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Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)

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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 gonorrhoea, 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...
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 trichomoniais 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 trichomoniais, 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 endocinial 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 trichomoniais 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 leucorrhoea.

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 gonorrhoea 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-diiasis, 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. Occasionally, 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 trichomoniais 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. gonorrhoea. 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

<table>
<thead>
<tr>
<th>Affected patients</th>
<th>Number</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with <em>Trichomonas vaginalis</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with <em>Trichomonas vaginalis</em></td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>and <em>Candida albicans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with <em>Trichomonas vaginalis</em></td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>and <em>Neisseria gonorrhoea</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 laying in the air or keeping on the table to dry horizontally. Gieniea’s thin film technique was used, using already prepared Gieniea’s stain, method and distilled water. *N. gonorrhoea* and *C. albicans* were cultivated using blood agar medium and Sabouraud dextrose agar, respectively. They were prepared according to the manufacturer’s instructions.

Stain procedure

Already prepared Gieniea’s stain was diluted 1 in 30 before use. One milliliter of Gieniea’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 Gieniea’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. 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 Gieniea’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. gonorrhoea* 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. gonorrhoea*. 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 gonorrhoea (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, whilst others use water after sexual contact. This is in concordance with findings of Joesoef et 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 trichomoniasis, candidiasis 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 trichomoniasis 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 trichomoniasis 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, premartial 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 wares, 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.

ACKNOWLEDGEMENTS
Our appreciation goes to the entire staff of Medical Microbiology Department and the ethical group of the organization for giving us the opportunity to collect the samples.

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


Influence of acetylsyringone 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 acetylsyringone (AS) tested, addition of 150 µM acetylsyringone was found to enhance the transformation frequency up to 75(±2.60)* and 60 (±2.08)* percentage by *A. rhizogenes* MTCC-532 and MTCC 2364 strains, respectively. Transformation efficiency was highly dependent on the acetylsyringone 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*, acetylsyringone, 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 achieved. 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 inducing, Gram-negative soil bacterium, which is able to induce hairy roots rapidly at the infection site (Baransi, 2008). The *agrobacterium*
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 carota 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 (Sawasaki 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, K2HPO4-0.5 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% HgCl2 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-GGCTATCCCGAGGCGAT-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 apices 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
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 acetylsyringone 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\%), number of lateral roots (12±0.42\%) and number of negative geotropic roots (9±0.31\%) was observed from carrot discs simmered with 48 h old *A. rhizogenes* MTCC-532 on MS plates supplemented with 150 µM acetylsyringone (Table 1). In the *A. rhizogenes* MTCC 2364 strain used for same condition only (60±2.08\%) percentage of hairy root initiation, (9 (0.31\%)) number of lateral roots and (5 (0.17\%)) number of negative geotropic roots was observed. The transformation frequency declined at both lower (<150 µM) and higher concentrations of acetylsyringone (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 transformed 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

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**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.

**Figure 5.** Negative geotropism of hairy root .

numerous lateral roots (Figure 4.), which grew both on the surface and penetrated into the media, exhibiting the
Table 1. Effect of *A. rhizogenes* (MTCC-532) strain with their ability to induce carrot hairy roots on various concentrations of acetosyringone.

<table>
<thead>
<tr>
<th>Acetosyringone (µM)</th>
<th>Total no. of carrot discs</th>
<th>No of discs inducing hairy root</th>
<th>Transformation rate (%)</th>
<th>Number of lateral roots</th>
<th>No of roots showed negative geotropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>03</td>
<td>15 (±0.52)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2 (±0.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (±0.00)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>05</td>
<td>25 (±0.87)&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>4 (±0.14)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1 (±0.03)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>07</td>
<td>35 (±1.21)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>7 (±0.24)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3 (±0.10)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>150</td>
<td>20</td>
<td>15</td>
<td>75 (±2.60)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 (±0.42)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9 (±0.31)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>08</td>
<td>40 (±1.39)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 (±0.35)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6 (±0.21)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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<0.05).

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

<table>
<thead>
<tr>
<th>Acetosyringone (µM)</th>
<th>Total no. of carrot discs</th>
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<tr>
<td>Control</td>
<td>20</td>
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<td>10 (±0.35)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0 (±0.00)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>05</td>
<td>15 (±0.52)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3 (±0.10)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1 (±0.03)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>07</td>
<td>30 (±1.04)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (±0.17)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 (±0.07)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>150</td>
<td>20</td>
<td>15</td>
<td>60 (±2.08)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9 (±0.31)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 (±0.17)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>08</td>
<td>35 (±1.21)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 (±0.24)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3 (±0.10)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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<0.05).

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).
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 \( \text{vir} \) 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 \( \text{vir} \) gene expression in Muskelon explants. Many other reports also pointed out that the \( \text{vir} \) 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 \textit{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 \( \text{gene and confirmed the} \) transformation at 341 bp segment. Similar primer was also used by Garland et al. (2001) and Rajkumar and Murugesan (2014) in \textit{Ulmus procera, Psoralea corylifolia} hairy root respectively to confirm the transformation. The confirmation of \text{roIC} gene transfer from \textit{A. rhizogenes} R1000 by PCR detection at 557 bp was also reported in \textit{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 \textit{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


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 carboxymethylcellulase 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 show 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 microcolonies with water channels in-between and an assortment of cells and extracellular polymers (polysaccharides, glycoproteins and proteins (Christensen, 1989;
Table 1. Different media composition involved in enzymes production.

<table>
<thead>
<tr>
<th>Different media used</th>
<th>Composition (g/l sea water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Med.1 (Hansen et al., 1984)</td>
<td>Yeast extract 5.0, sodium alginate 5.0, 0.6mM MgCl₂</td>
</tr>
<tr>
<td>Med.2 (Hansen et al., 1984)</td>
<td>Peptone 5, Meat extract 1.0, Yeast extract 2, Sodium chloride 5.0, sodium alginate 5.0</td>
</tr>
<tr>
<td>Med.3 (Alexeeva et al., 2002)</td>
<td>Bacto-peptone 2.0, casein hydrolysate 2.0, yeast extract 2.0, glucose 1.0, K₂HPO₄ 2.0, MgsO₄ 0.05, 50% sea water, 50% distilled water</td>
</tr>
<tr>
<td>Med.4 (Manyak et al., 2004).</td>
<td>Yeast extract 1.0, sodium alginate 2.0</td>
</tr>
<tr>
<td>Med.5 (Hansen and Nakamura, 1985).</td>
<td>Peptone 5.0, Meat extract 1.0, Yeast extract 2, Sodium chloride 5.0, sodium alginate 1.0, 30 mM MnSO₄</td>
</tr>
<tr>
<td>Med.6 (Oldak and Trafny, 2005; Jain and Ohman, 2005).</td>
<td>Tryptone 1.0, yeast extract 0.5, NaCl 0.5</td>
</tr>
</tbody>
</table>

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; Johansen 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 7×10⁶ 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.

### 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 (alginate, 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 dinitrosalicilic acid reagent (2.14% NaOH, 0.63% 3, 5-dinitrosalicilic 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 liberated 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 aeruginosa* ATCC 27953, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *B. subtilis* NRRRL-B-4219 were grown according to de Queiroz and Day (2007), a standardized suspension of *P. aeruginosa* (2 × 10⁶ cfu/ml) and *E. coli*, *S. aureus* and *B. subtilis* (3 × 10⁶ 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 CO₂ (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 alginate 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.

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

*Control

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

<table>
<thead>
<tr>
<th>Sodium alginate (g%)</th>
<th>Total protein (mg/ml)</th>
<th>Enzymes activity (U/ml)</th>
<th>Alginase</th>
<th>Dextrinase</th>
<th>Pectinase</th>
<th>CMCase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.16±0.07</td>
<td>22.8±0.15</td>
<td>18.2±0.09</td>
<td>104.8±1.05</td>
<td>46.8±0.04</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.18±0.09</td>
<td>35.9±1.30</td>
<td>30.7±0.06</td>
<td>172.9±1.08</td>
<td>71.6±0.09</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>0.25±0.04</td>
<td>39.9±0.70</td>
<td>45.8±0.19</td>
<td>197.2±0.05</td>
<td>75.6±1.50</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>0.33±0.10</td>
<td>32.1±1.07</td>
<td>43.2±0.57</td>
<td>334.3±0.12</td>
<td>19.2±1.04</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.38±0.08</td>
<td>27.8±2.05</td>
<td>30.7±1.03</td>
<td>112.4±2.08</td>
<td>17.5±1.10</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>0.35±0.15</td>
<td>20.2±0.14</td>
<td>13.4±0.02</td>
<td>67.8±0.09</td>
<td>13.8±0.09</td>
<td></td>
</tr>
</tbody>
</table>

*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 polysaccharide 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 polysaccharide 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
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 pH 5. 

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.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Total protein (mg/ml)</th>
<th>Enzymes activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>25</td>
<td>0.29±0.09</td>
<td>59.7±6.25</td>
</tr>
<tr>
<td>*30</td>
<td>0.38±0.05</td>
<td>78.1±3.09</td>
</tr>
<tr>
<td>35</td>
<td>0.37±0.12</td>
<td>69.3±1.42</td>
</tr>
<tr>
<td>40</td>
<td>0.32±0.07</td>
<td>32.1±2.97</td>
</tr>
</tbody>
</table>

*Control.

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

<table>
<thead>
<tr>
<th>Incubation period (h)</th>
<th>Total protein (mg/ml)</th>
<th>Enzymes activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>24</td>
<td>0.10±0.06</td>
<td>55.7±3.05</td>
</tr>
<tr>
<td>*48</td>
<td>0.38±0.05</td>
<td>78.8±5.67</td>
</tr>
<tr>
<td>72</td>
<td>0.24±0.08</td>
<td>35.9±1.10</td>
</tr>
</tbody>
</table>

*Control.

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

<table>
<thead>
<tr>
<th>pH value</th>
<th>Total protein (mg/ml)</th>
<th>Enzymes activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>4</td>
<td>0.30±0.05</td>
<td>21.2±0.64</td>
</tr>
<tr>
<td>5</td>
<td>0.34±0.09</td>
<td>56.2±2.83</td>
</tr>
<tr>
<td>6</td>
<td>0.35±0.08</td>
<td>81.3±6.08</td>
</tr>
<tr>
<td>*7</td>
<td>0.38±0.03</td>
<td>78.8±5.06</td>
</tr>
<tr>
<td>8</td>
<td>0.37±0.10</td>
<td>55.0±5.09</td>
</tr>
</tbody>
</table>

*Control.

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

<table>
<thead>
<tr>
<th>Inoculum size (ml %)</th>
<th>Total protein (mg/ml)</th>
<th>Enzymes activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>4</td>
<td>0.27±0.08</td>
<td>25.0±0.09</td>
</tr>
<tr>
<td>6</td>
<td>0.33±0.12</td>
<td>31.1±0.02</td>
</tr>
<tr>
<td>8</td>
<td>0.34±0.10</td>
<td>83.6±0.03</td>
</tr>
<tr>
<td>*10</td>
<td>0.35±0.09</td>
<td>81.0±0.07</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Agitation speed (rpm)</th>
<th>Total protein (mg/ml)</th>
<th>Enzymes activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>0</td>
<td>0.27±0.06</td>
<td>30.0±5.6</td>
</tr>
<tr>
<td>150</td>
<td>0.33±0.08</td>
<td>59.3±0.09</td>
</tr>
<tr>
<td>*200</td>
<td>0.34±0.04</td>
<td>83.2±0.07</td>
</tr>
<tr>
<td>250</td>
<td>0.38±0.09</td>
<td>64.5±0.03</td>
</tr>
</tbody>
</table>

*Control.

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

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>25</td>
<td>70.4</td>
</tr>
<tr>
<td>*30</td>
<td>100.0</td>
</tr>
<tr>
<td>35</td>
<td>105.4</td>
</tr>
<tr>
<td>40</td>
<td>52.3</td>
</tr>
</tbody>
</table>

*Control.

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

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>30</td>
<td>85.5</td>
</tr>
<tr>
<td>*60</td>
<td>100.0</td>
</tr>
<tr>
<td>90</td>
<td>112.1</td>
</tr>
<tr>
<td>120</td>
<td>93.4</td>
</tr>
<tr>
<td>150</td>
<td>76.3</td>
</tr>
</tbody>
</table>

*Control.

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

<table>
<thead>
<tr>
<th>Buffers</th>
<th>pH</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginase</td>
</tr>
<tr>
<td>Acetate</td>
<td>4</td>
<td>103.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>126.0</td>
</tr>
<tr>
<td>*6</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7</td>
<td>89.7</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>88.3</td>
</tr>
<tr>
<td>Carbonate- bicarbonate</td>
<td>9</td>
<td>83.4</td>
</tr>
</tbody>
</table>

*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, Cu²⁺, Co²⁺, Pb²⁺ are considered as enzyme
Table 15. Effect of different enzymes protein concentrations on the relative activity of partially purified enzyme.

<table>
<thead>
<tr>
<th>Enzyme concentration (mg/ml)</th>
<th>Relative activity (%)</th>
<th>Enzyme concentration (mg/ml)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alginase</td>
<td>Dextrinase</td>
<td>Pectinase</td>
</tr>
<tr>
<td>0.1</td>
<td>78.1</td>
<td>83.6</td>
<td>93.6</td>
</tr>
<tr>
<td>0.3</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>0.5</td>
<td>74.2</td>
<td>82.1</td>
<td>87.3</td>
</tr>
</tbody>
</table>

*Control.

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

<table>
<thead>
<tr>
<th>Substrate concentration (g %)</th>
<th>Relative activity (%)</th>
<th>Substrate concentration (g %)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alginase</td>
<td>Dextrinase</td>
<td>Pectinase</td>
</tr>
<tr>
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<td>100.0</td>
<td>100.0</td>
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<tr>
<td>0.8</td>
<td>86.1</td>
<td>91.2</td>
<td>86.1</td>
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</table>

*Control.

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

<table>
<thead>
<tr>
<th>Metal ions (0.02 M)</th>
<th>Relative activity (%)</th>
<th>Metal ions (0.02 M)</th>
<th>Relative activity (%)</th>
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</tbody>
</table>

*Control.

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 inhibitors (Table 17).
pathogenic bacteria. The enzymes mixture was more effective in degrading EPS and biofilm removal of S. aureus 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 carboxymethyl cellulase produced by marine B. cereus NRC-20, whereas some investigations reported more and more microorganisms from marine habitats that can produce polysaccharide lytic 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 alginic 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 (MnSO₄ and
MgCl<sub>2</sub>) 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
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.
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 Stoutd 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 et 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. aeruginosa, 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 (alginate, dextrinase, pectinase and carboxymethyl 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


Full Length Research Paper

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 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...
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 Nigr (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, which are 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-tillizers, pulp and coffee husk compounds, and earthworm castings (Theodoros 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 a cooperative 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 KH2PO4; 0.5 g of MgSO4·7H2O; 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 K2HPO4; 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 NaNO3; 5 g of KCl; 5 g of MgSO4·7H2O; 0.1 g of FeSO4·7H2O; 0.1 g of ZnSO4·7H2O; 0.05 g of CuSO4·5H2O 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 KH2PO4; 1 g of KNO3; 1 g of Czapek concentrate; 5 g of fungal extract; 15 g of agar and 1 L of distilled water).
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 fungal species 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 (\( H' \)), \( H' = -\sum (pi \ln pi) \), 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 Chaufloun (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
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,
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<td>Rhizopus stolonifer</td>
<td>8</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trichoderma harzianum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
</tbody>
</table>

*: 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 species and 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 Chaulfon, 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 Versiculares) according to Klich (2002), whereas five species of Penicillium were identified (Penicillium brevihumatum, 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 brevihumatum. 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 brevihumatum, 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 (Abrunhsosa et al., 2006; Abrunhsosa 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
samples 1 and 3. However, *Rhizopus stolonifer*, *Fusarium oxysporum* and *Aspergillus flavus* 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 bioprotector in 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* 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**


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

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Received 19 April, 2014; Accepted 2 June, 2014

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 value 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 subtilis*, 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
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 (Hg²⁺ to Hg⁰) 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 (Cd²⁺), arsenic (As) and copper (Cu²⁺), 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 Chirkoort, 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 Fe³⁺, 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 Fe³⁺ (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 Cd²⁺, Pb²⁺ and Cu²⁺, 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 As⁵⁺ when complexed with Fe³⁺, that is, Fe-bac performed well in the adsorption and reduction of As⁵⁺.

Thus, the aim of this work was to select bacteria tolerant to Fe³⁺ 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 (FeCl₃) (0.005, 0.5 and 1 g/L). The medium CD consisted of: 10 g/L of glucose, 0.5 g/L (NH₄)₂SO₄, 0.1 g/L KCl, 0.1 g/L NaCl, 0.5 g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂·2H₂O, 1.0 g/L KH₂PO₄, 0.005 g/L H₂BO₃, 0.002 g/L CuSO₄·5H₂O, 0.01 g/L ZnSO₄·7H₂O, 0.002 g/L Na₂MoO₄·2H₂O, 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 x 10⁸ 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 Fe³⁺ 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, 0.05 and 0.995 g/L), X₄= concentration of the inoculum (10⁵, 10⁶ and 10⁷ 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 = ecocomplex (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 Fe³⁺**

The production of biomass was measured in dry weight at 60°C
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\textsuperscript{®} version 8.0 software (Stat-Ease Inc., Minneapolis, MN, USA).

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Code</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>X1</td>
<td>3.295 3.5 4.5 5.5 5.705</td>
</tr>
<tr>
<td>Iron ferric (mg/L)</td>
<td>X2</td>
<td>147 250 500 750 852</td>
</tr>
</tbody>
</table>

### 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 Karnovsky 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 Karnovsky 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 aluminium 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 *Arabidopsis 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 CES127, *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...
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 \(2^5\), 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 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.

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

\(Q D [1 g/L] = Q D \text{ media added, 1 g/L of FeCl}_3 \text{ solution.}\)
The mathematical model is based on the significant variables. The prediction of the optimal operating conditions for the 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.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Maineffect</th>
<th>Value F</th>
<th>Value P Prob&gt; F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>384.07</td>
<td>74.93</td>
<td>&lt;0.0001</td>
<td>***</td>
</tr>
<tr>
<td>pH</td>
<td>-61.92</td>
<td>18.86</td>
<td>0.0074</td>
<td>**</td>
</tr>
<tr>
<td>Glucose</td>
<td>14.08</td>
<td>0.98</td>
<td>0.3686</td>
<td>NS</td>
</tr>
<tr>
<td>Iron ferric</td>
<td>338.58</td>
<td>563.97</td>
<td>&lt;0.0001</td>
<td>***</td>
</tr>
<tr>
<td>Inocullum</td>
<td>-24.25</td>
<td>2.89</td>
<td>0.1497</td>
<td>NS</td>
</tr>
<tr>
<td>NH4NO3</td>
<td>36.08</td>
<td>6.41</td>
<td>0.0525</td>
<td>NS</td>
</tr>
<tr>
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(***): 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.

<table>
<thead>
<tr>
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<th>pH</th>
<th>Iron ferric (g/L)</th>
<th>RESPONSE</th>
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<td>100</td>
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<td>2</td>
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<td>0.771</td>
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<tr>
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Table 6. ANOVA analysis of DCCR for the experimental results of the isolated Bacillus subtilis UFLASCF590 of culture for 7 days.

<table>
<thead>
<tr>
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<th>Value F</th>
<th>Value P Prob&gt; F</th>
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<td>7.11</td>
<td>0.1258</td>
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</tr>
</tbody>
</table>

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

The response surface was plotted against two independent variables (pH and concentration of Fe³⁺) for the predicted response Y (accumulation of Fe³⁺). 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 (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*...
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.

ACKNOWLEDGMENTS
The authors thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPQ), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and CAPES for scholarships and financial support.

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Coba MSM, Magalhães-Guedes KT, Silva CF, Dias DR, Duarte WF, Schwan RF (2012). Culturable bacteria from soils of the Brazilian...
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
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 reduce 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 (CC1021mt+) 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 (NH₄Cl 15 g/l, MgSO₄·7H₂O 4 g/l, CaCl₂·2H₂O 2 g/l) and 1 ml from the second stock solution (Phosphate buffer: K₂HPO₄ 28.8 g/100 ml and KH₂PO₄ 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.
Ultra violet (UV) mutagenesis

According to Harris (1989), 5 ml of the liquid culture with a density of $1 \times 10^7$ 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/m² 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 $C. \text{reinhardtii}$ ($CC1021mt^+$) to UV irradiation for 1 min. The three others ($PCMut2$, $PCMut3$ and $PCMut4$) were obtained from exposure of $P. \text{kessleri}$ 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 light inverted microscope of the OLYMPUS series. The initial cell density was about 1 x 10^6 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 NH4 removal was done by PCMut4, PC, PCMut3, CC1021Mut1 and CC1021 (90.1, 90, 88.8, 87.8, 87.6, 87.38, respectively). Highest PO4 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, CC1021Mut1 and CC1021 (69.7, 69, 68, 67.6, 67.5, 67.3, respectively). In sample No. 4, highest NH4 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 PO4 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 NH4, PO4 and Zn than the control except PC for NH4 and PCMut2 and PCMut3 for PO4. 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 (99.8, 99.7, 99.7, 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.
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; Abellovich, 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 O2 to heterotrophic aerobic bacteria to mineralize organic pollutants, using in turn the CO2 released from bacterial respiration (Munoz and Gueysse, 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 CC1021 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 1. The analysis of water before using algae.

<table>
<thead>
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<th>Control of dilution 2:3 (C2:3)</th>
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<td>30</td>
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<td>6.02</td>
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<td>2.16</td>
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### Table 2. Analysis of water samples after bioremediation for one week using CC1021 and CC1021Mut1 strains.

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<tr>
<td>Zinc (mg/l)</td>
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</table>
Table 3. Analysis of water samples after bioremediation for one week using Pc, PCMut2, PCMut3 and PCMut4 strains.

<table>
<thead>
<tr>
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<td>7.02</td>
<td>6.02</td>
<td>6.02</td>
<td>6.8</td>
<td>6.02</td>
<td>7.2</td>
<td>6.02</td>
<td>7.1</td>
<td>6.02</td>
</tr>
<tr>
<td>BOD5 (mg/l)</td>
<td>2.16</td>
<td>0.179</td>
<td>2.16</td>
<td>0.12</td>
<td>2.16</td>
<td>21.07</td>
<td>2.16</td>
<td>0.255</td>
<td>2.16</td>
<td>0.03</td>
<td>2.16</td>
<td>27.03</td>
</tr>
<tr>
<td>COD (mg/l)</td>
<td>32.34</td>
<td>0.533</td>
<td>32.34</td>
<td>0.097</td>
<td>32.34</td>
<td>29.568</td>
<td>32.34</td>
<td>2.22</td>
<td>32.34</td>
<td>0.058</td>
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<td>83.88</td>
</tr>
<tr>
<td>Ammonia (mg/l)</td>
<td>225.9</td>
<td>23</td>
<td>75.46</td>
<td>8.5</td>
<td>1</td>
<td>0.765</td>
<td>225.9</td>
<td>27</td>
<td>75.46</td>
<td>10</td>
<td>1</td>
<td>3.765</td>
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<tr>
<td>Phosphate (mg/l)</td>
<td>259.48</td>
<td>136.62</td>
<td>86.67</td>
<td>55.36</td>
<td>2.488</td>
<td>2.629</td>
<td>259.48</td>
<td>134.22</td>
<td>86.67</td>
<td>40.06</td>
<td>2.488</td>
<td>4.696</td>
</tr>
<tr>
<td>Cobalt (mg/l)</td>
<td>0.9611</td>
<td>0.2916</td>
<td>0.3210</td>
<td>0.1359</td>
<td>0.0005</td>
<td>0.0031</td>
<td>0.9611</td>
<td>0.2978</td>
<td>0.3210</td>
<td>0.1428</td>
<td>0.0005</td>
<td>0.0082</td>
</tr>
<tr>
<td>Zinc (mg/l)</td>
<td>13.21</td>
<td>1.363</td>
<td>4.414</td>
<td>1.496</td>
<td>0.0154</td>
<td>0.0507</td>
<td>13.21</td>
<td>1.454</td>
<td>4.414</td>
<td>1.504</td>
<td>0.0154</td>
<td>0.122</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCMut2</th>
<th>PCMut3</th>
<th>PCMut4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>pH</td>
<td>6.02</td>
<td>7</td>
</tr>
<tr>
<td>BOD5 (mg/l)</td>
<td>2.16</td>
<td>0.164</td>
</tr>
<tr>
<td>COD (mg/l)</td>
<td>32.34</td>
<td>4.2</td>
</tr>
<tr>
<td>Ammonia (mg/l)</td>
<td>225.9</td>
<td>22.6</td>
</tr>
<tr>
<td>Phosphate (mg/l)</td>
<td>259.48</td>
<td>116.16</td>
</tr>
<tr>
<td>Cobalt (mg/l)</td>
<td>0.9611</td>
<td>0.307</td>
</tr>
<tr>
<td>Zinc (mg/l)</td>
<td>13.21</td>
<td>2.427</td>
</tr>
</tbody>
</table>

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, PCMut3 and PCMut4 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 CCG1021 Mut1 strain. This is in line with the study of Ikeda 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 to xenobiotics (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 caused an increase of removal rate. Generally, the result...
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

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**Figure 6.** Decrease % of the selected parameters in sample No. 2 after using the algal strains for one week.
Table 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%; 92.4, 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) NH₄ contents were removed more effectively than phosphate in all algal strains.
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 $CC1021Mut1$ ranked the second order in lowering the levels of COD and Zn (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 $CC1021Mut1$ 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.

ACKNOWLEDGEMENTS

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Aziz MA, Ng WJ (1993). Industrial wastewater treatment using an
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 lanceolata* 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
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, osteomyellitis and food poisoning (Mylo 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, coloectyitis or septicaemia (Singh et al., 2000).

Many compounds were isolated previously from genus Lonicera including iridoids, bisiriodoids, 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 Abbottabad, 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 (Leven 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 (Leven 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, Pseudomonas aeruginosa (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 Plates. 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.
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. Ethyl acetate fraction exhibited best activity against these bacteria. Besides that, chloroform, n-hexane and ethanol fractions have lowest activity against these bacteria. Ethyl 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. pneumoniae* were not detected.

This investigation is probably the first to exhibit 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
Table 3. MIC and MBC of *Lonicera lanceolata* of ethyl acetate fraction.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>&gt;10</td>
<td>14</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>&gt;10</td>
<td>N.d</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>&gt;10</td>
<td>12</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td>0.625</td>
<td>1.877</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.312</td>
<td>1.260</td>
</tr>
</tbody>
</table>

N. d = not detected.

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

Figure 2. MIC and MBC of *Lonicera lanceolata* of ethyl acetate fraction.
Table 4. Antifungal activities of Lonicera lanceolata crude extracts.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane</td>
<td>1(±0.5)</td>
</tr>
<tr>
<td>chloroform</td>
<td>6(±0.11)</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>7(±0.17)</td>
</tr>
<tr>
<td>ethanol</td>
<td>1(±0.14)</td>
</tr>
<tr>
<td>Standard</td>
<td>8(0)</td>
</tr>
</tbody>
</table>

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


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$_2$S 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$_2$S 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$_2$S, 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...
are complex mixture of ammonia (NH₃), volatile sulfur (H₂S, SO₂) 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 (NH₃, H₂S, and SO₂) 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 x 10⁶ 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 m²) 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 gas detector tube (No. 3M for NH₃; No. 4HM and 4LT for H₂S; No. 5LA for SO₂). 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:

1. BA1 (spray with 1% BA)
2. BA2 (spray with 5% BA)
3. BA3 (spray with 10% BA)
4. BA4 (spraying with 100% BA)
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).

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 NH3 from slurry are shown in Figure 1. The NH3 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 NH3 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 H2S emission are shown in Figure 2. The only significant difference observed in H2S 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, SO2 emissions from slurry were not affected by treatment with water or BA.

Experiment 2

The effects of direct application of 10% BA on NH3 emission from pig slurry over 7 day are documented in Table 1. There were no significant differences among treatments in NH3 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 H2S emissions from pig slurry at day 1 and 2. On day 3, the H2S 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 H2S 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.

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Control</th>
<th>Water</th>
<th>BA one time/day</th>
<th>BA one time/ two day</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>41.67</td>
<td>50.33</td>
<td>74.67</td>
<td>65.33</td>
<td>10.31</td>
<td>0.24</td>
</tr>
<tr>
<td>Day 2</td>
<td>143.33</td>
<td>95.33</td>
<td>108.00</td>
<td>11.67</td>
<td>18.32</td>
<td>0.42</td>
</tr>
<tr>
<td>Day 3</td>
<td>118.00</td>
<td>126.67</td>
<td>66.67</td>
<td>81.33</td>
<td>16.30</td>
<td>0.11</td>
</tr>
<tr>
<td>Day 4</td>
<td>153.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>103.33&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>51.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.89</td>
<td>0.03</td>
</tr>
<tr>
<td>Day 5</td>
<td>163.33</td>
<td>98.00</td>
<td>48.33</td>
<td>63.33</td>
<td>28.49</td>
<td>0.11</td>
</tr>
<tr>
<td>Day 6</td>
<td>236.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>121.67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>43.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Day 7</td>
<td>213.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>164.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.45</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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

Table 2. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture media (BA) on hydrogen sulfide (H₂S) emission from pig slurry.

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Control</th>
<th>Water</th>
<th>BA one time/day</th>
<th>BA one time/ two day</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>339.33</td>
<td>430.00</td>
<td>346.67</td>
<td>403.33</td>
<td>75.32</td>
<td>0.83</td>
</tr>
<tr>
<td>Day 2</td>
<td>400.00</td>
<td>406.67</td>
<td>325.67</td>
<td>350.00</td>
<td>52.23</td>
<td>0.69</td>
</tr>
<tr>
<td>Day 3</td>
<td>25.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.33&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.89</td>
<td>0.01</td>
</tr>
<tr>
<td>Day 4</td>
<td>550.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>360.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>400.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>55.36</td>
<td>0.001</td>
</tr>
<tr>
<td>Day 5</td>
<td>533.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>373.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.39</td>
<td>0.02</td>
</tr>
<tr>
<td>Day 6</td>
<td>333.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>183.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>223.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.16</td>
<td>0.02</td>
</tr>
<tr>
<td>Day 7</td>
<td>216.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>133.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>173.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.15</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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

Table 3. Effects of spraying 10% of *Bacillus amyloliquifaciens* culture media (BA) on sulfur dioxide (SO₂) emission from pig slurry.

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Control</th>
<th>Water</th>
<th>BA one time/day</th>
<th>BA one time/ two day</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.53</td>
<td>0.50</td>
<td>0.70</td>
<td>0.60</td>
<td>0.10</td>
<td>0.58</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.83</td>
<td>0.70</td>
<td>0.77</td>
<td>0.73</td>
<td>0.09</td>
<td>0.79</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.87</td>
<td>0.90</td>
<td>0.67</td>
<td>0.60</td>
<td>0.09</td>
<td>0.14</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.77</td>
<td>0.60</td>
<td>0.50</td>
<td>0.67</td>
<td>0.07</td>
<td>0.16</td>
</tr>
<tr>
<td>Day 6</td>
<td>0.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.80</td>
<td>0.67</td>
<td>0.43</td>
<td>0.47</td>
<td>0.11</td>
<td>0.14</td>
</tr>
</tbody>
</table>

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

The SO₂ emissions from pig slurry in response to spraying with 10% BA are shown in Table 3. During the 7 day measurement period, the SO₂ 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, cellulase, 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 (NH3) 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 NH3 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 NH3 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-gestation 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 NH3 emissions in the present experiment.

Hydrogen sulfide (H2S) and sulfur dioxide (SO2) 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 H2S in vitro. They also demonstrated that soil isolates belonging to the genera Bacillus, Pseudomonas effectively decomposed H2S. Ushida et al. (2003) isolated a VS degrading Bacillus spp. (Strain KPU 0013) and reported their ability to reduce H2S emission from pig slurry. The possible explanation of reduction in H2S emission in this experiment is that, BA may decompose the H2S in vitro. Nakada and Ohta (1997) also reported removal of H2S 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 (SO2) is one of the six criteria pollutants defined in the US Clean Air Act. However, information of livestock related SO2 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 SO2 from pig slurry. In experiment 1, we found no significant effect of BA on SO2 emissions from pig slurry. However, in experiment 2, slightly reduced SO2 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 NH3, H2S, and SO2 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.

ACKNOWLEDGEMENT

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REFERENCES


Full Length Research Paper

Optimization of solid-state fermentation conditions for the production of cellulase and its hydrolytic potentials by *Trichoderma viride* 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$_2$PO$_4$, 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 substrates 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.

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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 by using various nutrients as substituted 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$_2$PO$_4$, 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$^4$ 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$_2$PO$_4$ (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$_2$PO$_4$ supply were undertaken using the response surface methodology, in which MATLAB was used. A 3x3 fractional factorial design was employed to optimize medium components. The factors and levels used are shown in Table 1.

<table>
<thead>
<tr>
<th>Variables (% w/w)</th>
<th>Coded</th>
<th>Range and levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat bran</td>
<td>$X_1$</td>
<td>-1</td>
</tr>
<tr>
<td>peptone</td>
<td>$X_2$</td>
<td>0</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>$X_3$</td>
<td>+1</td>
</tr>
</tbody>
</table>

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

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{2} \sum_{j=1}^{3} \beta_{ij} X_i X_j + \sum_{i=1}^{3} \beta_{i+1} X_i + \varepsilon$$

Where, $\beta_0$ was an intercept; $\beta_i$, first-order model coefficient; $\beta_{ij}$, quadratic coefficient for the variable; $\beta_{i+1}$ interaction coefficient for the interaction of variables i and j, and $\beta_0$, $\beta_i$, and $\beta_{ij}$, were evaluated by
Table 2. Cellulase production by *T. viride* in solid-state fermentation of residue from Chinese herb carried out in pot fermenters.

<table>
<thead>
<tr>
<th>Enzyme activities (IU/g dry-weight substrate)</th>
<th>Fermentation days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Endo-glucanase</td>
<td>2.32</td>
</tr>
<tr>
<td>Filter-paper cellulase</td>
<td>1.23</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>1.37</td>
</tr>
</tbody>
</table>

Hydrolysis experiments

In this study, hydrolysis efficiency was defined as cellulose and hemicellulose 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$_3$PO$_4$ 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. viride*

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. viride* 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-cel lulase 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. viride* 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$_2$PO$_4$ 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. viride* 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$_2$PO$_4$. 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$_2$PO$_4$, 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$_2$PO$_4$ 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$_2$PO$_4$ also have significant interaction. However, the interaction between peptone and KH$_2$PO$_4$ was insignificant.

We can learn that the response surface has a maximum point. The maximum FPase production by *T. viride* Sn-9106 was obtained in the optimized medium when the initial concentration of wheat bran, peptone and
KH$_2$PO$_4$ 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.

<table>
<thead>
<tr>
<th>Number</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>Measured value</th>
<th>$Y$</th>
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<td>1</td>
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<td>0</td>
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<td>12.60333</td>
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<td>0</td>
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<td>12.60333</td>
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Table 4. Model Summary Statistics.

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<thead>
<tr>
<th>Source</th>
<th>Standard Deviation</th>
<th>R-Squared</th>
<th>Adjusted R-Squared</th>
<th>Predicted R-Squared</th>
<th>PRESS</th>
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<td>0.01667</td>
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<td>0.073595</td>
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<tr>
<td>Quadratic</td>
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<td>0.933365</td>
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<tr>
<td>Cubic</td>
<td>0.337244</td>
<td>0.997751</td>
<td>0.984259</td>
<td>+</td>
<td>Suggested</td>
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Table 5. Anova results for cellulase production obtained from RSM.

<table>
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<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value</th>
<th>Prob &gt; F</th>
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</thead>
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<td>7.781795</td>
<td>0.0180</td>
<td>significant</td>
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<td>0.490518</td>
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<tr>
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<td>Cor Total</td>
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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$_2$PO$_4$ 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$_2$PO$_4$ on FPase production. C. Effect of interaction between the peptone and KH$_2$PO$_4$ on cellulase FPase production.
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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- Journal of Bacteriology Research
- Journal of Evolutionary Biology Research
- Journal of Yeast and Fungal Research
- Journal of Brewing and Distilling