(1):61- 65.

Quinn TC, Holmes KK (1984). Trichomoniasis. Published In: Tropical and Geographical Medicine 41: 335- 341.

Rosemarie MH (1985). Human Sexuality, A nursing Perspective (2). Apple Ton Cenccrofts. A Publishing Division of Prentice - Hall. Inc. p. 52.

Smyth JD (1994). Animal Parasitololgy, Cambridge University Press UK, pp. 256- 270.

Sobel JD (1997). Vaginitis. Published In: N Engl. J. Med. 337(26): 1896 - 1903.

Taub W (1976). Sex and Infection : Veneral Diseases. Published In : Sadock B J. 21 :318-325.

World Health Organisation (WHO) (2001). Global Prevalence and Incidence of selected curable sexually transmitted infections. p. 10.

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Full Length Research Paper

Influence of acetosyringone concentration on induction of carrot hairy root by *Agrobacterium rhizogenes*

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Carrot hairy root induction by two different strains of *Agrobacterium rhizogenes*- MTCC- 532 and MTCC- 2364 grown in yeast extract mannitol agar medium (YEMA) was studied in carrot (*Daucus carota*). The maximum hairy root induction was observed when carrot was simmered with 48 h old culture of *A. rhizogenes* MTCC-532. With different concentrations of acetosyringone (AS) tested, addition of 150 µM acetosyringone was found to enhance the transformation frequency up to 75(±2.60)[a] and 60 (±2.08)[a] percentage by *A. rhizogenes* MTCC-532 and MTCC 2364 strains, respectively. Transformation efficiency was highly dependent on the acetosyringone concentrations, type of bacterial strains and carrot genotype. Transfer of Ri Ti-DNA was confirmed by polymerase chain reaction (PCR) analysis, the detection of *ags* gene in transformed carrot hairy root. *A. rhizogenes* transformed hairy roots had the ability to form copious lateral roots as well as a negative geotropic growth habit in a shorter period of time. The Murashige and Skoog (MS) medium was found to be the best medium for hairy root mass multiplication, which induced high root biomass production and rapid root tip elongation.

Key words: Carrot, *Agrobacterium rhizogenes,* acetosyringone, hairy roots, Murashige and Skoog (MS) medium, arbuscular mycorrhizae.

INTRODUCTION

Abundant studies have demonstrated that arbuscular mycorrhizal (AM) fungi are obligatory symbionts which colonize the roots of approximately 80% of terrestrial plants (Lekberg and Koids, 2005) and improve the nutrient mobilization from soil, plant growth and disease tolerance (Douds and Siedel, 2012; Elsen et al., 2008).The presently available open pot culture methods for mass production of arbuscular mycorrhizae is having limitations like high cross contamination, being time consuming process; besides, only small amount of inoculum production is acheived. To overcome this problem, *in vitro* cultivation of AM fungi by root organ culture seems to be promising. Mosse and Hepper (1975) first proposed the use of root organ cultures with excised roots as host partner in AM symbiosis. The *Agrobacterium rhizogenes* is a well known tumor inducting, Gram-negative soil bacterium, which is able to induce hairy roots rapidly at the infection site (Baranski, 2008). The *agrobacterium*

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mediated transformation frequency is based on *vir* gene expression (Mohiuddin et al., 2011). Transcription of high level *vir* gene expression is induced by acetosyringone released by wounded plant cells and it has been reported that the compound increases *agrobacterium* mediated transformation frequencies in a number of plant species (Kumar et al., 2006). Genetically modified carrot (*Daucus carrota* L.) roots, show profuse lateral branching and rapid root tip elongation within two to three weeks. Transformed hairy roots are genetically and biosynthetically stable for long periods (Sawsan et al., 2012). The negative geotropism of transformed roots facilitates contacts with hyphae of AM fungi. The success of *in vitro* cultivation on AM fungi depends on host partner growth.

Therefore, the objective of the study was to optimize the acetosyringone concentration, a wound response molecule known for enhanced hairy root formation for maximum transformation efficiency and also to select suitable *A. rhizogenes* strains for maximum hairy root induction in carrot.

MATERIALS AND METHODS

Preparation of bacterial strain

The strains of *A. rhizogenes* such as MTCC-532 and MTCC-2364 were used for induction of hairy root. The *A. rhizogenes* strains obtained from microbial type culture collection (IMTCC), Chandigarh, India, were grown in yeast extract mannitol agar medium (YEMA), which comprised (g/l) of mannitol -10, Yeast extract -0.5 g, Sodium chloride - 0.1, magnesium sulfate- 0.2, $K_2H PO_4$- 0.5 g, agar - 20, distilled water- 1000 ml, finally adjust pH-6.8.

Carrot discs preparation

The commonly cultivated carrot cultivar Ooty-1 obtained from Kavi farm, Santhur, Nilgiris, Tamil Nadu, India, was used as an experimental material. Freshly harvested carrots were washed 2 to 4 times thoroughly with tap water. Then they were surface sterilized with 0.1% $HgCl_2$ for 10 min with continuous stirring. They were further rinsed three times (each for 5 min) with sterile distilled water and dipped in 70% ethanol for 30 s and superficially flamed and peeled out. Each carrot was sliced into 0.5 cm thick discs and was placed on 0.5% MS (half strength) (Murashige and Skoog, 1962) plates with the basal sides facing upwards (Figure 1). The sterile needle was used to prick manually for wounding on carrot surface. For pre-cultivation, the plates were incubated at 28°C in dark for 24 h.

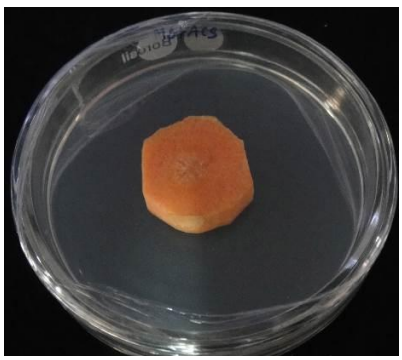

Figure 1. Pre-cultivation of carrot discs.

Co-cultivation and *agrobacterium* mediated transformation

After completion of pre-cultivation, the carrot discs were placed on sterilized Whatman No 1 filter paper in Petri dish to remove excess moisture present on the surface of the carrot discs. The carrot discs were simmered with a loopful of 48 h old bacterial suspension MTCC-532 and MTCC 2364, inoculated on the basal side of carrot discs for infection. Control carrot discs were simmered in an uninoculated YEMA broth. Then the discs were transferred to 1% MS (full strength) basal medium containing four different concentrations of acetosyringone (AS) (Sigma Aldrich) *viz.,* 50, 100, 150 and 200 µM, which were added separately to the media before plating. The control treatment plate was kept without addition of acetosyringone compound. Then the plates were incubated at 28°C under darkness for two to three weeks.

Conformation of transformed hairy root by PCR analysis

Carrot hairy root were cut into the small pieces and the genomic DNA was isolated from transformed carrot hairy root line by CTAB methods (Doyle and Doyle, 1990). The PCR was performed to amplify T-DNA *agropine synthase* (ags) transformed hairy root. The specific primer used to amplify *ags* genes were forward primer (5-GCGCATCCCGAGGCGAT-3) and reverse primer (5-AGGTCTGGCGATCGCAGGA-3). PCR amplification was performed with a program of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min. annealing at 55°C for 1 min, extension at 72°C for 1 min. and a final extension at 72°C for 7 min and storage at 4°C. The amplification was analyzed by agarose gel electrophoresis. Plasmid DNA from *A. rhizogenes* strains MTCC-532 and 2364 was used as a positive control.

Mass production of hairy roots

After two to three weeks of incubation, carrot discs showed profuse white, turgescent and non-ramified apexes hairy roots. Fresh hairy roots were cut into minimum 3 cm long and then transferred to MS medium containing plates and incubated in inverted position under dark at 27°C. Bacteria free hairy root were obtained by subsequent subculture three times in fresh MS medium containing, cefotaxime at 250 mg/l (HiMedia, Mumbai, India) to make it free of *A. rhizogenes.* The bacterial free hairy roots could be used for *in vitro* mass production of AM fungi.

Statistical analyses

All the data were subjected to statistical analysis with software, Microsoft Excel for Windows 2007 add-in with XLSTAT Version 2010.5.05 (XLSTAT, 2010). Statistically significant differences between the treatments were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at a 5 % significance level.

RESULTS

Transformation

After 5 to 10 days of co-culture with *A. rhizogenes*, callus initiation (Figure 2.) was observed on the surface of carrot discs, followed by appearance of the transformed roots on the side wall of discs (Figure 3). Hairy root initiation continued to occur from 10 days to two to three weeks. A typical hairy root was formed quickly from

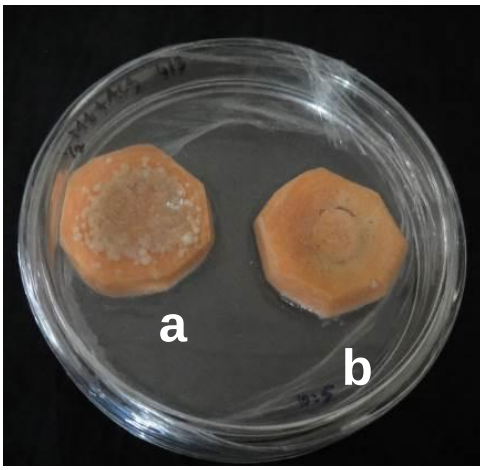

Figure 2. a- Co-cultivation of carrot with *A. rhizogenes and* callus formation; b- Uninoculated control.

Figure 3. Initiation of hairy root on carrot discs.

Figure 4. Growth of hairy root on carrot discs.

numerous lateral roots (Figure 4.), which grew both on the surface and penetrated into the media, exhibiting the

Figure 5. Negative geotropism of hairy root .

negative geotropic growth habit (Figure 5). Some carrot discs were observed without any hairy root induction. This may be due to instability of the new genome or due to non-expression of genes involved in root induction. The carrot discs produced hairy roots up to three weeks and later on rotting was noticed. Among the four different concentrations of acetosyringone tested, 150 µM was the most effective in enhancing hairy root induction from both strains, as compared to the control. The maximum hairy root induction percentage (75±2.60[a]), number of lateral roots (12±0.42[a]) and number of negative geotropic roots (9±0.31[a]) was observed from carrot discs simmered with 48 h old *A. rhizogenes* MTCC-532 on MS plates supplemented with 150 µM acetosyringone (Table 1). In the *A. rhizogenes* MTCC 2364 strain used for same condition only (60±2.08[a])) percentage of hairy root initiation, (9 (0.31[a])) number of lateral roots and (5 (0.17[a])), number of negative geotropic roots was observed. The transformation frequency declined at both lower (<150 µM) and higher concentrations of aceto-syringone (Table 2). These transformed hairy roots were cut around 3 cm long roots and transferred to a sterile hormone-free MS medium (Figure 6). The trans-formed carrot hairy root was multiplied as per the above procedure described in Materials and Methods (Figures 7 and 8). Hairy roots were maintained with regular sub culturing at three weeks interval.

Detection of *agropine synthase* (*ags*) gene in transformed carrot hairy root

PCR analysis was done using a pair of gene specific primer (forward and reverse) which amplifies the T-DNA *agropine synthase* gene. The total genomic

Table 1. Effect of *A. rhizogenes* (MTCC-532) strain with their ability to induce carrot hairy roots on various concentrations of acetosyringone.

Acetosyringone (µM)	*Agrobacterium rhizogenes* (MTCC-532)				
	Total no. of carrot discs	No of discs inducing hairy root	Transformation rate (%)	Number of lateral roots	No of roots showed negative geotropism
Control	20	03	15 (±0.52)^{d}	2(±0.07)^{e}	0 (±0.00)^{d}
50	20	05	25 (±0.87)^{cd}	4(±0.14)^{d}	1 (±0.03)^{d}
100	20	07	35 (±1.21)^{bc}	7(±0.24)^{c}	3 (±0.10)^{d}
150	20	15	75 (±2.60)^{a}	12(±0.42)^{a}	9 (±0.31)^{a}
200	20	08	40 (±1.39)^{b}	10(±0.35)^{b}	6 (±0.21)^{d}

Values are mean (±SE) (N=20) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$).

Table 2. Effect of *A. rhizogenes* (MTCC-2364) strain with their ability to induce carrot hairy roots on various concentrations of acetosyringone.

Acetosyringone (µM)	*Agrobacterium rhizogenes* (MTCC-2364)				
	Total no. of carrot discs	No. of discs inducing hairy root	Transformation rate (%)	Number of lateral roots	No. of roots showed negative geotropism
Control	20	03	10 (±0.35)^{c}	1 (±0.03)^{e}	0 (±0.00)^{d}
50	20	05	15 (±0.52)^{c}	3 (±0.10)^{d}	1 (±0.03)^{d}
100	20	07	30 (±1.04)^{b}	5 (±0.17)^{c}	2 (±0.07)^{d}
150	20	15	60 (±2.08)^{a}	9 (±0.31)^{a}	5 (±0.17)^{a}
200	20	08	35 (±1.21)^{b}	7 (±0.24)^{b}	3 (±0.10)^{d}

Values are mean (±SE) (N=20) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$).

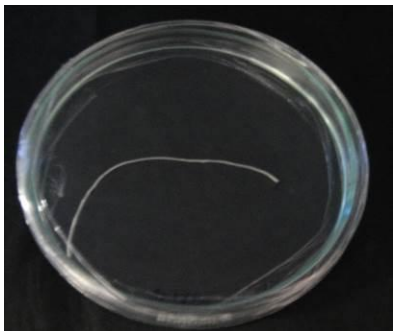

Figure 6. Transformed hairy root on MS medium.

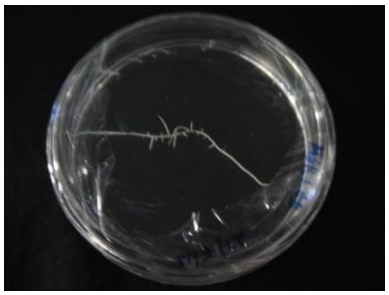

Figure 7. Lateral branches formation after 3 days of incubation.

Figure 8. Hairy root proliferation.

DNA was extracted from the transformed carrot hairy roots to observe the presence of Ri Ti-DNA. Also, for positive control of PCR analysis, the total genomic DNA was extracted from *A. rhizogenes* and resolved in agarose gel electrophoresis. The primer showed amplification, which confirmed the successful transformation of T-DNA *agropine synthase* gene, which produced the band of size approximately 341 bp (Figure 9).

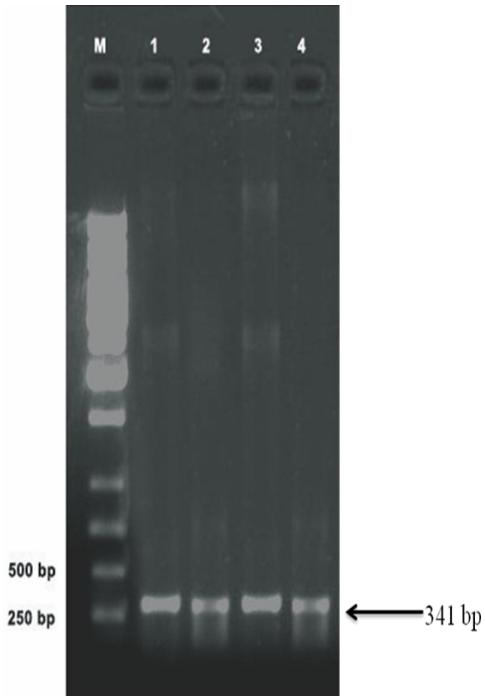

Figure 9. Confirmation of hairy root induction by *A. rhizogenes* using *ags* gene detection by PCR. M-1 kb ladder; 1- *A. rhizogenes* 532 genomic DNA (positive control); 2- *A. rhizogenes* 532 mediated carrot hairy root; 3- *A. rhizogenes* 2364 genomic DNA (positive control);4- *A. rhizogenes* 2364 mediated carrot hairy root.

DISCUSSION

Carrot is one of the most suitable and well known model plant species for hairy root production (Bidondo et al., 2012). So far, the co-cultivation of arbuscular mycorrhizal fungi with carrot hairy root was used because of their easy propagation and fast growth rate over normal root (Yinli et al., 2004). Carrot hairy root are better adapted to low level of nutrient content and survive for long time without sub culturing than normal root (Saravanakumar et al., 2012).

Carrot hairy roots have been used to initiate monoxenic culture of AM fungi since 1988. Suitable methods for their maximum hairy root induction in shorter period of time and mass multiplication has not been described yet. In this present study, it was observed that *A. rhizogenes* strain MTCC-532 showed higher transformation efficiency and higher lateral branching, more number of negative geotropic roots as compared to MTCC-2364 strain, so that transformation efficiency is highly dependent on the type of bacterial strain used. This result was supported by Ahlawat et al. (2012), who used different *A. rhizogenes* strains like LBA 9402, LBA 920, LBA 301, MTCC 532, NRRL B193, A4 for induction of *Artemisia annua* L. hairy root. Among the strains, LBA 9402 showed 100% transformation frequency within five to six days of hairy root induction. Inoculation of carrot discs with 48 h old culture and incubation in darkness at 28°C provided a suitable condition for bacterial strains to insert their maximum copies of Ri t-DNA.

The present findings is in line with those reported by Ridgway et al. (2004), who used 24 h old culture of *A. rhizogenes* (A4T) strain and achieved 53% of hairy root induction when freshly harvested carrot was used as explants. Among the different concentration of acetosyringone used in our study, specific concentration (150 µM), which played a role in enhancement of virulence *vir* gene activity of two different *A. rhizogenes* strains, resulted in enhanced transformation frequency of carrot explants. These findings are supported by Mohiuddin et al. (2011), who reported that acetosyrin-gone is an amino acid derivative and a phenolic compound, which must be constant for biological activity and maintenance of *vir* gene expression in Muskmelon explants. Many other reports also pointed out that the *vir* genes are inducible in response to the monocyclic phenolic compound like acetosyringone (Shaw et al., 1988, Ridgway et al., 2004). The results obtained from the higher (200 µM) and lower (100 µM) concentration of acetosyringone indicated an inhibitory action rather than stimulatory.

Similar result was also observed by Kumar et al. (2006) in *Nicotiana tabacum* with high concentration (>200 µM) of acetosyringone to reduces the transformation efficiency. Freshly harvested carrots are invariably better in initiating hairy roots overall. This may be due to the active nature or less dormant nature of the carrots. However, hairy root induction also depends on the carrot genotype and origin of plant (Danesh et al., 2006). Molecular analysis of carrot hairy root through PCR was done using a pair of primer, which amplified the T DNA *agropine synthase* gene and confirmed the transformation at 341 bp segment. Similar primer was also used by Gartland et al. (2001) and Rajkumar and Murugesan (2014) in *Ulmus procera, Psoralea corylifolia* hairy root respectively to confirm the transformation. The confirmation of *rolC* gene transfer from *A. rhizogenes* R1000 by PCR detection at 557 bp was also reported in *Withania somnifera* L. (Saravanakumar et al., 2012). The MS medium has been used for growing transformed roots, because it allows significantly better growth and rapid root tip elongation.

The same trends was observed by Pratap Chandran and Potty (2010), where in different media like modified whites' medium, MSR medium, MS medium were used for hairy root multiplication. Among the media, MS salts with B5 vitamins were used for successful mass production of carrot hairy root. Similar result also observed by Diop (2003) pointed out that MS medium is the most suitable medium used for mass production of hairy root for *in vitro* culture of AM fungi.

Conclusion

This study demonstrates the ability of *A. rhizogenes* (MTCC-532) strain and 150 µM concentration of acetosyringone combination showed maximum hairy root induction and growth under *in vitro* conditions. The potential role of *Agrobacterium* strain and host explant genotype in hairy root induction is of great scientific interest, which may allow the rational manipulation of hairy root biomass production on large scale to develop monoxenic culture of AM Fungi.

Conflict of Interests

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

REFERENCES

Ahlawat Sl, Saxena P, Ram M, Alam P, Nafis T, Mohd A, Abdin Z (2012). Influence of *Agrobacterium rhizogenes* on induction of hairy roots for enhanced production of artemisinin in *Artemisia annua* L. plants. Afr. J. Biotechnol. 11(35):8684-8691.

Baranski R (2008). Genetic transformation of carrot (*Daucus Carota*) and other apiaceae species. Transgenic Plant J. 12(1):19-36.

Bidondo LF, Pergola M, Silvani V, Colombo R, Bompadre J, and Godeas A (2012). Continuous and long-term monoxenic culture of the arbuscular mycorrhizal fungus *Gigaspora decipiens* in root organ culture. Fungal Biol. 116(6):729-735.

Danesh YR, Mohammadi E, Goltapeh Alizadeh A, Modarres Sanavy M (2006). Optimizing carrot hairy root production for monoxenic culture of arbuscular mycorrhizal fungi in Iran. J. Biol. Sci. 6:87-91.

Diop TA (2003). *In vitro* culture of Arbuscular mycorrhizal fungi: advances and future prospects. Afr. J. Biotechnol. 2:692-697.

Douds DD, Seidel R (2012). The contribution of arbusclar mycorrhizal fungi to the success or failure of agricultural practices In C.A. Edwards et al.(eds.) Microbial ecology in sustainable Agroecosystems. Boca Raton, FL: Taylor Francis Group. pp. 133-152.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Elsen AD, Gervasio R, Swennen S, Waele D (2008). AMF-induced biocontrol against plant parasitic nematodes in *Musa* sp.: a systemic effect. Mycorrhiza 18 (5):251-256.

Gartland JS, Brasier CM, Fenning TM, Birch R, Gartland KMA (2001) Ri-plasmid mediated transformation and regeneration of *ulmus procera.* Plant Growth Regul. 33:123-129.

Kumar V, Sharma A, Prasad BCN, Gururaj HB, Ravishankar GA (2006). *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone treatment. Plant Biotechnol. 9:1-8.

Lekberg Y, Koide RT (2005). Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2000. New Phytol. 168:189-204.

Mohiuddin KM, Abdullah C, Harikrishna K, Chowdhury K, Napis S (2011). Enhanced Virulence Gene Activity of *Agrobacterium* in Muskmelon (Cucumis melo L.) cv. 'Birdie'. Not. Sci. Biol. 3(2):71-79

Mosse B, Hepper CM (1975). Vesicular- arbuscular infections in root-organs cultures. Physiol. Plant. Pathol. 5:215-223.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Pratap Chandran R, Potty VP (2010). *In vitro* co culture of VAM fungi *clomus microcarpum* in Ri T-DNA transformed hairy root of *vigna vacillate*. Int. J. Agric. Environ. Biotechnol. 3:333-338.

Rajkumar D. and Murugesan R (2014). Hairy root induction of *Psoralea corylifolia* for enhanced production of antifungal compound against red rots pathogen *Colletotrichum falcatum*. Afr. J. Agric. Res. 9:755-765.

Ridgway HJ, Kandula K, Stewart A (2004).Optimizing production of carrot hairy roots. N. Z. Plant Prot. 57:77-80

Saravanakumar A, Aslam A, Shajahan A (2012). Development and optimization of hairy root culture systems in *Withania somnifera* (L.) Dunal for withaferin-A production. Afr. J. Biotechnol. 11(98):16412-16420.

Sawsan AAE, Abdel Rahman RA, Mazen MBH, El-Enany AE, Allam N (2012). Biotechnological Aspects for VAM Aseptic Mass Production. World Appl. Sci. J. 17 (1):20-28.

Shaw CH, Ashby AM, Brown A, Royal C, Loake GJ (1988). *VirA* and *virG* are the Ti-plasmid functions required for chemotaxis of *Agrobacterium tumefaciens* towards acetosyringone. Mol. Microbiol. 2:413-417.

XLSTAT (2010) XLSTAT. Addinsoft SARL, Paris. http://www.xlstat.com

Yinli BL, Xiaolin L, Honggang W, Christie P (2004). Establishment of monoxenic culture between the arbuscular mycorrhizal fungus *Glomus sinuosum* and Ri T-DNA-transformed carrot roots. Plant Soil 261:239-244.

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African Journal of Microbiology Research

Full Length Research Paper

Production, purification and characterization of polysaccharide lytic enzymes of a marine isolate, *Bacillus cereus* NRC-20 and their application in biofilm removal

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In screening for marine bacterial polysaccharide lytic enzymes, the most potent isolate was identified as *Bacillus cereus* NRC-20. This strain showed high alginase, dextrinase, pectinase and carboxymethyle-cellulase productivity when production medium was adjusted at pH 6.0 and contains (g/l): 3.0 sodium alginate, 3.0 malt extract, 30.0 jatropha cake and inoculated with 8% (v/v) of a 24 h old cell suspension and incubated at 30°C for 48 h on a rotary shaker at 200 rpm. These enzymes mixture were precipitated by 60% acetone and purified by using sephadex G-100, whereas SDS-PAGE suggested a molecular weight of approximately 17, 20, 89 and 279 KDa, respectively. The optimum partially purified enzymes activity when enzyme protein concentration was 27.4 mg/ml and substrate concentration 5.0 g/l shown after 90 min of incubation at 30°C and pH 5 and stable at 60°C for 60 min. The enzymes activities were enhanced in the presence of 0.02 M Na^{+} and K^{+} ions and completely inactivated by Mg^{+2}, Cu^{+2}, Co^{+2} and Pb^{+2} The enzymes mixture affected the matrix integrity of different microbial biofilms artificially grown on stainless steel sheets of *Bacillus subtilis* NRRL B-4219, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27953 and *Escherichia coli* ATCC 25922.

Key words: *Bacillus cereus*, polysaccharide lytic enzymes production, fermentation parameters, enzyme characterization, biofilm removal.

INTRODUCTION

The structure of biofilm matrix is a mixture of micro-colonies with water channels in-between and an assortment of cells and extracellular polymers (polysaccharides, glycoproteins and proteins (Christensen, 1989;

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Abbreviations: DNSA, Di-nitrosalicylic acid; **EPS,** extracellular polysaccharides; **PPE,** partially purified enzymes; **CMC,** carboxymethyle cellulose.

Table 1. Different media composition involved in enzymes production.

Different media used	Composition (g/l sea water)
Med.1 (Hansen et al., 1984)	Yeast extract 5.0, sodium alginate 5.0, 0.6mM $MgCl_2$
Med.2 (Hansen et al., 1984).	Peptone 5, Meat extract 1.0, Yeast extract 2, Sodium chloride 5.0,sodium alginate 5.0
Med.3 (Alexeeva et al., 2002)	bacto-peptone 2.0, casein hydrolysate 2.0, yeast extract 2.0, glucose 1.0, K_2HPO_4 2.0, $MgSO_4$ 0.05, 50% sea water, 50% distilled water
Med.4 (Manyak et al., 2004).	Yeast extract 1.0, sodium alginate 2.0
Med.5 (Hansen and Nakamura, 1985).	Peptone 5.0, Meat extract 1.0, Yeast extract 2, Sodium chloride 5.0, sodium alginate 1.0, 30 mM $MnSO_4$
Med.6 (Oldak and Trafny, 2005; Jain and Ohman, 2005).	Tryptone 1.0, yeast extract 0.5, NaCl 0.5

Flemming et al., 1992; Costerton et al., 1995). Biofilms can cause many problems in industry such as, increased frictional resistance to fluids on water conduits and in ship hulls (fouling), decreased heat transfer from heat exchangers, corrosion of metallic substrata, contamination of foods in food industry (Johansen et al., 1997; Xiong and Liu, 2010).

Mechanical cleaning (scrubbing, sonication, freezing and thawing) of biofilms can be costly and biocides are ineffective due to the reduced susceptibility and acquired resistance of microorganisms in biofilms to antimicrobial agents (Walker et al., 2007). Also, excessive use of antimicrobials could be toxic and damaging to the environment (de Carvalho, 2007). The use of polysaccharide lytic enzymes for biofilm removal is one of the attractive strategies for biofilm problem elimination. Enzymes remove biofilms by destroying the physical integrity of the extracellular polysaccharides (EPS) matrix where a mixture of hydrolytic enzymes could be employed to degrade the biofilm matrix resulting in complete removal of biofilms and enhancement of the antimicrobial activity (Manyak et al., 2004; Xavier et al., 2005).

Numerous bacteria can produce polysaccharide lytic enzymes, but most are marine bacteria (Manyak et al., 2004). Marine bacteria of the genus *Bacillus* are known to produce both antimicrobial metabolites and enzymes (Ivanova et al., 1999; Peterson et al., 2006).

Microbial EPS lytic enzymes are a wide range of enzymes most of which are highly specific. These specific polysaccharide-degrading enzymes, when applied on a polysaccharide matrix can yield oligosaccharide fragments, which are amenable to NMR and other analytical techniques. Complex systems containing various mixtures of enzymes may also be effective in many applications such as biofilm removal from different surfaces preventing health problems (Chen and Stewart, 2000; Donlan and Costerton, 2002; Manyak et al., 2004; Xavier et al., 2005; Johanson et al., 2008; Häussler and Parsek, 2010).

The aim of this study was to obtain marine bacterial isolates from different localities in Egypt that have the maximum production and activity of polysaccharide lytic enzymes which are effective in the degradation and removal of bacterial biofilm that are most commonly found causing problems in many medical and industrial fields.

MATERIALS AND METHODS

Microorganisms and culture conditions

Twenty eight (28) bacterial isolates were obtained from different localities of marine sea water in Egypt (Ain-Sokhna-Hurghada-Sharm Elsheikh) and were tested for their productivity of extracellular polysaccharide lytic enzymes. The growth medium used was nutrient agar which is composed of (g/l): yeast extract 2.0, meat extract 1.0, peptone 5.0, NaCl 5.0 (0.8 M), agar 15.0 and supplemented with sodium alginate 5.0 and adjusted at pH 7. For the production of alginase enzyme, the following minimal medium (Manyak et al., 2004) was used (g/l sea water): yeast extract 1.0 and sodium alginate 2.0 and pH was adjusted to 7.0. 5 ml of the bacterial isolate suspension (72 h old focus 7×10^6 cfu/ml) was added to 250 ml shake flask containing 45 ml of the medium, the culture was incubated at 30°C for 48 h under shaking (200 rpm). The culture supernatant was collected by centrifugation at 6000 rpm for 10 min and is considered to be the crude polysaccharide lytic enzymes mixture. Supernatant was frozen until use and culture was run in duplicate.

Pseudomonas aeruginosa ATCC 27953, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* NRRL B-4219 as biofilm producing bacteria were obtained from American Type Culture Collection, Rockville, MD and Northern Regional Research Laboratory, Peoria, IL, United State. The strains were maintained in nutrient agar.

Optimization for maximum enzyme production

The different parameters were tested to obtain the maximum enzyme production including: different fermentation media (Table 1), different carbon source concentrations of the most potent fermentation medium and different nitrogen sources (yeast extract, casein hydrolysate, peptone, beef extract, urea, malt extract, tryptone, soy bean, N-acetylglucosamine, ammonium chloride, ammonium sulphate and/or sodium nitrate (at equimolecular basis to yeast extract present in the original medium) and different medium pH (4-9) was adjusted by acetate, phosphate and/or carbonate-bicarbonate buffers. Different agricultural waste material additives (rice shell, rice stem, molasses, jatropha cake, apple

peels, carrot peels and/or potato peels) were used as an inducing waste material. Finally the optimum medium was inoculated with different inoculums size. Also, incubation temperature (25-40°C), time of incubation (24-27 h) under different agitation speeds were evaluated.

Determination of polysaccharide lytic enzyme activity

All enzymatic activities in culture supernatant were determined at 30°C. All assays were run in triplicate, standard deviations of mean of subsamples was calculated by Microsoft excel program for all results obtained.

To assay the activity of polysaccharide lytic enzymes (alginase, dextrinase, pectinase and carboxymethyl cellulase (CMCase): 0.2 g of one of the following polysaccharides separately and respectively, sodium alginate, dextrin, pectin and CMC dissolved in 100 ml of 0.2 M phosphate buffer (pH 7) were used as substrates (Sumner and Sisler, 1944). The reaction mixtures was started by mixing 0.3 ml of enzyme source with 0.7 ml of substrate solution, and was stopped after 1 h of incubation, by adding 1 ml of dinitrosalicylic acid reagent (2.14% NaOH, 0.63% 3, 5-dinitrosalicylic acid, 0.5% phenol, all from Sigma) and immersion of the test tubes in a boiling water bath for 5 min. After cooling in tap water, absorption was measured at 575 nm. One unit enzyme was expressed as 1 µg/ml reducing sugar equivalent to D-glucose librated as an indication of enzyme activity per reaction time (Manyak et al., 2004).

Estimation of total protein

The total protein of the enzymes mixture was determined using the method of Lowry et al. (1951) with Bovine Serum Albumin (BSA) as a standard.

Partial purification and characterization of the lytic enzymes

Fractional precipitation with acetone was carried out by slowly adding with stirring a certain volume of cold acetone to 1 L of the culture filtrate (kept in an ice bath) until the required concentration of acetone was reached. Then after removing the precipitated fraction by centrifugation at 10.000 rpm for 15 min in a cooling centrifuge at 4°C, further acetone was added to the remaining supernatant to reach the next desired concentration and the process was repeated until the final concentration of the precipitant reached 100% (v/v). Several enzyme fractions were thus obtained at the corresponding concentration. The precipitate was dissolved in 1 ml of phosphate buffered solution. Enzyme activity and protein content of the concentrates were determined.

To examine the influence of several factors affecting their activities, the partially purified enzyme at different protein concentrations (13.7-48 mg/ml) were incubated each with the different specific substrates separately at different incubation temperatures ranging from 25-40°C for different time intervals (30-150 min) and at different degrees of pH (4-9) by using different buffer systems(0.2 M) such as acetate buffer (pH 4.0 and/or 5.0), phosphate buffer (pH 6.0, 7.0 and/or 8.0) and carbonate-bicarbonate buffer (pH 9.0). The enzymes mixture was added at different protein concentrations (13.7-48 mg/ml) to the respective substrates, evaluating the effect of enzymes protein concentration on enzymes activities.

The effect of substrate concentration on enzymes activities was tested whereas, the enzymes mixture was added to each of the specific substrates separately (sodium alginate, dextrin, pectin and/or CMC), in concentrations ranging from 2.0 to 10.0 g/l. Also, the effect of addition of metal ions: Na^{+}, K^{+}, Mg^{++}, Cu^{++}, Co^{++} and Pb^{++} as chloride salts (0.02 M) on enzymes activities were tested.

The molecular weights of the different polysaccharide lytic enzymes (after fractionation of enzymes on sephadex column G-100) were determined by native SDS-PAGE using standard bovine serum albumin protein marker (18-116 KDa), protein contents were adjusted to 2 mg/ml per sample.

Biofilm formation and removal

Pseudomonas aeruginose ATCC 27953, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *B. subtilis* NRRL B-4219 were grown according to de Queiroz and Day (2007), a standardized suspension of *P. aeruginosa* (20×10^4 cfu/ml) and *E. coli*, *S. aureus* and *B. subtilis* (30×10^4 cfu/ml) was inoculated into flasks, containing 100 ml sterile trypton soy broth (TSB). Standard biofilms were obtained by attachment of bacterial cells on sterile stainless steel sheets (ALSI 304) (1 × 1 cm) that are used in the hospitals, clamped vertically to a Teflon carousel, which was placed inside the flasks.

The flasks were incubated at 30°C for six days with continuous agitation at 200 rpm. During the biofilm growth, about 25 ml of the inoculating medium was replaced and fed with an equal volume of sterile TSB every 48 h to keep the bacterial cells at the exponential phase. At 6 days, bacterial suspensions containing the planktonic cells were discarded from the flasks. The sheets were withdrawn and dipped into saline solution (0.9%, w/v) to discard loosely attached cell, before being used as standard biofilm samples in removal tests.

To assay the biofilm removal efficiency, an standardized *P. aeruginosa* and *E. coli, S. aureus* and/or *B. subtilis* biofilm sheets were incubated into a falcon tube with 10 ml of the partially purified enzymes mixture at 30°C for 30 min without agitation, the biofilms with no enzymes were used as control. After incubation period, the sheets were then soaked for 1 min in saline solution (0.9%, w/v) and the effect of enzymatic activity on the biofilm was evaluated using the scanning electron microscope.

Samples preparation for scanning electron microscopy

Stainless steel sheet samples were fixed with 6% glutaraldehyde in 0.2 mol l^{-1} cacodylate buffer, then samples were dehydrated in ethanol. After the drying step, samples were critically dried with CO_2 (Molobela et al., 2010). Samples were coated with gold/palladium in an Edwards S-150 sputter coater and visualized using a scanning electron microscope (Quanta EFG 250).

RESULTS

In this work, some local marine bacterial strains were tested for their productivity of extracellular polysaccharide lytic enzymes. From the positive strains, the bacterial isolate NRC-20 was selected for further studies whereas it grew well and produce high level of alginase activity (23.1 µg/ml), and this strain was identified as *B. cereus* NRC-20.

Interaction between production of polysaccharide lytic enzymes and environment

Different fermentation media were used for polysaccharide lytic enzymes production (Table 1) and results

Table 2. Effect of different fermentation media on the polysaccharide lytic enzymes production by *Bacillus cereus*NRC-20.

Medium no.	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
1 (Hansen et al.,1984)	0.18±0.10	33.3±0.03	23.9±0.1	164.4±0.04	59.0±0.06
2*(Hansen et al., 1984)	0.13±0.15	23.9±0.07	16.9±0.05	148.0±0.06	47.4±0.13
3 (Alexeeva et al.,2002)	0.37±0.18	30.9±0.12	25.8±0.05	170.9±0.03	55.6±0.06
4 (Manyak et al., 2004) (minimal medium)	0.18±0.09	35.5±0.08	30.7±0.07	172.6±0.10	71.8±0.09
5 (Hansen and Nakamura, 1985)	0.40±0.06	27.3±0.14	17.6±0.13	162.8±0.09	52.2±0.07
6 (Jain and Ohman,2005)	0.17±0.04	27.2±0.09	23.2±0.09	98.6±0.05	55.4±0.14

*Control

Table 3. Effect of different concentrations of sodium alginate on the polysaccharide lytic enzymes production medium by *B. cereus* NRC-20.

Sodium alginate (g%)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
0.1	0.16±0.07	22.8±0.15	18.2±0.09	104.8±1.05	46.8±0.04
*0.2	0.18±0.09	35.9±1.30	30.7±0.06	172.9±1.08	71.6±0.09
0.3	0.25±0.04	39.9±0.70	45.8±0.19	197.2±0.05	75.6±1.50
0.4	0.33±0.10	32.1±1.07	43.2±0.57	334.3±0.12	19.2±1.04
0.5	0.38±0.08	27.8±2.05	30.7±1.03	112.4±2.08	17.5±1.10
0.6	0.35±0.15	20.2±01.45	13.4±02.06	67.8±0.09	13.8±0.09

*Control.

indicated that, minimal medium no.4 containing only sodium alginate as a carbon source, yeast extract and sea water exhibited the highest potential for the production of alginase, dextrinase, pectinase and CMCase (35.5, 30.7, 172.6 and 71.8 µg/ml), respectively (Table 2).

The addition of the sodium alginate at concentration of 0.3% (w/v), as a sole carbon source to the fermentation medium resulted in a maximum production of alginase, dextrinase, pectinase and CMCase (39.9, 45.8, 197.2, 75.6 µg/ml) respectively. Increasing the concentration of sodium alginate up to 0.4 g % resulted in decreasing the enzymes production, while the amount of pectinase increased reaching 334.3 µg/ml (Table 3).

Results presented in Table 4 reveal that, organic nitrogen sources yielded better enzymes production than inorganic nitrogen sources. Malt extract (1g %) is the best nitrogen source used, producing values 78.1, 92.5, 282.1, 98 µg/ml for the activity of alginase, dextrinase, pectinase and CMCase, respectively.

The effect of addition of seven different industrial and agro-waste materials as an inducer agents on polysac-charide lytic enzymes production by *B. cereus* NRC-20 were studied using seven different agriculture and industrial waste materials rich in polysaccharides. The results indicated that the maximum enzymes production was obtained in the presence of jatropha cake (Table 5). Thus, different concentrations of jatropha cake (10-50 g/l) were used to evaluate the best concentration, maximum enzymes yield for the production of polysac-charide lytic enzymes were obtained at concentration of 30 g/l (Table 6).

Results from Tables 7, 8, 9 and 10 showed that the environmental condition for the maximum enzymes production was obtained when the fermentation medium was adjusted at pH 6.0 and inoculated with 8% (V/V) of the bacterial cell suspension and incubated at 30°C and 200 rpm for 48 h. The enzyme production was 83.6, 124.6, 292.6, 97.5 µg/ml for alginase, dextrinase, pectinase and CMCase, respectively (Table 11).

Partial purification of enzymes and its properties

The enzymes mixture was precipitate by using acetone 60%. For partially purified enzyme (PPE) assay conditions, increasing the reaction temperature up to 35°C increased the PPE activities (209.2, 229.4, 339.7 and 220.3 µg/ml) for alginase, dextrinase, pectinase and CMCase, respectively (Table 12).

Incubation time of PPE with their respective substrates influences the enzymes activities to a great extent as demonstrated in Table 13. Whereas, each enzyme activity of the PPE mixture reached its maximum after

Table 4. Effect of different nitrogen sources on polysaccharide lytic enzymes production medium by *Bacillus cereus* NRC-20.

Nitrogen source	Enzymes activity (U/ml)			
	Alginase	Dextrinase	Pectinase	CMCase
*Yeast extract	39.9±0.07	45.2±0.06	199.1±0.12	75.62±0.04
Peptone	35.0±0.07	65.7±0.03	279.2±0.09	112.3±0.07
Casein hydrolysate	20.3±0.03	21.2±0.08	59.8±0.03	28.7±0.12
Beef extract	40.6±0.06	84.8±0.05	310.2±0.06	43.1±0.09
Urea	24.6±0.08	19.2±0.08	62.5±0.04	18.4±0.07
Malt extract	78.1±0.04	92.5±0.14	282.1±0.05	98±0.05
Tryptone	36.8±0.09	91.2±0.09	266.3±0.08	75.6±0.13
Soy bean	55.0±0.04	79.3±0.07	151.2±0.02	40.3±0.09
N.acetylglucoseamine	18.4±0.05	21.2±0.06	33.6±0.05	17.9±0.04
Ammonium Sulphate.	23.4±0.03	26.4±0.03	64.4±0.02	26.1±0.05
Ammonium Chloride	27.1±0.12	21.9±0.10	49.7±0.04	30.4±0.08
Sodium Nitrate	22.8±0.10	23.1±0.08	57.7±0.06	26.5±0.07

*Control.

Table 5. Effect of addition of different waste materials on the production medium of polysaccharide lytic enzymes by *Bacillus cereus* NRC-20.

Waste material (20 g/l)	Enzymes activity (U/ml)			
	Alginase	Dextrinase	Pectinase	CMCase
Rice shell	26.6±0.07	42.5±0.04	150±0.06	20.9±0.09
Rice stem	82.8±0.09	64.1±0.09	112.0±0.08	55.3±0.07
Jatropha cake	120.0±0.03	138.8±1.3	292.0±0.09	95.5±0.08
Molasses	22.8±1.00	23.4±0.08	168.0±1.10	33.7±0.05
Carrot peels	50.6±0.05	63.6±1.2	149.68±0.06	29.68±0.08
Apple peels	47.5±0.05	60.1±0.09	147.18±1.60	33.75±1.10
Potato peels	47.8±0.08	68.7±0.07	153.75±0.09	30.63±1.40
*control	83.2±0.07	124.9±0.09	292.4±0.10	92.4±0.09

*Control.

Table 6. Effect of different concentrations of jatropha cake on the production medium of polysaccharide lytic enzymes by *Bacillus cereus* NRC-20.

Jatropha cake concentration (g/L)	Enzymes activity (U/ml)			
	Alginase	Dextrinase	Pectinase	CMCase
10	69.6±0.09	43.4±0.07	286.9±0.07	36.9±1.2
*20	120±0.05	140.2±1.6	292.7±1.04	98±0.09
30	255±0.02	118.9±0.06	392.2±0.09	61.2±0.04
40	202.5±0.09	120.7±0.08	250.8±0.05	54.8±0.05
50	176.2±0.08	93.6±0.03	216.4±0.04	49.7±0.09

*Control.

90 min of incubation giving the values of 227.1, 237.4, 384.6 and 217.7(µg/ml) for alginase, dextrinase, pectinase and CMCase, respectively and the highest activity of enzymes mixture has been achieved at pH5.

Data in Table 14 reveals that the enhancement of the lytic enzymes activities was proportional to its concentration up to 27.4 mg/ml, whereas the PPE activities were 250.3, 249.1 and 648.1 µg/ml for alginase, dextrinase

Table 7. Effect of different incubation temperatures on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Temperature (ºC)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
25	0.29±0.09	59.7±6.25	72.6±0.49	150.4±14.93	43.8±3.03
*30	0.38±0.05	78.1±3.09	92.3±9.13	282.8±6.55	98.5±12.12
35	0.37±0.12	69.3±1.42	89.1±2.07	234.3±2.39	79.5±2.09
40	0.32±0.07	32.1±2.97	50.4±0.75	134.5±17.06	23.8±1.07

*Control.

Table 8. Effect of different incubation periods on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Incubation period (h)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
24	0.10±0.06	55.7±3.05	50.2±1.02	151.2±10.05	46.3±8.08
*48	0.38±0.05	78.8±5.67	92.7±7.06	282.8±16.47	98.6±12.10
72	0.24±0.08	35.9±1.10	66.8±3.03	156.2±18.04	38.2±6.19

*Control.

Table 9. Effect of different pH values on the polysaccharide lytic enzymes production by *B. cereus* NRC-20.

pH value	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
4	0.30±0.05	21.2±0.64	29.4±3.33	239.4±17.07	38.2±4.14
5	0.34±0.09	56.2±2.83	39.2±4.10	265.1±9.05	68.4±4.08
6	0.35±0.08	81.3±6.08	98.2±1.77	299.8±24.09	98.9±3.08
***7**	0.38±0.03	78.8±5.06	92.7.8±8.11	292.7±14.03	98.3±0.53
8	0.37±0.10	55.0±5.09	45.9±3.05	150.4±8.06	34±1.11

*Control.

Table 10. Effect of different inoculum sizes on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Inoculum size (ml %)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
4	0.27±0.08	25.0±0.09	52.1±0.05	187.4±0.04	27.4±0.02
6	0.33±0.12	31.1±0.02	116.5±0.12	268.7±0.07	43.6±0.05
8	0.34±0.10	83.6±0.03	124.6±0.08	299.6±0.09	98.8±0.04
*10	0.35±0.09	81.0±0.07	98.1±0.06	299.3±0.07	98.9±0.08

*Control.

and pectinase, respectively, while CMCase had the highest activity (316.2 µg/ml) at protein concentration of 48 mg/ml (Table 15). On the other hand, by increasing the substrate concentration up to 5 g/l enhanced the activities of the different enzymes giving the values 247.8, 250.1, 422.7 and 239.2 µg/ml for alginase, dextrinase, pectinase and CMCase, respectively, while the further increase in substrate concentrations resulted

Table 11. Effect of different agitation speeds on the polysaccharide lytic enzymes production by *Bacillus cereus* NRC-20.

Agitation speed (rpm)	Total protein (mg/ml)	Enzymes activity (U/ml)			
		Alginase	Dextrinase	Pectinase	CMCase
0	0.27±0.06	30.0±5.6	30.0±2.1	200.53±0.9	65±12.4
150	0.33±0.08	59.3±0.09	80.5±0.05	235.1±0.05	67.4±2.06
*200	0.34±0.04	83.2±0.07	124.9±0.69	298.4±0.10	98.6±7.09
250	0.38±0.09	64.5±0.03	62.3±1.9	189.2±0.13	45.7±0.08

*Control.

Table 12. Effect of different incubation temperatures on the relative activity of partially purified enzyme.

Temperature (°C)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
25	70.4	81.8	88.8	76.8
*30	100.0	100.0	100.0	100.0
35	105.4	124.3	113.7	144.5
40	52.3	72.9	67.3	61.5

*Control.

Table 13. Effect of different assay incubation times on the relative activity of partially purified enzyme.

Time (min)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
30	85.5	69.8	68.7	72.1
*60	100.0	100.0	100.0	100.0
90	112.1	110.0	125.1	114.3
120	93.4	94.9	124.2	106.0
150	76.3	54.2	82.8	98.6

*Control.

Table 14. Effect of different assay pH values on the relative activity of partially purified enzyme.

Buffers	pH	Relative activity (%)			
		Alginase	Dextrinase	Pectinase	cmcase
Acetate	4	103.8	102.9	109.1	46.5
	5	126.0	115.2	119.7	105.4
	*6	100.0	100.0	100.0	100.0
Phosphate	7	89.7	91.6	89.4	84.2
	8	88.3	84.7	79.8	84.1
Carbonate- bicarbonate	9	83.4	74.8	72.7	79.1

*Control.

in the decrease in enzyme activity (Table 16). Among different added metal ions, K^+ was a strong activator of the enzymes mixture activities, Na^+ was a moderate activator while, Cu^{++}, Co^{++}, Pb^{++} are considered as enzyme

Table 15. Effect of different enzymes protein concentrations on the relative activity of partially purified enzyme.

Enzyme concentration (mg/ml)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
0.1	78.1	83.6	93.6	86.3
0.2	91.2	96.8	98.7	94.8
*0.3	100.0	100	100.0	100
0.4	85.5	94.9	94.9	103.1
0.5	74.2	82.1	87.3	125.4
0.6	67.8	81.5	62.2	125.3

*Control.

Table 16. Effect of different substrates concentrations on the relative activity of partially purified enzyme.

Substrate concentration (g %)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
*0.2	100.0	100.0	100.0	100.0
0.5	122.0	118.7	128.1	136.2
0.8	86.1	91.2	86.1	91.6
1.1	71.3	76.6	56.8	71.0

*Control.

Table 17. Effect of addition of different metal ions on the relative activity of partially purified enzyme.

Metal ions (0.02 M)	Relative activity (%)			
	Alginase	Dextrinase	Pectinase	CMCase
Na^{+}	138.1	156.0	171.5	136.6
K^{+}	110.0	144.1	193.5	92.9
Mg^{++}	100.2	66.6	108.8	77.1
Cu^{++}	61.4	67.2	50.1	80.8
CO^{++}	70.4	60.9	58.4	78.3
Pb^{++}	59.1	59.7	64.0	70.5
*control	100.0	100.0	100.0	100.0

*Control.

inhibitors (Table 17).

Thermal exposure of the PPE to different temperatures for different time intervals (not tabulated) demonstrated that, the enzyme mixture alginase, dextrinase, pectinase and CMCase are stable with increasing the reaction temperature, whereas the pectinase enzyme retained only 82.7% and dextrinase enzyme retained 98.1% of the relative activity after being exposed to 60°C for 120 min, while alginase and dextrinase enzymes started losing their activities after exposure to 70°C for 60 min, retaining 99.3, 96.3% relative activities, respectively. The molecular weights determined in the PPESDS-PAGE with Coomassie-brilliant blue staining mixture were: 17, 89, 279 KDa, respectively (Figure 1).

Effect of partially purified enzymes on biofilm removal

The microscopic studies of the effect of PPE mixture on *P. aeruginosa*, *E. coli*, *S. aureus* and *B. subtilis* biofilms revealed that the biofilms treated showed a reduction in the biofilm cells and substantial degradation of the extracellular polymeric substances (EPS) protecting the

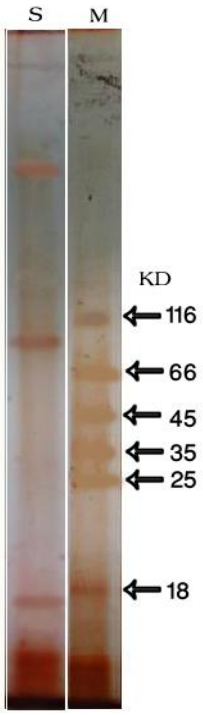

Figure 1. Molecular weight of partially purified enzymes of B. cereus. NRC-20 M: Protein marker (18-116 KDa); S: Bands of PPE.

pathogenic bacteria. The enzymes mixture was more effective in degrading EPS and biofilm removal of *S. aures* followed by *E. coli*, *P. aeruginosa* and then *B. subtilis* (Figures 2, 3, 4 and 5)

DISCUSSION

In response to environmental and health concerns on the extended use of antibiotics, it seems inevitable that greater reliance will be placed on alternative biological control techniques including the use of natural microbial derived substances like microbial enzymes. Marine derived microbial enzymes are used for many purposes including both medical and industrial fields, one of the most interesting uses of marine microbial enzymes is the use of enzymes for biofilm removal.

The application of enzymes for control of protein biofilm on surfaces and in closed pipelines is well known (Aldridge et al., 1994), while the application of enzymes to degrade EPS is promising and attractive in medical sciences and many industries where complete biofilm removal is essential. It is interesting in this study to focus on the removal of polysaccharide components of the extracellular polymeric substance (EPS) of *P. aeruginosa* ATCC 27953, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *B. subtilis* NRRL B-4219 biofilms as well studied bacterial organisms for biofilm formation using enzymes mixture isolated from marine *B. cereus* NRC-20.

Exopolysaccharides are a major component of most biofilm matrices (Orgaz et al., 2006; Fernández et al., 2012), the absence of exopolysaccharide synthesis and export, bacteria can adhere to the surfaces but unable to form multilayer biofilms and due to the heterogeneity of the extracellular polysaccharides within the biofilms, a mixture of exopolysaccharide lytic enzymes is necessary for sufficient degradation of bacterial biofilm (Xavier et al., 2005; Kaplan, 2010). Therefore, the investigated enzymes production and properties in this study are, a mixture of alginase, dextrinase, pectinase and carboxy-methyl cellulase produced by marine *B. cereus* NRC-20, whereas some investigations reported more and more microorganisms from marine habitats that can produce polysaccharide lyric enzymes (Kaplan, 2010) and they have been attracting more attention as a resource for new enzymes, because the microbial enzymes are relatively more stable and active than the corresponding enzymes derived from plant or animal (Gupta et al., 2003; Byrd et al., 2011).

The screening medium containing alginate as the only carbon source supported growth and best production of the multiple specificity enzyme mixture, this agrees with the findings of Manyak et al. (2004) who reported that, marine-derived microorganisms are useful sources of polysaccharide lyases, since they evolved powerful enzyme systems to take advantage of the ubiquitous marine nature. The best alginase producing marine bacterial isolate was identified using API 50CHB system and was found to be *B. cereus***.** Heterotrophic *Bacillus* strains are considered to be species of a variety of habitats. Species of *Bacillus marinus, Bacillus badius Bacillus subtilis, B. cereus, Bacillus licheniformis, Bacillus firmus* and *Bacillus lentus* are often isolated from marine habitats as reported by Ivanova et al. (1999). The capacity of the *Bacillus* sp. to produce and secrete large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers (Namasivayam et al., 2011).

The production conditions of the lytic enzymes responsible for the degradation of the most common biofilm matrix polysaccharides have been investigated. Commercially available enzyme preparations have seldom proved the capability of degrading microbial heteropolysaccharides (Sutherland, 1999; Loiselle and Anderson, 2003; Chaignon et al., 2007).

The best fermentation medium for the different polysaccharide lytic enzymes production was the minimal medium containing only sodium alginate as a carbon source along with yeast extract and sea water. On the other hand, the rest of the enzymes production media containing other mineral supplements ($MnSO_4$ and

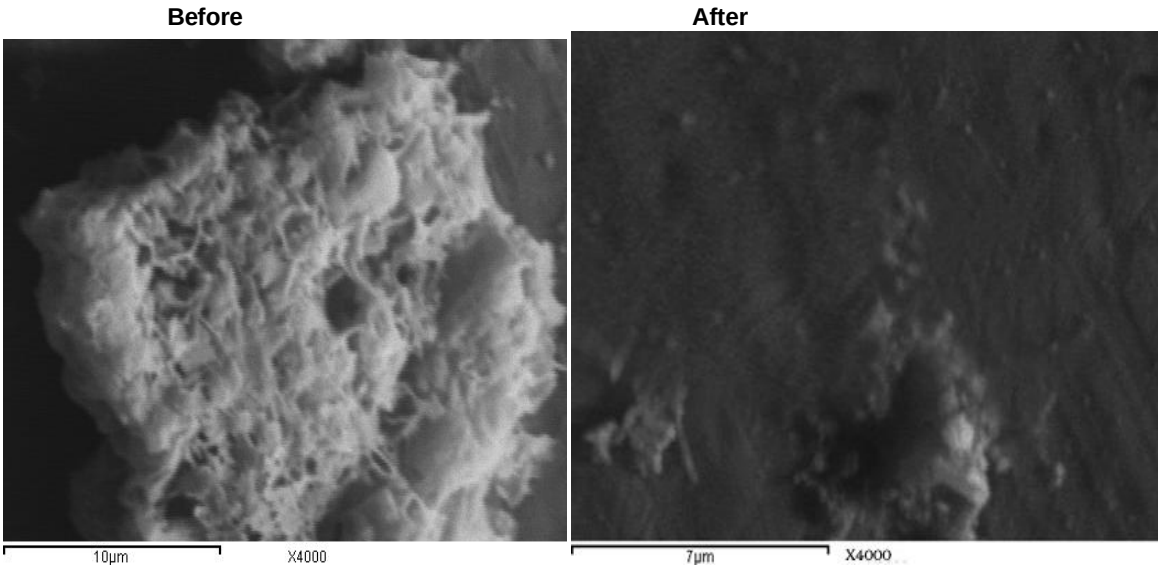

Figure 2. Six days old *P. aeruginosa* biofilm on stainless steel before and after partially purified enzyme treatment.

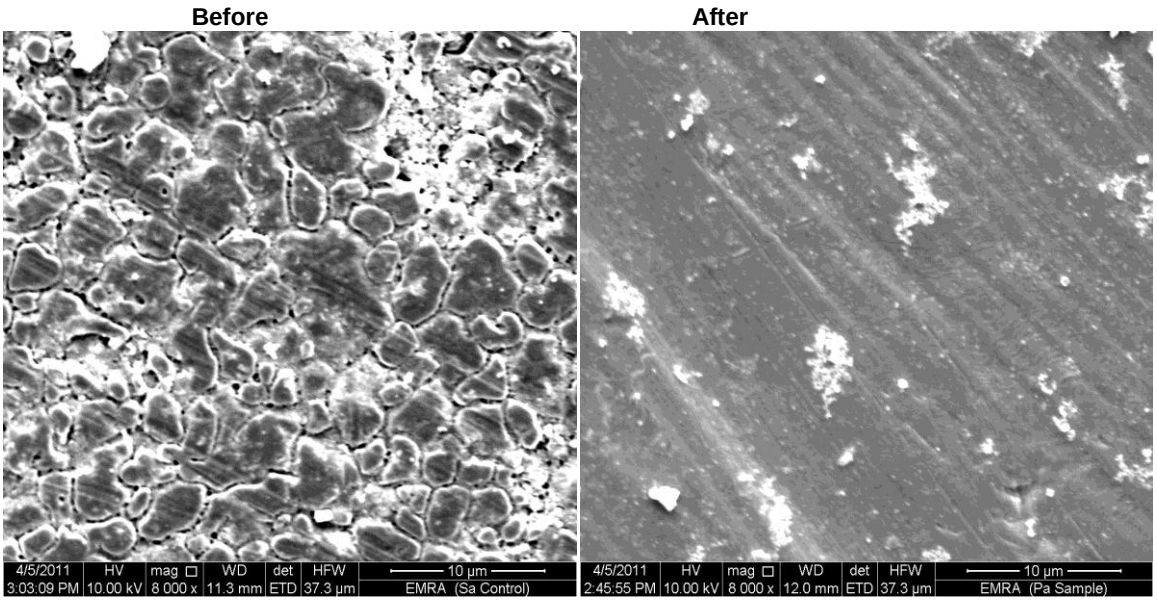

Figure 3. Six days old biofilm of *S. aureus* on stainless steel sheets before and after partially purified enzyme treatment.

$MgCl_2$) and other rich nitrogen sources (peptone, tryptone, casein hydrolysate) seemed to minimize the enzymes production. The best concentration of sodium alginate is 0.3 g%, best nitrogen source is malt extract 0.3 g%, provided the organic nitrogen sources yielded better polysaccharide lytic enzymes productivity than inorganic sources. This is in agreement with the results reported by Stoudt and Nollstadt (1982) and Namasivayam et al. (2011), indicating that the use of organic nitrogen sources such as yeast extract, corn steep liquor are better than inorganic ingredients together with the addition of the trace substances.

Bacillus cereus NRC-20 was found to give maximum enzyme production at 30°C.This agrees with the results by Namasivayam et al. (2011) that, most *Bacillus* sp. need 32-37°C for pectinase enzyme production. Optimal pH for enzyme production in this study was found to be pH 6.0. This agrees with the reported data of Liu and Chi-Li (1988), indicating that the best alginase production is at pH 5-6.5, using sodium alginate as the carbon source.

The best inoculum size was found to be 8% (v/v), incubated for 48 h on a rotary shaker adjusted at 200

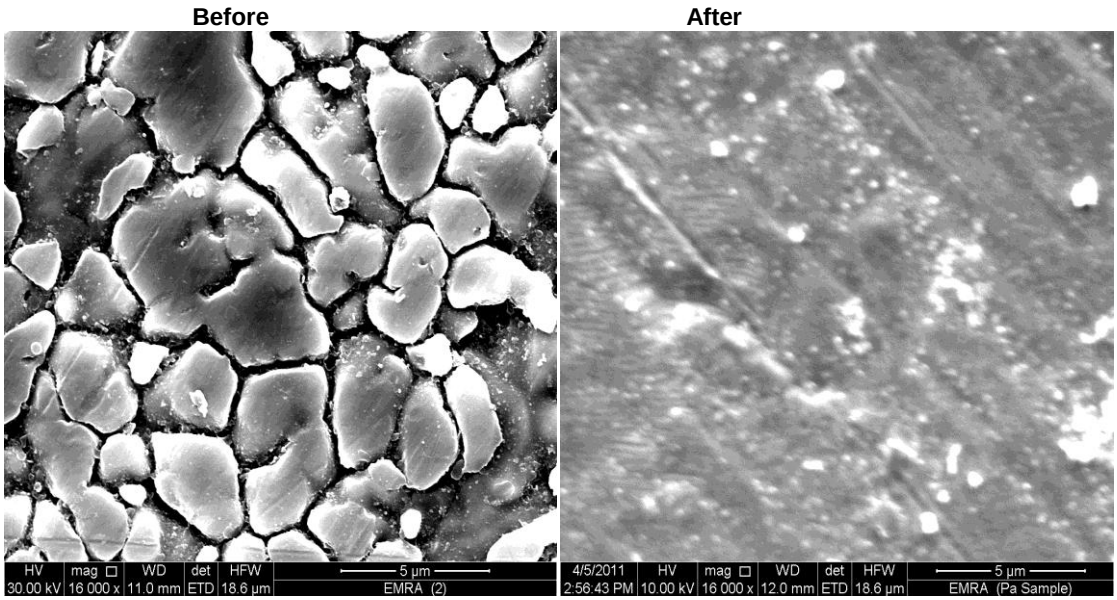

Figure 4. Six days old biofilm of *E.coli* on stainless steel sheets before and after partially purified enzyme treatment

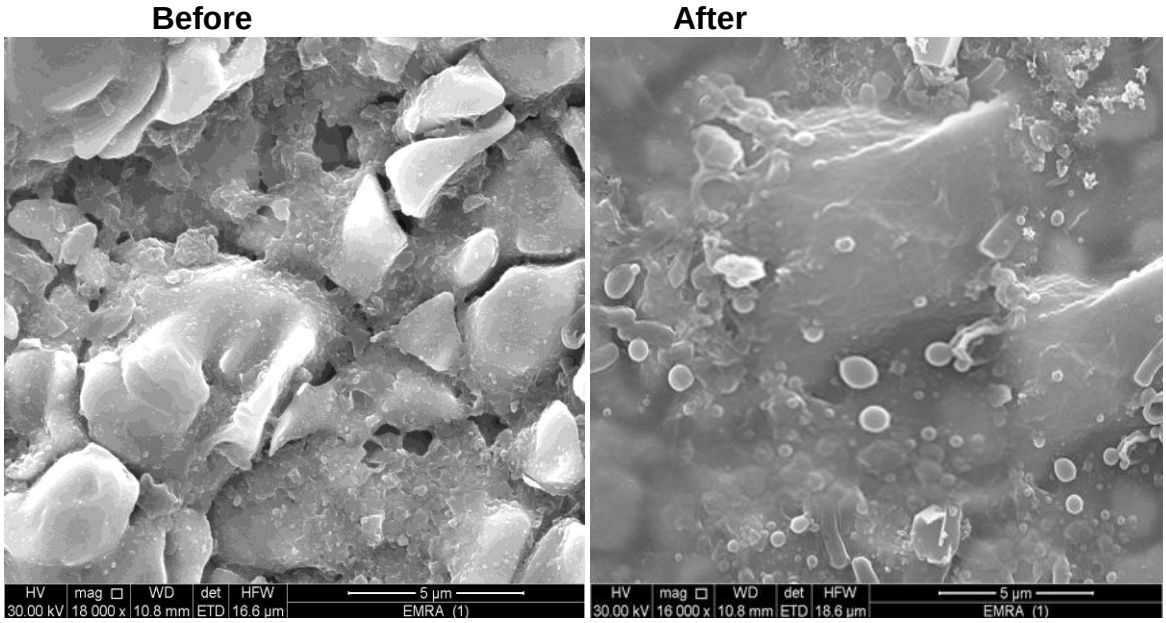

Figure 5. Six days old *Bacillus subtilis* biofilm on stainless steel sheets before and after partially purified enzyme treatment.

rpm. This is in agreement with reported data by Manyak et al.(2004) who harvested his polysaccharide lytic enzyme mixture after 48 h.

Several tested waste material additives were tested as inducer agents for the production of polysaccharide lytic enzymes. Jatropha cake which is the wastes of vegetable oil seed extraction produced promising amounts of polysaccharide lytic enzymes since, the plant seed will contain a number of lipoproteins and lipopolysaccharides like amylose, glucan and pectin which will increase the productivity of polysaccharide lytic enzymes (Achten et al., 2008). On the other hand, wheat bran acts as a better agro substrate and magnesium chloride was supplement for better production of pectinase

(Namasivayam et al., 2011).

Precipitation of the PPE from the supernatant of the enzyme production medium by *Bacillus c.*-NRC-20 was carried out by 60% acetone, which resulted in the highest yield of enzymes activities. The previous results are in agreement with the results reported by Stoudt and Nollstadt (1982), they found that the enzymes hydrolyzing the plaque matrix were precipitated by 60% acetone. On the other hand, pectinase from *B. cereus* isolated from solid waste was precipitated by 80% ammonium sulphate (Lopez et al., 2010; Namasivayam et al., 2011); also, the polysaccharide lytic enzymes mixture, reported by Manyak et al. (2004), was precipitated by 80% ammonium sulphate.

The PPE had the best activity when 27.4 mg/ml of the enzymes mixture protein was incubated with 5.0 g/l of the corresponding polysaccharide substrates at 35°C for 90 min at pH 5. Wang et al. (2004) reported that in an enzyme mixture from *Penicillium roquefort*, the dextrinase enzyme activity was stable in a pH range of 5.0 to 5.3 and when SDS-PAGE was applied, three enzyme protein bands appeared (17, 89, 279 KDa) while, a bifunctional alginate lyase was separated and polyacrylamide gel electrophoresis separation resulted in two alginases having molecular weights of 23.0 and 33.9 KDa.

Many antimicrobial agents fail to penetrate the biofilm of bacterial pathogens due to EPS which acts as barrier protecting the bacterial cells within. The alternative will be the use of compounds which can degrade the EPS of the biofilm (Loiselle et al., 2003; Walker et al., 2007). Enzymes have been proven to be effective for the EPS degradation of the biofilms (Lequette et al., 2010) and remove biofilms directly by destroying the physical integrity of the EPS by weakening the carbohydrate, proteins and lipid components making up the structures of the EPS through the degradation process (Xavier el al., 2005).

Interestingly, the electron microscope photos of stainless steel sheets of biofilms under test were examined before and after PPE mixture treatment and the enzymes mixture under test was effective for the removal of the different biofilms, showing almost complete removal of *P. aeruginoa, S. aureus* and *E. coli* biofilms. The major components of the polysaccharide matrices for these biofilms are alginate for *P. aeruginosa* (May et al., 1991), cellulose for both *S. aureus* and *E. coli* (da Re and Ghigo, 2006; Seidl et al., 2008). On the other hand, the lytic enzymes mixture from *B. cereus* NRC-20, showed little effect on the biofilm of *B. subtilis,* since the major component of this biofilm matrix is γ-glutamate (Morikawa et al., 2006), and the enzyme mixture does not contain a lytic enzyme for this polysaccharide

Conclusion

The present study clearly indicates that the *B. cereus* NRC-20 can be used for the production of polysaccharide lytic enzymes mixture (alginase, dextrinase, pectinase and carboxymethyle cellulase) used for biofilm removal of a number of pathogenic bacteria including: *P. aeruginosa, S. aureus, E. coli* and to a lesser extent *B. subtilis*. These enzymes represent a method to eliminate biofilms through naturally derived microbial products, minimizing the possible hazards of large quantities and extended use of antibiotics which can be damaging and not effective in many cases. The constant periodical use of these polysaccharide lytic enzymes is recommended for eliminating existing biofilms and preventing the formation of new ones.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Achten WMJ, Verchot L, Mathijs E,Singh VP, Aerts R, Muys B (2008). Jatropha bio-diesel production and use. Biomass and Bioenergy. 32:1063-1084.

Aldridge IY, Chmurny AB, Durham DR, Roberts RL, Fan LD (1994). Proteases to inhibit and remove biofilm. European patent, 0 590 746 A1.

Alexeeva YV, Ivanova EP, Bakunina IY, Zvayginsteva TN (2002).Optimization of glucosidase production by *Pseudoalteromonas issachenkonii*. Lett. Appl. Microbiol. 35(4):343-346.

Byrd SM, Pang B, Hong W, Waligora AE, Juneau AR, Chelsie E, Parsek RM, Kock DN, Wozniak JD, Swords WE (2011). Direct evaluation of *Pseudomanas aeruginosa* biofilm mediators in a chronic infection model. J. Infect. Immun. 79:3087-3095.

Chaignon P, Sadovskaya I, Ragunah CH, Ramasubbu N, Kaplan JB, Jabbouri S (2007). Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Appl. Microbiol. Biotechnol. 75:125-132.

Chen X, Stewart PS (2000). Biofilm removal caused by chemical treatments. Water Res.34: 4229-4233.

Christensen BE (1989). The role of extracellular polysaccharide in biofilms. J. Biotechnol. 10:181-202.

Costerton JWZ, Lewandowski DE, Caldwell DR, Korber and Lappin-Scott HM (1995).Microbial biofilms. Annu. Rev. Microbiol. 49:711-745.

da Re S, Ghigo JM (2006). A CsgD-independent pathway for cellulose Production and biofilm formation in *Escherichia coli*. J. Bacteriol. 188:3073-3087.

de Carvalho CCCR (2007). Biofilms: recent developments on an old battle. Recent Patents Biotechnol. 1:49-57.

de Queiroz G, Day DF(2007). Antimicrobial activity and effectiveness of a combination of sodium hypochlorite and hydrogen peroxide in killing and removing *Pseudomonas aeruginosa* biofilms from surfaces. J. Appl. Microbiol. 103(4):794-802.

Donlan MR, Costerton JW (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 167-193.

Fernández L, Breidenstein EM, Song D, Hancock ER (2012). Role of intracellular proteases in the antibiotic resistance, motility and biofilm formation of *Pseudomanas aeruginosa*. J. Antimicrob. Agents. Chemother. 56:1128-1132.

Flemming HC, Schaule G, McDonogh RG (1992).Biofouling on membranes. A short review, In. Melo LF, BottTR, FletcherM, Capdeville B. (ed.), Biofilms science and technology. Kluwer Academic Press, Dordrecht, The Netherlands. Pp. 487-497.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial α-amylases: A biotechnological perspective. J. Proc. Biochem. 38:1599-1616.

Hansen BJ, Nakamura KL (1985). Distribution of alginate lyase enzyme

activity among strains of *Bacillus circulans*. Appl. Environ. Microbiol. 122:1019-1021.
Hansen BJ, Scott Doubet R, Ram J (1984). Alginase enzyme production by *Bacillus circulans*. Appl. Environ. Microbiol. 47(4):704-9.
Häussler S, Parsek RM (2010). Biofilms, new perspectives at the heart of surface-associated microbial communities. J. Bacteriol. 192(12):2941-2949.
Ivanova EP, Vysotskii MV, Svetashev VI, Nedashkovskaya OI, Gorshkova MN, Mikhailov VV, Yumoto N, Shigeri Y, Tagushi T, Yushikawa S (1999). Characterization of *Bacillus* strains of marine origin. Int. Microbiol. 2:267-271.
Jain S, Ohman DE (2005). Alginate secretion in *Pseudomonas aeruginosa*. Infect. Immun.73:6429-6436.
Johansen C, Falholt P, Gram L (1997). Enzymatic removal and disinfection of bacterial biofilms. Appl. Environ. Microbiol. 63(9):3724-3728.
Johanson TB, Lindsey NS, Nelson CD, Mayo AJ (2008). Extracellular proteolytic activities expressed by *Bacillus pumilus* isolated from endodontic and periodontal lesions. J. Med. Microbiol.57:643-651.
Kaplan JB (2010). Biofilm dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. JDR89(3):205-218.
Lequette Y, Boels G, Clarisse M, Faille C (2010). Using enzymes to remove biofilms of bacterial isolates samples in the food industry. J. Biofoul. 4:421-431.
Liu L, Chi-Li M (1988). Thermostable alginate lyase from *Bacillus stearothermophilus*. US patent 5139945.
Loiselle M, Anderson K (2003). The use of cellulose in inhibiting biofilm formation from organisms commonly found on medical implants. Biofoul. 2:77-85.
Lopez D, Valmakis H, Kolter R (2010). Biofilms. Cold Spring Harb. Perspect. Biol.27 May, 2010.
Lowry 0H, Rosenbrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
Manyak DM, Weiner RM, Carlson PS, Quintero EJ (2004). Preparation and use of biofilm degrading, multiple specificity enzyme mixture. US patent,6759040.
May BT, Shinabarger D, Maharaj R, Kato J, Chu L, De Vault DJ, Roychoudhury S, Zielinski AN, Berry A, Rothmel KR, Misra KT, Chakrabarty MA (1991). Alginate eynthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. Clin. Microbiol. Rev. 4 (2):191-206.
Morikawa M, Kagihiro S, Haruki M, Tanako K, Branda S, Kolter R, Kanaya S (2006). Biofilm formation by a *Bacillus subtilis* strain that produces γ-glutamate. Microbiol. 152:2801-2807.
Namasivayam E, Ravindar DJ, mariapan K, Jiji A, Kumar M, Jayaraj R (2011). Production of extracellular pectinase by *Bacillus cereus* isolated from market solid waste. J. Bioanal. Biomed. 3: 070-075.
Oldak E, Trafny AE (2005). Secretion of protease by *Pseudomonas aeruginosa* biofilm exposed to ciprofloxacin. Antimicrob. agents of chemo. 49(8):328-3288.
Orgaz B, Kives J, Pedregosa AM, Monistrol IF, Laborda F, SanJose C (2006). Bacterial biofilms removal using fungal enzymes. J. Enz. Microb. Technol. 40:51-56.
Peterson SB, Dunn AK, Klimowicz AK, Handelsman J (2006). Peptidoglycan from *Bacillus cereus* mediates commensalism with rhizosphere bacteria from the *Cytophaga-flavobacterium* group. Appl. Environ. Microbiol. 72(8):5421-5427.
Seidl K, Goerke C, Wolz C, Mack D , Berger-Bächi D, Bischoff M (2008). *Staphylococcus aureus* CcPA affects biofilm formation. Infect. Immun. 76 (5):2044-2050.
Stoudt TH, Nollstadt KH (1982). Oral hygiene enzymes and methods for preparation. US patent 4335101.
Sumner JB, Sisler EB (1944). A simple method for blood sugar. Arch. Biochem. 4:333-336.
Sutherland IW (1999). Polysaccharases for microbial exopolysaccharides. Carb. Polymers. 38 (4):319-328.
Walker SL, Fougialakis M, Gerezo B, Livens S (2007). Removal of microbial biofilms from dispense equipment: the effect of enzymatic pre-digestion and detergent treatment. J. Inst. Brew. 113:61-66.
Wang Y, Suzuki A, Tanaka T, Kumura H, Shimazaki K (2004). Partial characterization of dextrin degrading enzyme obtained from blue cheese. J. Dairy Sci. 87:1627-1633.
Xavier JB, Picioreanu C, Abdul Rani S, Van Loosdricht MCM, Stewart SP (2005). Biofilm control strategies based on enzymic disruption of biofilm. A model study. Microbiol. 151:3817-3832.
Xiong Y, Liu Y (2010). Biological control of microbial attachment: a promising alternative for mitigating membrane biofouling. Appl. Microbiol. Biotechnol. DOI10.1007.

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Diversity and association of filamentous fungi in coffee beans under organic and conventional cultivation

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Brazil is a country with great biodiversity; however, knowledge of this microbiological wealth is currently insufficient for its utilization in a sustainable manner. Agricultural expansion represents one of the largest current dangers to biodiversity and threatens to cause the extinction of a variety of species. This study therefore aimed to isolate and identify the species of fungi present in coffee beans cultivated in organic and conventional systems. Eighteen (18) samples of coffee beans from southern Minas Gerais were analyzed, and 346 fungal isolates were obtained from the analyzed coffee beans. These isolates belonged to 32 species in the following 14 genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Mucor*, *Rhizopus*, *Trichoderma*, *Epicoccum*, *Phoma*, *Bipolaris*, *Glomerella*, *Colletotrichum*, *Alternaria* and *Gliocladium*. Organic coffee bean samples exhibited the highest indices of fungal diversity. Two species identified in this study, *Aspergillus flavus* and *Aspergillus ochraceus*, are extremely important for their toxigenic characteristics. We utilized simple correspondence analysis to evaluate the interaction of the identified fungi with the toxigenic species. An association of toxigenic fungi with other fungi is important because some microorganisms can degrade mycotoxins. In the organic coffee beans, *A. flavus* was associated with *Cladosporium cladosporioides*, *A. ochraceus*, and *Penicillium brevicompactum*. In contrast, in the conventional coffee beans, *A. ochraceus* was only associated with *C. cladosporioides*. These results demonstrate that greater fungal diversity exists in organic coffee beans.

Key words: Interactions, microorganisms, mycotoxins, *Aspergillus.*

INTRODUCTION

Fungi exhibit greater species richness than most other organisms and, thus, are of significant environmental and economic importance (Varga et al., 2011; Blackwell, 2011). Recent predictions based on molecular methods

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have suggested that there are 5.1 million fungal species (O'Brien et al., 2005); however, only approximately 5% of the predicted filamentous fungal species have been described (Hawksworth, 1991).

The biodiversity of Brazil is among the greatest in the world (Agostinho et al., 2005), and studies to estimate the risk of mycotoxins, biological control, or fungal diversity in agricultural systems are therefore critical for the utilization of these microorganisms in biotechnological processes. However, global warming and the need to expand agriculture significantly threaten microbial diversity (Hole et al., 2005). Pesticides that are intensively used in conventional cultivation can lead to a diminished ecosystem, reducing biodiversity while allowing for pathogen increase (Lima and Vianello, 2011).

In agricultural products, such as coffee, interactions between different fungal species represent a natural phenomenon that affects the development of these microorganisms and their subsequent production of mycotoxins. Several studies have been undertaken to assess the growth of mycotoxigenic fungi and mycotoxin degradation (Noonim et al., 2008; Batista et al., 2009, Abrunhosa et al., 2010). These interactions are influenced by environmental conditions, microorganism diversity (Logrieco et al., 2007), agricultural products (Perrone et al., 2007), the type of processing (Batista et al., 2003), and the cultivation system used (Schneider et al., 2010). Interactions between mycotoxigenic and nontoxic species can lead to the appearance of diseases, the production of different types of mycotoxins, or their degradation. The results of these interactions are influenced by environmental conditions (Logrieco et al., 2007).

Ochratoxin A (OTA) has been detected in various agricultural products, including coffee (Clouvel et al., 2008; Batista et al., 2009; Duarte et al., 2010). According to Petzinger and Weidenbach (2002), coffee drinks significantly contribute to the ingestion of OTA in the human diet. OTA is primarily produced by *Aspergillus* section *Circumdati* (*A. westerdijkiae* and *A. ochraceus*) and section *Nigri* (*A. carbonarius* and *A. niger*) species (Batista et al., 2009; Gil- Serna et al., 2011).

In Brazil, conventional farming is dependent on the use of herbicides, pesticides, and inorganic nutrient applications, whichare less beneficial to the environment than organic methods (Bettiol, et al., 2002).In contrast, organic coffee is produced without the use of pesticides and soluble fertilizers, which are replaced by recycling organic by-products such as animal manure, biofer-tilizers, pulp and coffee husk compounds, and earthworm castings (Theodoro and Guimarães, 2003). For the production of coffee to be considered organic, the crop must be free from the use of pesticides and chemical fertilizers for at least three years (Bakutis et al., 2006).

The objectives of this study were therefore to evaluate the diversity of filamentous fungi in coffee beans cultivated in organic and conventional systems and to assess the ecological interactions between species of fungi that produce toxins and those that do not.

MATERIALS AND METHODS

Samples

Samples of stored coffee beans (500g per sample) were obtained from acooperative in southern Minas Gerais. The samples consisted of both conventional and organic coffee beans (*Coffea arabica* L.), which were harvested from three districts in Southern Minas Gerais. Two types of coffee were analyzed: coffee harvested onto cloth and coffee swept from the ground. The harvested fractions were as follows: organic coffee (two samples) from a district at latitude 20° 56' and longitude 44° 55', with a mean annual temperature of 20°C, a mean annual rainfall of 1597.6 mm, and an altitude of 1013 m (IBGE, 2013); organic coffee (eight samples) and conventional coffee (seven samples) from a second district located at latitude 21° 46' and longitude 45° 57', with a mean annual temperature of 20°C, a mean annual rainfall of 1592.7 mm and an altitude of 836 m (IBGE, 2013); and conventional coffee (one sample) from a district located at latitude 21° 42' and longitude 46° 14', with a mean annual temperature of 18.2°C, a mean annual rainfall of 1605 mm, and an altitude of 1076 m (IBGE, 2013). These 18 samples were sent to the Laboratory for Food Mycotoxins and Mycology of the Department of Food Science of the Federal University of Lavras in Minas Gerais for analysis.

Isolation of filamentous fungi

The fungi associated with green coffee beans were isolated by direct plating on Dichloran Rose Bengal Chloramphenicol HiMedia medium (10 g of glucose; 5 g of bacteriological peptone; 1 g of KH_2PO_4; 0.5 g of $MgSO_4 \cdot 7H_2O$; 15 g of agar; 1 L of distilled water; 25 mg of Rose Bengal and 2 mg of dichloran) as described by Samson et al. (2000). The isolates were selected according to morphological differences in the color and appearance of their colonies.

From each sample, 200 beans were randomly collected; 100 beans were plated following surface disinfection, and 100 beans were plated without surface disinfection. For disinfection, the samples were immersed in a solution of 70% alcohol followed by a solution of 1% sodium hypochlorite for 30 s. The samples were then washed with distilled water. After seven days of culture, the cultures were purified using malt agar (MA) medium.

Identification of filamentous fungi

Samples of the fungal species were removed from the pure cultures and cultured in specific media. Standard identification manuals were used for each genus. *Aspergillus* and *Penicillium* isolates were cultured in Czapek yeast agar (CYA: 1 g of K_2HPO_4; 10 mL of Czapek concentrate; 5 g of fungal extract; 15 g of agar and 1 L of distilled water), Czapek concentrate (30 g of $NaNO_3$; 5 g of KCl; 5 g of $MgSO_4 \cdot 7H_2O$; 0.1 g of $FeSO_4 \cdot 7H_2O$; 0.1 g of $ZnSO_4 \cdot 7H_2O$; 0.05 g of $CuSO_4 \cdot 5H_2O$ and 100 mL of distilled water) and MEA (20 g of malt extract; 1 g of peptone; 30 g of glucose; 20 g of agar and 1 L of distilled water) at 25 and 37°C, respectively. After seven days of incubation, macroscopic and microscopic characteristics were observed. *Aspergillus* isolates were identified according to Klich (2002), and *Penicillium* species were identified according to Pitt (2000), as these identification processes have been supported by Pitt and Hocking (1997) and Samson et al. (2000). Synthetic nutrient-poor agar medium (SNA: 1 g of KH_2PO_4; 1 g of KNO_3; 1 g

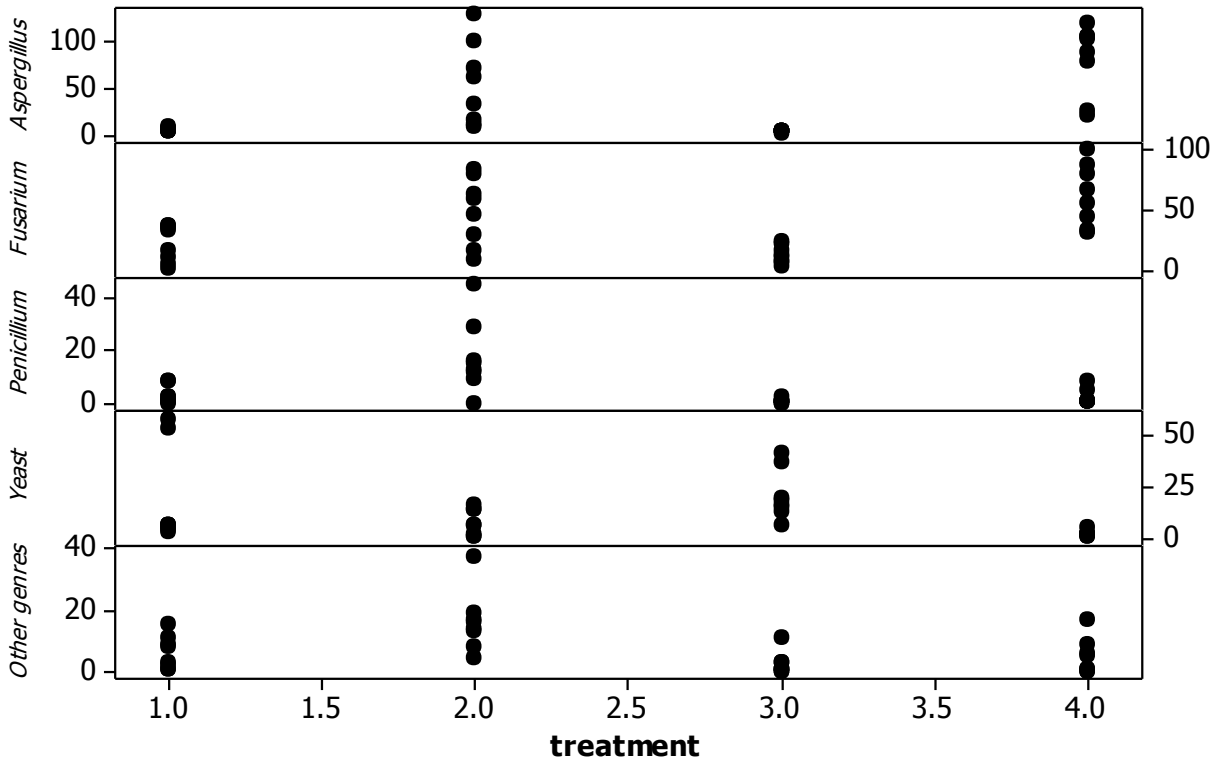

Figure 1. 1) Organic coffee with surface disinfection; 2) organic coffee without surface disinfection; 3) conventional coffee with surface disinfection; and 4) conventional coffee without surface disinfection.

of $MgSO_4 \cdot 7H_2O$; 0.5 g of KCl; 0.2 g of glucose; 0.2 g of saccharose;20 g of agar and 1 L of distilled water) was used for isolates of the *Fusarium* genus, and MA (malt extract) was used for the analysis of microscopic characteristics. Potato dextrose agar (PDA) was used to observe the colony color. The isolates were maintained in a photoperiod for 10 days at 21°C. Isolates belonging to the genera *Mucor*, *Rhizopus*, *Cladosporium* and *Trichoderma* were cultured in malt extract (MEA) at 25°C for seven days, and isolate identification was accomplished according to Samson et al. (2000). *Colletotrichum*, *Glomerella*, *Bipolaris* and *Epicoccum* were cultured in MA, whereas *Phoma* and *Alternaria* were cultured in OA medium (oatmeal agar: 30 g of oats, 1 L of distilled water and 15 g of agar) at 25°C for seven days. Fungal identification was performed as previously described by Ellis (1971).

Statistical analyses

The statistical methodology used in this study involved the construction of a 95% confidence interval for the standard deviation, aiming to infer the dispersion of the occurrence of filamentous fungi in organic and conventional coffee samples. To identify the relationship between toxigenic and nontoxigenic fungi through grouping, a simple correspondence analysis was utilized according to the methodology described by Hair et al. (1998).

Biodiversity indices

Margalef's richness index (Rm) was used to determine the diversity and richness of the filamentous fungalspecies isolated from organic and conventional coffee beans. In this index, $Rm = (S - 1)/(\ln n)$; where, S is the number of species and n is the number of identified individuals. The Shannon-Weiner diversity index (H'), $H' = -\Sigma (pi \ln pi)$, was also used, as described by Magurran (1988); where, pi is the proportion of individuals in each species with respect to the total number of individuals.

RESULTS AND DISCUSSION

Coffee sample contamination

Filamentous fungi were detected in all coffee bean samples from both the conventional and the organic cultivation systems. The highest contamination indices were found in the samples that were not disinfected with 1% sodium hypochlorite.

Aspergillus, *Fusarium* and *Penicillium* (Figure 1) represent the major identified fungal genera and are primarily responsible for mycotoxin production in agricultural products (Cast, 2003). These genera were also identified in coffee bean samples by Batista et al. (2009), Batista and Chaulfoun (2007), and Rezende et al. (2013). The presence of these genera in coffee is of concern given their potential production of mycotoxins, such as aflatoxin B1, aflatoxin B2 and OTA (Batista et al. 2003; Amézqueta et al., 2009).

Organic and conventional coffee bean samples that were not disinfected with 1% sodium hypochlorite were

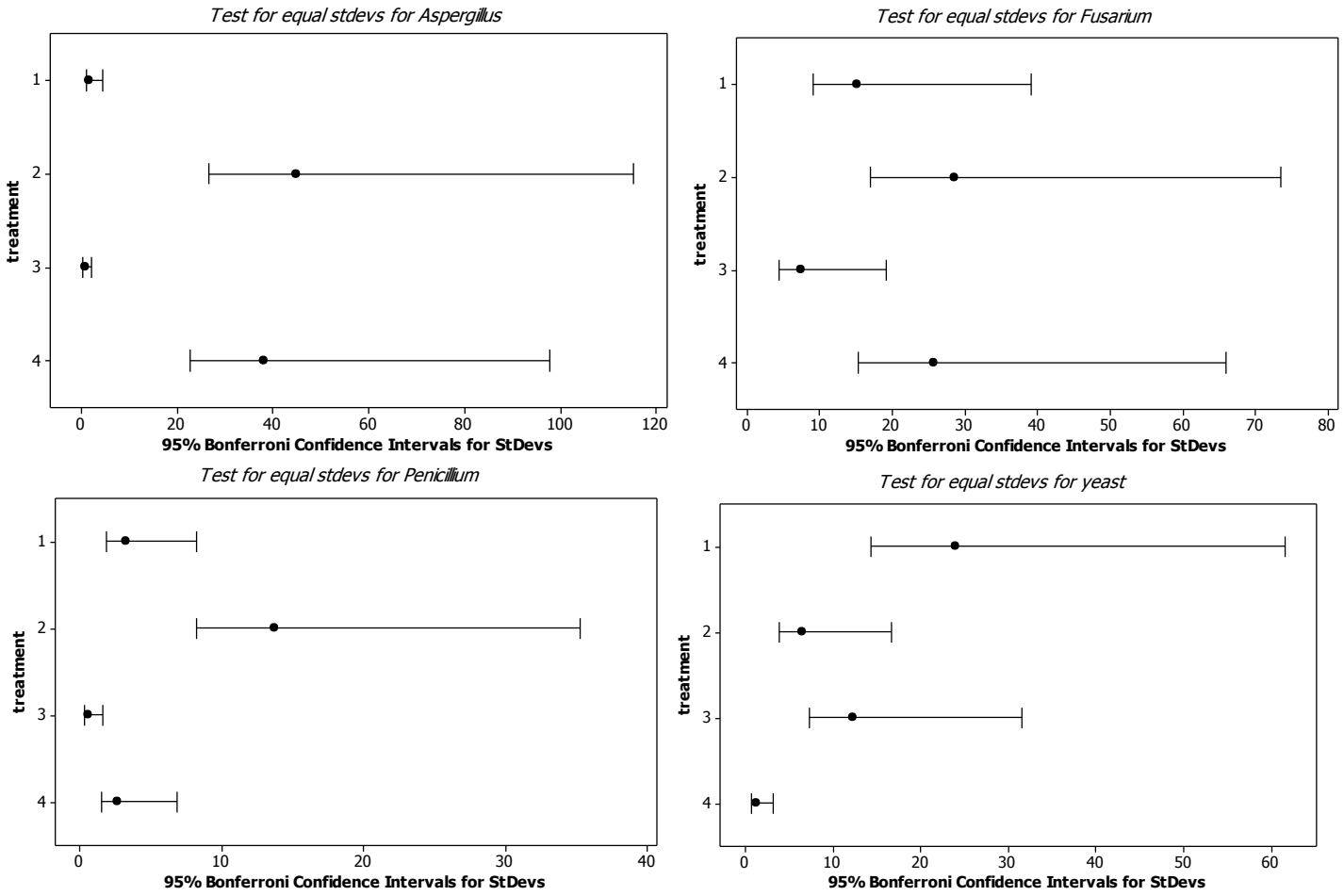

Figure 2. The frequency of filamentous fungi in processed coffee beans cultivated under organic and conventional systems, with and without surface disinfection. Treatment 1: organic coffee with surface disinfection. Treatment 2: organic coffee without surface disinfection. Treatment 3: conventional coffee with surface disinfection. Treatment 4: conventional coffee without surface disinfection.

found to exhibit a higher frequency of filamentous fungi (Figure 1). The analysis of coffee beans with and without surface disinfection accounts for fungal spores present inside and outside of the beans, respectively. In Figure 1, the vertical axis represents the fungal counts (in arbitrary units according to the data distribution) for each treatment. The distribution of the fungal counts for each treatment indicates the scatter between observations; a shorter distance between the fungal counts indicates a greater homogeneity between the scores of each fungus for each treatment.

Based on the fact that the variability within the samples cannot be directly observed (Figure 1), the homogeneity of the coffee bean samples was analyzed (Figure 2). A high diversity of *Aspergillus*, *Fusarium* and *Penicillium* was identified in the coffee bean samples with and without disinfection (Figures 2A, 2B, and 2C). Organic coffee samples exhibited a larger species variability,which was indicated by a higher contamination index for filamentous fungi.

The presence of the fungal genus *Aspergillus* in coffee is not desirable because the species *A. ochraceus*, *Aspergillus sclerotiorum*, *Aspergillus sulphureus*, *Aspergillus steynii*, and *Aspergillus westerdijkiae* have been associated with OTA production in coffee. The presence of the *Fusarium* genus was confirmed in coffee samples that had not been disinfected. Similar results were also reported by Pasin et al. (2009), who observed that the *Fusarium* genus was found with high incidence on the external portion of the beans under different coffee cultivation conditions.

Identification of filamentous fungi

Three hundred and forty-six (346) fungal isolates were obtained from all analyzed coffee beans, with 32 species belonging to the following 14 genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Mucor*, *Rhizopus*, *Trichoderma*, *Epicoccum*, *Phoma*, *Bipolaris*, *Glomerella*,

Table 1. Number of isolates of filamentous fungi present in coffee beans from organic and conventional farming

Species	Conventional				Organic			
	Cloth*		Sweeping**		Cloth*		Sweeping**	
	With disinfection	Without disinfection	With disinfection	Without disinfection	With disinfection	Without disinfection	With disinfection	Without disinfection
Alternaria alternata	6	-	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	7	6	4	2
Aspergillus foetidus	-	-	-	-	-	9	-	3
Aspergillus niger	3	-	-	-	-	-	-	-
Aspergillus ochraceus	-	-	-	-	5	14	-	4
Aspergillus oryzae	-	-	-	-	-	-	-	2
Aspergillus sulphureus	-	-	-	-	2	-	-	-
Aspergillus tubingensis	-	5	-	-	-	-	-	-
Aspergillus versicolor	-	-	-	-	-	3	-	-
Cladosporium cladosporioides	4	-	-	3	5	5	-	-
Colletotrichum gloeosporioides	-	-	-	-	3	4	-	-
Epicoccum purpurascens	6	-	-	-	-	-	-	-
Fusarium oxysporum	1	-	-	6	-	-	-	2
Fusarium semitectum	-	5	-	8	-	-	-	8
Fusarium solani		6						9
Gliocladium sp.	-	-	-	-	-	-	1	-
Mucorhiemalis	-	-	-	-	4	-	-	7
Penicillium brevicompactum	8	11	-	-	1	11	-	-
Penicillium citrinum	2	-	-	-	-	-	-	-
Penicillium hirsutum	-	-	-	-	-	-	3	-
Penicillium solitum	-	-	-	-	-	-	2	-
Rhizopus stolonifer	8	1	-	-	-	-	-	-
Trichoderma harzianum	-	-	-	-	-	-	-	4

*: Coffee harvested onto cloth; **: harvested coffee swept from the ground.

Colletotrichum, *Alternaria* and *Gliocladium* (Table 1). Various fungal genera have also been reported in studies on conventional coffee beans, notably species of *Aspergillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, *Rhizopus* and *Trichoderma*, among others (Chalfoun and Batista, 2003; Joosten et al., 2001; Prado et al., 2004; Leong et al., 2007; Rezende et al., 2013).

Fungi of the genus *Aspergillus* were detected in all samples that were not subjected to disinfection. The section *Circumdati* accounted for 49.35% of the observed contamination, followed by section *Nigri* (47.26%) and section *Flavi* (3.37%).*A. ochraceus* of section *Circumdati* was the predominant speciesand accounted for 73.17% of the total contamination. This fungal species has been identified in other studies on coffee beans (Batista et al., 2003; Silva et al., 2008;

Suarez-Quiroz et al., 2004; Batista and Chaulfoun, 2007). Additional species were also identified in the coffee samples, including *A*. *sulphureus* (17.7%) (Batista et al., 2003) and *A*. *ostianus* (9.75%). Studies using coffee beans harvested in Minas Gerais reported that 80% of the identified isolates belonged to the genus *Aspergillus* section *Circumdati* at all harvesting and processing stages (Batista et al., 2009). In comparison to the coffee swept from the ground, the coffee harvested onto cloth exhibited a high level of mold contamination. The coffee swept from the ground was found to contain the highest level of microorganisms, which is undesirable in a high-quality coffee (Batista et al., 2007). However, the quality of coffee swept from the ground is influenced by geographical location and climatic conditions. In high altitude regions without rain during the harvest, the level of microorganism contamination for coffee swept from the ground would be equivalent to that for coffee harvested onto cloth.

Fungal richness and diversity

Twelve (12) of the thirty-two species identified in organic and conventional coffee beans were present only in organic coffee beans and include *Fusarium solani*, *Colletotrichum gloeosporioides*, *Aspergillus tamarii*, *A*. *oryzae*, *A*. *parasiticus*, *A*. *versicolor*, *Penicillium hirsutum*, *Trichoderma harzianum*, *Phoma* sp., *Bipolaris* sp., *Glomerella cingulata* and *Mucor hiemalis*. The highest richness index was observed in the organic coffee bean samples (Rm = 4.18) in comparison to the conventional coffee bean samples (Rm = 3.24). This result indicates that the species richness is greater in the organic coffee than in the coffee from conventional cultivation. Several studies have indicated that organic farming allows for greater species richness than conventional farming (Mader et al., 2002; Stokstad, 2002; Delate and Cambardella, 2004; Bengtsson et al., 2005).

Regarding species diversity, the Shannon index for the organic coffee was 5.18, whereas the Shannon index for the conventionally cultivated coffee was 4.60. Thus, consistent with both the Margalef and the Shannon indices, the species richness and diversity of filamentous fungi are greater in the organic system. In organic agriculture, it is possible to observe an increase in soil biodiversity and biological activity. The greater organism diversity in this system maintains the biological equilibrium, promoting a reduction in disease- and pest-related problems (Hyde, 2001; Lima and Vianello, 2011).

The genera with the greatest number of represented species were *Aspergillus* and *Penicillium*. Twelve (12) *Aspergillus* species were divided into four groups (sections *Nigri*, *Circumdati*, *Flavi* and *Versicolores*) according to Klich (2002), whereas five species of *Penicillium* were identified (*Penicillium brevicompactum*, *Penicillium hirsutum*, *Penicillium crustosum*, *Penicillium citrinu* and *Penicillium solitum*).

Association of fungi with toxigenic species

The existence of different fungal species and their ability to produce different classes of secondary metabolites has enabled these microorganisms to become strong competitors within several ecosystems (Logrieco et al., 2007). Although the functions of mycotoxins have not been entirely elucidated, they are believed to participate in the elimination of other competing microorganisms from the environment (Brase et al., 2009). Therefore, the investigation of the association between different species of filamentous fungi is necessary to understand their complex ecological relationship and potential interactions that occur to promote the synthesis or degradation of mycotoxins in the environment.

The filamentous fungi that are associated with toxigenic species are shown in Figure 3. For these analyses, samples contaminated with *A*. *ochraceus*, which is one of the major OTA-producing species in coffee beans and *A*. *flavus*, which is known to produce aflatoxin (Chalfoun and Parizzi, 2008), were used for each cultivation system.

The association between toxigenic fungi and filamentous fungi in both the organic and the conventionally cultivated coffee beans was determined using a correspondence analysis technique (Hair et al., 1998), in which the homogeneity criteria included the location of profiles next to the center and their contribution to other components. Accordingly, in Figure 3A, an association between the toxigenic fungus *A. flavus* and species of filamentous fungi in the organic coffee bean samples can be observed. These *A. flavus*-associated species include *Cladosporium cladosporioides*, *Aspergillus ochraceus* and *Penicillium brevicompactum*. However, according to the established criterion for grouping, an association with *A. flavus* was not observed for *Colletotrichum gloeosporioides*, *Fusarium equiseti* and *Aspergillus foetidus* for either coffee cultivation system.

Using the coordinates and contributions obtained from the correspondence analysis, the organic coffee samples S1, S2, S3, and S4 did not confirm a clear association between toxigenic fungal species and *Aspergillus foetidus*, *Aspergillus flavus*, *Penicillium brevicompactum*, and *Cladosporium* (Figure 3B). Regarding group formation among the species *A. flavus*, *A*. *ochraceus* and *C. cladosporioides* in conventional coffee, the results illustrated in Figure 3C demonstrate that these fungi were associated with sample S2.

Microorganisms such as protozoa, bacteria, yeast, and filamentous fungi are capable of degrading mycotoxins through the production of proteolytic enzymes (Abrunhosa et al., 2006; Abrunhosa et al., 2010). Although a report from Shantha (1999) revealed that the fungus *Cladosporium* sp. represented the least efficient species to degrade aflatoxin, producing an inhibition of less than 10%, Figure 3 D indicates that only *C*. *cladosporioides* was associated with *A*. *ochraceus* in

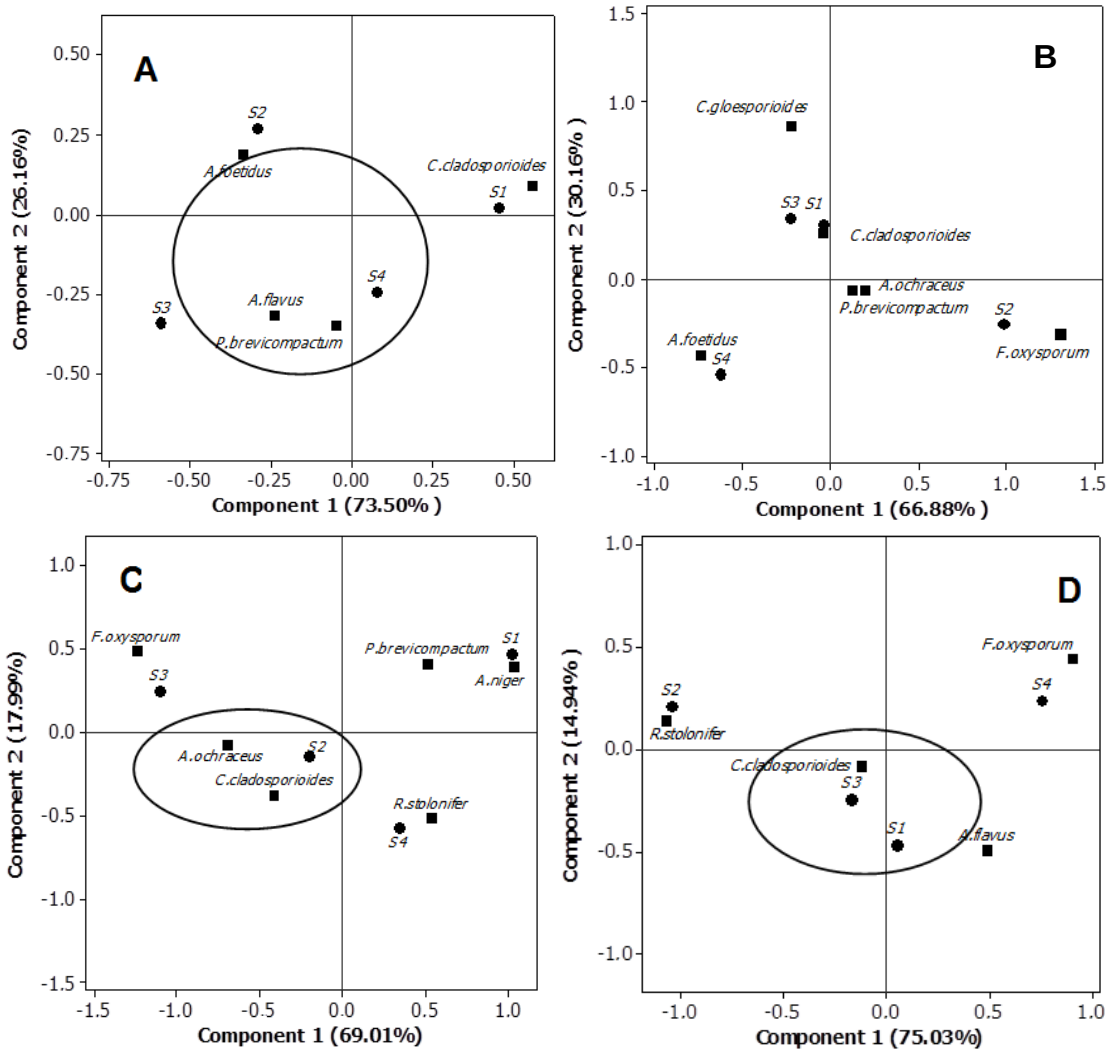

Figure 3. The association profile of toxigenic fungi in organic and conventionally cultivated coffee beans. A: *A. flavus* in organic coffee x fungi. **B:** *A. ochraceus* in organic coffee x fungi. **C:** *A. flavus* in conventional coffee x fungi. **D:** *A. ochraceus* in conventional coffee x fungi.

samples 1 and 3. However, *Rhizopus stolonifer*, *Fusarium oxysporum* and *Aspergillu sflavus* were not associated with this toxigenic fungus. Hence, a comparison of these associations with the results of Abrunhosa et al. (2002) suggests that *C. cladosporioides* exhibits a significant ability to degrade OTA. The occurrence of potentially toxigenic species in coffee represents a significant threat to the economy of this agricultural production addition to human and animal health.

The results of the association of fungal species with toxigenic species indicate that in both conventional and organic coffee, *A. ochraceus* and *A. flavus* are associated with *C. cladosporioides*. In the presence of *A. ochraceus*, *C. cladosporioides* can serve as a bioprotectorin organic and conventional coffee due to its ability to degrade mycotoxins. According to Martins et al. (2001), *Cladosporium* growth functions as a barrier to the growth of other fungi that are considered detrimental to coffee quality. Therefore, the interaction between *A. ochraceus* and *C. cladosporioides*is considered to demonstrate a positive effect on the safety and quality of the produced coffee.

Conflict of Interests

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

REFERENCES

Abrunhosa L, Paterson RRM, Venâncio A (2010).Biodegradation of ochratoxinA for food and feed decontamination. Toxins 2: 1078-1099.
Abrunhosa L, Santos L, Venancio A (2006). Degradation of ochratoxin A by proteases and by crude enzyme of *Aspergillus niger*. Food

Biotechonol. 20: 231-242.
Abrunhosa L, Serra R, Venâncio A (2002). Biodegradation of ochratoxin A by fungi isolated from grapes. J. Agric. Food Chem. 50: 7493-7496.
Agostinho AA, Thomaz SM, Gomes LC (2005).Conservation of the Biodiversity of Brazil's Inland Waters. Conserv. Biol. 19:646-652.
Amezqueta S, Gonzales-Penas E, Murillo-Arbizu M, Ceraina A (2009).Ochratoxin A decontamination: A review. Food Control 20:326-333.
Bakutis B, Baliukoniene V, Lugauskas A (2006). Factors predetermining the abundance of fungi and mycotoxins in grain from organic and conventional farms. Ekologija 3:122-127.
Batista LR, Chalfoun SM (2007).Incidência de Ocratoxina A emdiferentes frações do café (*Coffeaarabica* L.): bóia, mistura e varrição após secagem em terreiros de terra, asfalto e cimento. Ciência e Agrotecnologia 31:804-813.
Batista LR, Chalfoun SM, Prado G, Schwan RF, Wheals AE (2003). Toxigenic fungi associated with processed (green) coffee beans (*Coffeaarabica*L.). Int. J. Food Microbiol. 85: 293-300.
Batista LR, Chalfoun SM, Silva CF, Cirillo M, Varga EA, Schwan RF (2009).Ochratoxin A in coffee beans (*Coffeaarabica* L.) processed by dry and methods. Food Control 20:784-790.
Bengtsson J, Ahnstrom J, Weibull A (2005). The effects of organic agriculture on biodiversity and abundance: a met analysis. J. Appl. Ecol. 42: 261-269.
Bettiol W, Ghini R,Galvão, JAH, Ligo MAV, Mineiro JL (2002). Soil organisms in organic and conventional cropping systems. Scientia Agricola 59: 565-572.
Blackwell M (2011). The Fungi: 1,2,3 … 5,1 million species? Am. J. Bot. 98:426-438.
Brase S, Encinas A, Keck J, Nising CF (2009). Chemistry and Biology of mycotoxins and related fungal metabolites. Chem. Rev. 109: 3903-3990.
Cast (2003).Mycotoxins: risks in plant, animal and human systems. Council for Agricultural Science and Technology Task Force Report 139:139, 2003.
Chalfoun SM, Batista LR (2003).Fungosassociados a frutos e grãos de café *Aspergillus*&*Penicillium*. Brasília: Embrapa 69 p.
Chalfoun SM, Parizzi FC (2008). Fungostoxigênicos e Micotoxigênicosem Café. In: Borém, FM. (Eds.). Pós-colheitado café. Lavras 14: 509-543.
Clouvel P, Bonvarlet L, Martinez A, Lagouarde P, Diengi, Martin P, (2008).Wine contamination by ochratoxin A in relation to vine environment. Int. J. Food Microbiol. 123:74-80.
Delate K, Cambardella CA (2004).Agroecosystem performance during transition to certified organic grain production. Agron. J. 96: 1288-1289.
Duarte SC, Pena A, Lino CM (2010). Review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Food Microbiol. 27:187-198.
Ellis MB (1971). *Dematiaceous hyphomycetes*. Kew: Commonwealth Mycological Institute, 608p.
Gil-Sernaa J, Vázqueza C , Sardiñasa N, González-Jaénb MT, Patiñoa B (2011).Revison of ochratoxin a production capacity by the main species of *Aspergillus* section *Circumdati*– *Aspergillus steynii* revealed as the main risk of OTA contamination. Food Control 22:343-345.
Hair JF, Anderson RE, Tham RL, Black WC (1998). Multivariate Data Analysis, 5 ed. Prentice Hall. New Jersey.
Hawksworth DL (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol. Res. 95: 641-655.
Hole DG, Perkins AJ, Wilson JD (2005). Does organic farming benefit biodiversity? Biol. Conserv. 122:113-130.
Hyde KD (2001). Where are the missing fungi? Does Hong Kong have any answers? Mycol. Res. 105:1514-1518.
IBGE, InstitutoBrasileirode Geografia e Estatística (2013). IBGE Cidades@, 2013.Disponível em: <http://www.ibge.gov.br/cidadesat/painel/painel.php?315170#/>Acessoem: 2dez. 2013.
Joosten HMLJ, Goetz J, Pittet A, Schellenberg M, Bucheli P (2001). Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. Int. J. Food Microbiol. 65:39-44.
Klich MA (2002).Identification of common *Aspergillus* species. Amsterdam: Centraalbureauvoor Schimmelautures 116 p.
Leong SL, Hien LT, Trang NT, Hooking AD, Scott ES (2007).Ochratoxin A: producing *Aspergilli* in Vietnamese green coffee beans. Lett. Appl. Microbiol. 45:301-306.
Lima GP, Vianello (2011). Review on the main differences between organic and conventional plant-based foods. Int. J. Food Sci. Technol. 46: 1-13.
Logrieco A, Moretti A, Perrone G, Mulé G (2007). Biodiversity of complexes of mycotoxigenic fungal species associated with *Fusarium* ear rot of maize and *Aspergillus* rot of grape. Int. J. Food Microbiol. 119:11-16.
Mader P, Fliebach A, Dubois D, Gunst L, Fried P, Niggli U (2002). Soil fertility and biodiversity in organic farming. Science 296: 1694-1697.
Magurran AE (1988).Ecological diversity and its measurements. Sydney: Croom Helm, 179p.
Martins NA, Silveira AP, Silva RJN (2001).Avaliação da microbiota presente no café armazenado e recém beneficiado. In: SIMPÓSIO DE PESQUISA DOS CAFÉS DO BRASIL, 2., 2001, Vitória. Resumos... Brasília: CBP&D-Café/Embrapa Café, 2001. p. 59.
Noonim P, Ahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA (2008). Isolation, identification and toxigenic potential of ochatoxin A-producing *Aspergillus* species from coffee beans grown in two regions of Thailand. Int. J. Food Microbiol. 128:197-202.
O'Brien BL, Parrent JL, Jackson JA, Moncalvo JM, Vilgalys R (2005). Fungal community analysis by large-scale sequencing of environmental samples. Appl. Environ. Microbiol. 71: 5544-5550.
Pasin LAP, Almeida JR, Abreu MS (2009).Fungo sassociados a grãos de cinco cultivares de café (*Coffeaarábica* L.). Acta botânica brasileira 23:1129-1132.
Perrone G, Susca A, Cozzi G, Ehrlich J, Varga J, Frisvad JC, Meijer A, Noonim P, Mahakarnchanakul W, Samson RA (2007). Biodiversity of *Aspergillus* species in some important agricultural products. Stud. Mycol. 59:53-66.
Petzinger E, Weidenbach A (2002). Mycotoxins in the food chain: the role of ochratoxins. Livest. Prod. Sci. 76:245-250.
Pitt JI (2000). Toxigenic fungi: which are important. Med. Mycol. 38:17-22.
Pitt JI, Hocking AD (1997). Fungi and food spoilage. 2. ed. London: Blackie Academic and Professional, 540 p.
Prado E, Marín S, Ramos AJ, Sanchis V (2004). Occurrence of ochratoxigenic fungi and ochratoxinaA in green coffee from different origins. Food Sci. Technol. Int. 10: 45-49.
Rezende EF, Borges JG, Cirillo MA, Prado G, Paiva LC, Batista LR (2013).Ochratoxigenic fungi associated with green coffee beans (*Coffea arabica* L.) in conventional and organic cultivation in Brazil. Braz. J. Microbiol. 44:377-384.
Samson RA, Hoekstra ES, Frisvad JC (2000).Introduction to food and airborne fungi. 7. ed. Utrecht: Centraalbureauvoor Schimmelcultures, 389 p.
Schneider S, Hartmann M, Enkerli J, Widmer F (2010).Fungal community structure in soil of conventional and organic farming systems. Fungal Ecol. 3: 215-224.
Shantha T (1999).Fungal degradation of aflatoxin B1. Nat. Toxins 7:175-178.
Silva CF, Batista LR, Schwan RF (2008).Incidence and distribution of filamentous during fermentation drying and storage of coffee (*Coffee Arabica* L) beans. Braz. J. Microbiol. 39:238-240.
Stokstad E (2002). Organic farms many benefits. Science, Washington, 1589p.
Suárez-Quiroz M, Gonzalez-Rios O, Barel M, Guyot B, Solarr-galiwdo S, Gauduir J P (2004). Study of ochratoxin: producing strains in coffee processing. Int. J. Food Sci. Technol. 39:501-507.
Theodoro CGC, Guimarães JBC (2003). Avaliação do estadonutricional de agroecossitemas de café orgânico no estado de Minas Gerais. Ciência e Agrotecnologia 27:1222- 1230.
Varga J, Frisvad JC, Kocsubé S, Brankovics B, Tóth B, Szigeti G, Samson RA (2011). New and revisited species in *Aspergillus* section *Nigri*. Stud. Mycol. 69:1-17.

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Full Length Research Paper

Bioaccumulation of Fe^{3+} by bacteria isolated from soil and fermented foods for use in bioremediation processes

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It is known that in areas where mining activity exist, a great ecological imbalance with accumulation of iron particles in the soil can occur, which in high concentrations damages cellular structures of plants and microorganisms. Bacteria resistant to high concentrations of iron with the ability to reduce Fe^{3+} can make it bioavailable as an electron acceptor. The aim of this work was to select bacteria resistant to Fe^{3+} and to evaluate the accumulation of this element in the bacterial biomass of selected strains. Of 183 isolates tested using three different iron concentrations (0.005, 0.5 and 1 g/L) in culture medium chemically defined, 12 bacterial strains showed better growth in 1 g/L of iron. The three best isolates (*Bacillus simplex* UFLA CESB127, *B. subtilis* UFLA SCF590 and *Acetobacter tropicalis* UFLA DR6.2) were selected for further experiments. The selection of physical factors was performed using the Plackett-Burman design in order to assess the effect of the parameters on the accumulation of iron, followed by a central composite rotational design. A pH valuev of 3.5 and iron concentration of 0.750 g/L presented the best conditions for the accumulation of iron by bacterial isolates. After the optimization step, the bioaccumulation of Fe^{3+} was 99.22%. The validation confirmed the model of the experimental results. These results indicate the potential use of these isolates in the removal of iron, which in turn may be a promising alternative to conventional methods of treatment of contaminated soils.

Key words: Bioaccumulation, ferric iron, *Bacillus subtillis*, response surface methodology.

INTRODUCTION

Contamination of soils and industrial effluents with trace elements such as cadmium, lead, iron, manganese, mercury, chromium, copper, nickel, zinc has become a serious problem worldwide due to their high toxicity and bioaccumulative potential (Lodeiro et al., 2006). In undisturbed environments these elements are found in concentrations below 5 g/mL, but in contaminated areas they are often found above the permissible concentrations (Volesky, 2001; Lodeiro et al., 2006; Li et al., 2011).

The persistence of trace elements in the environment compromises the quality of soil, water and air with direct consequences on macro and microorganisms. Some

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species of bacteria are naturally resistant to higher concentrations of these elements making them the focus of approach to use non-pathogenic microbial biomass as an innovative and promising alternative to clean up contaminated areas. Some studies on bacteria of the phyla Firmicutes, Actinobacteria and Proteobacteria showed that they were capable of resisting and reducing mercury (Hg^{2+} to Hg^{0}) in the soil with high concentrations of this metal (Chakravarty et al., 2007). Strains of *Bacillus thuringiensis*, *Bacillus* sp. and *Paenibacillus polymyxa* are capable of absorbing cadmium (Cd^{2+}), arsenic (As) and copper (Cu^{2+}), respectively, due to the high affinity that these microorganisms demonstrated to these metals. The configuration of their surface polymers have been explored due to the ability to link to these metals (El-Helow et al., 2000; Nakamura et al., 2000; Acosta et al., 2005; Zhang et al., 2008; Kumar et al., 2010).

Iron is the fourth most abundant element found naturally in the earth's crust potentially making it the largest acceptor of electrons present in this environment (Stucki and Kostka, 2006; Dong et al., 2009; Marschner et al., 2011). However, mining activities mean that iron is the main polluter found in soil in particulate form and can compromise the integrity of molecules such as DNA, proteins and lipids (Naidoo and Chirkoot, 2004; Kuki et al., 2009). Many microorganisms have the ability to grow in high concentrations of trace elements. In general, this ability may be the result of intrinsic or induced mechanisms and may also be influenced by cell concentration, concentration of metal, composition of the culture medium, ionic strength and the presence of other ions in solution (Ledin, 2000). Among these factors, the pH value and stage of development of biomass are the factors that most influence the accumulation of elements (Guan et al., 1993).

Bacteria belonging to the genus *Bacillus* have the ability to reduce Fe^{3+}, when it is in excess in soils. These bacteria are widespread in natural systems such as soils, waters and marine sediments, and some of these microorganisms in these environments are potentially amenable to reducing Fe^{3+} (Lovley, 2000; Scheid et al., 2004; Liu et al., 2011). *Bacillus subtilis*, is a bacterium that is easily manipulated and has no or only a low level of pathogenicity. It has been studied for biotechnological potential of bioaccumulation (Diderichsen and de Boer, 1991). *B. subtilis* and *Bacillus licheniformis* show the capacity to adsorb various heavy metals, such as Cd^{2+}, Pb^{2+} and Cu^{2+}, and even compete for these metals (Daughney et al., 1998; Fein et al., 2005). Yang et al. (2012) reported the ability of *B. subtilis* to adsorb As^{5+} when complexed with Fe^{3+}, that is, Fe-bac performed well in the adsorption and reduction of As^{5+}.

Thus, the aim of this work was to select bacteria tolerant to Fe^{3+} and evaluate the nutritional and physical parameters that increase metal accumulation in bacteria with potential for use in bioremediation processes *in situ*.

MATERIALS AND METHODS

Microorganisms

The bacterial isolates tested belong to the Collection of Microorganisms from the Laboratory of Physiology and Genetics of Microorganisms, Department of Biology, Federal University of Lavras (UFLA, Brazil). These bacteria were isolated from Cerrado soils of Minas Gerais, from coffee fruit and cocoa fermentation (Silva et al., 2008; Coba et al., 2012; Pereira et al., 2012).

Cultivation of isolates

One hundred and eighty-three isolates were reactivated in a nutrient broth: 3 g/l of beef extract, 5 g/l peptone) and after 24 h, plated on nutrient agar (nutrient broth plus 15 g/l agar), incubated at 28°C for 24 h to check for purity by microscopic observation.

Selection of bacterial isolates under different conditions of iron

The evaluation of the growth of the isolates was performed using chemically defined culture medium (CD) (Pas et al., 2004) supplemented with different concentrations of ferric chloride ($FeCl_3$) (0.005, 0.5 and 1 g/L). The medium CD consisted of: 10 g/L of glucose, 0.5 g/L $(NH_4)_2SO_4$, 0.1 g/L KCl, 0.1 g/L NaCl, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl_2.2H_2O$, 1.0 g/L KH_2PO_4, 0.005 g/L H_3BO_3, 0.002 g/L $CuSO_4.5H_2O$, 0.01 g/L $ZnSO_4.7H_2O$, 0.002 g/L $Na_2MoO_4.2H_2O$, 0.001 g/L KI, 15 g/L Agar (pH 5.0 and no added vitamins). The bacterial inoculum was standardized according to the Mc Farland No. 5 scale (1.5×10^9 CFU/ml) and 10 µl of bacterial suspension added to each concentration tested in triplicate. The CD medium without the addition of iron was used as a positive control for each isolate. The colony diameters of the different isolates tested was performed after 48 h of incubation at 28°C, and the isolates with greater than or equal to 0.2 mm diameter were selected for the experimental design.

Experimental design

Identification of variables with significant effect on the growth of isolates: Delineation Plackett-Burman

Isolates selected under different conditions of iron were subjected to experimental design of Plackett and Burman (1946) for assessment of growth and the concentration of Fe^{3+} on microbial biomass (dependent variables) to fifteen different physicochemical and nutritional conditions generated from the 8 independent variables X1= pH (3.5 , 4.5 and 5.5), X2= concentration of glucose (5.0, 10.0 and 15.0 g/L), X3= iron concentration (0.005, 0.5 and 0.995 g/l), X4= concentration of the inoculum (10^6, 10^7 and 10^8 CFU/ml), X5 = nitrogen concentration (0.0 , 1.0 and 2.0 g/L), X6 = concentration of potassium (0.0 , 1.0 and 2.0 g/L), X7 = concentration of phosphate (0.0, 4.0 and 8.0 g/L), and X8 = ecoenzyme (enzyme complex whose function is the effect of buffer solution) (0.0, 5.0 and 10.0 g/L). The CD culture medium was used as base culture medium for all the different conditions tested. All cultures were maintained at 28°C for up to 7 days.

Determination of microbial biomass and content of Fe^{3+}

The production of biomass was measured in dry weight at 60°C

Table 1. Condition variables at different levels using CCRD for evaluation of the absorption of iron ferric isolate selected.

Variable	Code	Levels				
		-1.41	-1	0	+1	+1.41
pH	X1	3.295	3.5	4.5	5.5	5.705
Iron ferric (mg/L)	X2	147	250	500	750	852

after 7 days of growth for each test performed. The content of Fe^{3+} residual was assessed by an atomic absorption spectrophotometer (Varian SpectrAA 10 Plus) at 320 and 0.2 nm slit (Ewing, 1989; AOAC, 1997). The sample was obtained from the culture medium in the different conditions tested by rotary shaker at 150 rpm at intervals of 24 h and 7 days of and growth. The concentration of bioaccumulation by bacterial biomass was calculated by subtracting the initial concentration in each assay and the values of residual Fe^{3+}obtained from culture media by analysis in a spectrophotometer.

After identifying the independent variables that had a significant effect on the dependent variables, a central composite rotational design (CCRD) was applied for the purpose of optimizing the process of bioaccumulation by the isolates.

Central composite rotational design (CCRD)

Eight CCRD experiments were performed, grown in the culture medium CD (Pas et al., 2004) by varying the pH and the concentration of Fe^{3+} ($FeCl_3$ solution) (Table 1). The concentration of Fe^{3+} in the biomass was measured as described in determination of microbial biomass and content of Fe^{3+}.

The validation of the methodology used in the optimization process was performed by testing the best conditions obtained in the optimization and comparing them with the values predicted by the model. The experiment was done in triplicate, based on five points under conditions of interest within the surface and applying the same experimental procedures used to build the models. Statistical analyzes and graphs were performed using Design Expert® version 8.0 software (Stat-Ease Inc., Minneapolis, MN, USA).

Scanning electron microscopy with energy dispersive spectroscopy X-ray (SEM -EDS)

The samples of biomass previously centrifuged at 6000 g, from the validation stage were lyophilized. The samples were deposited on 1.0 mm diameter glass slides and immersed in a modified Karnovisky fixative solution (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.05 M cacodylate buffer pH 7.2, 0.001 M $CaCl_2$)/1 h. Subsequently, the slides were washed in 0.005 M cacodylate buffer to remove the glutaraldehyde residue of the Karnovisky solution and 5 drops of osmium tetroxide solution was added for fixation. After 4 h, the samples were washed in distilled water and dehydrated in an acetone series (25, 50, 75, 90 and 100%) and then placed in a desiccator to dry for 1 h. After drying, the samples were mounted on aluminum supports (stubs) and metalized by vacuum precipitation to a micrometric film of conductive material (carbon) on the surface of the material analyzed. Samples were observed in a scanning electron microscope (LEO EVO 40XVP) coupled with an energy dispersive system (X-ray microanalysis-EDS) and analyses of results were performed using the Genesis software under conditions: 30.000 kV, spot ranging from 5 to 7 and high vacuum (Alves, 2004).

RESULTS AND DISCUSSION

Selection of bacterial isolates under different conditions of iron

Of the 183 strains tested in different concentrations of Fe^{3+}, 12 showed higher tolerance to iron than the control, that is, colony diameter >0.4 mm in the CD medium with 0.5 g/L Fe^{3+} (data not shown). Of the 12 isolates with higher growth, three grew similarly with the control in the absence of iron (0.6 mm), and with a diameter of 0.3-0.5 mm when grown on CD medium with 1 g/L of Fe^{3+} (Table 2). The selection of bacteria capable of growing in higher concentrations of trace elements has the potential for bioremediation processes. This biotechnology may involve stimulation of the microbial community, the native population (biostimulation), introduction of a viable population (bioaugmentation), bioaccumulation (performed by living cells) or the use of biomass from dead cells (biosorption) (Abou-Shanab, 2011). In this work, bacterial strains from different origins were able to grow in high ferric iron concentrations; especially the species *B. subtilis*. Hsieh et al. (2009) studied the tolerance and accumulation of Hg^{2+} by *Bacillus megaterium* (and the successful transfer of the gene responsible for to *Arabdopsis thaliana* plant) *B. subtilis* previously grown in 8 mM solution of Fe^{3+} was capable of absorbing arsenic (Yang et al., 2012). This suggests that bacteria may be tolerant and/or resistant to multi-metals and thus can be used in soil contaminated by these elements. Because *B. subtilis* is tolerant to metals, this makes it a promising organism for the process of bioremediation of soil, since, along with *Corynebacterium* and other Firmicutes, dominate soils contaminated with metals (Ellis et al., 2003).

Variables having a significant effect on the accumulation of Fe^{3+}

Bacillus simplex UFLA CESB127, *B. subtilis* UFLA SCF590 and *Acetobacter tropicalis* UFLA DR6 were the three isolates that showed higher growth (Table 2). Twelve isolates from soil of the Cerrado of Minas Gerais state, from fermenting coffee fruit and from the fermentation of cocoa underwent 15 trials, with 8 independent variables analyzed using the Plackett and Burman design (1946) (Table 3). The results of the trial are given in Table 4.

Fe^{3+} is an element that can be chemically reduced or used as an electron acceptor, in an anaerobic response. Iron can be classified as essential, but is toxic to organisms in general when in high concentrations (Valls and Lorenzo, 2002). Operating costs in the bioremediation

Table 2. Evaluation of growth (mm) of 12 strains of bacteria isolated from different sources, QD cultured in medium at concentration of 1 g/L. Maximum diameter for each species are in bold.

Strain	Source	Species/genera	Media	
			QD (Without iron)	QD [1] g/L
			mm	
UFLA CESB127	Cerrado Soil	***Bacillus simplex***	**0.6**	**0.5**
UFLA CESB135	Cerrado Soil	*Bacillus simplex*	0.5	0.3
UFLA SCF590	Fermentation Coffee	***Bacillus subtilis***	**0.6**	**0.4**
UFLA SCF747	Fermentation Coffee	*Bacillus subtilis*	0.6	---
UFLA BR2.11	Fermentation Cocoa	*Acetobacter ghanensis*	0.5	0.3
UFLA ER5.15	Fermentation Cocoa	*Acetobacter* sp.	0.5	0.3
UFLA DR2.31	Fermentation Cocoa	*Acetobacter syzygii*	0.4	---
UFLA DR3.11	Fermentation Cocoa	*Gluconobacter* sp.	0.6	0.3
UFLA DR4.13	Fermentation Cocoa	*Acetobacter* sp.	---	---
UFLA DR6.12	Fermentation Cocoa	***Acetobacter tropicalis***	**0.6**	**0.4**
UFLA DR13.1	Fermentation Cocoa	*Acetobacter* sp.	---	---
UFLA DR3.21	Fermentation Cocoa	*Acetobacter* sp.	---	---

QD [1g/L] = QD media added, 1 g/L of $FeCl_3$ solution.

process should be considered, and thus the optimal conditions for bioaccumulation should be previously established in the laboratory in order to reduce these costs. Therefore, applying the methodology of an experimental design such as Plackett-Burman and CCRD help to establish the physical and chemical factors that increase the efficiency of bioaccumulation. Of the eight factors used to perform the Plackett-Burman, pH and concentration of Fe^{3+} were those that had the most significant effect on the accumulation of iron. Previous studies for adsorption of Cr^{6+} in a consortium of denitrifying bacteria determined that the pH and the amount of active (living) biomass were the determining factors for increased accumulation of this metal (Guan et al., 1993).

Among the independent variables tested only the pH and concentrations of Fe^{3+} showed significant negative and positive effects, respectively on bioaccumulation by bacterial biomass. Other factors showed no significant effects, but the magnitude of the effect of inoculum concentration (-24.25) was interesting showing that the lowest concentration of cells may be used in subsequent studies (Table 4).

The statistical significance of the model was checked by F-test. The analysis of variance (ANOVA) for bioaccumulation showed that the regression model was significant and the lack of fit was not significant (Table 4). The statistical model relate to biomass lack of fit and therefore was not significant, not allowing the optimization of this response. The cell biomass density is also a factor that previously was shown to influences the bioaccumulation. Inoculums with high cell density accumulated less metal due to the electrostatic interactions of the functional groups of the cell surface (Ledin, 2000). In our study, the cell concentration presented negative effect on the bioaccumulation of Fe^{3+}, indicating that the lower cell density should be used in the process (Table 4). In some cases, both organic and inorganic electron donor compounds may be used for the reduction of iron, for example when the availability of the metal for the microorganism occurs when it is in soluble form (Weber et al., 2006).

Central composite rotational design (CCRD)

B. subtilis UFLA SCF590 showed the best accumulation capacity of Fe^{3+} (highest accumulation of 0.828 g/l) and was selected for the optimization process using the CCRD. The experimental design was 2^2, with 11 trials being conducted, including four axial tests and another three central points. It was observed that the best conditions occurred in test 3 (pH= 3.5 and concentration of Fe^{3+} of 0.750 g/L) showing maximum accumulation of Fe^{3+} of 0.771 g/L (Table 5). Other tests also showed a high degree of accumulation of Fe^{3+} by biomass, but these conditions involved raising the pH and/or lowering the concentration of Fe^{3+}.

The model fit was assessed by the coefficient of determination R^2. The regression equation obtained indicated R^2 of 0.9922, with their predicted and fitted values of 0.9480 and 0.9845, respectively, suggesting an adequate fit of the model to quadratic experimental data and indicating that the model can explain 99.22% of the variability in response. The experimental results were modeled with a polynomial equation of second order to explain the dependence of the growth of the microorganism on the two analyzed factors

Table 3. Ferric iron accumulation (g/L) determined in Plackett-Burman design experiments after incubation of 3 bacterial isolates for 7 days. Absorption maxima are in bold and the percentage of biomass accumulation are in parenthesis.

Assay	pH	Glucose (g/L)	$FeCl_3$ (g/L)	Inoculum (UFC/mL)	NH_4NO_3 (g/L)	K_2HPO_4 (g/L)	Na_2PO_4 (g/L)	Ecoenzyme (g/L)	Iron ferric accumulation (g/L)		
									***B. simplex* UFLA CESB127**	***B. subtilis* UFLA SCF590**	***A. tropicalis* UFLA DR6.12**
B1	7.5	15	0.005	10^8	2	2	0	0	NA (0%)	NA (0%)	NA (0%)
2	3.5	15	0.995	10^6	2	2	8	0	**795 (79.5%)**	**828 (82.8%)**	**588 (58.8%)**
3	7.5	5	0.995	10^8	0	2	8	10	441 (44.1%)	527 (52.7%)	577 (57.7%)
4	3.5	15	0.005	10^8	2	0	8	10	NA (0%)	NA (0%)	NA (0%)
5	3.5	5	995	10^6	2	2	0	10	585 (585%)	818 (81.8%)	775 (77.5%)
6	3.5	5	0.005	10^8	0	0	8	0	NA (0%)	NA (0%)	NA (0%)
7	7.5	5	0.005	10^6	0	2	8	10	NA (0%)	NA (0%)	NA (0%)
8	7.5	15	0.005	10^6	2	2	0	10	NA (0%)	NA (0%)	NA (0%)
9	7.5	15	0.995	10^6	0	0	8	0	529 (52.9%)	0.1 (53.1%)	479 (47.9%)
10	3.5	15	0.995	10^8	0	0	0	10	566 (56.6%)	757 (75.7%)	742 (74.2%)
11	7.5	5	0.995	10^8	2	0	0	0	534 (53.4%)	602 (60.2%)	559 (55.9%)
12	3.5	5	0.005	10^6	0	0	0	0	NA (0%)	NA (0%)	NA (0%)
13	5.5	10	0.5	10^7	1	1	4	5	533 (53.3%)	557 (55.7%)	554 (55.4%)
14	5.5	10	0.5	10^7	1	1	4	5	526 (52.6%)	572 (57.2%)	558 (55.8%)
15	5.5	10	0.5	10^7	1	1	4	5	603 (60.3%)	569 (56.9%)	559 (55.9%)

NA – Not accumulated.

(pH and concentration of Fe^{3+}).

$$Y = 548.33 - 32.39X_1 + 230.28X_2 - 17.00X_1X_2 - 5.79X_1^2 - 58.29X_2^2,$$

where Y is the estimated accumulation of Fe^{3+} and X1 and X2 are the coded values, respectively, for pH and concentration of Fe^{3+} making it possible to confirm that the accumulation of iron can be estimated on the basis of quadratic effect on both factors .

The statistical significance of the model was checked by F-test (Table 6). The analysis of variance (ANOVA) for the accumulation of iron showed that the regression model was significant and lack of fit is not-significant, which can then describe the mathematical model based on the significant variables. The mathematical model is capable of describing the accumulation of iron based on the significant variables.

Regression analysis between X1 and X2 (Table 6), evaluated after 7 days of growth was significant for the confidence interval of 95 % ($p<0.05$). As noted, X1 and X2 factors showed values of -32.39 and 230.28, that is, negative and positive effects, respectively. This means that the lower the pH value in conjunction with the higher concentration of Fe^{3+} induced a greater accumulation of this metal by the bacteria. It was also noted that the quadratic effect of X2 was significant but other values for the interaction and the quadratic for X1 were not significant. This analysis of variance result showed the existence of significant differences between the effects caused by each factor analyzed.

The prediction of the optimal operating conditions for the accumulation of Fe^{3+} was determined experimentally using response surface methodology (RSM). The interaction effect of the parameters that significantly affect the accumulation of Fe^{3+} by *B. subtilis* UFLA SCF590 is shown in Figure 1. The curve in the

Table 4. ANOVA analysis showing each variable described for iron ferric absorption on 7 days of culture in Plackett-Burman experiments design.

Factors	Maineffect	Value F	Value P	Significance
			Prob> F	
Model	384.07	74.93	<0.0001	***
pH	-61.92	18.86	0.0074	**
Glucose	14.08	0.98	0.3686	NS
Iron ferric	338.58	563.97	<0.0001	***
Inocullum	-24.25	2.89	0.1497	NS
NH4NO3	36.08	6.41	0.0525	NS
K2HPO4	23.58	2.74	0.1590	NS
Na2PO4	-24.25	2.89	0.1497	NS
Ecoenzime	11.75	0.68	0.4474	NS
Lackoffit		6.86	0.1155	NS

(***): Significant at >99.9% (for 0.0001 <P value <0.001); (**) Significant levels between 99 and 99.9% (for 0.001 <P <0.01); NS: not significant (NS P <0.05 was considered to be non-significant).

Table 5. Two variable central composite rotational design (CCRD) and their responses to the iron ferric accumulated from isolated *Bacillus subtilis* UFLASCF590.

Assays	pH	Iron ferric (g/L)	RESPONSE	
			Iron ferric accumulation (g/L)	Accumulation percentage
1	3.5	0.25	0.255	100
2	5.5	0.25	0.204	81.6
3	3.5	0.75	0.771	100
4	5.5	0.75	0.652	86.9
5	3.3	0.50	0.582	100
6	5.7	0.50	0.519	100
7	4.5	0.1475	0.135	91.5
8	4.5	0.852	0.756	88.7
9	4.5	0.50	0.545	100
10	4.5	0.50	0.562	100
11	4.5	0.50	0.538	100

Table 6. ANOVA analysis of DCCR for the experimental results of the isolated *Bacillus subtilis* UFLASCF590 of culture for 7 days.

Factor		Effect	ValueF	Value P	Significance
				Prob> F	
Regressionmodel		548.33	127.64	<0.0001	***
X_1	pH	-32.39	11.80	0.0185	***
X_2	Ferro	230.28	596.66	<0.0001	***
X_1X_2		-17.00	1.63	0.2583	NS
X_1^2		-5.79	0.27	0.6278	NS
X_2^2		-58.29	26.99	0.0035	***
Lackoffit			7.11	0.1258	NS

(***) Significant at >99.9% (for 0.0001 <P value <0.001); NS: not significant (NS P <0.05 was considered to be non-significant).

response surface was plotted against two independent variables (pH and concentration of Fe^{3+}) for the predicted response Y (accumulation of Fe^{3+}).

A RSM showed the model proposed for the

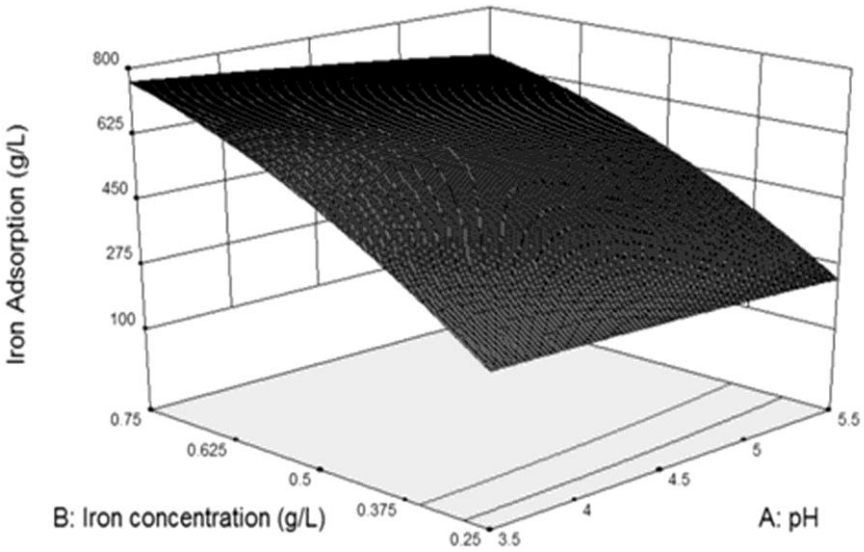


Figure 1. Effect of pH and ferric iron concentration on absorption by *Bacillus subtilis*. Response surface for the ferric iron absorption during the central composite rotational design–CCRD.

accumulation of Fe^{3+} after 7 days of testing. Table 5, indicates that the maximum accumulation of Fe^{3+}occurs with lower pH values (A) and with maximum concentration of Fe^{3+} tested (B) (Figure 1). By analyzing the response surface, we obtained a pH of 3.5 and concentration of Fe^{3+} of 0.750 g/l in optimal conditions resulting in a cumulative total biomass of 0.771 g/L. This validation was conducted with new experiments carried out under the optimum conditions shown in the CCRD, with the aim of confirming the proposed model. The comparison between the experimental results and those predicted by the model, confirmed the effect of the factors evaluated and the close agreement of the model with experimental results. Fe^{3+} solubility increases with a decrease of pH (Weber et al., 2006), which explains the higher bioaccumulation with lower pH values. The degree of bioaccumulation in this work by *B. subtilis* (UFLA SCF590) is reliable since there was no precipitate in the culture medium ensuring that the decrease in metal concentration in the medium corresponded to the accumulation of microbial biomass.

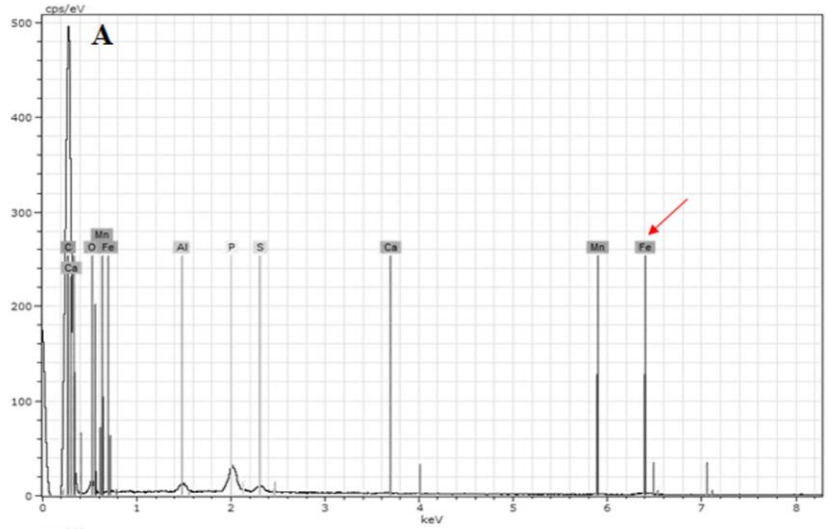
The transport of Fe to the interior of cells involves mobilization strategies (in this case reduction) in addition to a transport of high affinity (Miethke et al., 2013). The presence of other metals such as Cu^{2+}, Ni^{2+} and Cr^{2+} can stimulate the production of siderophores (is an organic compound that acts on iron uptake by organisms, p.e., bacteria) even in the presence of high concentrations of iron, acting as a system of extracellular resistance mechanism (Schalk et al., 2011) to toxic metals. Recently, it was observed that Fe^{3+} can be transported to the interior of the bacterial cell by specific transporters even without suffering prior reduction (Miethke et al., 2013).

Scanning electron microscopy with energy dispersive spectroscopy X-ray (SEM-EDS)

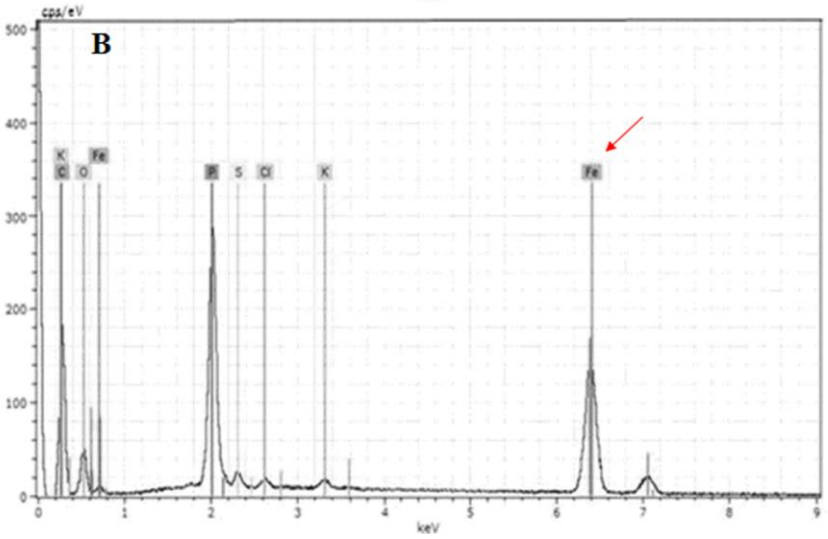
The energy dispersive X-ray (EDS) was used to qualitatively determine the Fe^{3+} produced from biomass in the control culture (no addition of iron) and in the culture after validating the optimal culture condition (Figure 2). Spectra of chemical composition by energy dispersive X-ray, are presented in Figure 2. The peaks for Ca and Mn disappeared and there was a decrease in peak S after the biomass was exposed to Fe^{3+} at a concentration of 0.750 g/L. Conversely, there was an increase in Cl and K peaks and no change in the Al and P peaks. These results indicated that the accumulation of Fe^{3+} by *B. subtilis* UFLASCF590 also includes ion exchange mechanisms. It was observed that the presence of Fe^{3+} in the biomass (peak of Fe^{3+} at 6.25 Kev) was approximately 5 times higher than in the control, that is, 34.3% concentration of atomic mass as compared to the control which showed 7.04%, confirming the accumulation of Fe^{3+} by *B. subtilis* (Figure 2). Spectroscopy energy dispersive X-rays (EDS) are thus a useful tool to evaluate the chemical characteristics of the biomass. From the analysis by EDS, we could see that the transport of Fe^{3+} involved the displacement of other intra and extracellular ions such as Ca and Mn, Cl and K, respectively. The exchange in the ion concentration was also observed after the absorption of Cu and Pb by *Bacillus* sp. (Tunali et al., 2006).

Conclusion

These results demonstrated that the isolate *B. subtilis*



ELEMENT	ATOMIC NUMBER	[Atomic Mass] standard %
Carbon (C)	6	0
Oxygen (O)	8	40,78007
Aluminium (Al)	13	7,486932
Phosphorus (P)	15	22,09808
Sulfur (S)	16	1,257599
Chlorine (Cl)	17	0,59126
Potassium (K)	19	0,970376
Calcium (Ca)	20	0
Manganese (Mn)	25	0
Iron (Fe)	26	34,30262
Copper (Cu)	29	0
Total	---	100



ELEMENT	ATOMIC NUMBER	[Atomic Mass] standard %
Carbon (C)	6	0
Oxygen (O)	8	49,05615
Aluminium (Al)	13	7,486932
Phosphorus (P)	15	22,34158
Sulfur (S)	16	7,015405
Chlorine (Cl)	17	0
Potassium (K)	19	0
Calcium (Ca)	20	0,816485
Manganese (Mn)	25	2,40733
Iron (Fe)	26	7,042312
Copper (Cu)	29	3,833808
Total	---	100

Figure 2. Analyzes micrograph and EDS taken by SEM and EDS of the *Bacillus subtilis* with the presence of Fe III (a) and control (b).

UFLA SCF590 is potentially useful in bioremediation processes in environments contaminated with Fe^{3+}. The process of removal of Fe^{3+} by this isolate was confirmed to be an active process of bioaccumulation involving the displacement of other ions. The maximum accumulation was obtained at pH 3.5 and a concentration of Fe^{3+} of 0.770 g/l with 100% efficiency.

Further studies are being developed using experimental microorganisms in the best conditions in order to evaluate the characteristics of bioaccumulation in soils previously contaminated with Fe^{3+}.

Conflict of Interests

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

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REFERENCES

Abou-Shanab RAI (2011). Biomanagement of Metal-Contaminated Soil. In: Khan MS, Zaidi A, Coel R, Musarrat J (Ed.). Bioremediation: new approaches and trends (Chapter 3), Springer. pp. 65-94.

Acosta MP, Valdman E, Leite SGF, Battaglini, Ruzal F (2005). Biosorption of copper by *Paenibacillus polymixa* cells and their exopolysaccharide. World J. Microbiol. Biotechnol. 21:1157-1167.

Alves E (2004). Introdução à microscopia eletrônica de varredura. Lavras: UFLA/FAEPE. p. 43.

AOAC (1997). Official methods of analysis of the Association of Official Analytical Chemists: 16.ed. Washington, p. 850.

Chakravarty S, Bhattacharjee S, Gupta KK, Singh M, Chaturvedi HT, Maity S (2007). Adsorption of zinc from aqueous solution using chemically treated newspaper pulp. Bioresour. Technol. 98:3136-3141.

Coba MSM, Magalhães-Guedes KT, Silva CF, Dias DR, Duarte WF, Schwan RF (2012). Culturable bacteria from soils of the Brazilian

Cerrado in Minas Gerais State. Prime J. Microbiol. Res. 2:131-136.
Daughney CJ, Fein JB (1998). The effect of ionic strength on the adsorption of H^+, Cd^{2+}, Pb^{2+}, and Cu^{2+} by *Bacillus subtilis* and *Bacillus licheniformis*: a surface complexation model. J. Colloid Interface Sci. 198:53-77.
Diderichsen B, De Boer AS (1991). On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Appl. Microbiol. Biotechnol. 36:1-4.
Dong H, Jaisi DP, Kim J, Zhang G (2009).Microbe-clay mineral interactions. Am. Miner. 94:1505-1519.
El-Helow ER, Sabry SA, Amer RM (2000). Cadmium biosorption by a cadmium resistant strain of *Bacillus thuringiensis*: regulation and optimization of cell surface affinity for metal cations. BioMetals 13:273-280.
Ellis RJ, Morgan P, Weightman AJ, Fry JC (2003). Cultivation Dependent and Independent Approaches for determining bacterial diversity in heavy metal contaminated soil. Appl. Environ. Microbiol. 69:3223-3230.
Ewing GW (1989). Instrumental Methods of Chemical Analysis, 5th edition, McGraw – Hill Book Company, New York. pp 1-7.
Fein JB, Boily JF, Yee N, Gorman-Lewis D, Turner BF (2005). Potentiometric titrations of *Bacillus subtilis* cells to low pH and a comparison of modelling approaches. Geochimica et Cosmochimica Acta 69:1123-1132.
Guan L, Petersen J, Johnstone D, Yonge D, Brouns T (1993). Equilibrium sorption of $Cr^{+6}$by a consortia of denitrifying bacteria. Biotechnol. Lett. 15:727-732.
Hsieh J, Chen CY, Chiu M, Chein MJC, Endo G, Huang, CC (2009). Expressing a bacterial mercuric ion binding protein in plant for phytoremediation of heavy metals. J. Hazard. Mater. 161:920–925.
Kuki KN, Oliva MA, Costa AC (2009). The simulated effects of iron dust and acidity during the early stages of establishment of two coastal plant Species. Water Air Soil Pollut. 196:287–295.
Kumar A, Bisht BS, Joshi VD (2010). Biosorption of Heavy Metals by four acclimated microbial species, *Bacillus* species, *Pseudomonas* species, *Staphylococcus* species and *Aspergillusniger*. J. Biol. Environ. Sci. 4:97-108.
Ledin M (2000). Accumulation of metal by microorganisms - processes and importance for soil systems. Earth Sci. Rev. 51:1-31.
Li T, Di Z, Islam E, Jiang H, Yang X (2011). Rhizosphere characteristics of zinc hyperaccumulator*Sedumalfredii* involved in zinc accumulation. J. Hazard. Mater 185:818-823.
Liu D, Dong H, Bishop ME, Wang H, Agrawal A, Tritschler S, Eberl DD, Xie S (2011). Reduction of structural Fe(III) in nontronite by methanogen *Methanosarcinabarkeri*. Geochimica et Cosmochimica Acta 75:6994-7010.
Lodeiro P, Barriada JL, Herrero R (2006). The marine macroalga*Cystoseirabaccata* as biosorbent for cadmium (II) and lead (II) removal: kinetic and equilibrium studies. Environ. Pollut. 142:264-273.
Lovley DR (2000). Environmental microbe-metal interactions. ASM, Washington, pp. 408.
Marschner P, Crowley D, Rengel Z (2011). Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis: model and research methods. Soil Biol. Biochem. 43:883-894.
Miethke M, Monteferrante CG, Marahiel MA, Van Djil JM (2013). The *Bacillus subtilis*EfeUOB transporter is essential for hight-affinity acquisition of ferrous and ferric-iron. Biochim. Biophys. Acta 1833:2267-2278.
Naidoo G, Chirkoot D (2004). The effects of coal dust on photosynthetic performance of the mangrove, *Avicennia marina* in Richards Bay, South Africa. Environ. Pollut.127:359-366.
Nakamura K, Aoki J, Morishita K, Yamamoto M (2000). Mercury volatilization by the most mercury-resistant bacteria from the seawater of Minamata Bay in various physiological conditions. Clean Technol. Environ. 2:174-178.
Pas M, Milacic R, Draslar K, Pollak N, Raspor P (2004).Uptake of chromium (III) and chromium (IV) compounds in the yeast cell structure. BioMetals 17:25-33.
Pereira GV, Miguel MG, Ramos CL, Schwan RF (2012). Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. Appl. Environ. Microbiol. 78:5395-5405.
Plackett RL, Burman JP (1946).The design of optimum multifactorial experiments. Biometrika 33:305-325.
Schalk IJ, Hannauer M, Braud A (2011). New Roles for bacterial siderophores in metal transport and tolerance. Environ. Microbiol.13:2844-2854.
Scheid D, Stubner S, Conrad R (2004). Identification of rice root associated nitrate, sulfate and ferric iron reducing bacteria during root decomposition. FEMS Microbiol. Ecol. 50:101-110.
Silva CF, Batista LR, Abreu LM, Dias ES, Schwan RF (2008). Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation. Food Microbiol. 25:951-957.
Stucki JW, Kostka JE (2006). Microbial reduction of iron in smectite. Compt. Ren. Geosci. 338:468-475.
Tunali S, Çabuk A, Akar T (2006). Removal of lead and cooper ions from aqueous solutions by bacterial strain isolated from soil. Chem. Eng. J. 115:203-211.
Valls M, de Lorenzo V (2002). Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiol. Rev. 26:327-338.
Volesky B (2001). Detoxification of metal-bearing efflents: biosorption for the next century. Hydrometallurgy 59:203-216.
Weber KA, Achenbach LA, Coates JD (2006). Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. Nat. Rev. Microbiol. 4:752-764.
Yang T, Chen ML, Liu LH, Wang JH, Dasgupta PK (2012). Iron (III) modification of *Bacillus subtilis* membranes provides record sorption capacity for arsenic and endows unusual selectivity for As(V). Environ. Sci. Technol. 46:2251-2256.
Zhang X, Jia Y, Wnag X, Xu L (2008). Phylogenetic analysis and arsenate reduction effect of the arsenic-reducing bacteria enriched from contaminated soils at an abandoned smelter site. J. Environ. Sci. 20:1501-1507.

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Bioremediation of Al-Sayyadin Lagoon polluted water using wild and mutant strains of microalgae

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In this study, an attempt was made to reduce the pollutants of Al-Sayyadin Lagoon water, which is still the main site of open fishing in Ismailia, Egypt, using the wild and mutant strains of two green microalgae called *Chlamydomonas reinhardtii CC1021* and *Parachlorella kessleri PC*. Four mutant strains were obtained from UV mutagenesis of the two wild types. One mutant strain was from *C. reinhardtii CC1021 (CC1021Mut1)* and the 3 other strains ((*PCMut2*, *PCMut3* and *PCMut4*) were from *P. kessleri PC*. Reduction of nutrients like phosphate, ammonia, chemical oxygen demand (COD), biological oxygen demand (BOD) as well as some heavy metals like Co, Zn by the six microalgal strains was studied. The results obtained showed that the treated wild and mutant strains with mixture of TAP medium and polluted water showed highest growth rate and were more efficient in improving water quality than that treated with polluted water only in phycoremediation. The dose of UV radiation used in this study had no negative impact on the efficiency of bio-remediation potentials of the tested mutant strains but in some results it enhanced their growth rate and removal efficiency. The statistical analysis indicated that there was significant differences ($p<0.05$) found in bioremediation of water parameters between the selected algae. The wild and mutant strains of *P. kessleri* had higher efficiency in phycoremediation than the wild and mutant strains of *C. reinhardtii CC1021*. The mutant algae of *P. kessleri PC* exhibited significant higher growth rate and removal efficiency than the wild type.

Key words: Bioremediation, *Chlamydomonas reinhardtii*, *Parachlorella kessleri*, ultra violet (UV) mutagenesis, wild and mutant strains.

INTRODUCTION

Pollution of surface water has become one of the most important environmental problems. Two types of large and long-lasting pollution threats can be recognized at the global level: organic pollution leading to high organic content in aquatic ecosystems and eutrophication. It is well-known that polluted water can reduce water quality, thus restricting the use of water bodies for many purposes. Organic pollution occurs when large quantities of organic compounds from many sources are released into the receiving running waters, lakes and also seas. Organic pollutants originate from domestic sewage farm water and could negatively affect water quality in many

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Figure 1. Geographical location of Al-Sayyadin Lagoon.

ways. During the decomposition process of organic water, dissolved oxygen in the water may be used up at greater rate than it can be replenished, thus giving rise to oxygen depletion which has severe consequences on aquatic biota. Organic effluents also frequently contain large quantities of suspended solid which reduces the light available to photosynthetic organisms, mainly algae. In addition to organic wastes from people and animals, these may also be rich in disease causing (pathogenic) organisms (Xu and Nirmalakhandan, 1998; Altenburger et al., 2000). Numerous natural and chemical substances have been used and released without knowledge of the possible impact on the structure and function of aquatic ecosystems (Häder et al., 2006). There are many physico-chemical methods available in treating polluted waters, but recent progress in bioremediation suggests that algae can play dual role in increasing biomass by utilizing waste as nutrients and can help in solving problems of pollution created by effluents (Mehta and Gaur, 2005). Efforts have been made to apply intensive microalgal cultures to remediate different wastes that have been described by de-Bashan et al. (2002), Rao et al. (2011) and Kshirsagar (2013).

The aim of the present investigation is to examine the efficiency of microalgae strains in the removal of inorganic nutrient and some heavy metals to prevent further deterioration of water quality of El Sayyadin Lagoon. It also aims to boost published literatures on mutagenesis in this area, which is far more limited.

MATERIALS AND METHODS

Collection of wastewater

The polluted water samples used in this study were collected from Al Sayyadin Lagoon located in Ismailia City, Egypt (Figures 1 and 2). This lagoon is polluted by sanitary and agricultural wastewater from El Bahtini and Mahsama drains.

Microalgal strains

The microalgal strains of *Chlamydomonas reinhardtii (CC1021*mt+) and *Parachlorella kessleri PC* (the wild type strain SAG 211-11h) were formerly referred to as *Chlorella vulgaris* but were later reassigned into *Chlorella kessleri* (Fott and Nováková, 1969). Today, these species are referred to as *P. kessleri* (Krienitz et al., 2004), obtained from the Biotechnology Department, Kazakh National University- Al-Farabi culture collection. Both of these strains were cultured in TAP medium in 250 ml Erlenmeyer flask at 28°C and were exposed to continuous illumination at a light intensity of 120 μE $m^{-2}s^{-1}$. TAP medium composed of 25 ml from the first stock solution (NH_4Cl 15 g/l, $MgSO_4.7H_2O$ 4 g/l, $CaCl_2.2H_2O$ 2 g/l) and 1 ml from the second stock solution (Phosphate buffer; K_2HPO_4 28.8 g/100 ml and KH_2PO_4 14.4 g/100 ml) in addition to Tris-base (2.42 g), 1 ml of glacial acetic acid, 1 ml of microelements and pH = 6-7.

Figure 2. Al-Sayyadin Lagoon.

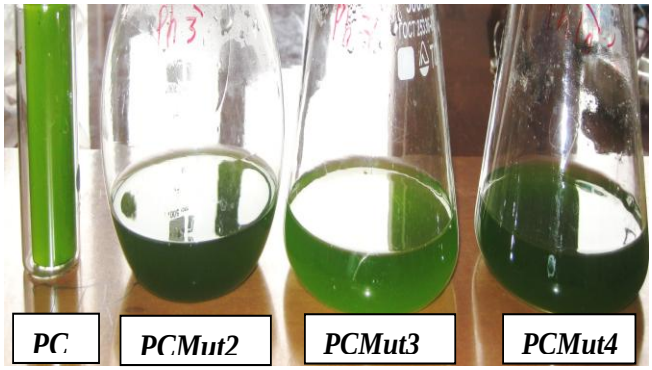

Figure 3. Growth of the wild type *Parachlorella kessleri PC, PC Mut2, PCMut3* and *PCMut4*.

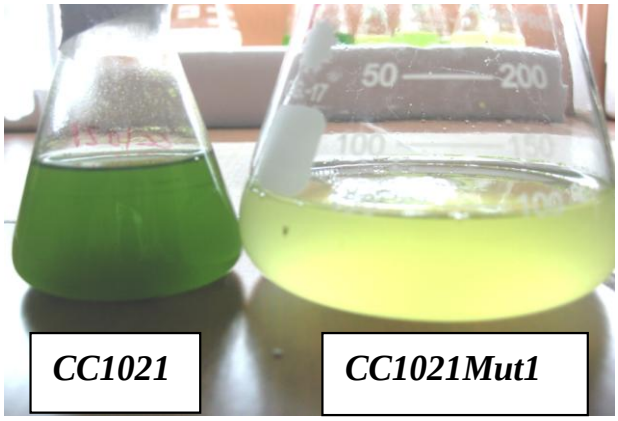

Figure 4. The wild type *CC1021* (dark green colour) and *CC1021Mut1* (light green colour).

Ultra violet (UV) mutagenesis

According to Harris (1989), 5 ml of the liquid culture with a density of $1x10^6$/ ml of algal cells was placed in 9 cm Petri dish, forming a thin layer covering the bottom. The dish was placed on shaker with 20 rpm and exposed to UVC lamp of 254 nm and 5 W/m^2 at a distance equal to 15 cm for 0, 1, 3, 5, 7 and 10 min, respectively. After UV irradiation, the cells were inoculated in solid TAP medium and incubated in the dark for 24 h to prevent photo-reactivation. After 24 h, some dishes were incubated in light under photoautotrophic condition and the others incubated in the dark under heterotrophic condition for a period of 15 days.

Selection of the mutant strains

After the incubation period, four mutant strains were selected based on the phenotypic characteristics which appeared in the color and size of colonies that differ from the wild type colonies. These mutant strains were *CC1021Mut1* obtained from exposure of *C. reinhardtii (CC1021mt+*) to UV irradiation for 1 min. The three others (*PCMut2, PCMut3* and *PCMut4)* were obtained from exposure of *P. kessleri* to UV irradiation for 3, 7 and 10 min, respectively. These mutants were transferred from solid to liquid medium and kept under phototrophic growth conditions to be tested for their efficiency in the bioremediation of polluted water (Figures 3 and 4).

Design of the bioremediation experiment

To study the efficiency of microalgae in bioremediation of polluted water, the following methods were employed: (i) Cultural media inoculated with algal strain (negative control), (ii) polluted water inoculated with algal strain (positive control), (iii) series of diluted polluted water and cultural media inoculated with algal strain as follows:

Sample number 1: Negative control (1000 ml of the culturing medium) inoculated with the algal strain.

Sample number 2: Dilution 4:1 (800 ml culturing medium + 200 ml of the polluted water).
Sample number 3: Dilution 3:2 (600 ml culturing medium + 400 ml of the polluted water).
Sample number 4: Dilution 2:3 (400 ml culturing medium + 600 ml of the polluted water) inoculated with the algal strain.
Sample number 5: Dilution 1:4 (200 ml culturing medium + 800 ml of the polluted water) inoculated with the algal strain.
Sample number 6: Positive control (1000 ml of the polluted water) inoculated with the algal strain.

The total number of samples for the six algal strains was 36 and these samples were incubated for one week at the same growth condition of light and temperature.

Investigation of algal growth strains in the samples

Examination of the algal cells was done by using light inverted microscope of the OLYMPUS series. The initial cell density was about 1×10^5 cells/ml. The number of cells was counted everyday for one week by using UTERMÖHL's technique (1958) under light microscope (Wetzel and Likens, 1979). The growth of algal strains was plotted for the six samples for each strain. The algal growth percentage of the six samples for each algal strain was calculated (Figure 5).

Analytical method

Water samples were filtered onto Whatman GF/C glass fiber filters to get rid of the algal cells. The physical and chemical parameters and some heavy metals of polluted water were analyzed before (Table 1) and after using the selected algae (Tables 2 and 3) according to the Standard Methods for Examination of Water and Waste Water (APHA, 1989). The parameters under study were ammonia, phosphate, biological oxygen demand (BOD), chemical oxygen demand (COD) and cobalt and zinc. After one week, these parameters were analyzed in samples numbers 2, 4 and 6 for each of the algal strain.

RESULTS

Algal growth rate

It is clear from Figure 5 that the algal growth rate of the two wild types and their mutants in both selected dilutions are lower in the positive controls which contain the polluted water only as compared to other dilution samples and the negative control. On the other hand, the negative control of *C. reinhardtii CC1021, P. kessleri* and *CC1021 Mut1* strains, respectively achieved a higher percentage of cell numbers as compared to their dilutions and decreased with the increase of polluted water content. In contrast, there is a significant increase of the growth rate recorded in sample numbers 2, 3, 4 for *PCMut2* and in samples 3 and 4 for *PCMut3* as compared to the negative control. *PCMut4* strain growth rate percentage was high in samples 2, 3, 4, 5 as compared to the control and showed the best growth rate of 152.2% in sample number 3 (dilution 3:2).

Water analysis

The results obtained from water analysis (Tables 2 and 3; Figures 6, 7, 8) showed that, the initial pH of the waste water was 6.02, and after being treated with the algal strains, it increased from 7 to 7.3. In respect of nutrient salts, in sample No. 2, the highest NH_4 removal was done by *PCMut3,PC, PCMut2*, *PCMut4, CC1021Mut1* and *CC1021* (90.1, 90, 88.8, 87.8, 87.6, 87.38, respectively). Highest PO_4 removal was done by *PCMut3, PCMut2*, *PC*, *PCMut4, CC1021Mut1* and *CC1021* (55.2, 48.3, 47.3, 45.2, 41.5, 38.6, respectively). Highest Zn removal was done by *CC1021, CC1021Mut1*, *PC, PCMut2*, *PCMut4* and *PCMut3* (92.38, 90.7, 89.7, 89, 87.6 and 83). Highest Co removal was done by *PC, PCMut2*, *PCMut3, PCMut4*, *CC1021 Mut1* and *CC1021* (69.7, 69, 68, 67.6, 67.5, 67.3, respectively). In sample No. 4, highest NH_4 removal was done by *PC, PCMut3, PCMut2, CC1021Mut1, PCMut4 and CC1021*, (88.7, 88.1, 86.7, 80.6, 80.2, 73.5, respectively). Highest PO_4 removal was done by *PCMut2, PC, PCMut3, PCMut4, CC1021 and CC1021Mut1* (53.8, 36.1, 34.2, 22, 12.2, 9). Highest Zn removal was done by *CC1021*, *CC1021Mut1, PCMut3*, *PC, PCMut2 and PCMut4* (90.4, 89.8, 66.9, 66, 65.1 and 63.4). Highest Co removal was done by *PCMut2*, *PC, PCMut3*, *CC1021Mut1, PCMut4* and *CC1021* (60.8, 57.7, 55.5, 51, 49.9 and 48.6). In sample number 6, the algal strains showed an obvious percent increase in the level of NH_4, PO_4 and Zn than the control except *PC* for NH_4 and *PCMut2* and *PCMut3* for PO_4. The highest Co removal was done by *PCMut3*, *PCMut4, PC, CC1021Mut1, PCMut2* and *CC1021* (92.7, 85, 83.2, 62.2, 54.45 and 34.45).

Chemical oxygen demand (COD) and biological oxygen demand (BOD)

It is clear from Tables 2 and 3 and Figure 9a and b that the levels of COD and BOD of treated samples reduced significantly in both samples number 2 and 4 and the reduction percentage was nearly the same values for both. In sample number 2, the highest COD removal was done by *CC1021, PC, CC1021Mut1, PCMut2 PCMut3 and PCMut4* (99.5, 98.35, 96.5, 93.14, 87, 86.4, respectively), while the highest BOD removal was done by *CC1021Mut1, CC1021*, *PCMut3*, *PC, PCMut2* and *PCMut4* (94.8, 94.8, 92.4, 91.7, 88.8, 86.2). In sample number 4, the highest COD removal was done by *PCMut2*, *CC1021, CC1021 Mut1*, *PC, PCMut3* and *PCMut4* (99.8, 99.75, 99.75, 99.7, 99.5 and 99.45) while the highest BOD was by *PCMut2*, *CC1021, CC1021 Mut1*, *PCMut4*, *PCMut3* and *PC* (98.6, 97.5, 97.3, 96.9, 95.8 and 94.45).

DISCUSSION

Microalgae usually play an important role during the

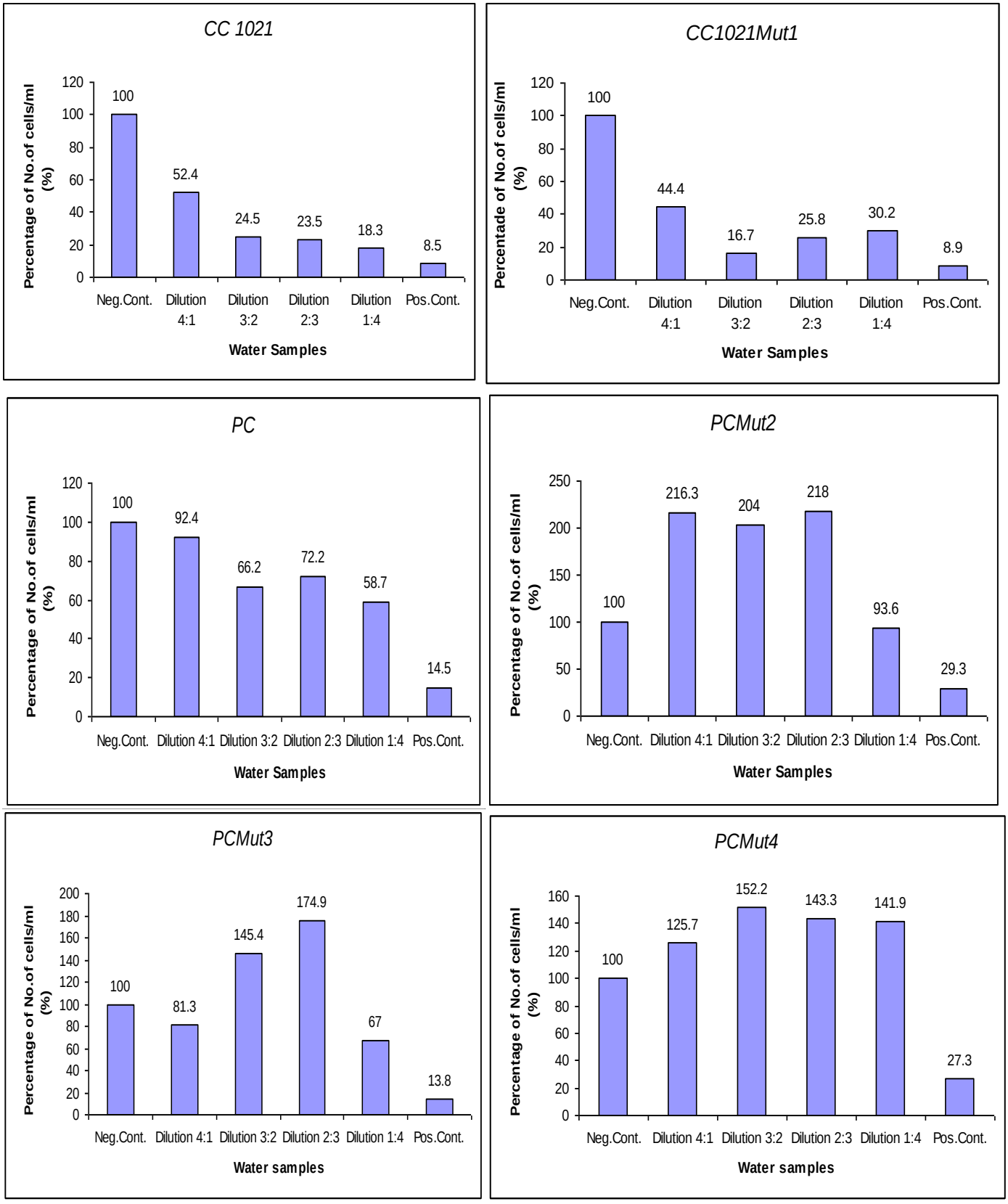

Figure 5. The growth percent of the six algal strains in different selected samples after 7 days.

Table 1. The analysis of water before using algae.

Parameter	Control (C)	Control of dilution 4:1 (C4:1)	Control of dilution 2:3 (C2:3)
Temperature (°C)	30	30	30
pH	6.02	6.02	6.02
BOD_5 (mg/l)	2.16	2.16	2.16
COD (mg/l)	32.34	32.34	32.34
Ammonia (mg/l)	1	225.9	75.46
Phosphate (mg/l)	2.488	259.48	86.67
Cobalt (mg/l)	0.0005	0.9611	0.3210
Zinc (mg/l)	0.0154	13.21	4.414

Table 2. Analysis of water samples after bioremediation for one week using *CC1021* and *CC1021Mut1* strains.

Parameter	*CC1021*						*CC1021Mut1*					
	C-4:1	No. 2	C-2:3	No. 4	C	No. 6	C-4:1	No. 2	C-2:3	No. 4	C	No. 6
Temperature (°C)	30	30	30	30	30	30	30	30	30	30	30	30
pH	6.02	7.2	6.02	7.2	6.02	6.3	6.02	7.3	6.02	7.3	6.02	6.7
BOD_5 (mg/l)	2.16	0.1125	2.16	0.055	2.16	8.911	2.16	0.112	2.16	0.058	2.16	29.287
COD (mg/l)	32.34	0.1716	32.34	0.0825	32.34	10.65	32.34	1.1375	32.34	0.08	32.34	10.75
Ammonia (mg/l)	225.9	28.5	75.46	20	1	2.665	225.9	28	75.46	14.6	1	3.265
Phosphate (mg/l)	259.48	158.86	86.67	76.07	2.488	5.248	259.48	151.84	86.67	78.83	2.488	4.72
Cobalt (mg/l)	0.9611	0.3138	0.3210	0.1649	0.0005	0.0118	0.9611	0.3125	0.3210	0.1573	0.0005	0.0068
Zinc (mg/l)	13.21	1.006	4.414	0.4235	0.0154	0.1658	13.21	1.219	4.414	0.449	0.0154	0.1121

tertiary treatment of domestic wastewaters in maturation ponds or the treatment of small/middle scale municipal wastewater in facultative or aerobic ponds (Aziz and Ng, 1993; Abeliovich, 1986; Mara and Pearson**,** 1986; Oswald et al., 1996). Algae utilize wastes as nutritional source and enzymatically degrade pollution. They enhance the removal of nutrients, heavy metals and pathogens and furnish O_2 to heterotrophic aerobic bacteria to mineralize organic pollutants, using in turn the CO_2 released from bacterial respiration (Munoz and Guieysse, 2006). The present work aims to describe the screening of 6 microalgal strains to select a good candidate for the removal of pollutants from waste water. In this study, two different species of green microalgae- *C. reinhardtii CC*1021 strain and *P. kessleri* were used as target organisms because they were used as model for phototrophic organisms and are green algae in particular.

It is well known that genus *Chlamydomonas* was considered as a significant step toward the use of algae for remediation of contaminated sites and waters (Hallmann, 2007). The mutations in this study were induced by using UV radiation. UV mutagenesis offers many advantages such as less pollution, simple operation and sterile cultivation condition (Huang et al., 2010). UV-induced physiological effects such as declining photosynthetic rates can be related not only to damaged biomolecules, but also to ultrastructural changes in organelles or membranes (Holzinger and Lütz, 2006). The two microalgae were treated with the mutagen UV and the result was 4 mutant strains and such mutagenically treated algae were compared with untreated wild types in terms of growth and remediation efficiency. The results showed that all the cultures of the selected strains showed highest growth percent as compared to that of the positive control (Figure 6). The less growth of the algal strains in the positive control is an indication of highly polluted water (Afkar et al., 2010). This may be attributed to the presence of other microorganisms and bacteria that compete with our strains and lowered their growth as compared to the others that contained mixture of polluted water and culturing medium. It is well known that the role of culture media is to make algal growth to be faster than the growth of the other microorganisms which is not adapted to this culture media. This result was confirmed by reports of Cho et al. (2011) and Olumayowa et al. (2013) that the growth of microalgae increases by 1.5 and 2.5 fold in autoclaved wastewater to get rid of other microorganisms and bacteria, even though some bacteria have been found to secrete algal growth promoters (Mazur et al., 1995).

The growth rates of the wild type strain of *CC1021*, *CC1021* mutant and *PC* wild type in this study are nearly the same, that is, they attained the higher growth

Table 3. Analysis of water samples after bioremediation for one week using *Pc, PCMut2, PCMut3* and *PCMut4* strains.

Parameter	C-4:1	No.2	C-2:3	No.4	C	No.6	C-4:1	No.2	C-2:3	No.4	C	No.6
				PC						***PCMut2***		
Temperature (°C)	30	30	30	30	30	30	30	30	30	30	30	30
pH	6.02	7.02	6.02	7.0	6.02	6.8	6.02	7.2	6.02	7.1	6.02	6.6
BOD_5 (mg/l)	2.16	0.179	2.16	0.12	2.16	21.07	2.16	0.255	2.16	0.03	2.16	27.03
COD (mg/l)	32.34	0.533	32.34	0.097	32.34	29.568	32.34	2.22	32.34	0.058	32.34	83.88
Ammonia (mg/l)	225.9	23	75.46	8.5	1	0.765	225.9	27	75.46	10	1	3.765
Phosphate (mg/l)	259.48	136.62	86.67	55.36	2.488	2.629	259.48	134.22	86.67	40.06	2.488	4.696
Cobalt (mg/l)	0.9611	0.2916	0.3210	0.1359	0.0005	0.0031	0.9611	0.2978	0.3210	0.1428	0.0005	0.0082
Zinc (mg/l)	13.21	1.363	4.414	1.496	0.0154	0.0507	13.21	1.454	4.414	1.504	0.0154	0.122
				PCMut3						***PCMut4***		
Temperature (°C)	30	30	30	30	30	30	30	30	30	30	30	30
pH	6.02	7	6.02	7.2	6.02	6.9	6.02	7.2	6.02	7.1	6.02	6.9
BOD_5 (mg/l)	2.16	0.164	2.16	0.09	2.16	3.029	2.16	0.297	2.16	0.067	2.16	19.43
COD (mg/l)	32.34	4.2	32.34	0.15	32.34	7.123	32.34	4.4	32.34	0.179	32.34	25.98
Ammonia (mg/l)	225.9	22.6	75.46	9	1	2.765	225.9	27.6	75.46	14.9	1	2.77
Phosphate (mg/l)	259.48	116.16	86.67	56.93	2.488	1.039	259.48	142.26	86.67	65.88	2.488	1.888
Cobalt (mg/l)	0.9611	0.307	0.3210	0.1258	0.0005	0.0013	0.9611	0.3127	0.3210	0.1609	0.0005	0.0027
Zinc (mg/l)	13.21	2.427	4.414	1.461	0.0154	0.0438	13.21	2.238	4.414	1.613	0.0154	0.0727

percentage in the negative control sample, and decreased with the increase of polluted water in the dilutions (Figure 5). In contrast, the growth rate of mutants *PCMut2, PC*Mut3 and *PC*Mut4 exhibited significant higher growth values in the presence of cultural media and polluted water than the wild type. This demonstrates that UV radiation had a negative potential effect on the mutant growth of *CC1021 Mut1* strain. This is in line with the study of Ikehata and Ono (2011) that showed it had no negative potential effect on the growth of the three mutants of *PC* but it enhanced their growth and resistance to pollutants. This result contradicts that of Michler et al. (2002) who reported that UV can have a strong effect on nutrient uptake, motility, reproduction and growth. About the resistance of microalgae, it was found that, microalgae are able to survive short-term unpredictable environmental stress by means of physiological acclimatization as a result of the modification of gene expression (Bradshaw and Hardwick, 1989; Fogg, 2001; Costas et al., 2008). However, when environmental stress exceeds physiological limits, only the occurrence of mutations that confers resistance can allow adaptation (Costas et al., 2001). More and more examples accumulate on rapid adaptation of microalgae to extreme environments through the selection of rare spontaneous mutations conferring resistance which affects only one gene. By means of these mechanisms, microalgae achieve adaptation toxenobiotics (García-Villada et al., 2002). In addition, Chlorophytes species were more capable of rapid adaptation to extreme environments than other algae phyla (de-Godos et al., 2010). The obtained results (Tables 1, 2 and 3; Figures 6, 7 and 8) demonstrate that in samples number 2 and 4 (dilution 4:1 and dilution 2:3), all the algal strains did not only attain higher growth percent, but also achieved high efficiency of removal percent; this result may be in accordance with that of Wang et al. (2009) and Choi and Lee (2012) who reported more quantitatively increasing *Chlorella c*aused an increase of removal rate. Generally, the result

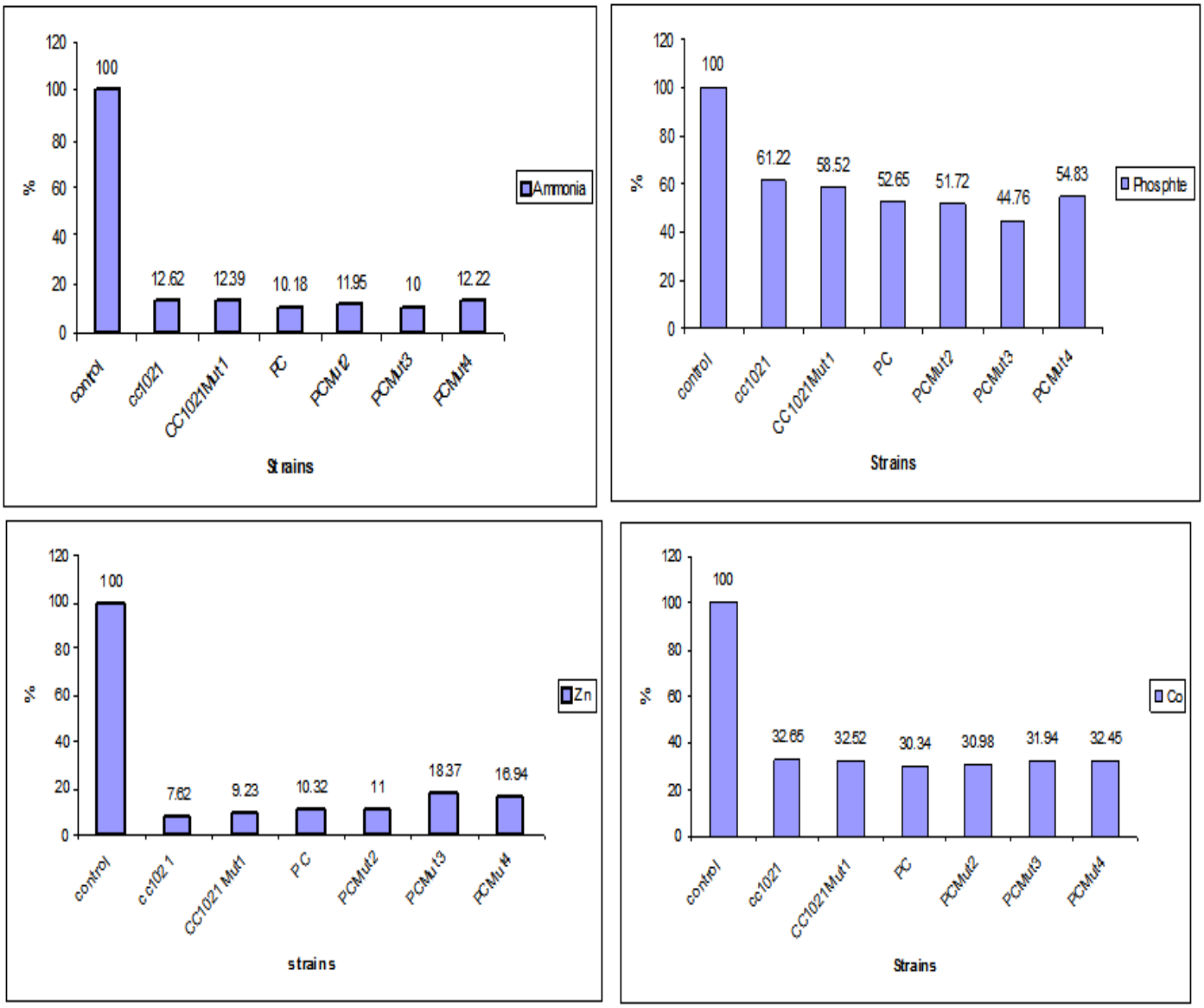

Figure 6. Decrease % of the selected parameters in sample No. 2 after using the algal strains for one week.

showed that NH_4-N contents were removed more effectively than phosphate and this is in agreement with Kshirsagar (2013). The above results are in agreement with many researchers with respect to phycoremediation especially *Chlorella* and *Chlamydomonas* (Cho et al., 2011; Johnson and Wen, 2010; Kong et al., 2010). The mechanism involved in algal nutrient removal was an uptake by the cells and stripping ammonia through elevated pH (Aslan and Kapdan, 2006; Kong et al., 2010). The initial pH in this study was 6.02 and reached 7.2 after bioremediation. Microalgae were reported to be more efficient in sequestering metal species from solution than bacterial and fungal biomass (Khoshmanesh et al., 1996). The mechanism of the effectiveness in removing heavy metals from waste water by microalgae is related to their large surface area and high binding affinity (Roy et al., 1993). Different algal species have different sizes, shapes and cell wall compositions which affect their metal binding (Wehrheim and Wettern, 1994; Cai et al., 1995). Generally in the pH (6 to 7) most of metal ions can be significantly removed (Rivas, et al., 2007; Sannasi and Salamija, 2011).

It was remarkable that *CC1021* showed the best removal of Zn and this was similar to that reported by Jennett et al. (1980). The effect of BOD and COD reduction in the presence of 6 algal strains is seen in

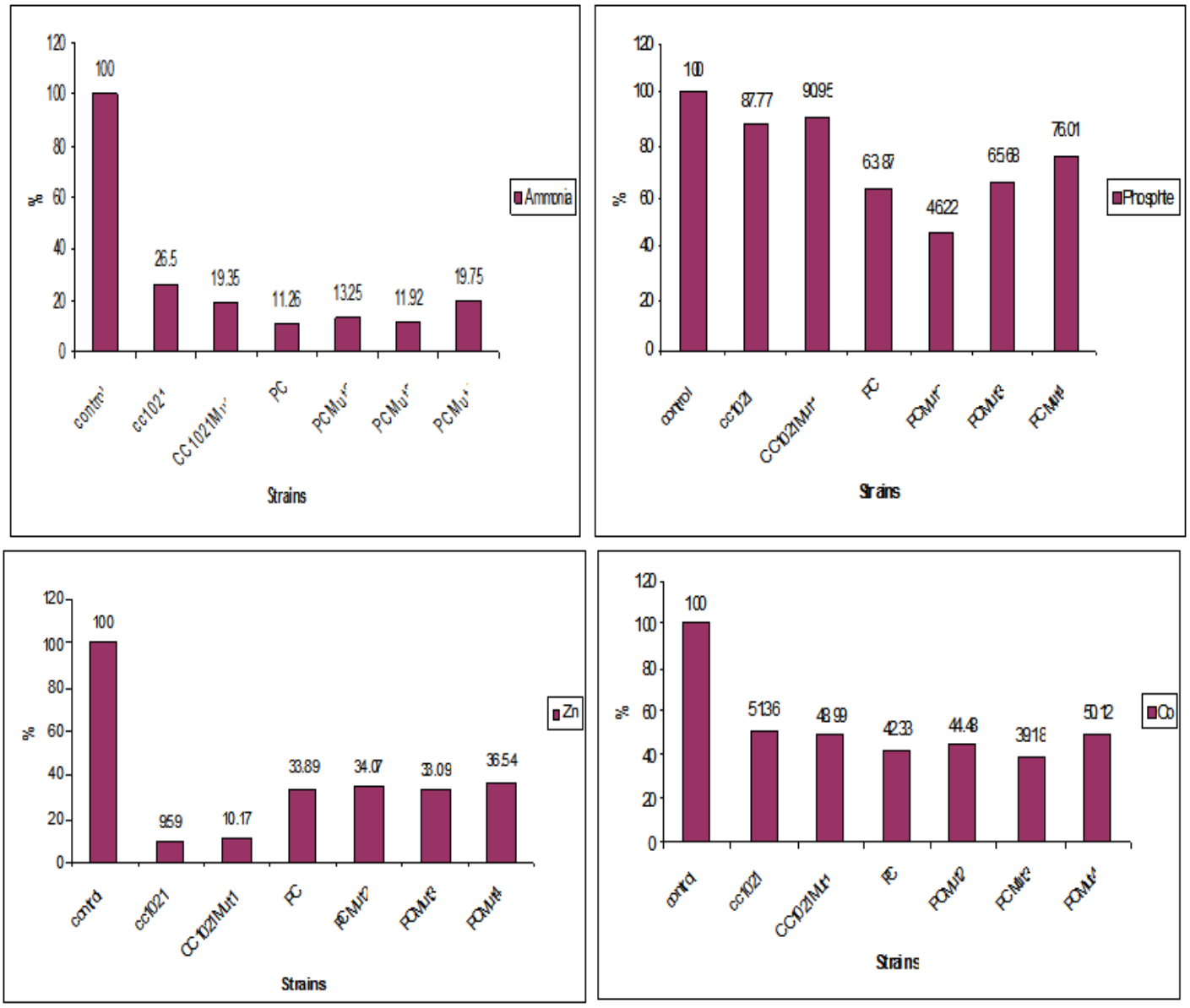

Figure 7. Decrease % of the selected parameters of sample No.4 after using the algal strains.

Tables 2 and 3 and graphically represented in Figures 9a and b. The percentage of BOD and COD reduction in *CC1021* reached 94.8, 99.5 and 97.4, 99.7%, respectively; for the mutant strain, *CC1021Mut1,* 91.7, 98.3 and 94.4, 99.7%; for the wild type *PC*, 91.7, 98.3 and 94.4, 99.7%; while the removal percents, 88.2, 93.1 and 98.6, 99.8%; 9 2.4, 87% and 95.8, 99.5%; 86.2, 86.2% and 96.9, 99.4% were achieved for *PCMut2, PCMut3* and *PCMut4*. A similar result was observed by Kshirsagar (2013), Kotteswari et al. (2012) and Wang et al. (2009); *C. vulgaris* mostly showed that COD and BOD removal efficiency was 88 and 89.60% (Valderrama et al., 2002), which is in agreement with our results.

Conclusion

1) The dose of UV radiation used in this study had no negative impact on the efficiency of bio-remediation potentials of the tested mutant strains; however, in some results, it enhanced the resistance of the mutants to polluted water, increasing their growth rate and in turn their removal efficiency.
2) NH_4 contents were removed more effectively than phosphate in all algal strains.

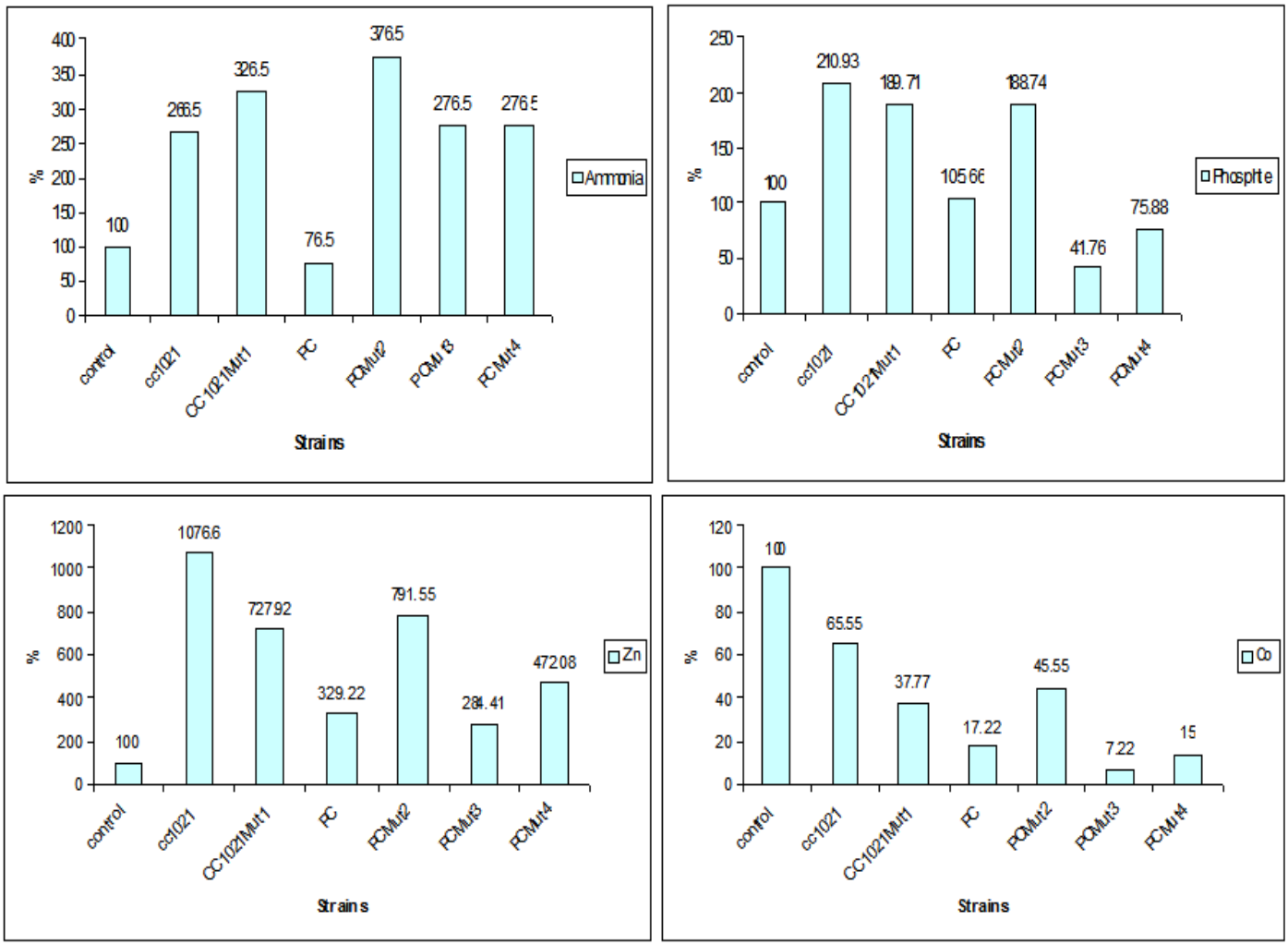

Figure 8. Increase % of the selected parameters of sample No. 6 after using the algal strain except Cobalt.

3) Statistical analysis using a *post hoc* multiple test showed that the wild and mutant strains had convergent efficiency in treatment of polluted water, as there was significant difference (p<0.05) found in bioremediation of water parameters between the six algae in both dilutions of 4:1 and 2:3.

4) The mutant strain, *PCMut2* showed the best removal capacity for BOD, COD and phosphate, while the mutant strain, *PCMut3* showed the best result for Co; the wild strain *PC* has the best removal of ammonia (Duncan, p< 0.05). On the other hand, the wild type *CC1021* was the best efficient strain in lowering the levels of Zn. However, the mutant strain *CC1021Mut*1 ranked the second order in lowering the levels of COD and Zn (Duncan, p< 0.05).

5) The sample number 4, dilution 2:3 (400 ml culturing medium + 600 ml of the polluted water) had better concentration than the others for effective nutrients removal.

6) The wild and mutant strains of *P. kessleri* had higher efficiency in phycoremediation than the wild and mutant strains of *C. reinhardtii CC1021*.

7) The mutant strains of *Parachlorella PC* appeared more efficient in bioremediation than the wild type as they have fast growth rates by mutagenesis. This removable efficiency exhibited by these mutants makes them excellent candidates for the removal of polluted water.

In summary, there is a possibility of producing mutants of *Chlorella* that have higher growth rate and removal efficiency.

Conflict of Interests

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

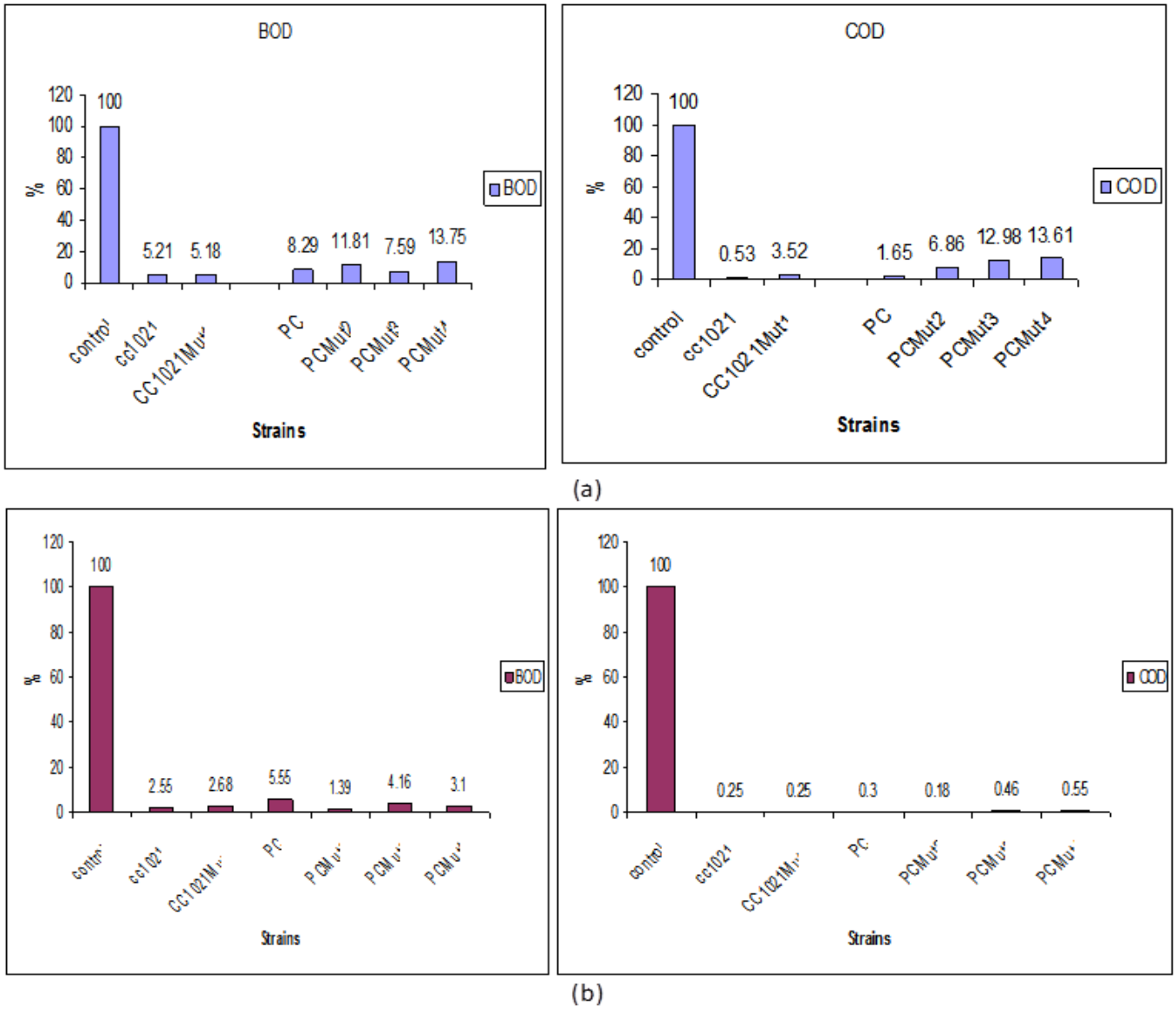

Figure 9. Decrease % of the BOD and COD after using the algal strains (a) in sample No. 2 (b) in sample No. 4.

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REFERENCES

Abeliovich A (1986). Algae in wastewater oxidation ponds. In: Richmond A. (Ed.), Handbook of Micro algal Mass Culture, CRC Press, Boca Raton, FL. pp. 331–338.

Afkar E, Ababna H, Fathi AA (2010). Toxicological Response of the Green Algae *Chlorella vulgaris* to Some Heavy Metals. Am. J. Environ. Sci. 6(3):230-237.

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M, Grimme LH (2000). Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals. Environ. Toxicol. Chem. 19:2341-2347.

APHA (1989). Standard Method for the Examination of Water and Waste Water. Clesceri LS, Greeberg AE, Rhodes TR. Standard Method for the Examination of Water and Waste Water. Joint Ed Board American Public Health Association, Washington, DC 20005, 10-223.

Aslan S, Kapdan IK (2006). Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecol. Eng. 28:64–70.

Aziz MA, Ng WJ (1993). Industrial wastewater treatment using an

activated algae-reactor. Water Sci. Technol. 28(5):71–76.
Bradshaw AD, Hardwick K (1989). Evolution and stress genotypic and phenotypic components. Biol. J. Linn. Soc. 37:137-155.
Cai XH, Logan T, Gustafson T, Traina S, Sayre RT (1995). Applications of eukaryotic algae for the removal of heavy metals from water. Mol. Mar. Biol. Biotechnol. 4:338.
Cho S, Luong TT, Lee D, Oh YK, Lee T (2011). Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production. Bioresour. Technol. 102(18):8639-8645.
Choi JH, Lee M (2012). Effect of micro algae on removal of nutrients from wastewater. Environ. Eng. Res. 17(51):53-58.
Costas E, Carrillo E, Ferrero LM, Agrelo M, Garcia-Villada L, Juste J, López-Rodas V (2001). Mutation of algae from sensitivity to resistance against environmental selective agents: The ecological genetics of Dictyosphaerium chlorelloides (Chlorophyceae) under lethal doses of 3-(3,4-dichlorophenyl)-1,1 dimethylurea herbicide. Phycologia 40:391-398.
Costas E, Flores-Moya A, López-Rodas V (2008). Rapid adaptation of algae to extreme environments (geothermal waters) by single mutation allows “Noah’s Arks” for photosynthesizers during the Neoproterozoic “Snowball Earth”. New Phytol. 189:922-932.
de-Bashan LE, Moreno M, Hernandez JP, Bashan J (2002). Ammonium and phosphorus removal from continuous and semi-continuous cultures by the microalgae *Chlorella vulgaris* coimmobilized in alginate *Chlorella vulgaris* coimmobilized in alginate beads with Azospirillum brasilense”. Water Res. 36:2941-2948.
de-Godos I, Vargas VA, Blanco S, Garcia-Gonzalez MC, Soto R, Garcia-Encina PA, Becares E, Muioz R (2010). A comparative evaluation of microalgae for the degradation of piggery wastewater under photosynthetic oxygenation. Bioresour. Technol. 101:5150–5158.
Fogg GE (2001). Algal adaptation to stress-some general remarks. In: RaiLC, Gaur, J.P,eds. Algal adaptation to environmental stresses: Physiological, biochemical and molecular mechanisms. Berlin, Germany: Springer. pp. 1–20.
Fott B, Nováková M (1969). A monograph of the genus *Chlorella*: The fresh water species. In Fott, B., Studies in Phycology. Academia, Prague. pp. 10-74.
García-Villada L, López-Rodas V, Bañares E, Flores-Moya A, Costas E (2002). Evolution of microalgae in highly stressing environments: An experimental model analyzing the rapid adaptation of Dictyosphaerium chlorelloides (Chlorophyceae) from sensitivity to resistance against 2,4,6,-trinitrotoluene (TNT) by rare preselective mutation. J. Phycol. 38:1074-1081.
Häder D, Millán de Kuhna R, Strebb C, Breitera R, Richterb P, Neeßea T (2006). Screening for unicellular algae as possible bioassayorganisms for monitoring marine water samples. Water Res. 40:2695-2703.
Hallmann GE (2007). Algal adaptation to stress-some general remarks. In: Rai LC, Gaur JP eds. Algal adaptation to environmental stresses: Physiological, biochemical and molecular mechanisms. Berlin, Germany, Springer. pp. 1–20.
Harris EH (1989). The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego, CA.
Holzinger A, Lütz C (2006). Algae and UV irradiation: Effects on ultrastructure and related metabolic functions. Micron 35:190–207.
Huang GH, Chen G, Chen F (2010). Rapid screening method for lipid production in alga based on Nile red fluorescence. Biomass Bioenergy 33:1386-1392.
Ikehata H, Ono T (2011). The Mechanisms of UV Mutagenesis. J. Radiat. Res. 52:115–125.
Jennett CJ, Hassett MJ, Smith EJ (1980). The use of algae to control heavy metals in the environment. Mater. Environ. 2(1):26-31.
Johnson MB, Wen ZY (2010). Development of an attached microalgal growth system for biofuel production. Appl. Microbiol. Biotechnol. 85(3):525-34.
Khoshmanesh A, Lawson F, Prince IG (1996). Cadmium uptake by unicellular green microalgae. Chem. Eng. J. Biochem. Eng. J. 62(1):81–88.
Kong QX, Li L, Martinez B, Chen P, Ruan R (2010). Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. Appl. Biochem. Biotechnol. 160(1):9-18.
Kotteswari M, Murugesan S, Ranjith Kumar R (2012). Phycoremediation of Dairy Effluent by using the Microalgae Nostoc sp. Int. J. Environ. Res. Dev. 2(1):35-43. ISSN 2249-3131.
Krienitz L, Hegewald EH, Hepperle D, Huss VAR, Rohr T, Wolf M (2004). Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). Phycologia 43:529-542.
Kshirsagar AD (2013). Bioremediation of wastewater by using microalgae: An experimental study. Int. J. Life Sci. Biotechnol. Pharma Res. 2(3).
Mara DD, Pearson HW (1986). Artificial freshwater environments: Waste stabilization ponds. Biotechnology 8:177-206.
Mazur T, Hadas E, Iwanicka I (1995). The duration of the cyst stage and the viability and virulence of Achanthamoeba Isolates. Trop. Med. Parasitol. 46:106-108.
Mehta SK, Gaur JP (2005). Use of algae for removing heavy metal ions from wastewater: Progress and prospects. Crit. Rev. Biotechnol. 5(3):113-152.
Michler T, Aguilera J, Hanelt D, Bischof K, Wiencke C (2002). Long term effects of ultraviolet radiation on growth and photosynthetic performance of polar and cold-temperate marcoalgae. Mar. Biol. 140:1117-1127.
Munoz R, Guieysse B (2006). Algal-bacterial processes for the treatment of hazardous contaminants: A review. Water Res. 40(15):2799-2815.
Olumayowa O, Helena D, Jon KP (2013). Oxidative stress-tolerant microalgae strains are highly efficient for biofuel feedstock production on wastewater. Biomass Bioenergy 56:284-294.
Oswald WJ, Green FB, Bernstone LS, Lundquist TJ (1996). Advanced Integrated Wastewater Pond Systems for Nitrogen Removal. Wastewater Pond Systems for Nitrogen Removal. Wat. Sci. Technol. 33(7):207-217.
Rao HP, Kumar RR, Raghavan BG, Subramanian VV, Sivasubramanian V (2011). Application of phycoremediation technology in the treatment of wastewater from a leather-processing chemical manufacturing facility. Water Soil Air 37(1):7-14.
Rivas BL, Villegas S, Ruf B, Peric IM (2007). Removal of metal ions with impact on the environment by water insoluble function copolymers: Synthesis and metal ion uptake properties. J. Chil. Chem. Soc. 52(2): 1164-1168.
Roy D, Greenlaw PN, Shane BS (1993). Adsorption of heavy metals by green algae and ground rice hulls. J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng. 28(1):37–50.
Sannasi IP, Salamija S (2011). Preliminary Adsorption study for Heavy Metal Removal with Ion-Exchange Resins in the Teaching Laboratory. Orient. J. Chem. 27(2):461-467.
Utermöhl H (1958). Zur Vervollkommnnung der quantitative n Phytoplankton. Mhodik. Mitt. Inter. Verein. Limnol. 9:1-38.
Valderrama LT, Del Campoa CM, Rodrigueza CM, de-Bashana LE, Bashanb Y (2002). Recalcitrant wastewater from ethanol and citric acid production using the microalgae *Chlorella vulgaris* and the macrophyte Lemna minuscule. Water Res. 36:4185-4192.
Wang L, Min M, Liang Y, Paul Chen P, Chen Y, Liu Y, Ruan R (2009). Cultivation of Green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. Appl. Biochem. Biotechnol.
Wehrheim B, Wettern M (1994). Biosorption of cadmium, copper and lead by isolated mother cell walls and whole cells of *Chlorella fusca*. Appl. Microbiol. Biotechnol. 8(4):227-232.
Wetzel RG, Likens GE (1979). Limnological analysis. Second edition. Pub. Springer-Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo, Hong Kong, Barcelona, Budapest.
Xu S, Nirmalakhandan N (1998). Use of QSAR models in predicting joint effects in multicomponent mixtures of organic chemicals. Water Res. 32:2391-239.

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Full Length Research Paper

Antimicrobial assay of methanolic crude of *Lonicera lanceolata*

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The objective of this study was to collect information about medicinal plant *Lonicera lanceolata.* The antimicrobial assay of four fractions (n-hexane, chloroform, ethyl acetate and ethanol) obtained from *Lonicera lanceolata* were screened against two human Gram-positive *(Staphylococcus aureus, Micrococcus luteus)* and four Gram-negative pathogen (*Escherichia coli, Pseudomonas aeruginosa, Enterobacter cloacae, Klebsiella pneumoniae*). The antimicrobial and antifungal activities were performed by Agar well diffusion method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by Agar well dilution method and Viable cell count method respectively. Ethyl acetate fraction exhibited maximum antibacterial activity while the other fractions that is, hexane, chloroform, ethanol also showed standard antibacterial activities. MIC and MBC were determined for ethyl acetate fraction. MICs ranged from 0.312 to >10 µg/ml and MBCs from 0.260 to 15 µg/ml. The ethyl acetate and chloroform fractions showed excellent antifungal activity while other fractions have least antifungal activity.

Key words: *Lonicera lanceolata,* antimicrobial activity, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC).

INTRODUCTION

Lonicera lancolata belong to a genus *Lonicera* usually found in Bhutan, Nepal, China and mountains area of Pakistan. The genus *Lonicera* belong to the family Caprifoliaceae, which have about 12 genera and 450 species (Mabberley et al., 1997), found usually in moderate region of Northern Hemisphere. Most of the plants of genus *Lonicera* are used against variety of diseases like acute fever, headache, respiratory infections (Houghton et al., 1993), antioxidant (Ali et al., 2013), cytoprotective (Chang et al., 1992), hepatoprotective (Ya-Ping et al., 1992; Shi et al., 1999), antiviral (Chang et al., 1995), antitumor (Wang et al., 2009; Yip et al., 2006) and anti-inflammatory activities (Yoo et al., 2008). Microbial assay is a principal way of deterioration of foods and is usually responsible for the loss of quality and safety of foods. The pathogenic

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microorganisms in foods are increasing with the increase in occurrence of food-borne diseases. The *Staphylococcus aureus*is mainly involved for post-operative wound infections, toxic shock syndrome, endocarditic, osteomyelitis and food poisoning (Mylotte et al., 1987). *Listeria monocytogenes* is usually responsible for the harsh food-borne illness, listeriosis, which has been one of the rising zoonotic diseases during the last two decades (Farber et al., 2000). The *Escherichia coli* is found in human intestines and is responsible for urinary tract infection, coleocystitis or septicaemia (Singh et al., 2000).

Many compounds were isolated previously from genus *Lonicera* including iridoids, bisiridoids, sulphur containing monoterpenoids, alkaloidal glycosides, triterpenoids, saponins, coumarin glycosides and flavones glycosides (Machida et al., 1995; Bailleul et al., 1981; Souzu et al., 1969; Souzu et al., 1970) which have good activities against pathogens.

The aim of this study is to investigate the antimicrobial activity of the four fractions obtained from the crude of *L. lanceolata.* The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also carried out.

MATERIALS AND METHODS

Plant material

The whole plant material of *L. lanceolata* was collected from Hazara division, in May 2012. The plant was identified by Professor Manzoor Ahmad Plant Taxonomist on the basis of its morphology in Government Degree College Abbotabad, Pakistan, where a voucher specimen was deposited in herbarium (Accession No. D-056).

Preparation of crude extracts

Ninety grams of each powdered plant material were extracted with 80% methanol by maceration for 2 days with repeated stirring and the resulting liquid was filtered with filter paper (Whatman No. 3 filter paper, Whatman Ltd., England). This process was repeated several times. The solvent was evaporated by Rota-vapor (BU¨ CHI Rota-vaporR-205, Switzerland) at about 50°C. The gummy filtrate was placed in an oven at 50°C for about 2 days to evaporate the water. The dehydrated mass was change into powdered, packed into a glass vial and stored in a desiccator using silica gel (Ieven et al., 1979).

Preparation of fractions

Extraction of about 90 g of *L. lanceolata* was carried out with n-hexane, chloroform, ethyl acetate and ethanol using Soxhlet apparatus. The solvent was removed under reduced pressure and the fractions were then transferred to vacuum oven at 50°C for about one day to remove any residual solvent. The resulting semisolid mass of each fraction was stored in a desiccator using silica gel (Ieven et al., 1979).

Microorganisms

Six bacterial species, two human Gram-positive and four Gram-negative that is, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomona saeruginosa* (clinical strain/PIMS), *Enterobacter cloacae* (clinical strain/PIMS), *S. aureus* (MRSA, clinical strain/PIMS) and *Micrococcus luteus* (clinical strain/PIMS) were used in antimicrobial test. Strains were obtained from Microbiology Research Laboratory School of Life Science Beijing University of Chemical Technology (BUCT), China and their identification and characterization were carried out. These cultures were retained on agar slants at 4°C for antimicrobial screening. Microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.4. Ofloxacin (10 µg) and Ampicillin (10 µg) (Oxoid) were used as reference antibiotics (Table 1).

Anti-microbial screening

Screening for antibacterial activity by Agar well diffusion method

The antibacterial assay was carried out by means of Agar well diffusion method (Hadacek et al., 2000). All bacterial strains were first grown-up in nutrient broth at 37°C for 22-24 h in incubator till turbidity that became comparable to McFarland 0.5 turbidity standard was obtained. The inocula of the relevant bacteria were streaked on to the Muller Hinton Agar (oxoid) plates using a disinfected swab in order to confirm a uniform thick lawn of growth following incubation. Wells of 6 mm in diameter were produced on to nutrient agar plates by using a germ-free cork borer. The wells were filled with the given test agents (100 µl) and the plates were then allowed to keep on for 1:30 h at 25°C. At last, the plates were incubated at 37°C for 22-24 h and the resulting diameters of zones of inhibition were measured.

Determination of minimum inhibitory concentration (MIC)

Agar dilution method

Minimum inhibitory concentration of the crude extracts was found out by agar dilution method (EUCAST Definitive Document., 2000; Mukherjee, 2002; Anon, 2000). The sterilized Muller Hinton Agar (oxoid) was retained to cool to 50°C and approximately 20 ml of this was added to clean test tubes which contained 1 ml of dissimilar concentration of crude extract. This mixture was thoroughly mixed and poured into pre-labelled disinfected Petri Dishes. Petri dishes having only growth media were prepared in the same way so as to serve for comparison with Petri plate including crude extract. The concentrations of the extracts used in this test ranged from 0.312 µg/ml to 2000 µg/ml. The suspensions of the respective microorganisms having density adjusted to 0.5 McFarland turbidity standards were inoculated on to the chain of agar plates using standard loop. The plates were then incubated at 37°C for 22-24 h. The lowest possible concentration which inhibited the growth of the respective organisms was taken as MIC. All tests were done in triplicate.

Determination of minimum bactericidal concentration (MBC)

Viable cell count method

Minimum bactericidal concentration of the *L. lanceolata* was calculated by the viable cell count method (Toda et al., 1989; Anon,. 2003), and the results were written as number of viable cells as a percentage of the control.

Table 1. Zone of inhibition of reference antibiotics.

Reference	Microorganisms and their zone of inhibition (mm)					
Antibiotics	**Ec**	**Kp**	**Ps**	**Ent**	**Ml**	**Sta**
Ofloxacin	15.1(±0.02)	14(±0.01)	11.9(±0.01)	13(±0)	10(±0.11)	9.6(±0.05)
Ampicillin	14.1(±0.05)	12.3(±0.5)	11(±0)	14(±0.11)	13(±0.05)	14(±0.05)

Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Ps: *Pseudomonas aeruginosa*; Ent: *Enterobacter cloacae*; Ml: *Micrococcus luteus*; Sta: *Staphylococcus aureus* (methycillin resistant); mm: millimeter.

Table 2. Inhibition zones of *Lonicera lanceolata* plant extracts.

Fraction	Zone of inhibition (mm)					
	Ec	**Kp**	**Ent**	**Ps**	**Ml**	**Sta**
n-hexane	2(±0.4)	6(±0.3)	6(±0)	3(±0.3)	2(±0.7)	5(±0.4)
Chloroform	8(±0.4)	6(±0.6)	11(±0.4)	7(±0.4)	7(±0.5)	9(±0.2)
Ethyl acetate	14(±0.3)	14(±0.4)	12(±0.2)	11(±0.2)	13(±0.5)	15(±0.5)
Ethanol	2(±0.2)	4(±0.1)	6(±0.2)	4(±0.6)	4(±0.3)	3(±0.5)

Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Ps: *Pseudomonas aeruginosa*; Ent: *Enterobacter cloacae*; Ml: *Micrococcus luteus*; Sta: *Staphylococcus aureus* (methycillin resistant); mm: millimeter

Screening for antifungal activity

These fractions were screened against *Aspergillus niger*: The required quantity of each fungal culture was placed in 2 ml of sabauraud dextrose broth. This suspension was constantly spread on Petri plates containing sabauraud dextrose agar media using germ-free swabs. Samples were applied into wells using same technique for bacteria and incubated at room temperature for 3 days. The plates were checked for the presence of zones of inhibition and the results were recorded. Itraconazole was used as a positive control.

RESULTS

Almost all fractions of *L. lanceolata* presented hopeful activity against both Gram positive and negative bacterial pathogens. Ethyl acetate fraction has high antibacterial assay as compared to other fractions which is cleared from Table 2 and Figure 1. Therefore it was further considered for determination of MIC and MBC, respectively. The MIC values ranged from 0.312 to > 10 µg/ml for all tested strains while the MBC values reported were so many times higher than MIC (Table 3), (Figure 2). The MBC value for *K. pneumoniae* was not detected.

In the similar way nearly same pattern of defencelessness was examined against fungal strain *A. niger*. The maximum antifungal activity was presented by chloroform and ethyl acetate fractions. Ethnanol and n-hexane fractions have lowest antifungal activity which is represented in Table 4 and Figure 3.

DISCUSSION

The antimicrobial activities of four fractions obtained from the crude of *L. lanceolata* were tested against six bacteria species *E. coli, K. pneumoniae, P. aeruginosa, E. cloacae, S. aureus,* and *M. luteus.* Almost all fractions exhibited more or less antimicrobial activity against the test cultures. Ethyle acetate fraction exhibited best activity against these bacteria. Besides that, chloroform, n-hexane and ethanol fractions have lowest activity against these bacteria. Ethyle acetate and chloroform fractions obtained from the crude of *L. lanceolata* were primarily reported as active against the *A. niger*.

The MIC of the ethyl acetate fraction was taken as 0.312 µg/ml. It is important that the MIC value is too high to be taken in susceptible ranges (Paul et al., 2006). The MBC value of ethyl acetate fraction is many times higher than MIC. The antibacterial and antifungal assays were carried out by Agar well diffusion method. The MIC was carried out by using Agar well dilution method while MBC was performed by viable cell count method.The MBC values for *K. pneumonia* were not detected.

This investigation is probably the first to exibit the antimicrobial assay of four fractions obtained from crude of *L. lanceolata*. As a comprehensive literature review to the best of our knowledge there is no information about the antimicrobial activity of these four fractions obtained from this plant.

Conclusion

It is clear from the present studies that four fractions were obtained from the crude of *L. lanceolata* which shows prominent antimicrobial activity. The ethyl acetate fraction showed highest antimicrobial activities. The MIC of ethyl acetate fraction was taken as 0.312 µg/ml. More

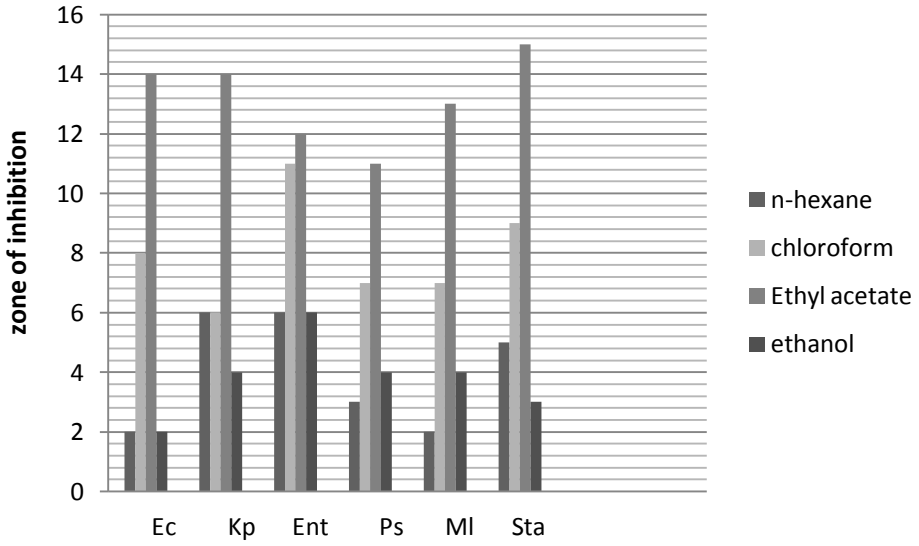

Figure 1. Inhibition zones of *Lonicera lanceolata* plant extracts. *E. coli, K. pneumoniae, P. aeruginosa, E. cloacae, M. luteus, S. aureus.*

Table 3. MIC and MBC of *Lonicera lanceolata*of ethyl acetate fraction.

Microorganism	MIC (µg/ml)	MBC (µg/ml)
E. coli	>10	14
K. pneumoniae	>10	N.d
P. aeruginosa	>10	12
E. cloacae	5	15
M. luteus	0.625	1.877
S. aureus	0.312	1.260

N. d = not detected.

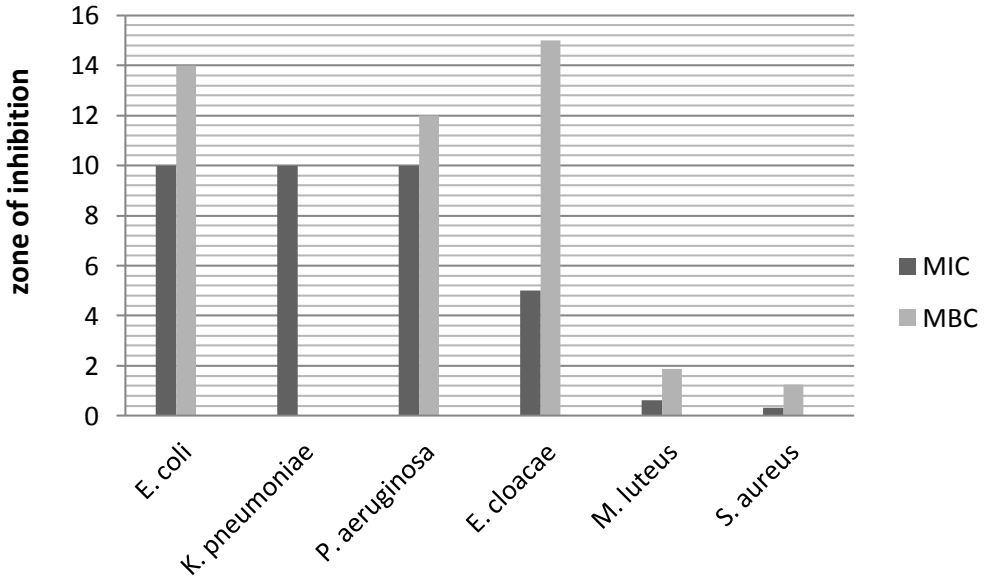

Figure 2. MIC and MBC of *Lonicera lanceolata* of ethyl acetate fraction.

Table 4. Antifungal activities of *Lonicera lanceolata* crude extracts.

Compounds	Zone of inhibition (mm)
n-hexane	1(±0.5)
chloroform	6(±0.11)
Ethyl acetate	7(±0.17)
ethanol	1(±0.14)
Standard	8(0)

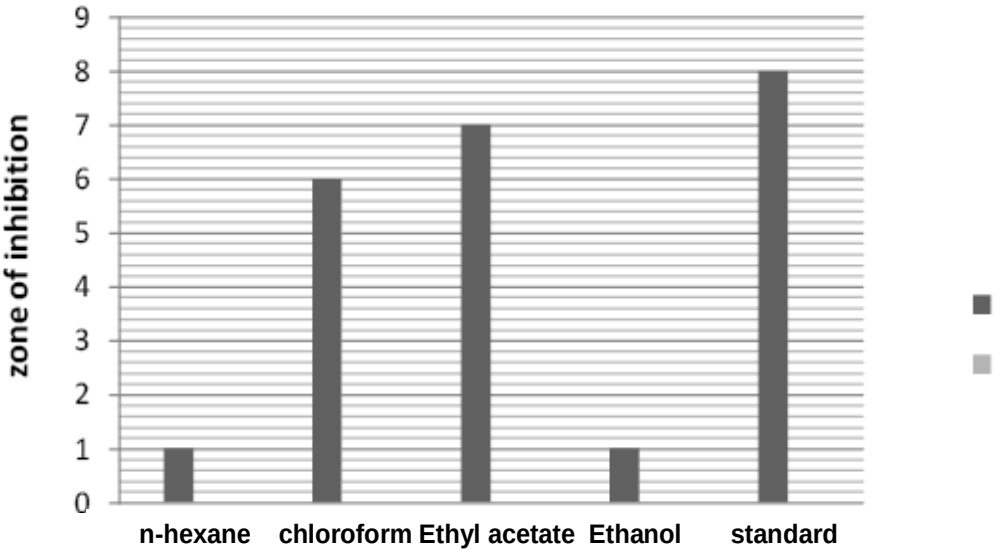

Figure 3. Antifungal activities of *Lonicera lanceolata* crude extracts.

investigations are mandatory to expose the hidden medicinal importance of plant *L. lanceolata*

Conflict of Interests

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

REFERENCES

Ali I, Khan D, Ali F, Bibi H, Malik A (2013). Phytochemical, antioxidant and antifungal studies on the constituents of Lonicera Quinquelocularis. J. Chem. Soc. Pak. 35:139-143.

Anon (2002). Traditional Medicine Strategy 2002-2005. Edited by WHO/EDM/TRM. World Health Organization, Geneva, 74.

Anon (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin. Microbiol.Infect.9:1-7Bailleul F, Leveau A, Durand, M (1981). New iridoid from the fruits of Lonicera alpigena. J. Nat. Prod. 44:573-575.

Chang WC, Lin TM, Lee SS, Karin SC, Liu C, Hsu FL (1995). Differential inhibition of reverse transcriptase and cellular DNA polymerase α-activities by lignans isolated from Chinese herbs, Phyllanthus myrtifolius Moon, and tannins from Lonicera japonica Thumb and Castanopsis hystrix. Antiviral Res. 27:367-374.

Chang WC, Hsu FL (1992). Inhibition of platelet activation and endothelial cell injury by polyphenolic compounds isolated from Lonicera japonicaThumb. Prostaglandins, Leukotrienes and Essential Fatty Acids. 45:307.

EUCAST Definitive Document (2000). Determination of Minimum Inhibitory Concentrations (MICs) of Antimicrobial Agents by Agar Dilution. EUCAST Definitive Document. E. 6:509-515.

Farber JM (2000). Present situation in Canada regarding Listeria monocytogenes and ready-to-eat seafood products. Inter. J. Food Microbiol. 62:247–251.

Hadacek F, Greger H (2000). Testing of antifungal natural products: methodologies,comparability of results and assay choice. Phytochem. Analys., 11:137-147.

Houghton PJ, Boxu Z, Xisheng Z (1993). A clinical evaluation of the Chinese herbal mixture “Aden-I” for treating respiratory infections. Phytother. Res. 7:384-386.

Ieven M, Berghe V, Mertens DA, Vlietinck F, Lammens AE (1979). Screening of higher plants forbiological activities. *Plant. Medic.,* 36:311-321.

Machida K, Asano J, Kikuchi M (1995). Caeruleosides A and B, bis-iridoid glucosides from Lonicera caerulea. Photochemistry. 39:111-114.

Mabberley DJ (1997). The plant-book, a Portable Dictionary of the Vascular Plants, Cambridge University Press.

Mukherjee PK (2002). Quality Control of Herbal Drugs, an Approach to Evaluation of Botanicals. Business Horizons, New Delhi India. p.256.

Mylotte JM, McDermott C, Spooner JA (1987). Prospective study of 114 consecutive episodes of Staphylococcus aureus bacteremia. Review of Infectious Diseases. 9:891–907.

Paul CJ, Arnold L, Vlietinck VBD, Vanden ML (2006). Anti-infective potential of natural products: How to develop a stronger in vitro ‘proof-of-concept. J. Ethnopharmacol. 106:290–302.

Shi JZ, Chen XF, Wan L (1999). Hepatoprotective effect of several constituents of Lonicera fulvotomentosa and L. macranthoide on CCl4 and D-galactosamine induced liver injuries in mice and rats.

China J. China Mat. Med. 24:363-365.
Singh R, Chandra R, Bose M, & Luthra PM (2000). Antibacterial activity of Curcuma longa rhizome extract on pathogenic bacteria. Current Science., 83:25.
Souzu I, Mitsuhashi H (1969). Structures of iridoids from lonicera morrowii a. gray I. Tetrahedron lett. 32:2725-2728.
Souzu I, Mitsuhashi H (1970). Structures of iridoids from lonicera morrowii A. Gray II. Tetrahedron lett. 11:191-192.
Toda M, Okubo S, Hiyoshi R, Shimamura T (1989). The bactericidal activity of tea and coffee. Leter. Appl.Microbiol. 8: 123-125.
Wang J, Zhao XZ, Qi Q, Tao L, Zhao Q, Mu R, Gua HY, Wang M, Feng X, Guo QL (2009). Macranthoside B, a hederagenin saponin extracted from Lonicera macranthoides and its anti-tumour activities in vitro and in vivo. Food Chem. Toxicol. 47:1716-1721.
Ya-Ping L, Jie L, Xian-Sheng J, Qing M, Cherukury M, Curtis DK (1992). Protective effects of fulvotomentosides on cadmium-induced hepatotoxicity. Acta Pharmacol. Sin. 13 (3):209-212.
Yip ECH, Chan ASL, Pang H, Tam YK, Wong YH (2006). Protocatechuic acid induces cell death in HepG2 hepatocellular carcinoma cells through a c-Jun N-terminal kinase-dependent mechanism. Cell boil. Toxicol. 22:293-297.
Yoo HJ, Kang HJ, Song YS, Park EH, Lim CJ (2008). Anti-antigenic, antinociceptive and anti-inflammatory activities of Lonicera japonica extract. J. Pharm. Pharmacol. 60:779-786.

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Evaluation of *Bacillus amyloliquefaciens* as manure additive for control of odorous gas emissions from pig slurry

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Two *in-vitro* experiments were conducted to evaluate the efficacy of *Bacillus amyloliquefaciens* culture broth (BA) on reduction of odorous gas emission from pig slurry. In experiment 1, the treatments included control (no spray), water spray, and spray with 1% (BA1), 5% (BA2), 10% (BA3) and 100% (BA4) BA. Each treatment was replicated three times. The only significant difference in NH_3 emission was observed at 48 h, when BA1, BA3 and BA4 showed significant reduction compared to the control. The H_2S emission was significantly reduced only at 3 h in response to treatments with BA compared to control. The SO_2 emissions from slurry were not affected by the treatments. The treatments of experiment 2 were: control (no spray), water spray, 10% BA spray one time/day and 10% BA spray one time/two day. The NH_3 emissions were significantly reduced in response to treatments with BA at day 4, 6 and 7 compared to the control. Significant reduction in H_2S emissions were observed from day 3 to 7 from the BA one time/day treated slurry compared to the control slurry. The SO_2 emissions did not differ among treatments, with the exception of a tendency to decline in response to treatment with BA one time/day at day 4 and 6. Overall, treatment with 10% BA one time/day was effective in reducing NH_3, H_2S, and SO_2 from pig slurry and can be used as manure additive.

Key words: *Bacillus amyloliquefaciens,* pig slurry, *in-vitro* fermentation, ammonia, sulfur dioxide, hydrogen sulfide.

INTRODUCTION

The term manure was used in the past to describe excreta that was predominantly used as fertilizer and soil conditioner. However, increasing use of chemical fertilizer in crop, animal manure is no longer in demand for its fertilizer value, but with the development of intensive, confined housing and feeding practices, it is now considered a pollutant and odor nuisance. Odors from animal feeding operations are produced via an incomplete fermentation of manure by anaerobic bacteria. Schiffman et al. (2001) identified a total of 331 different compounds in the air and lagoon water from pig production facilities. Odor emissions from swine facility

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are complex mixture of ammonia (NH_3), volatile sulfur (H_2S, SO_2) and a large number of volatile organic compounds (VOCs). Exposure to high levels of odorous gases not only adversely affect the health and performance of animals but also affect the health of workers and cause environmental problems such as the nitrification and acidification of rain (Ushida et al., 2003). Therefore, reduction in odor nuisance plays an important role for strategies concerning where to permit pig production facilities to be located and determines the maximum size of the facilities. So far, strategies to reduce odor mainly focused on technical approaches such as bio-filter (Sheridan et al., 2002), bio-scrubbers (Hahne et al., 2003), manure storage covers (VanderZaag et al., 2008), mechanical aeration (Al-Kanani et al., 1992), diet manipulation (Sutton et al., 1999) and segregation of feces and urine. Some of these techniques are effective, but tend to be expensive (VanderZaag et al., 2008) and their effectiveness period is short. To date, limited information is available on whether direct application of microbial additives is effective in reducing odor and noxious gas emissions from pig slurry (Kim et al., 2008; Rahman et al., 2011).

Pig manure is primarily a mixture of urine and feces, and it contains undigested dietary components, endogenous end products, and indigenous bacteria from the lower gastrointestinal tract (Sutton et al., 1999). Generation of odors from stored swine slurry is a complex process that involves many bacterial species, producing an extensive array of volatile compounds. Hence, microorganisms play a major role in both production and reduction of malodors (Zhu, 2000). A number of studies have been conducted to investigate the effects of direct fed microbials (DFM) on reduction of noxious gas from pig slurry. *Bacillus*-based DFM were effective at breaking up manure solids, and demonstrates its effectiveness in odor control (Davis et al., 2008; Wang et al., 2009). Direct application of microorganisms have been extensively practiced in municipal wastewater to degrade organic matter (Low and Chase, 1999) since degradation of organic matter in wastewater relies on microorganisms (Sund et al., 2001). A previous study (Rahman and Mukhtar, 2008) suggested that direct application of microbial additive is also effective in reducing solids and nutrient contents in manure from anaerobic dairy lagoons. This study was designed to evaluate the effectiveness of a microbial treatment technology; spraying of *Bacillus amyloliquefaciens* culture broth (BA) in reducing noxious gas (NH_3, H_2S, and SO_2) emissions from pig slurry under *in vitro* fermentation condition.

MATERIALS AND METHODS

This *in-vitro* study was conducted at Animal Nutrition and Feed Science Laboratory, Sunchon National University, Republic of Korea. The experimental protocols were approved by the Animal Care and Use Committee of Sunchon National University, Republic of Korea.

Source of bacterial stock culture

The bacterial stock culture used in this experiment was *B. amyloliquefaciens* KB3 culture broth provided by the Jeonnam Biodiversity Foundation, Jeonnam, Republic of Korea. It was isolated from bug feces and there were 1×10^9 cfu bacteria per ml.

Sample collection

A total of 12 crossbred (Landrace × Yorkshire × Duroc) growing pigs (average body weight 40 ± 0.12 kg) were housed for a period of 8 days in individual elevated solid-sided stainless steel metabolism cages (1.6×0.8 m^2) equipped with plastic slatted floors. Pigs were allowed to consume feed and water *ad libitum* and feces and urine were collected on day 6, 7 and 8 of the period. The slurry samples were collected from the tray placed below the cage, 3 times in the morning (half an hour apart) and 3 times in the afternoon (half an hour apart). Each time, about 200 g of fresh feces was collected from each pig and was put into plastic sample bag. The sample of day 6 and 7 were stored at 4°C to avoid pre-fermentation and loss of water. After completion of day 8 collection, all samples were homogenized, mixed well and brought to room temperature (24 to 28°C) before the commencement of experiment.

In vitro fermentation and measurement of noxious gas concentration

Two experiments were conducted to investigate the effects of BA on odorous gas emissions from pig manure under anaerobic condition. The *in-vitro* trials were carried out in glass reaction chamber to facilitate anaerobic fermentation with air circulation and stirring device.

Experiment 1

There were six treatments including: control (no spray), water (water spray), BA1 (spray with 1% BA), BA2 (spray with 5% BA), BA3 (spray with 10% BA), and BA4 (spraying with 100% BA). The stock culture was diluted with distilled water (DW) to prepare 1, 5, 10 and 100% culture solution. Approximately 2 kg of the stock slurries was stored in each glass fermentation chamber in triplicate for each treatment. The glass chambers had a small hole at one side of the top cover to facilitate gas measurement which was equipped with a small tube with cover. A circulating fan run by electricity was used for uniform distribution of heavy and light gas in each fermentation chamber. Following one pretreatment sampling at 0 h, gas samples were again recorded at 0 h following sprayed with 100 ml of bacterial culture. The gas was sampled using a Gastec gas sampling pump (model AP-20; Gastec Corp., Japan) and Gastec detector tube (No. 3M for NH_3; No. 4HM and 4LT for H_2S; No. 5LA for SO_2). Prior to measurement, the slurry samples were shaken manually for approximately 30 s in order to disrupt any crust formation on the surface of the slurry sample and to homogenize the samples. The cap of the adjacent tube was open and headspace air was sampled within 10 s, approximately 2.0 cm above the slurry surface at a rate of 100 ml/min. After sampling, the tube was closed using the cover and allowed to ferment at 32°C, with additional samples being collected at 3, 6, 12, 24 and 48 h following sprayed with 100 ml of each bacterial culture.

Experiment 2

In Experiment 2, a 10% dilution of the stock culture was prepared by mixing with 90 ml of DW. There were four treatments including:

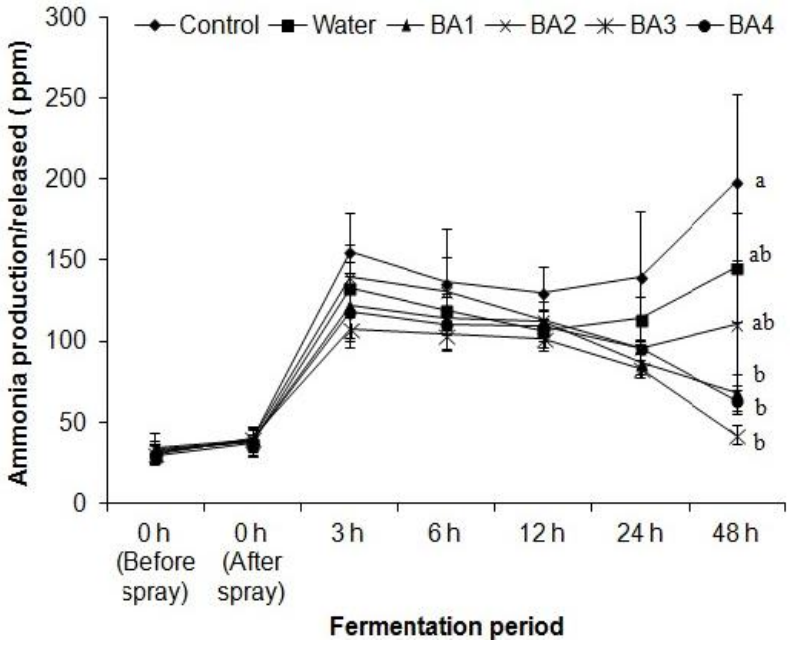

Figure 1. Effects of spraying different concentration of *Bacillus amyloliquifaciens* culture broth (BA) on ammonia emission from pig slurry for 48 h. Control, no spray; Water, water spray; BA1, BA 1%; BA2, BA 2%; BA3, BA 10%; BA4, BA 100%. Different letters at a particular time points indicates significant difference ($P < 0.05$).

control (no spray), water (water spray), 10% BA spray one time/day and 10% BA spray one time/two day. Approximately, 2 kg of stock slurry was stored in each fermentation chamber and allowed to ferment for 7 days at 32°C. The odorous gases were sampled on day 1 to 7, following spray with 100 ml of each treatment bacterial solution. The gas was sampled using a Gastec gas sampling pump (model AP-20; Gastec Corp., Japan) and Gastec detector tube (No. 3M for NH_3; No. 4HM and 4LL for H_2S; No. 5LA for SO_2). The gas measurement technique was same as experiment 1.

Statistical analysis

All experimental data was analyzed in accordance with the General Linear Model Procedure established by the Statistics Analysis Systems Institute (SAS, 2003). The variability of all of the data was expressed as the standard error (SE) and a probability level of $P < 0.05$ was considered to be statistically significant, whereas a $P < 0.10$ was considered to constitute a tendency. Treatment means were computed with the LSMEANS option of the SAS program.

RESULTS

Experiment 1

The effects of spraying with different levels of BA on the emission of NH_3 from slurry are shown in Figure 1. The NH_3 emission from the slurry in the control treatment was higher than that of slurry in the water, BA1, BA2, BA3, and BA4 treated groups throughout the entire experimental period. However, the only significant difference was observed at 48 h, when NH_3 emission was reduced in response to treatment with BA1, BA3 and BA4 compared to the control, with BA3 showing the highest efficacy ($P < 0.05$).

The effects of BA on slurry H_2S emission are shown in Figure 2. The only significant difference observed in H_2S emission was between that of slurry from the control group and the BA treated groups at 3 h ($P < 0.05$). As shown in Figure 3, SO_2 emissions from slurry were not affected by treatment with water or BA.

Experiment 2

The effects of direct application of 10% BA on NH_3 emission from pig slurry over 7 day are documented in Table 1. There were no significant differences among treatments in NH_3 emission at day 1 to 3 and day 5. However, it was significantly reduced in response to treatment with 10% BA one time/day and one time/two day at day 4, 6 and 7 compared to the control group ($P < 0.05$), with the lowest emissions being observed in the BA one time/day treated group.

As shown in Table 2, treatment with water and BA did not affect the H_2S emissions from pig slurry at day 1 and 2. On day 3, the H_2S emissions were significantly lower from both of the BA treated slurry compared to the control slurry ($P < 0.05$). From day 4 to 7 significant differences were observed in H_2S emissions between that of slurry from the control and 10% BA one time/day treatment

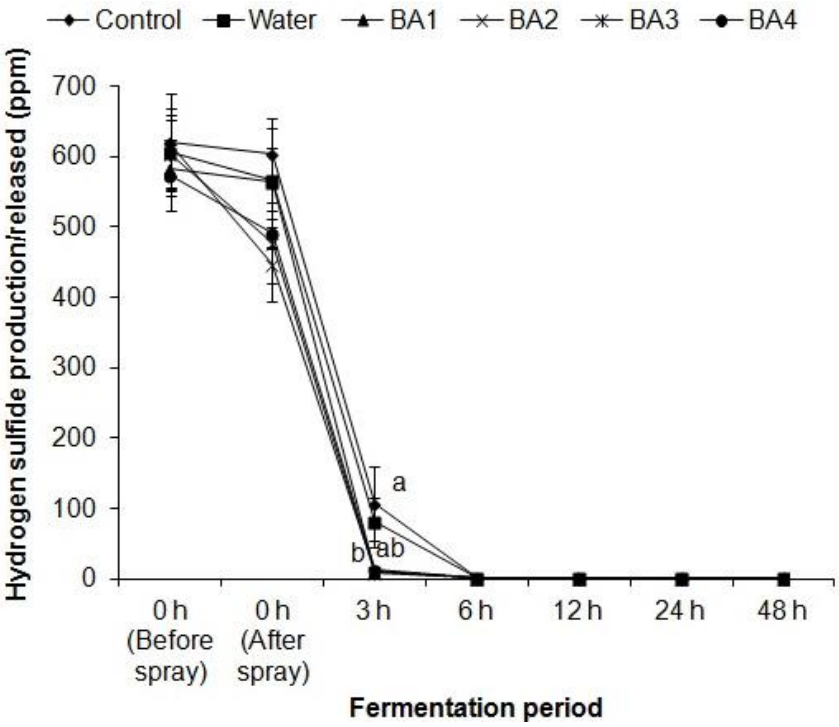

Figure 2. Effects of spraying different concentration of *Bacillus amyloliquifaciens* culture broth (BA) on hydrogen sulfide emission from pig slurry for 48 h. Control, no spray; Water, Water spray; BA1, BA 1%; BA2, BA 2%; BA3, BA 10%; BA4, BA 100%. Different letters at a particular time points indicates significant difference ($P < 0.05$).

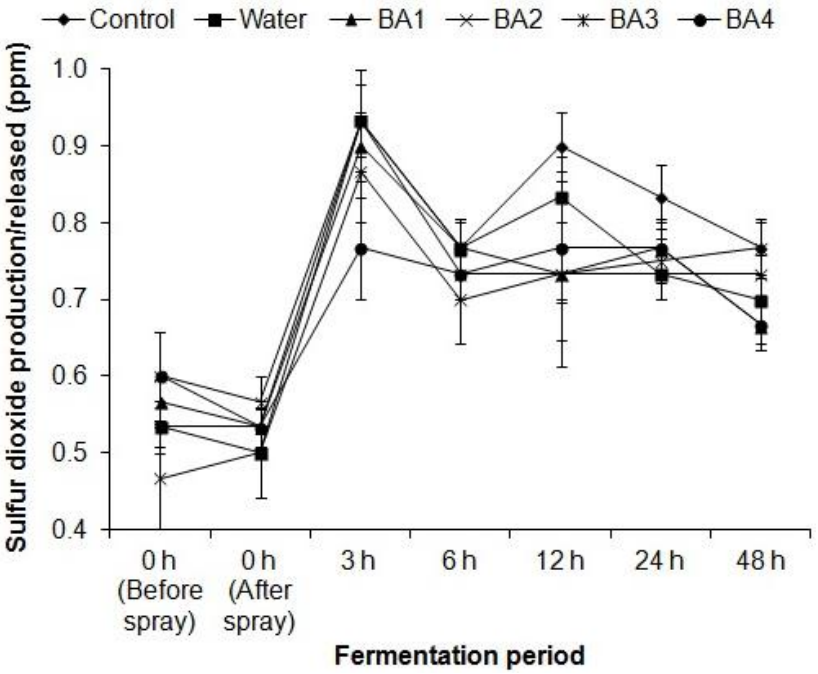

Figure 3. Effects of spraying different concentration of *Bacillus amyloliquifaciens* culture broth (BA) on sulfur dioxide emission from pig slurry for 48 h. Control, no spray; Water, Water spray; BA1, BA 1%; BA2, BA 2%; BA3, BA 10%; BA4, BA 100%.

Table 1. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture broth (BA) on ammonia emission from pig slurry[A].

Incubation period	Treatments				SEM[B]	P-value
	Control	Water	BA one time/day	BA one time/ two day		
Day 1	41.67	50.33	74.67	65.33	10.31	0.24
Day 2	143.33	95.33	108.00	11.67	18.32	0.42
Day 3	118.00	126.67	66.67	81.33	16.30	0.11
Day 4	153.33[a]	103.33[ab]	51.67[b]	76.00[b]	18.89	0.03
Day 5	163.33	98.00	48.33	63.33	28.49	0.11
Day 6	236.67[a]	121.67[ab]	43.33[b]	59.33[b]	29.08	0.02
Day 7	213.33[a]	164.33[a]	39.33[b]	45.33[b]	23.45	0.01

[a,b]Means in a row with no common superscripts significantly differ (P < 0.05). [A]Each value represents the mean of 3 replicates. [B]Standard error of the means.

Table 2. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture media (BA) on hydrogen sulfide (H_2S) emission from pig slurry[A].

Incubation period	Treatments				SEM[B]	P value
	Control	Water	BA one time/day	BA one time/ two day		
Day 1	339.33	430.00	346.67	403.33	75.32	0.83
Day 2	400.00	406.67	325.67	350.00	52.23	0.69
Day 3	25.00[a]	14.33[ab]	0.33[b]	1.17[b]	2.89	0.01
Day 4	550.00[a]	360.35[a]	9.67[b]	400.07[ab]	55.36	0.001
Day 5	533.33[a]	320.00[a]	9.33[b]	373.33[a]	78.39	0.02
Day 6	333.33[a]	183.33[a]	4.83[b]	223.40[a]	42.16	0.02
Day 7	216.67[a]	133.33[a]	2.50[b]	173.33[a]	23.15	0.01

[a,b]Means in a row with no common superscripts significantly differ (P < 0.05). [A]Each value represents the mean of 3 replicates. [B]Standard error of the means.

Table 3. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture media (BA) on sulfur dioxide (SO_2) emission from pig slurry[A].

Incubation period	Treatments				SEM[B]	P value
	Control	Water	BA one time/day	BA one time/ two day		
Day 1	0.53	0.50	0.70	0.60	0.10	0.58
Day 2	0.83	0.70	0.77	0.73	0.09	0.79
Day 3	0.87	0.90	0.67	0.60	0.09	0.14
Day 4	0.80[a]	0.63[ab]	0.57[b]	0.63[ab]	0.05	0.10
Day 5	0.77	0.60	0.50	0.67	0.07	0.16
Day 6	0.90[a]	0.60[ab]	0.53[b]	0.63[ab]	0.09	0.10
Day 7	0.80	0.67	0.43	0.47	0.11	0.14

[a,b]Means in a row with no common superscripts tended to differ (P < 0.10). [A]Each value represents the mean of 3 replicates. [B]Standard error of the means.

group (P < 0.05).

The SO_2 emissions from pig slurry in response to spraying with 10% BA are shown in Table 3. During the 7 day measurement period, the SO_2 emissions from pig slurry did not differ significantly among treatments, with the exception of a decreasing tendency in response to treatment with 10% BA one time/day when compared with control group at day 4 and day 6 (P < 0.10).

DISCUSSION

Odor production and accumulation of manure solids are characteristics that manifest as a result of inadequate microbial decomposition of manure (Davis et al., 2008). Undigested carbohydrates and protein (nitrogen) that have passed through the gastro-intestinal and urinary tract undergo microbial anaerobic decomposition to produce odorous compounds. This is further compounded by swine diet formulations, which commonly contain high concentrations of trace minerals and antibiotics that have deleterious effects on the bacteria needed for effective manure decomposition (Gilley et al., 2000). Therefore, direct application of microbial culture to improve manure digestion would provide a convenient mean to reduce odor emission from pig slurry. *B. amyloliquefaciens* (BA) is a potent spore-forming *Bacillus,* produces a number of extracellular enzymes including α-amylase, cellulose, metalloproteases and proteases (Gould et al., 1975; Gracia et al., 2003) to promote manure digestion and thereby may attenuate odor generation (Schreier, 1993). Ohta and Ikeda (1978) identified *Bacillus* spp. as effective microorganisms for reducing malodors. This study demonstrates that direct application of BA is an effective means of reducing odorous gas emission from pig slurry.

Feed nitrogen which is not utilized as body protein is excreted with feces and urine. According to Muck and Steenhuis (1981), the main part of ammonia (NH_3) originated from the decomposition of urea nitrogen in the urine by urease producing bacteria such as *Bacteriodes*, *Bifidobacteria*, *Proteus* spp. and others. Others, notably *E. coli,* do not have urease activity, so that release ammonia by deamination of organic nitrogen other than urea (Vince et al., 1973). As soon as the urine comes in contact with feces, the urea is converted into NH_3 and carbon dioxide by the microbial urease enzyme present in feces in the presence of high pH (Stevens et al., 1989; Aarnink, 1997). Therefore, reduction in the concentration of ammonia-producing bacteria is a key aspect to reduce emission of ammonia. It has been reported that, BA generates antimicrobial bacteriocin (barnase) (Lisboa et al., 2006), which may reduce ammonia producing *Clostridium perfringens, E. coli* and *Yersinia* in the feces, thereby attenuating the release of NH_3 in the present experiment. By contrast to our result Rahman et al. (2011) reported no effect of microbial additives on odor and ammonia reduction from farrowing-gastation swine operation. Lim et al. (2011) reported that bacteriocin produced by BA has antimicrobial activity against a wide range of microorganisms. Another possibility is that, BA produces several extracellular enzymes including metalloproteases and proteases, which may improve the digestion of fecal organic nitrogen and thereby reducing the ammonia production. The pH of the slurry is another important factor influencing the ammonia emission (Freney et al., 1983). Bacillus has been reported to reduce the pH of slurry via the production of organic acid (Wang et al., 2009), which may cause a reduction in hydrolysis of urea and deamination of other forms of nitrogen, thereby reducing NH_3 emissions in the present experiment.

Hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) have been identified as the most dangerous volatile sulfur gases (VS) among the by-products of manure decomposition generated under simulated anaerobic fermentation conditions (Banwart and Brenmer, 1975). Production of VS by anaerobic bacteria involves dissimilatory sulfate reduction and metabolism of sulfur-containing amino acids (Ushida et al., 2003). VS could be removed from the air with the use of chemoautotrophic or heterotrophic bacteria (Kanagawa and Mikami, 1989). Sato et al. (1999) demonstrated that a range of heterotrophic bacteria could decompose H_2S *in vitro*. They also demonstrated that soil isolates belonging to the genera *Bacillus*, *Pseudomonas* effectively decomposed H_2S. Ushida et al. (2003) isolated a VS degrading *Bacillus* spp. (Strain KPU 0013) and reported their ability to reduce H_2S emission from pig slurry. The possible explanation of reduction in H_2S emission in this experiment is that, BA may decompose the H_2S *in vitro*. Nakada and Ohta (1997) also reported removal of H_2S by applying a deodorant bacterium *Bacillus* sp. BN53-1. Another possibility is that, BA reduced the pH of the feces, which prevent sulfate reduction by the sulfate reducing bacteria (Tuttle et al., 1969) and metabolism of sulfur-containing amino acids by anaerobic bacteria (Arakawa et al., 2000; Ushida et al., 2001).

Sulfur dioxide (SO_2) is one of the six criteria pollutants defined in the US Clean Air Act. However, information of livestock related SO_2 can hardly be found. To the best of our knowledge, no other studies have been carried out to evaluate the effects of microorganisms on the emissions of SO_2 from pig slurry. In experiment 1, we found no significant effect of BA on SO_2 emissions from pig slurry. However, in experiment 2, slightly reduced SO_2 emissions were found on days 4 and 6 in response to treatment with 10% BA one time/day, which may be due to degradation of VS by BA or reduced growth and activity of sulfur-reducing bacteria in the slurry.

Conclusion

The results of this study indicate that direct application of 10% BA one time/day is more proficient in reduction of fecal NH_3, H_2S, and SO_2 emissions. Therefore, this level can be used as manure additives for odor reduction in pig facility. However, the underlying mechanisms by which reduction occurred should be further assessed by evaluating community structure of fecal bacteria and fecal pH which may better explain the relationship between *B. amyloliquefaciens* and native bacterial populations in the slurry.

Conflict of Interests

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

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REFERENCES

Aarnink AJA (1997). Ammonia emission from houses for growing pigs as affected by pen design, indoor climate and behavior. Ph. D. Thesis. Wageningen Agricultural University, The Netherlands.

Al-Kanani T, Akochi E, Mackenzie AF, Alli I, Barrington S (1992). Odor control in liquid hog manure by added amendments and aeration. J. Environ. Qual. 21:704-708.

Arakawa T, Ishikawa Y, Ushida K (2000). Volatile sulfur production by pig cecal bacteria in batch culture and screening inhibitors of sulfate-reducing bacteria. J. Nutr. Sci. Vitaminol. 46:193-198.

Banwart WL, Brenmer JM (1975). Identification of sulfur gases evolved from animal manures. J. Environ. Qual. 4:363-366.

Davis ME, Parrott T, Brown DC, de Rodas BZ, Johnson ZB, Maxwell CV, Rehberger T (2008). Effect of a Bacillus-based direct-fed microbial feed supplement on growth performance and pen cleaning characteristics of growing-finishing pigs. J. Anim. Sci. 86:1459-1467.

Freney JR, Simson JR, Denmead OT (1983). Volatilization of ammonia. Dev. Plant Soil Sci. 9:1-31.

Gilley JE, Spare DP, Koelsch RK, Schulte DD, Miller PS, Parkhurst AM (2000). Phototrophic anaerobic lagoons as affected by copper and zinc in swine diets. Trans. ASAE 43:1853-1859.

Gould AR, May BK, Elliott WH (1975). Release of extracellular enzymes from *Bacillus amyloliquefaciens*. J. Bacteriol. 122:34-40.

Gracia MI, Araníbar MJ, Lázaro R, Medel P, Mateos GG (20030. *α*-Amylase Supplementation of Broiler Diets Based on Corn. Poult. Sci. 82:436-442.

Hahne J, Schicke R, Vorlop KD (2003). Möglichkeiten und Grenzen zur minderung von Ammoniak-, Staub-und Geruchsemissionen durch Abluftreinigungsverfahren (options and limitations for the reduction of ammonia, dust and odour emissions by waste gas cleaning systems), 6. Tagung: Bau, Technik und Umwelt in der landwirtschaftlichen Nutztierhaltung, Vechta, Germany. pp. 253-259.

Kanagawa T, Mikami E (1989). Removal of methanethiol, deimethyl sulfide, dimethyl disulfide, and hydrogen sulfide from contaminated air by *ThiBacillus thioparus* TK-m. Appl. Environ. Microbiol. 55:555-558.

Kim JR, Dec J, Bruns MA, Logan BE (2008). Removal of odors from swine wastewater by using microbial fuel cells. Appl. Environ. Microbiol. 74:2540-2543.

Lim JH, Jeong HY, Kim SD (2011). Characterization of the Bacteriocin J4 produced by *Bacillus amyloliquefaciens* J4 isolated from korean traditional fermented soybean paste. J. Korean Soc. Appl. Biol. Chem. 54:468-474.

Lisboa MP, Bonatto D, Bizani D, Henriques JAP, Brandelli A (2006). Characterization of a bacteriocin-like substance produced by Bacillus amyloliquefaciens isolated from the Brazilian Atlantic forest. Int. Microbiol. 9:111-118.

Low EW, Chase HA (1999). Reducing production of excess biomass during wastewater treatment. Water Resour. 33:1119-1132.

Muck RE, Steenhuis TH (1981). Nitrogen losses in free stall dairy barns. In: Proceedings of the 4th International Symposium on Livestock Wastes, ASAE, St. Joseph, MI. pp. 406-409.

Nakada Y, Ohta Y (1997). Hydrogen sulfide removal by a deodorant bacterium *Bacillus* sp. BN53-1 (In Japanese). Seibutsu Kogaku Kaishi. 75:425-431.

Ohta Y, Ikeda M (1978). Deodorization of pig feces by actinomycetes. Appl. Environ. Microbiol. 36:487-491.

Rahman S, DeSutter T, Zhang Q (2011). Efficacy of a microbial additive in reducing odor, ammonia, and hydrogen sulfide emissions from farrowing-gestation swine operation. Agric. Eng. Int. CIGR J. 13:1-9.

Rahman S, Mukhtar S (2008). Efficacy of Microbial Treatment to Reduce Phosphorus and Other Substances from Dairy Lagoon Effluent. Appl. Eng. Agric. 24:809-819.

SAS (2003). SAS User's Guide: Statistics. Version 9. SAS Institute Inc., Cary, NC.

Sato H, Takakuwa S, Kimura T, Shimomoto H, Hirose T, Moriyama Y (1999). Isolation from soil of aerobic chemoheterotrophic bacteria capable of decomposing methanethiol and hydrogen sulfide. Microb. Environ. 14:131-137.

Schiffman SS, Bennett JL, Raymer JH (2001). Quantification of odors and odorants from swine operations in N. Carolina. Agric. For. Meteorol. 108:213-240.

Schreier HJ (1993). Biosynthesis of glutamine and glutamate and the assimilation of ammonia. In: *Bacillus subtilis* and other gram-positive bacteria (Ed. A. L. Sonenshein, J. A. Hoch and R. Losick). ASM Press, Washington, DC. pp. 281-285.

Sheridan BA, TP Curran, Dodd VA (2002). Assessment of the influence of media particle size on the biofiltration of odorous exhaust ventilation air from a piggery facility. Bioresource Technol. 84:129-143.

Stevens RJ, Laughlin RJ, Frost JP (1989). Effect of acidification with sulphuric acid on the volatilization of ammonia from cow and pig slurries. J. Agric. Sci. 113:389-395.

Sund JL, Evenson CJ, Strevett KA, Nairn RW, Athay D, Trawinsk E (2001). Nutrient conversions by photosynthetic bacteria in a concentrated animal feeding operation lagoon system. J. Environ. Qual. 30:648-655.

Sutton AL, Kephart KB, Verstegen MWA, Canh TT, Hobbs PJ (1999). Potential for reduction of odorous compounds in swine manure through diet modification. J. Anim. Sci. 77:430-439.

Tuttle JH, Dugan PR, MacMillan CB, Randles CI (1969). Microbial dissimilatory sulfur cycle in acid mine water. J. Bacteriol. 97:594-602.

Ushida K, Hashizume K, Miyazaki K, Kojima Y, Takakuwa S (2003). Isolation of *Bacillus* spp. as a volatile sulfur-degrading bacterium and its application to reduce the fecal odor of pig. Asian-Australas. J. Anim. Sci. 16:1795-1798.

Ushida K, Ohshima N, Tanimura A, Miyazaki K, Kojima Y, Takakuwa S (2001). Evaluation of methanethiol and hydrogen sulfide production by standard strains of intestinal bacteria and isolates from pig feces. Biosci. Microflora 20:53-57.

VanderZaag AC, Gordon RJ, Glass VM, Jamieson RC (2008). Floating covers to reduce gas emissions from liquid manure storages: a review. Appl. Eng. Agric. 24:657-671.

Vince A, Dawson AM, Park N, O'Grady F (1973). Ammonia production by intestinal bacteria. Gut 14:171-177.

Wang Y, Cho JH, Chen YJ, Yoo JS, Huang Y, Kim HJ, Kim IH (2009). The effect of probiotic Bioplus 2B® on growth performance, dry matter and nitrogen digestibility and slurry noxious gas emission in growing pigs. Lives. Sci. 120:35-42.

Zhu J (2000). A review of microbiology in swine manure odor control. Agric. Ecosyst. Environ. 78:93-106.

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Full Length Research Paper

Optimization of solid-state fermentation conditions for the production of cellulase and its hydrolytic potentials by *Trichoderma virride* Sn-9106

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Trichoderma viride **Sn-9106 with high cellulase activity was used to produce enzyme on residues of Chinese herbs as substrate in solid state fermentation. Residues of Chinese herbs and peptone were found to be the best combination of carbon and nitrogen source for the production of cellulase. The nutrient composition of medium was optimized using response surface methodology. A fractional factorial design (3^3) was applied to elucidate the nutrient medium components that significantly affect cellulase production. The concentration of peptone and wheat bran in the medium was a significant factor. The composition of nutrient fermentation medium optimized with response surface methodology was in g/L: wheat bran, 19.8, peptone, 2.06 and KH_2PO_4, 2.9. Compared to the original medium, the cellulase activity increased from 3.8 to 7.5 IU/mL.**

Key words: cellulase, *Trichoderma viride* Sn-9106, response surface methodology (RSM), solid state fermentation (SSF), residues of Chinese herbs (RCH).

INTRODUCTION

In recent years, one of the most important biotechnological applications is the conversion of lignocellulosics wastes into products of commercial interest such as bioethanol (Den Haan et al., 2007; Lynd et al., 2005). Cellulase is responsible for the hydrolytic cleavage of β-glycosidic bonds in cellulose and plays a critical role in the processing of lignocellulosics. It is a complex made up of three classes of enzymes: exoglucanase, endoglucanase and β-glucosidase (Chandrasekharaiah et al., 2012; Salahuddin et al., 2012). Cellulase is produced by two methods: submerged fermentation and solid-state fermentation (SSF). Compared to submerged fermentation, SSF has high productivity and its cost is low. Furthermore, the sub-strates used in solid-state fermentation are always industrial and agricultural wastes, which can reduce the fermentation cost (Jecu, 2000; Mekala et al., 2008; Zhao et al., 2010). In China, 13 million tons of residues from the Chinese herbs are produced annually. This abundant but low value resource contains about 70% hydrolysable cellulose

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Abbreviations: RSM, response surface methodology; **SSF,** solid-state fermentation; **RCH,** residues of Chinese herbs.

Table 1. Factors and coded values of RSM.

Variables (% w/w)	Coded	Range and levels		
		-1	0	+1
Wheat bran	X_1	15	20	25
peptone	X_2	1.5	2	2.5
KH_2PO_4	X_3	0.25	0.3	0.35

and hemicellulose, crude protein and trace element (Li et al., 2010). However, 90% residues of Chinese herbs (RCH) litter the environment and constitute waste problem (Xu et al., 2007). The use of Chinese herbs residues as the basis of the cultivation media, for decreasing costs of energy production and meeting the increased awareness in energy conservation and recycling is a matter of great interest. Although the production of cellulase using various nutrients as substrates by microorganisms has been reported (Khaleel and Gilna, 2011; Gamarra et al., 2010), researches are seldom done on production of cellulase using RCH as substrate. The aim of the present study was to demonstrate the optimization of SSF conditions with RCH for the production of cellulase and its hydrolytic potentials by *Trichoderma virride*.

MATERIALS AND METHODS

Microorganisms

T. virride Sn-9106 was isolated from soil samples collected from the soils in Dongling Mountain in Shenyang, China. Identification of isolates was carried out by the method of Barnett (1960) and Domch et al., (1980). Liquid inoculum medium (per liter, used in mycelium culture) consisted of 5.0 g of wheat bran, 2.0 g of peptone, 1 g of KH_2PO_4, 1.0 g of $CaCl_2$, and 6.0 g of glucose.

Substrate treatment

Residue from China herb was kindly provided by the Liaoning Benxi third medicine co., LTD. This material was thoroughly washed, dried and milled to 20 mm particle size. It contained 39% cellulose, 20% lignin, 28% hemicellulose, 7.5% extractives, 3.5% ash and 2% protein, on a dry-wt basis.

Inoculum and solid-state fermentation

T. virride Sn-9106 was used for cellulase production (Chen et al., 2012). It was grown on potato/dextrose/agar slants. Spores were washed from a 3-day agar-slant culture with 10 ml sterile distilled water and 2 ml of the suspension (10^6 spores/ml) was added to 250 ml shake-flasks, each containing 100 ml liquid inoculum medium. The inoculated flasks were incubated at 30±2°C and 150 rpm as a source of mycelia inoculum for SSF for 2 days before use.

Fermentations were carried out with pan bioreactor containing 20 g (dry-wt basis) of RCH as fermentation medium. The mixtures were autoclaved at 126°C for 40 min. Then, each pan bioreactor was inoculated with 1% (w/v) mycelium of Sn-9106. The nutrient elements (in g/L) of fermentation medium were calculated by weight of polysaccharide (cellulose and hemicellulose) content of residue and added to the substrate. The moisture content of the substrate after inoculation was about 75% (dry-wt basis) and the final pH was adjusted to 5.4. The fermentation was maintained for 72 h on the conditions of 30±2°C temperature; duplicate pan bioreactors were set up for each experimental variation.

Enzyme extraction

According to each gram of initial substrate weight, 100 ml distilled water was used to dispense the fermented moldy pith. The dispensed pith was shaken at 30±2°C and 130 rpm for 1 h. The mixture was filtered through nylon cloth of 200 mesh. The pH of the collected solution was measured before it was centrifuged. The supernatant was assayed for cellulase activity .

Assay of enzyme activity

Filter paper activity (FPA) was determined according to the method of the International Union of Pure and Applied Chemistry (IUPAC) and expressed as international units (IU). One IU of cellulase activity is the amount of enzyme that forms 1 µmol glucose (reducing sugars as glucose) per minute during the hydrolysis reaction. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method (Ghose, 1987).

Optimization of enzyme production with SSF

Single factor experiment

Several single factors of nutrient elements influencing enzyme production were optimized. The effect of wheat bran (5% to 40%), peptone (0.5% to 4%) and KH_2PO_4 (0.1% to 0.4%) on cellulase synthesis was determined by growing the organism in SSF. The methods used in fermentation process experiment and enzyme assay are described above.

RSM for medium optimization

The optimal experiments for wheat bran, peptone and KH_2PO_4 supply were undertaken using the response surface methodology, in which MATLAB was used. A 3×3 fractional factorial design was employed to optimize medium components. The factors and levels used are shown in Table 1.

The responses were analyzed using MATLAB 14.0 software. In developing the regression equation, the test factors were coded according to the Equation:

$$x_i = (X_i - X_0) / \Delta X_i$$

Where, x_i was the coded value of the independent variable; X_i was the actual value of the independent variable; X_0 was the actual value of the independent variable at the central point and ΔX_i was the steep change value. A quadratic polynomial regression model was assumed to predict both Y responses. The model response of Y was expressed as:

$$Y = \square_0 + \sum_{i=1}^{3} \beta i Xi + \sum_{i}^{3} \beta ii\ X2i + \sum_{i=1}^{2} \quad \sum_{j=i+1}^{3} \beta ij Xi\ Xj$$

Where, β_0 was an intercept; β_I, first-order model coefficient; β_{ii}, quadratic coefficient for the variable; β_{ij}, interaction coefficient for the interaction of variables I and j, and β_I and β_φ were evaluated by

Table 2. Cellulase production by *T. viride* in solid-sate fermentation of residue from Chinese herb carried out in pot fermenters.

Enzyme activities (IU/g dry-weight substrate)	Fermentation days			
	2	3	4	5
Endo-glucanase	2.32	10.07	9.87	9.52
Filter-paper cellulase	1.23	3.87	3.88	3.83
β-Glucosidase	1.37	4.45	4.89	4.23

the coefficient of determination (R^2) and the SPSS.

Contour plots were developed using the fitted quadratic polynomial equations obtained by keeping one of the independent variables at a constant value and changing the levels of the other two variables.

Hydrolysis experiments

In this study, hydrolysis efficiency was defined as cellulose and hemi-cellulose conversion efficiency. Pre-treated straw mixture was centrifuged at 10 *g* for 25 min to separate the supernatant and solids parts. TS contents of solids were adjusted to 10% by mixing supernatant and solids part. The hydrolysis experiments were conducted in 100 ml reaction system containing 10 g concentrated straw at 20 FPU/g cellulase. During the hydrolysis, the temperature was kept at 50±2°C, the revolution was kept at 250 rpm and the pH was maintained at 5.0 by pH controller.

Samples were taken aseptically after 72 h. The released glucose and xylose were determined on HPLC analysis with an Aminex HPX-87H column (Bio-Rad Laboratories) operating at 50°C and a flow rate of 0.6 ml 4 mM H_2PO_4 min^{-1}, using the refractive index detector. Cellulose and hemicellulose contents before and after hydrolysis were analyzed by strong acid analysis.

RESULTS AND DISCUSSION

Enzyme production by residue from Chinese herb of *T. virride*

RCH contains phenolic compounds which can restrain the growth of fungi (Xu et al., 2007; Yang et al., 2009); thus, an anti-phenol strain, *T. virride* Sn-9106 was isolated to produce cellulase. The formation of various enzymes in RCH cultures, under the conditions of SSF is shown in Table 2. Enzyme production appeared to be growth-associated. Maximum endo-glucanase, FP-cellulase and β-glucosidase activities were reached on day 3 of SSF, whereas their activity did not continue to rise after that.

Optimization of enzyme production by RSM

T. virride Sn-9106 was further investigated for cellulase production with RCH containing different concentration of wheat bran, nitrogen sources and inorganic salts in SSF. Peptone and KH_2PO_4 were selected as the main nitrogen source and inorganic salts; also wheat bran was added for optimizing cellulase production.

As shown in Figure 1, when the wheat bran was at 20%, the *T. virride* Sn-9106 produced a maximal FPase activity of 4.74 IU/g (Figure 1A). Application of peptone to mixed medium also induced activities of the enzymes to increase. The top activity was observed at 1.5% of peptone when FPU had activity of 6.45 IU/g (Figure 1B). We tested the growth and enzyme release activity using KH_2PO_4. Our result shows a pattern of activities for both enzymes similar to those supplied with wheat bran and peptone (Figure 1C). Also, the change in activities of FPU was dependent on the concentrations of KH_2PO_4, which showed the maximum activities of the enzymes at concentration of 0.25%.Fig.2 B

To get insight into the interaction with the three factors and to see the multiple capability of inducing cellulase, a combination experiment was designed with 3 factors × 3 levels (Table 3).

Then, SPSS was applied to get ideal second-degree polynomial regression models of FPase activity.

$$Y=7.16-0.023\ X_1+0.435\ X_2-0.025\ X_3-0.498\ X_1\ X_2+0.168\ X_1\ X_3+0.198\ X_2\ X_3-1.229\ X_1^2+1.859\ X_2^2-1.504\ X_3^2$$

The results shown in Tables 3 to 5 show that the model for FPase production was significant ($p=0.0180<0.0500$) with a satisfactory value of coefficient of determination, R^2 Ad (0.813423). This indicated that 81.34% of the variability in the response could be explained by the second-order model equation given above. Probability value for the lack of fit (LOF) was 0.0502, which was not significant. The results showed that this model is appropriate.

The resulting response surface showed the effect of wheat bran, peptone and KH_2PO_4 concentration on the FPase production (Figure 2). Because the shape of contour could reflect the instance, elliptical contour means strong interaction. This result demonstrate that there is a significant interaction between wheat bran and peptone. Wheat bran and KH_2PO_4 also have significant interaction. However, the interaction between peptone and KH_2PO_4 was insignificant.

We can learn that the response surface has a maximum point. The maximum FPase production by *T. virride* Sn-9106 was obtained in the optimized medium when the initial concentration of wheat bran, peptone and

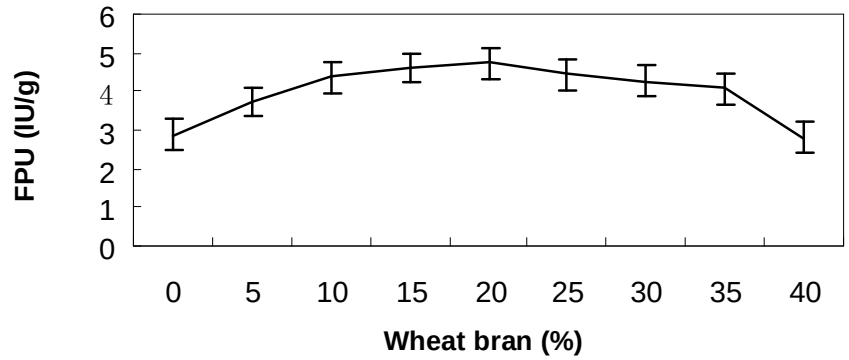

Figure 1A. Influence of wheat bran on FPase production of *T.virride* Sn-9106.

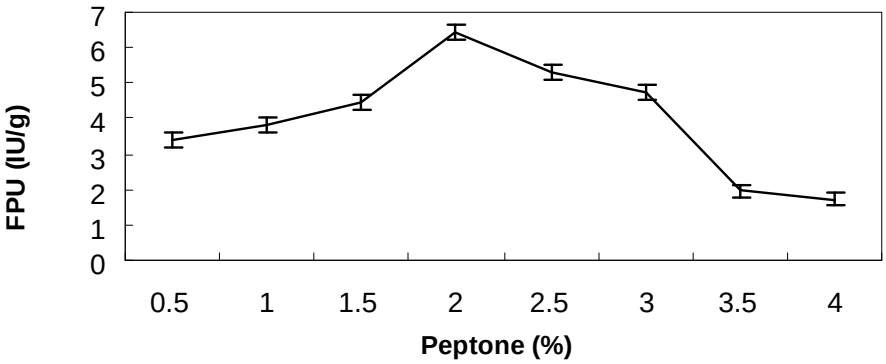

Figure 1B. Influence of peptone on FPase production of *T.virride* Sn-9106.

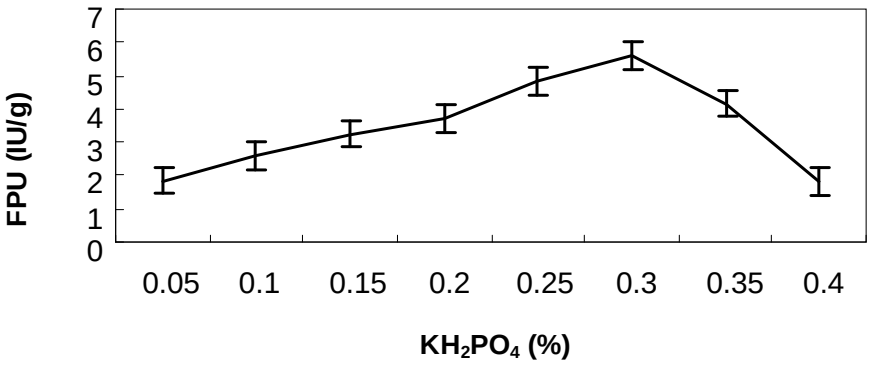

Figure 1C. Influence of KH_2PO_4 on FPase production of *T.virride* Sn-9106.

KH_2PO_4 was 19.8, 2.06 and 2.9% respectively. The maximum response predicted from the model was 7.5 IU/g. Repeated experiments were performed to verify the predicted optimum. The result from replications 7.4 IU/g was coincident with the predicted value and the model was proven to be adequate. Compared with the original medium, the FPase activity of *T. virride* Sn-9106 increased from 3.8 to 7.5 IU/g.

Enzymatic hydrolysis and conversion experiment

In this study, enzymatic hydrolysis of pre-treated straw mixture with TS content of 10% was studied. Two different cellulases, produced by *T. virride* Sn-9106 and Cellulast+Novozyme 188 (Purchased from Novozyme) were compared.

Figure 3 shows that the highest cellulose conversion

Table 3. Experimental design and results of RSM.

Number	X_1	X_2	X_3	Measured value	Y
1	-1	-1	0	6.16	4.9375
2	1	-1	0	5.9	6.1625
3	-1	1	0	7.7	7.4375
4	1	1	0	3.84	5.0625
5	-1	0	-1	6.8	7.7225
6	1	0	-1	8.05	7.4875
7	-1	0	1	7.35	7.9125
8	1	0	1	7.92	6.9975
9	0	-1	-1	7.09	7.39
10	0	1	-1	7.2	6.54
11	0	-1	1	5.03	5.69
12	0	1	1	8.24	7.94
13	0	0	0	12.99	12.60333
14	0	0	0	12.45	12.60333
15	0	0	0	12.37	12.60333

Table 4. Model Summary Statistics.

Source	Standard Deviation	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	3.007071	0.01667	0.25151	0.46262	147.9487	
2FI	3.42252	0.073595	0.62121	1.22904	225.4754	
Quadratic	1.161061	0.933365	0.813423	0.03523	104.7174	Suggested
Cubic	0.337244	0.997751	0.984259		+	Aliased

Table 5. Anova results for cellulase production obtained from RSM.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	94.41	9	10.49035	7.781795	0.0180	significant
A-A	0.66	1	0.66125	0.490518	0.5149	
B-B	0.98	1	0.98	0.726969	0.4328	
C-C	0.045	1	0.045	0.033381	0.8622	
AB	3.24	1	3.24	2.403448	0.1818	
AC	0.12	1	0.1156	0.085753	0.7814	
BC	2.4	1	2.4025	1.782186	0.2394	
A2	33.94	1	33.93601	25.1739	0.0040	
B2	49.78	1	49.7765	36.92445	0.0017	
C2	15.39	1	15.39103	11.41714	0.0197	
Residual	6.74	5	1.348063			
Lack of Fit	6.51	3	2.17095	19.08807	0.0502	not significant
Pure Error	0.23	2	0.113733			
Cor Total	101.15	14				

obtained was 86.3% at the enzyme loading of 20 FPU/g-cellulose, which is quite comparable with commonly cellulose conversion of 92.5% at commercial enzyme loading of 20 FPU/g-cellulose.

Previous study revealed that *Trichoderma* can grow on solid substrates, such as corn straw (Wang et al., 2005;

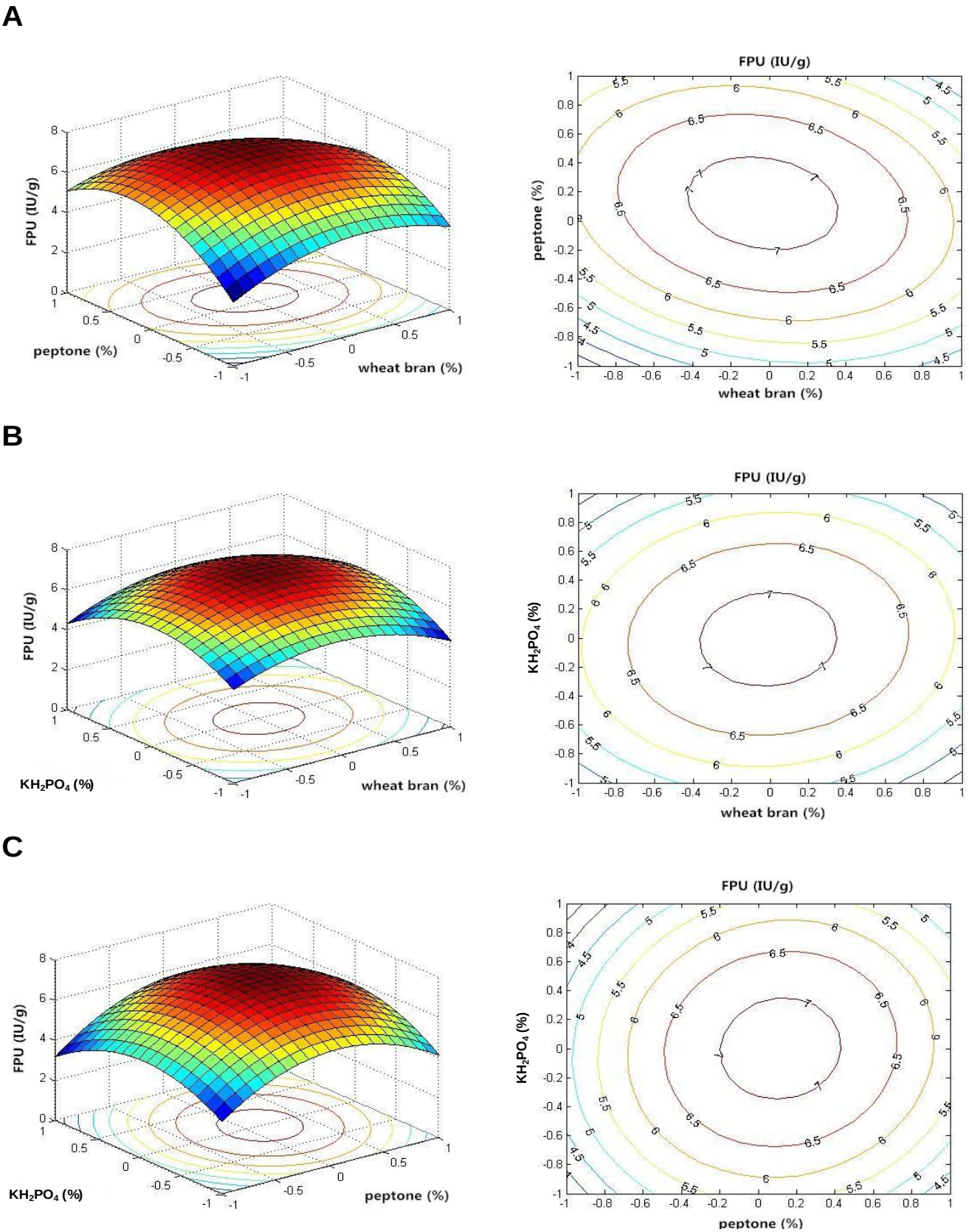

Figure 2. Response surface plot for the effect of wheat bran, peptone and KH_2PO_4 on FPase production. **A.** Effect of interaction between the wheat bran and peptone on FPase production. **B.** Effect of interaction between the wheat bran and KH_2PO_4 on FPase production. **C.** Effect of interaction between the peptone and KH_2PO_4 on cellulase FPase production.

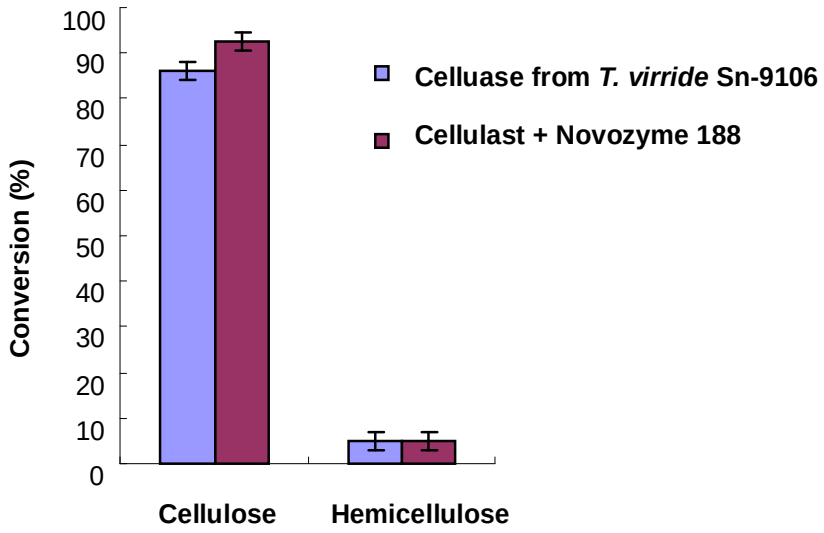

Figure 3. Comparison of hydrolysis efficiency with different enzyme loading.

Wang, 2006), wheat straw (Awafo et al., 1996) as well as sugar cane bagasse (Massadeh et al., 2001; Duenas et al., 1995) to promote cellulase production. But there is little concern on one of the lignocellulose residues-residues of Chinese herbs. In this study, a significant activity of cellulase was produced by the *T. virride* Sn-9106 grown on residues of China herbs. Enzymatic hydrolysis experiment showed that although the percentage of hydrolysis and conversion by *T.virride* Sn-9106 was lower than Celluclast+Novozyme188, a higher conversion was achieved in cellulose hydrolysis by *T. virride* Sn-9106.

Conflict of Interests

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

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REFERENCES

Awafo VA., Chahal DS, Simpson BK, Lê GB (1996). Production of cellulase systems by selected mutants of *Trichoderma reesei* in solid-state fermentation and their hydrolytic potentials. Appl. Biochem. Biotechnol. 57(1):461-470.

Barnett HL (1960). *Illustrated genera of imperfect fungi*. Burgen Publishing Co. Minnesota.

Chandrasekharaiah M, Thulasi A, Bagath M, Kumar DP, Santosh SS, Palanivel C, Jose Vazhakkala Lyju, Sampath KT(2012). Identification of Cellulase Gene from the Metagenome of *Equus burchelli* Fecal Samples and Functional Characterization of a Novel Bifunctional Cellulolytic Enzyme. Appl. Biochem. Biotechnol. 167(1): 132-141.

Chen HM, Zhang L, Li YQ, Kan GS, Ren DM (2012). Cloning and expression of recombinant EG Ifrom *Tricoderma viride*. China Brewing 7:033.

Den Haan R, Rose SH, Lynd LR, Van Zyl WH (2007). Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metab. Eng. 9(1):87-94.

Domch KH, Gams W, Anderson TH (1980). Compendium of soil fungi. Volume 2. Academic Press (London) Ltd..

Duenas R, Tengerdy RP, Gutierrez-Correa M (1995). Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. World J. Microbiol. Biotechnol. 11(3):333-337.

Gamarra NN, Villena GK, Gutiérrez-Correa M (2010). Cellulase production by *Aspergillus niger* in biofilm, solid-state, and submerged fermentations. Appl: Microbiol. Biotechnol., 87(2): 545-551.

Ghose TK (1987). Measurement of cellulase activities. Pure Appl. Chem. 59(2):257-268.

Jecu L (2000). Solid-state fermentation of agricultural wastes for endoglucanase production. Ind. Crops Prod. 11(1):1-5.

Khaleel KM, Gilna VV (2011). Cellulase enzyme activity of *Aspergillus fumigatus* from mangrove soil on lignocellulosic substrate. Recent Res. Sci. Technol. 3(1):132-134.

Li M, Yao R, Li H, Hu H (2010). Production cellulase from residues of the pony tree roots bark by solid fermentation and the saccharification. An Hui Chem. Eng. 51(1):57-60.

Lynd LR, Van Zyl WH, McBRIDE JE, Laser M(2005). Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol. 16(5):577-583.

Massadeh MI, Yusoff W MW, Omar O, Kader J (2001). Synergism of cellulase enzymes in mixed culture solid substrate fermentation. Biotechnol. Lett. 23(21):1771-1774.

Mekala NK, Singhania RR, Sukumaran RK, Pandey A(2008). Cellulase production under solid-state fermentation by *Trichoderma reesei* RUT C30: statistical optimization of process parameters. Appl. Biochem. Biotechnol. 151(2-3):122-131.

Salahuddin K, Prasad R, Gor SH, Visavadia MD, Soni VK, Hussain MD(2012). Biochemical characterization of thermostable cellulase enzyme from mesophilic strains of actinomycete. Afr. J. Biotechnol.

11(43):10125-10134.
Wang JS (2006). Cellulase preparation and alcohol production from corn straw through solid state fermentation. Hebei Agriculture University, China.
Wang JS, Wang J, Liu JB (2005). Studies on solid-state fermentation for cron stalk by *Trichoderma koningii* producing cellulase. Journal of Cellulose Science and Technology, 4: 004.
Xu GW, Ji WF, Wan YH, Liu CZ (2007). Energy production with light 2 industry biomass process residues rich in cellulose. Prog. Chem. 19(7/8):1164-1175.
Yang JF, Li H, Liu SH, Hu HW, Liu ZP, Zhao XY (2009). Fungistasis of three kinds of chinese herbs extracts against *Monilinia fructicola*. Chin. Agric. Sci. Bull. 25(12): 188-194.
Zhao XB, Zhou YJ, Zheng GJ, Liu DH (2010). Microwave pretreatment of substrates for cellulase production by solid-state fermentation. Appl. Biochem. Biotechnol. 160(5):1557-1571.

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