International Journal of Micro Biology, Genetics and Monocular Biology Research

Vol.1, No.3, pp.1-19, May 2017

Published by European Centre for Research Training and Development UK (www.eajournals.org)

OPTIMIZATION OF THE NUTRITIONAL REQUIREMENTS OF TWO NOVEL BACTERIAL CELLULOSE DECOMPOSERS ISOLATED FROM LEBANESE HABITAT

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ABSTRACT: *Two novel bacterial cellulose decomposers were isolated and identified phenotypically and morphologically as Gram negative short rods none spore formers. Genotypic identification using 16S rRNA was carried out for both isolates. Bacterial isolate 1 was identified as a member of the species Pseudomonas aeruginosa with 98% similarity thus; it was named Pseudomonas aeruginosa IZ. Bacterial isolate 2 was identified as a member of the species Serratia marcescens with 98% similarity thus; it was named Serratia marcescens IZ. Physiological, environmental and nutritional factors affecting Psuedomonas aeruginosa IZ and Serratia marcescens IZ cellulase activity were evaluated one factor at a time for optimum production and activity of their cellulase enzyme. The time course of cellulase activity by the selected bacterial isolates was monitored during 5 days. Maximum enzyme activity by Psuedomonas aeruginosa IZ was obtained at day 2 (0.35 U/ml) whereas maximum enzyme activity by Serratia marcescens IZ was obtained at day 1 (0.2 U/ml). The effects of Nitrogen source and fermentation technique on cellulase production by the bacterial isolates were studied. It was concluded that fermentation media CM2, which contains corn cobs as a sole source of both carbon and nitrogen, under shaken condition (150 rpm) revealed maximum Psuedomonas aeruginosa IZ cellulase activity after 2 days of incubation (0.71 U/ml) and Serratia marcescens IZ cellulase activity after 1 day of incubation 0.70 (U/ml). Results indicate that the enzymatic activity under these conditions increased 2.02 folds for Psuedomonas aeruginosa IZ, and 3.5 folds Serratia marcescens IZ. Optimization of the physiological and environmental factors affecting Psuedomonas aeruginosa IZ and Serratia marcescens IZ cellulase activity using Plackett-Burman revealed that optimized factors result in an increase of cellulase activity. Eleven different factors were chosen to perform this optimization process and the main effect of the examined factors affecting cellulase activity by both bacterial isolates was calculated. Data of the present investigation revealed that the rest of the factors studied didn't affect cellulase enzyme activity or production in a significant manner. By the end of the optimization part, the optimum nutritional conditions for Pseudomonas aeruginosa IZ cellulase production under submerged fermentation using shaken conditions were (g/l): KH_2PO_4 1.5, K_2HPO_4, 1.79, $ZnCl_2$, 0.0025, $MgSO_4.7H_2O$, 0.7, $CaCl_2$ 0.08 and corn cobs 100. The pH was adjusted to 5.0 and the flask was inoculated with 2% inoculum and incubated at 44˚C for 48 hours under shaken conditions (150 rpm). For Serratia marcescens IZ the optimum nutritional conditions for cellulase production under submerged fermentation using shaken conditions were (g/l): KH_2PO_4 0.5, K_2HPO_4, 0.5, $ZnCl_2$, 0.0025, $MgSO_4.7H2O$, 0.1, $CuCl_2$ 0.0025, $CaCl_2$ 0.08 and corn*

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

cobs 100. The pH was adjusted to 9.0 and the flask was inoculated with 10% inoculum and incubated at 44˚C for 24 hours under shaken conditions (150 rpm). On the basis of the high productivity of the enzyme at the end of the optimization experiments, the present work envisaged the production of cellulase from Pseudomonas aeruginosa IZ at high scale using corn cobs as a cheap carbon source under submerged fermentation to be exploited commercially for the use in industry.

KEYWORDS: Novel cellulose decomposers, Optimization, nutritional requirements

INTRODUCTION

Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited and inexpensive. Several lignocellulosic biomasses in the form of agro-residues are widely available. These include sugar cane bagasse, rice straw, wheat straw, cotton stock, bamboo and sugar cane tops (Sindhu et al., 2016). Due to its availability and renewability, lignocellulosic biomass can be considered as an appropriate substrate for the bioconversion to ethanol and other value added products (Zabed et al., 2016). Lignocellulosic biomass, composed of 30-35% cellulose (a homologous polymer of glucose linked by β-1,4 glycosidic bonds), is hydrolyzed by a complex enzyme system known as cellulase (exoglucanase, endoglucanase and β glucosidase) (Sandu & Maiti, 2013 and Achinas & Euverink, 2016). Cellulases contribute to 8% of the worldwide industrial enzyme demands (Sandu & Maiti, 2013).

Cellulase production appears to depend on a complex relationship involving a variety of factors like inoculum size, carbon source and cellulose quality, pH value, temperature, presence of inducers, medium additives, aeration, growth time, etc.(Immanuel et al., 2006 and Iqbal et al., 2011). Therefore, attention has been focused on studying the Cellulolytic activity and cellulase enzyme production by several microorganisms in various substrates as well as in various environments. To establish a successful fermentation process, it is necessary to make the environmental and nutritional conditions favorable for the microorganism for overproduction of the desired metabolite (El-Hadi et al., 2014).

Furthermore, the interaction of Congo red dye with intact β-D-glucans, such as cellulose, provides the basis for a rapid and sensitive assay system for bacterial strains possessing β-1,4-D-glucanohydrolase and β-1,3-D-glucanohydrolase activities. A close correspondence was observed between cellulolytic activity with β-1,4-D-glucanohydrolase and β-1,3-D-glucanohydrolase activities in the isolated bacterial strains (Teather & Wood, 1982). These characteristics informed our choice of the cellulose Congo red assay for the identification of cellulolytic microorganisms that can hydrolyze cellulose.

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

MATERIALS AND METHODS

Fresh dung samples were used to isolate Cellulolytic bacteria. These samples were collected in sterile plastic containers from a cattle barn located at Barja, Baaseer, Lebanon.

Microorganisms

Two gram –ve short rods bacterial strains namely: *Psuedomonas aeruginosa* IZ and *Serratia marcescens* IZ were isolated from the dung collected from Barja, Baaseer, Lebanon. They were identified using 16S rRNA and used in the present investigation.

Raw Material

Corn cobs were used as the substrate for the present work due to their cellulose rich nature and abundance in Lebanon. After removing the green husks, corn ears were boiled and the kernels were removed. The remaining cobs were dried at 60^0C until constant weight, grinded, and passed through a 1mm fine sieve and maintained in a tightly sealed container at room temperature.

Chemicals

All chemicals, solvents, reagents and media used throughout the present work were of analytical grade and obtained from recognized chemical suppliers

Isolation and Screening of Cellulolytic Bacteria

Dung samples collected from cattle barns were used to inoculate 10 ml tubes of basal salt media 1 (BS1) (g/L): $NaNO_3$, 2.5; KH_2PO_4 2; MgSO4, 0.2; NaCl 0.2; $CaCl_2.6H_2O$ 0.1; filter paper (Whatman filter paper no. 1 of area 70.541 cm^2) (Gupta et al., 2012). The cultures were incubated for 7 days in a shaker incubator at 37^0C at 100 rpm. Tubes that showed complete degradation of the filter paper were used to inoculate Carboxymethyl cellulose (CMC) agar plates. Bacterial colonies capable of utilizing cellulose as sole source of carbon are therefore isolated on the (CMC) agar plates. Morphologically different colonies were picked up and purified on the same medium. Confirmation of cellulose degrading ability of bacterial isolates was performed using a rapid plate assay method by sub-culturing on cellulose Congo-Red agar media. Colonies showing discoloration of Congo-Red were considered as positive cellulose degrading bacterial colonies (Lu Et al., 2004), and were selected for further study.

Cultivation Techniques

Submerged fermentation (SF) was the cultivation method used. Cultivation of the most promising cellulase producers was achieved in 250 ml Erlenmeyer flasks each containing 50 ml of the complex medium 1 (CM1) (g/L): Corn cob powder, 40; KH_2PO_4, 1; K_2HPO_4 1.45; $MgSO_4.7H_2O$,

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

0.4, $CaCl_2$, 0.05; $(NH_4)_2SO_4$, 5; $FeSO_4.7H_2O$, 0.00125; pH7.0. The medium was sterilized by autoclaving for 20 min at 121˚C. The flasks were received 6 % (v/v) seed culture as a standard inoculum (unless otherwise stated), and then incubated at 37˚C for 5 days under shaken conditions (150 rpm).

Maintenance of Bacteria

All the bacterial strains used throughout this present investigation were maintained on nutrient agar slants and stored at 4˚C with regular transfers at monthly intervals. For long preservation, the bacterial slants were folded with 25% sterile glycerol.

Seed Culture Preparation

Transfers from a bacterial slant (18 hrs old) were inoculated into 250 ml Erlenmeyer flasks containing 50 ml nutrient broth and then incubated at 37˚C under shaken conditions until reached an absorbance of 1 (A≤1). 6 % (v/v) were taken as a standard inoculum unless otherwise stated. Seed cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml nutrient broth medium with 3 ml of bacterial suspension (prepared from 18 hrs old cultures) and then incubated at 30˚ C ± 2 on a rotary shaker (180 rpm) till reaches O.D 600 ≤ 1.these were used as standard inocula (3ml/flask) unless otherwise stated.

Crude Enzyme Preparation (cell free extract)

At the end of the fermentation period, the fermented culture medium was subjected to cooling centrifugation for 20 min at 6000 rpm. The obtained cell free supernatant was used as the crude enzyme.

Identification of the Selected Bacterial Isolates
Phenotypic Characterization

Cell morphology was microscopically examined after Gram staining of 24 hrs old cultures. Gram reaction was confirmed by KOH test: a loopfull of bacterial colony was mixed with a drop of 3 % KOH on a clean glass slide. The absence or presence of slimy substances confirmed the result of Gram reaction (Halebian et al., 1981).

Molecular Characterization

Molecular characterization was further employed to obtain a full identification of selected bacterial isolates. DNA was isolated from the selected bacterial strains using Thermo Scientific Gene JET Genomic DNA Purification Kit according to manufacturer's instructions. Then, PCR was carried out using Maxima Hot Start PCR Master Mix (Thermo). The bacterial 16S rDNA was amplified from the total genomic DNA using universal Eubacteria specific primers, designated to amplify 1500 bp fragment of the 16S rDNA regions. The forward primer was 8F: 5' AGA GTTTGA TCC

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

TGG CTC AG 3' and the reverse primer was U1492R: 5' GGT TAC CTT GTT ACG ACT T 3', which yielded a product of approximately 1500 bp. A fraction of the PCR mixture was examined using agarose gel electrophoresis (Ausubel et al., 1999) and the remnant PCR product was purified using Gene JET TM PCR Purification Kit (Thermo) according to the kit manual. The purified product was directly sequenced on GATC Biotech Company (Germany) by using ABI 3730×1 DNA sequence with forward and reverse primers. The PCR product was sequenced using the same PCR primers. Blast program was used to access the DNA similarities, multiple sequence alignment and molecular phylogeny were performed using Bio Edit software (Hall, 1999). The phylogenetic tree was displayed using TREE VIEW program (Page, 1996).

Crude Enzyme Preparation (cell free extract)

At the end of the fermentation period, the fermented culture medium was subjected to cooling centrifugation for 20 min at 6000 rpm. The obtained cell free supernatant was used as the crude enzyme.

Enzyme Assay

Enzyme assay was determined in terms of carboxymethyl cellulase activity. The reducing sugars produced were measured as glucose using a modified technique described by Miller (1959). carboxymethyl cellulase activity (CMCase) was assayed as follows: 0.5 ml of crude enzyme was incubated with 0.5 ml 1% carboxymethyl cellulose solution in 0.1 M citrate buffer pH 4.8 at 50° C in a water bath for 30 min. 3 ml freshly prepared 3,5-dinitrosalycilic Acid Reagent (DNS) was added to stop the reaction and incubated in a boiling water bath for 5 min and cooled. The absorbance was measured at 540nm. The blank was prepared in a similar manner with the substitution of the crude enzyme with distilled water. One unit of enzyme activity is defined as the amount of protein (cellulase) required to liberate 1 µmol of reducing sugar (D- glucose) from CMC per min under the assay conditions. A calibration curve of glucose was used to convert the colorimetric readings into µmol of glucose.

Estimation of Protein Content

The protein content of the crude enzyme was determined according to Lowry's method (Ohnishi & Barr, 1978) which can be summarized as follows: to 1 ml of protein sample, 4 ml diluted Buiret reagent (1 ml Buiret reagent + 7 ml 2.3% Na_2CO) were added and left to stand for 10 minutes at room temperature; 0.125 ml of 2 N Folin-Ciocalteu phenol reagent was added immediately, mixed and left for 30 minutes at room temperature, thereafter the developed blue color was measured at 650 nm. A standard curve was prepared with bovine serum albumin (BSA).

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

Environmental and Nutritional Factors Affecting Cellulase Production Under Submerged Fermentation

Effect of Incubation Period

The time course of cellulase production was followed up to determine the maximum output of enzyme activity under SF technique along with determining bacterial growth by measuring the absorbance at 600nm. 50 ml of Fermentation media CM1 were dispensed in 250 ml Erlenmeyer flasks, sterilized, inoculated with 6% (v/v) standard inoculum, and incubated at 37˚C for different time intervals (1, 2, 3, 4 and 5 days) under shaken conditions (150 rpm).

Effect of Nitrogen source and Fermentation Technique

In order to determine the effect of Nitrogen source and fermentation technique on cellulase production by the bacterial isolates, 50 ml of complex media 2 (CM2) (g/L): Corn cob powder, 60; KH_2PO_4, 1; K_2HPO_4 1.45; $MgSO_4.7H_2O$, 0.4, $CaCl_2$, 0.05; $FeSO_4.7H_2O$, 0.00125; pH7.0 was dispensed in 250 ml Erlenmeyer flasks, sterilized, inoculated with 6% (v/v) standard inoculum and incubated at 37˚C for different time intervals (1 and 2 days) under static and shaken conditions (150 rpm).

Optimization of the Nutritional Factors Affecting Cellulase Production Using Multifactorial Statistical design: Plackett-Burman Design

The Plackett-Burman experimental design, a factorial design, (Plackett & Burman,1946 and Ghanem et al., 2000) was used to evaluate the relative importance of nutritional factors affecting cellulase enzyme production by the tested microorganisms. Eleven variables representing nutritional requirements and environmental factors were screened in twelve combinations organized according to Plackett-Burman design matrix described in the results section. For each variable, a high (+) and low (-) level was tested. All trials were performed in duplicates and the averages of obtained outputs were treated as the response for each trial. The main effect of each variable was determined with the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$$

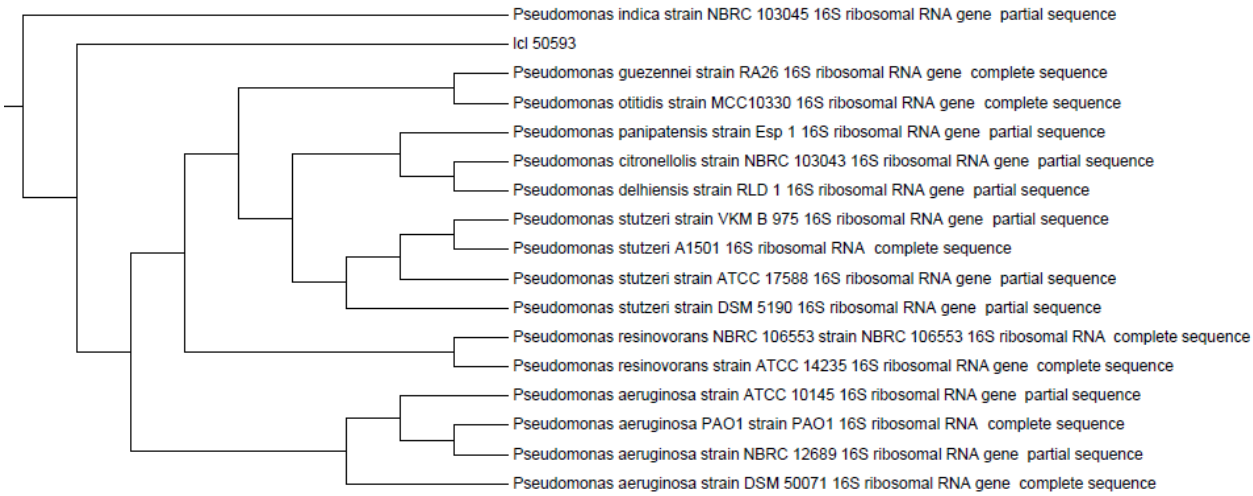
Where; E_{xi} is the variable main effect, M_{i+} and M_{i-} are cellulase activity in trials where the independent variable (xi) was present in high and low levels, respectively, and N is the number of trials divided by two. A main effect result with positive sign indicates that the high level of this variable is nearer to optimum and a negative sign indicates that the low level of this variable is nearer to optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance. Optimized conditions were predicted giving rise to maximum enzyme production.

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

RESULTS AND DISCUSSION

Identification of the Selected Bacterial Isolates

Two bacterial isolates (1 & 2) were selected showing partial discoloration of cellulose congo red plates due to the partial hydrolysis of congo red. Isolates 1 and 2 were later identified as *Pseudomonas aeruginosa* IZ (isolate 1) and *Serratia marcescens* IZ (isolate 2) based on the phylogenetic relationship of the bacterial isolates analyzed using the TREE VIEW program. 16S rRNA sequence comparison has been used as a powerful tool for establishing phylogenetic and evolutionary relationships among organisms (Lane et al., 1985 and Martinez-Murica & Collins, 1990). Polymerase chain reaction is routinely used for the detection of 16S rRNA specific genes for sequence analysis (Saiki et al., 1988). 16S rRNA gene sequence of isolate 1 showed 98% similarity with various *Pseudomonas* genotypes recorded in the computer database. Based on these phylogenetic characterization results, bacterial isolate 1 was identified as a member of the species *Pseudomonas aeruginosa*. Thus, it was named *Pseudomonas aeruginosa* IZ. 16S rRNA gene sequence of isolate 2 showed 98% similarity with various *Serratia* genotypes recorded in the computer database. Based on these phylogenetic characterization results, bacterial isolate 2 was identified as a member of the species *Serratia marcescens*. Thus, it was named *Serratia marcescens* IZ. These results were matched with the study of Hu et al. (2014) who reported that *Pseudomonas sp.* and *Serratia sp.* showed clear zones on cellulose Congo red agar plates. The potential isolates were also characterized and identified phenotypically and morphologically. Both isolates 1 and 2 were Gram negative rods none spore formers. Therefore, these Gram negative none spore forming short rods were identified as *Pseudomonas aeruginosa* IZ and *Serratia marcescens* IZ respectively. In line with these results, several cellulases have been isolated from a diversity of bacteria, *Pseudomonas* sp and *Serratia* sp. appear among the prominent cellulase producers namely: *Pseudomonas fluorescens* and *Serratia marcescens* (Sethi et al., 2013) (Fig. 1&2).



ISSN 2059-9609(Print)
ISSN 2059-9617(online)

Figure 1: Phylogenetic relationships among representative experimental isolate 1 and the most related bacteria based on 16S rRNA sequences

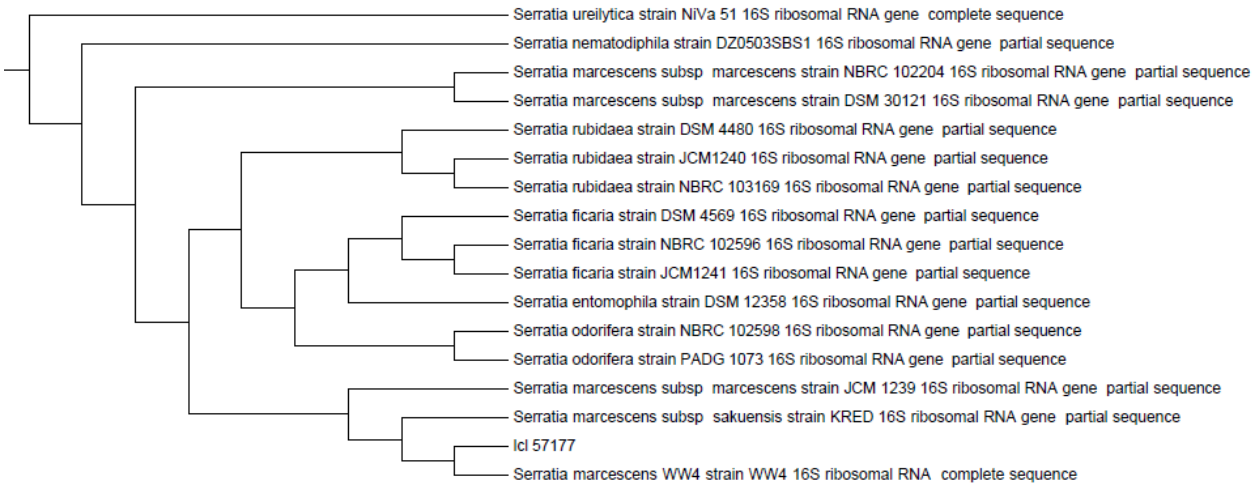


Figure 2: Phylogenetic relationships among representative experimental isolate 2 and the most related bacteria based on 16S rRNA sequences

Effect of Incubation Period

The present investigation revealed that the optimum incubation period for maximum cellulase activity was 48 hrs for *Pseudomonas aeruginosa* IZ and 24 hrs for *Serratia merscens* IZ Fig. (3 A&B). This can be probably attributed to the production of cellulase mainly during the late exponential phase of microbial growth (Di Pasqua et al., 2014). In agreement with the present findings, Kalaiselvi et al., reported a maximum cellulase activity at day 2 with *Pseudomonas aeruginosa*, and Sethi et al., showed maximum cellulase activity at day 1 with *Serratia marcescens* (Kalaiselvi et al., 2013 and Sethi et al., 2013).

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

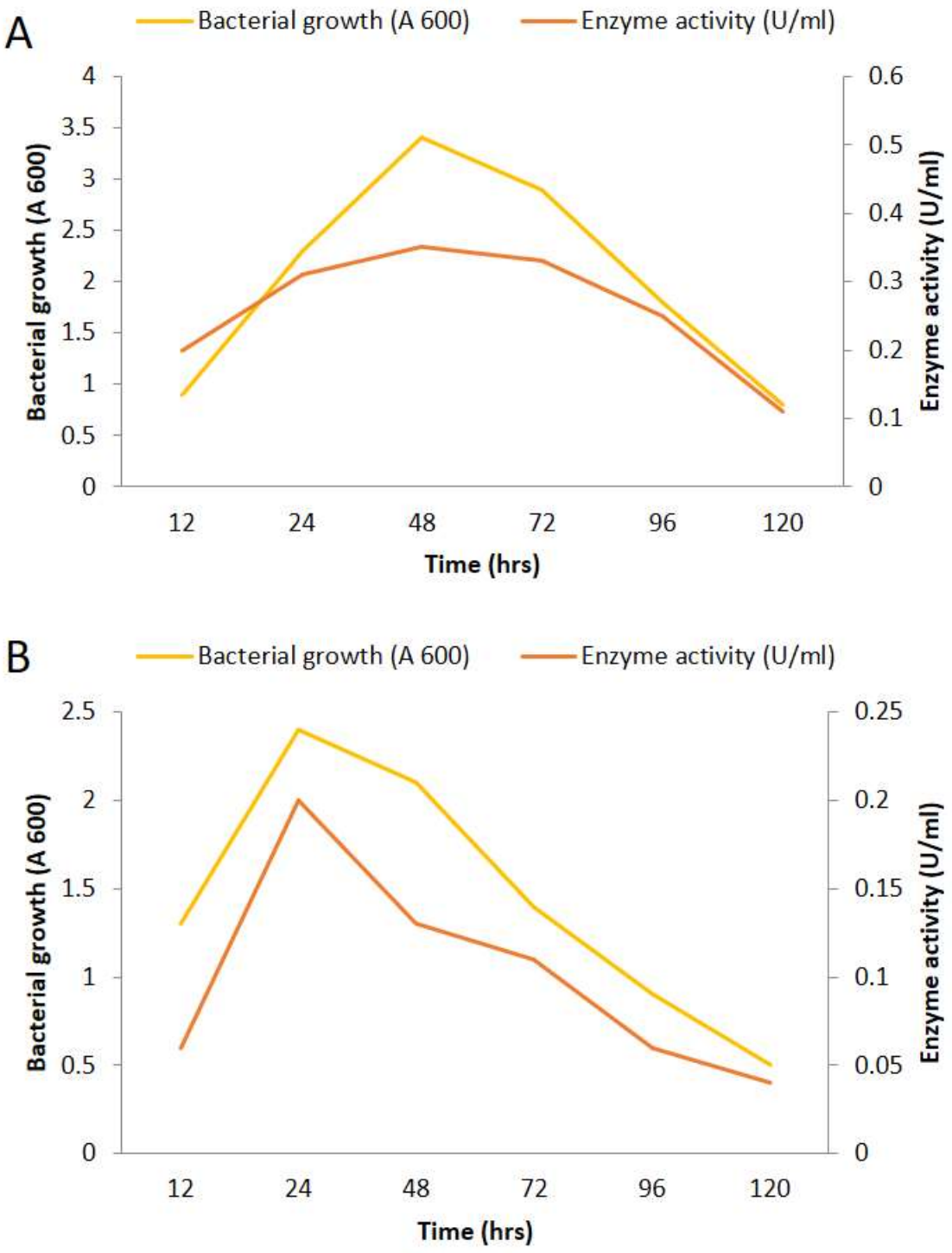


Figure 3: effect of incubation time on cellulase production by *Pseudomonas aeruginosa* IZ (A) and *Serratia marcescens* IZ (B)

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

Effect of nitrogen source and fermentation technique

The bacteria under investigation were cultivated using submerged fermentation technique under shaken and static conditions for cellulase production along with the elimination of ammonium sulfate as a nitrogen source and using corn cobs as sole carbon and nitrogen source. The present investigation revealed 2.02 and 3.5 fold increase for *Pseudomonas aeruginosa* IZ and *Serratia marcescens* IZ respectively under the used conditions. During fermentation, different levels of dissolved oxygen in the fermentation broth can be obtained by variations in the agitation speed. This can influence greatly the microbial cell growth and thereby production of extracellular enzymes (Ramanani & Gupta, 2004). In concurrence with these findings, *Psuedomonas flourescens* (Sethi et al., 2013), *Serratia merscens* (Sethi et al., 2013), *Bacillus pumilus* (Ariffin et al., 2006), have been reported to produce cellulase under submerged fermentation. On the contrary, *Trichoderma reesi* (Singhania et al., 2006), *Aspergillus niger* (Sadhu & Maiti, 2013) and *Trichoderma koningii* (Liu & Yang, 2007) have been reported to produce cellulase under solid state fermentation. Production of extracellular cellulase has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Sethi et al., 2013). Therefore, using corn cobs as the sole carbon and nitrogen source can explain the increase in cellulase activity by both bacterial isolates. The results of the present study are in accordance to El-Hadi et al. (2014) findings that organic nitrogen sources gave better cellulase activity than inorganic nitrogen. In screening and optimizing the factors affecting cellulase activity, it is very important to test as much factors as possible and to identify their significance.

Microbial cellulases possess a great diversity in their biochemical and biophysical properties. Therefore, cellulase production by microorganisms is greatly influenced by nutritional factors, especially carbon and nitrogen sources, and physiological factors such as incubation time and type of fermentation. Cellulases are inducible enzymes and the regulation of cellulase production is finely controlled by activation and repression mechanisms. The production of cellulolytic enzymes is induced only in the presence of substrates, and is repressed when easily utilizable sugars are available (Sukumaran et al., 2005). Thus, a lignocellulosic substrate (straw, corn cobs, rice or wheat bran and bagasse) is often added to the cultivation medium. Cellulose rich materials are produced in high amounts by agro-industrial activities and are normally discarded as wastes. Therefore, such microbial technology has a bidual function in the production of valuable products (cellulases and glucose) from low-cost substrates along with deriving an efficient way of dealing with and managing wastes (Zhang et al., 2006).

However, lignocellulosic substrate is not always required for cellulase production. Other non-lignocellulosic substrates, namely: starch, glycerol, glucose, maltose, lactose, and fructose have been reported to act as cellulase inducers (Sethi et al., 2013). Cellulose-containing media with different carbon and/or nitrogen sources might result in higher levels of cellulase production, namely: Glucose, fructose and starch (Sethi et al., 2013). El- Hadi et al, (2014) reported that maximum cellulase activity by *A. hortai* was observed when lactose was used as carbon source, and lower cellulase production was recorded when glucose, galactose or starch were used as carbon

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

sources. Ahmed et al. (2009) found that CMC induced cellulase production by *T. harzianum* whereas glucose repressed its synthesis. This might be attributed to the induction of enzymatic activity by lactose, or may be due to the high penetration rate of lactose through the cell membrane (Miyamoto et al., 2000). Nitrogen sources for cellulase production can be both inorganic (ammomium sulfate, ammomium nitrate, ammonium chloride, sodium nitrite and ammomiumdihydrogen phosphate) or organic (urea, yeast extract, beef extract, and peptone). El-Hadi et al. (2014) reported that peptone leads to maximum cellulase production by *A. horatai* and generally it was observed that organic nitrogen sources gave better cellulase activity than inorganic nitrogen. These results agreed with results of Dewsal et al. (2011) who found that urea enhanced cellulase production and inorganic nitrogen sources didn't exhibit any significant effect on the increase of enzyme production. Therefore, the effect of different growth substrates on cellulase production is highly variable, depending on the microorganism, the substrate, carbon and nitrogen concentration, implicating that the medium composition should be determined on a case-by-case basis (Brandelli & Riffel, 2005). Therefore, using corn cobs as the sole carbon and nitrogen source can explain the increase in cellulase activity by both bacterial isolates. The results of the present study are in accordance with El-Hadi et al. (2014) findings that organic nitrogen sources gave better cellulase activity than inorganic nitrogen.
Fig. (4A&B)

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

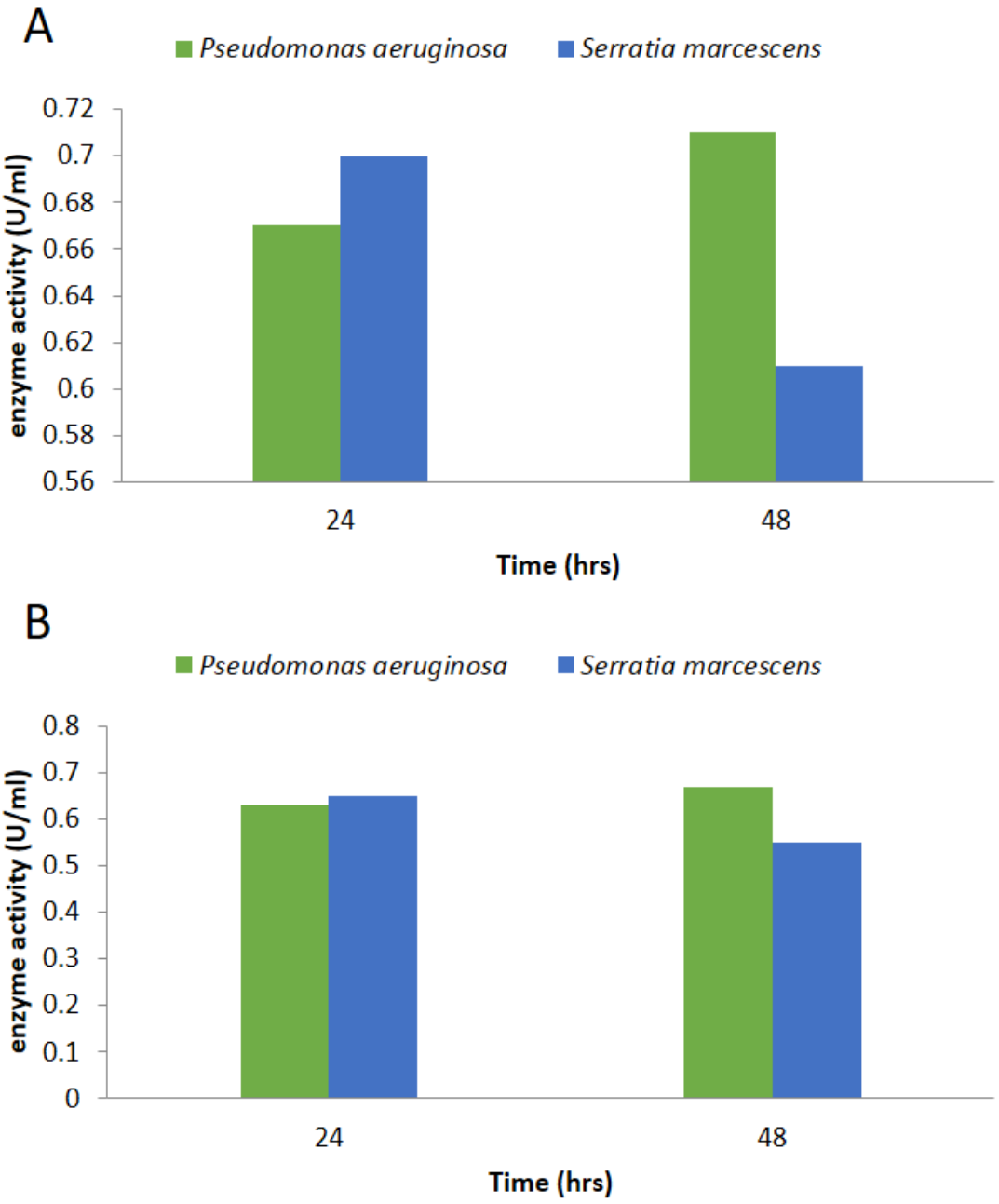


Figure 4: Cellulase production by *Psuedomonas aeruginosa* IZ and *Serratia marcescens* IZ using corn cobs as sole source of carbon and nitrogen under different fermentation techniques: (A) shaken conditions and (B) static conditions

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

Optimization of the Nutritional Factors Affecting Cellulase Production Using Multifactorial Statistical design: Plackett-Burman Design

In screening and optimizing the factors affecting cellulase activity, it is very important to test as much factors as possible and to identify their significance. Plackett-Burman design offers a good and fast screening procedure and mathematically computes the significance of a large number of factors in one experiment, which is time saving and maintains convincing information on each component (Srinivas et al., 1994). The design is recommended when more than five factors are under investigation (Lalliva et al., 1998 and Abdel-Fattah et al., 2002). The influence of eleven factors including: Corn cobs, KH_2PO_4, K_2HPO_4, $FeSO_4.7H_2O$, $ZnCl_2$, Temperature, Inoculum level, $MgSO4.7H_2O$, pH, $CuCl_2$ and $CaCl_2$ were tested (Table 1). The variation in cellulase activity ranged from 0.04 U/ml to 2.14 U/ml for *Pseudomonas aeruginosa* IZ and 0.08 U/ml to 1.5 U/ml for *Serratia merscens* IZ (table 2). The used factors have a strong influence on cellulase activity. It was shown that only corn cobs had a significant effect on cellulase activity by both bacteria, whereas the other factors slightly affected the enzyme activity. Data in the present investigation showed that cellulase activity increased with high level of corn cobs (100 g/l) for both bacteria. Murad & Azzaz (2013) reported that *Aspergillus flavus* NRRL 5521 cellulase was produced in the presence of different lignocellulosic waste material (corn stalks, rice straw and banana waste) as the sole carbon source which supported a maximum cellulase production. Since the addition of conventional carbon sources like glucose or glycerol to the culture medium resulted in considerable reduction in the cellulolytic activity for *Pseudomonas fluorescens* (Bakare et al., 2005), It has been reported that ligonocellulosic waste can act as sole carbon source for cellulose production (Murad & Azzaz, 2013). Data of the present investigation revealed that the rest of the factors studied didn't affect cellulase enzyme activity or production in a significant manner. By the end of the optimization part, the optimum nutritional conditions for *Pseudomonas aeruginosa* IZ cellulase production under submerged fermentation using shaken conditions (150 rpm) were (g/l): KH_2PO_4 1.5, K_2HPO_4, 1.79, $ZnCl_2$, 0.0025, $MgSO_4.7H_2O$, 0.7, $CaCl_2$ 0.08 and corn cobs 100. The pH was adjusted to 5.0 and the flask was inoculated with 2% inoculum and incubated at 44˚C for 2 days under shaken conditions. For *Serratia marcescens* IZ the optimum nutritional conditions for cellulase production under submerged fermentation using shaken conditions (150 rpm) were (g/l): KH_2PO_4 0.5, K_2HPO_4 0.5, $ZnCl_2$, 0.0025, $MgSO_4.7H_2O$ 0.1, $CuCl_2$ 0.0025, $CaCl_2$ 0.08 and corn cobs 100. The pH was adjusted to 9.0 and the flask was inoculated with 10% inoculum and incubated at 44˚C for 1 day under shaken conditions. On the basis of the high productivity of the enzyme at the end of the optimization experiments, the present work investigated the production of cellulase from *Pseudomonas aeruginosa* IZ at high scale using corn cobs as a cheap carbon source under submerged fermentation to be exploited commercially for the use in industry Fig. (5A&B)

In conclusion, it is evident from the present study that:
1. Two novel cellulolytic bacterial strains, namely *Psuedomonas aeruginosa* IZ and *Serratia marcescens* IZ, were isolated from Lebanon. They possess high cellulolytic activity and were

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

effective in the biodegradation of Lebanese lignocellulosic waste material, namely corn cobs, presenting a potential use for biotechnological processes.

2. A bioprocess which is a simple enzymatic treatment method was evaluated for the production of glucose using cellulase enzyme. This bioprocess addresses three important needs namely: ecological, economic and nutritional, resulting in a product with high economic and nutritional value. Thus, the bioconversion of lignocellulosic biomass from a waste material to a value-added product using microbial technology offers considerable opportunities in industrial applications: food processing, paper and pulp, detergent, textile pharmaceutical industries.

Table 1 Variables and their levels employed in Plackett-Burman design for screening of some nutritional and environmental factors affecting cellulase production by for *Psuedomonas aeruginosa* IZ and *Serratia marcescens* IZ

Variables	Code	Low level (-1)	Basal medium (0)	High level (+1)
Corn	CN	1 g/flask	3 g/flask	5 g/flask
Monopotassium phosphate KH_2PO_4	MP	0.5 g/L	1 g/L	1.5 g/L
Dipotassium phosphate K_2HPO_4	DP	0.5 g/L	1.145 g/L	1.79 g/L
Iron(II) Sulfate Heptahydrate FeSO4.7H2O	Fe^{2+}	-	0.00125 g/L	0.0025 g/L
Zinc Chloride $ZnCl_2$	Zn^{2+}	-	0.00125 g/L	0.0025 g/L
Temperature	T	30^0C	37^0C	45^0C
Inoculum level	IL	2 ml/50ml	5 ml/50ml	8 ml/50ml
Magnesium Sulfate Heptahydrate $MgSO_4.7H_2O$	Mg^{2+}	0.1 g/L	0.4 g/L	0.7 g/L
pH	pH	5	7	9
Copper(II) chloride $CuCl_2$	Cu^{2+}	-	0.00125 g/L	0.0025 g/L
Calcium chloride $CaCl_2$	Ca^{2+}	0.02 g/L	0.05 g/L	0.08 g/L

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

Table 2: Randomized Plackett-Burman experimental design for evaluating factors influencing cellulase production by *Pseudomonas aeruginosa* IZ and *Serratia marcescens* IZ

Variables / Trials	Corn	KH_2PO_4	K_2HPO_4	$FeSO_4.7H_2O$	$ZnCl_2$	T	Inoculum level	$MgSO_4$	pH	$CuCl_2$	$CaCl_2$	Enzyme activity *Pseudononas aeruginosa IZ*	Enzyme activity *Serratia marcescens* IZ
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.41	0.6
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.62	0.64
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.08	0.11
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.71	0.73
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	0.381	0.46
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	2.14	0.73
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	0.1	0.09
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.4	0.06
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	0.08	0.09
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.84	1.5
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	0.13	0.22
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.04	0.08
13	0	0	0	0	0	0	0	0	0	0	0	0.78	0.74

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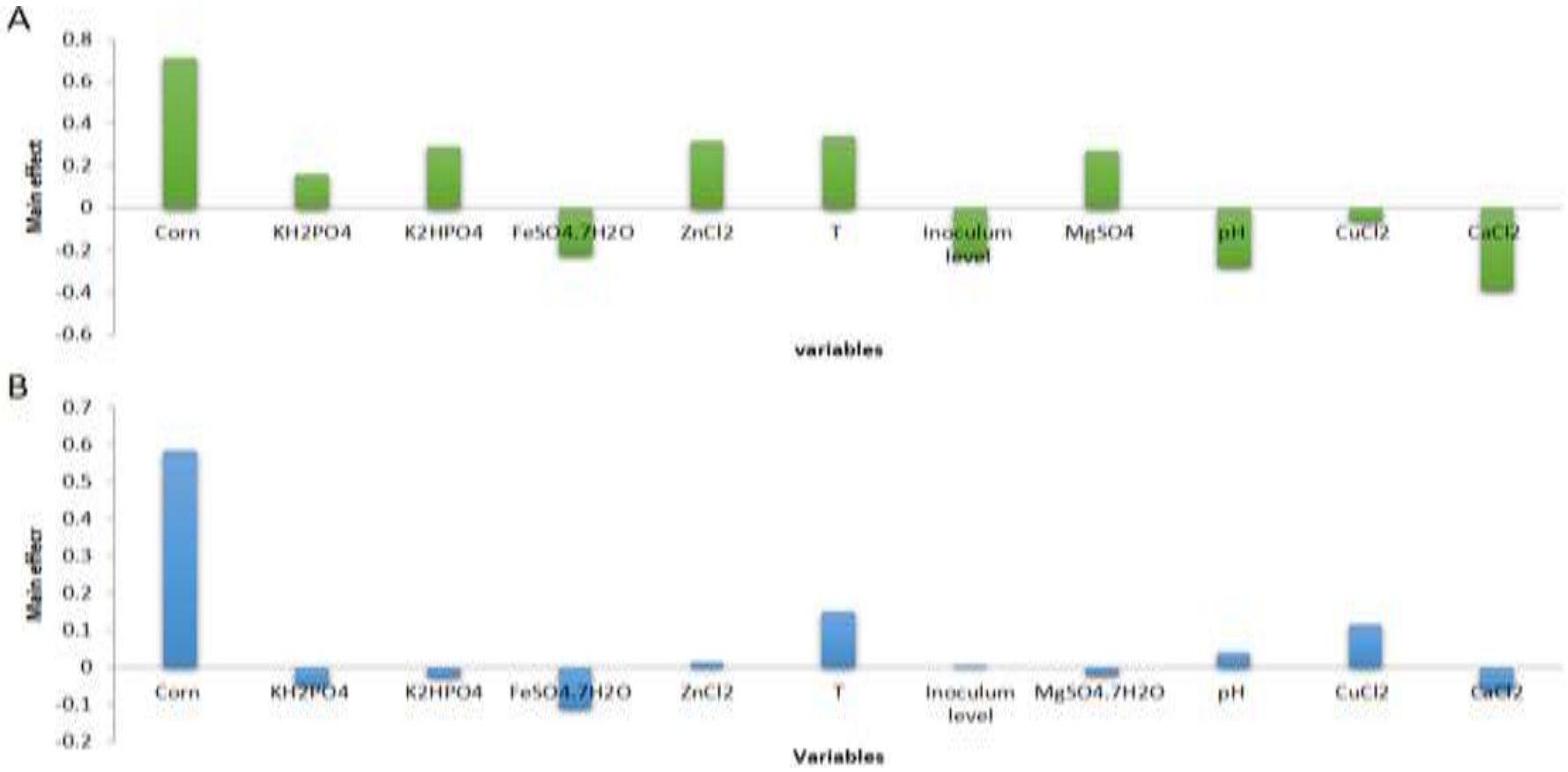

Figure 5. Main effect of variables on cellulase production by (A) Pseudomonas aeruginosa IZ and (B) Serratia marcescens IZ

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

REFERENCES

Abdel-Fattah, Y.; Soliman, N.; Gaballa, A.; Sabry, S. and El-Diwany, A. (2002). Lipase production from novel thermophilic *Bacillus* sp.: Application of Plackett-Burman design for evaluating culture conditions affecting enzyme formation. Acta. Microbiol. Pol., 51: 33-36.

Achinas, S. and Euverink, G.J.W. (2016). Consolidation briefing on biochemical ethanol production from lignocellulosic biomass. Electron. J. Biotechnol., 23:44-53.

Ahmed, S.: Bashir, A.; Saleem, H.; Saadia, M. and Jamil, A. (2009). Production and purification of cellulose-degrading enzymes from a filamentous fungus *Trichoderma harzianum*. Pak. J. Bot., 41:1411-1419.

Ariffin, H.; Abdullah, N.; Umi Kalsom, M.S.; Shiria, Y. and Hassan, M.A. (2006). Production and characterization of cellulase by *Bacillus pumilus* EB3. Int. J. Eng. Technol., 3: 47-53.

Ausubel, F.; Brent, R.; Kingston, R.; Moore, D.; Seidam, J.; Smith, J. and Struhl, K. (1999). Short Protocols in Molecular Biology. John Willey and Sons, Inc., New York.

Bakare, M.; Adewale, I. and Ajayi, A. (2005). Purification and characterization of cellulase from the wild type and two improved mutants of *Psuedonomas flourescens*. Afr. J. Biotechnol., 4:898-904.

Brandelli, A. and Riffel, A. (2005). Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers. Electronic J. Biotechnol., 8: 35- 42.

Deswal, D.; Khasa, Y. and Kuhad, R. (2011). Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation. Bioresour. Technol., 102:6065-6072.

Di Pasqua, R.; Ventorio, V.; Aliberti, A.; Robertiello, A.; Faraco, V.; Viscardi, S. and Pepe, O. (2014). Influence of Different Lignocellulose Sources on Endo-1,4-β-Glucanase Gene Expression and Enzymatic Activity of Bacillus amyloliquefaciens B31C. BioRes., 9:1303-1310.

El-Hadi, A.; Abu EL-Nour, S.; Hammad, A.; Kamel, Z. and Anwar, M. (2014). Optimization of cultural and nutritional conditions for carboxymethylcellulase production by *Aspergillus hortai*. J. Radiat. Res. Appl. Sci., 7:23-28.

Ghanem, N.; Yusef, H. and Mahrouse, H. (2000). Production of *Aspergillus terreus* xylanase in solid-state cultures: application of the Plackett-Burman experimental design to evaluate nutritional requirement. Bioresour. Technol., 73: 113-121.

Gupta, P.; Samant, K. and Sahu, A. (2012). Isolation of cellulase-degrading bacteria and determination of their cellulolytic potential. Int. J. Microbiol., DOI: 10.1155/2012/578925.

Halebian, S.; Harris, B.; Finegold, S. and Rolfe, R. (1981). Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. J. Clin. Microbiol., 3: 444-448.

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nuc. Acids Symp. Ser., 41: 95-98.

Hu, X.; Yu, J.; Wang, C. and Chen, H. (2014). Cellulolytic Bacteria Associated with the Gut of *Dendroctonus armandi* Larvae (Coleoptera: Curculionidae: Scolytinae). Forests., 5:455-465.

Immanuel, G.; Dhanusha, R.; Prema, P. and Palavesam, A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int. J. Environ. Sci. Technol., 3:25-34.

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

Iqbal, H.; Ahmed, I.; Zia, M. and Irfan, M. (2011). Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viridae* under SSF and its detergent compatibility. Adv. Biosci. Biotechnol., 2:149-156.

Kalaiselvi, V.; Jayalakshmi, S. and Lakshmi, R. (2013). Biofuel Production using Marine Microbes. Int.J.Curr.Microbiol.Appl.Sci., 2: 67-74.

Lane, D.; Pace, B.; Oslen, G.; Stahl, D.; Sogin, M. and Pace, N. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA, 82: 6955-6959.

Lavilla, I.; Perez-Cid, B. and Bendicho, C. (1998). Optimization of digestion methods for sewage sludge using Plackett-Burman saturated designs. Anal. Chem., 361: 164-167.

Liu, J. and Yang, J. (2007). Cellulase production by *Trichoderma Koningii* AS3.4262 in solid-state fermentation using lignocellulosic waste from the vinegar industry. Food Technol. Biotechnol., 45:420-425.

Lu, W.; Wang, H. and Nie, Y. (2004). Effect of inoculating flower stalks and vegetable waste with lingo-cellulolytic microorganisms on the composting process. J. Environ. Sci. Health Part B Pestic. Food Contam. Agric. Wastes., 39:871-887.

Martinez-Murica, A. and Collins, M. (1990). A phylogenetic analysis of the genus *Leuconostoc*, based on reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett., 70: 73-84.

Miller, G. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31:426-428.

Miyamoto, Y.; Ooi, T. and Kinoshita, S. (2000). Production of lactobionic acid from whey by *Pseudomonas* sp. LS13-1. Biotechnol. Lett., 22:427-430.

Murad, H. and Azzaz, H. (2013). Cellulase production from rice straw by *Aspergillus flavus*. Science International., 1:103-107.

Ohnishi, T. and Barr, J. (1978). A simplified method of quantitative protein using biuret and phenol reagent. Anal. Biochem., 86: 193-200.

Page, R. (1996). Treeview: An application to display phylogenetics trees on personal computers. Comp. App. Biosci., 12: 357-358.

Plackett, R. and Burman, J. (1946). The design of optimum multifactorial experiments. Biometrical., 33: 305-325.

Ramnani, P. and Gupta, R. (2004). Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG-1 using statistical methods involving response surface methodology. Biotechnol. Appl. Biochem., 40: 491-496.

Sadhu, S. and Maiti, T. (2013). Cellulase production by bacteria: A review. Br. Microbiol. Res. J., 3:235-258.

Saiki, R.; Gefland, D.; Stoffel, S.; Scharf, S.; Higuuchi, R.; Horn, G.; Mullis, K. and Erlich, H. (1988). Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Sci., 239: 487-491.

Sethi, S.; Aparna, D.; Gupta, B. and Gupta, S. (2013). Optimization of cellulase production from bacteria isolated from soil. ISRN Biotechnology., http://dx.doi.org/10.5402/2013/985685.

Sindhu, R.; Binod, P. and Pandey, A. (2016). Biological pretreatment of lignocellulosic biomass – An overview. Bioresour. Technol., 199: 76-82.

ISSN 2059-9609(Print)
ISSN 2059-9617(online)

Singhania, R.; Sukumaran, R. and Pandey, A. (2007). Improved cellulase production by *Trichoderma reesei* RUT C30 under SSF through process optimization. Appl. Biochem. Biotechnol., 142:60-70.

Srinivas, M.; Chand, N. and Lonsane, B. (1994). Use of Plackett-Burman design for screening the several nitrogen sources, growth/product promoters, mineral and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation system. Bioproc. Eng., 10: 139-144.

Sukumaran, R.; Singhania, R. and Pandey, A. (2005). Microbial cellulasesproduction, application and challenges. J. Sci. Ind. Res., 64:832-844.

Teather, R. and Wood, P. (1982). Use of congo red- polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol., 43:777-780.

Zabed, H.; Sahu, J.N.; Boyce, A.N. and Faruq, G. (2016). Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. Renew. Sustainable Energy Rev., 66: 751-744.

Zhang, X. and Zhang, Y. (2013). Cellulase: Charachteristics, sources, production, and application. In: Yang, S. El-Enshay, A. and Thongchul, N. Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals, and polymers, 1st ed. New York, NY: John Wiley & Sons, Inc

ISSN 2059-9609(Print)
ISSN 2059-9617(online)