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SCREENING, ISOLATION, OPTIMIZATION AND MOLECULAR IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* NRC-S4 AND INVESTIGATION OF ITS β-GLUCOSIDASE PRODUCTION

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Abstract

β-glucosidase producing microorganisms are potential sources that can be employed for bioconversion of biomass, agricultural wastes, into biofuels. In this study nine different bacterial strains were isolated from soil and agricultural wastes. The activity was tested by growth in medium supplemented with Carboxymethyl cellulose (CMC). The CMCase positive strains were checked for their ability to produce β-glucosidase, then, characterized morphologically, biochemically and genetically identification. Under this condition the efficient β-glucosidases producing NRC-S4 was identified genetically by 16s rRNA gene sequence as *Pseudomonas aeruginosa* and submitted on GenBank with accession number LC455963.1. Phylogenetic tree was determined the relationship of strain NRC-S4 with different strains of genus *Pseudomonas spp.* Growth medium and nutritional requirements for maximum growth were optimized. Examination of its growth characteristics showed that its optimum growth after 48h. at 40°C, the best carbon source for fermentation was 1% cellobiose, while 2% peptone and ammonium chloride compound were the best nitrogen sources. Also, the initial pH during fermentation was 7, with 1% inoculum size, resulting in a high amount of enzyme. Therefore, the maximum β-glucosidase production achieved was 35.23 IU. Conclusion, *Pseudomonas aeruginosa* strain NRC-S4 was identified as a potential β-glucosidase producer and the cultural conditions and the nutritional requirements for this strain have been optimized.

Key words : β-glucosidase, Optimization, Molecular identification, 16S rRNA.

Introduction

Recently, lignocellulosic materials are under intensive research due to depletion of fossil fuels and production of ecofriendly biofuels, cellulose is considered as one of the most important sources of carbon in this planet and its annual biosynthesis by both land plants and marine algae (Saha *et al.,* 2006). Cellulases are an important class of enzymes which are used for the hydrolysis of cellulosic materials for the production of glucose, alcohol, cellulose acetate oligosaccharides (Zaldivar *et al.,* 2001). These enzymes are also used for non-specific cleavage of chitosan to form low molar mass oligosaccharides (Xia *et al.,* 2008). β-glucosidase is one of the members of cellulase enzyme system, along with endoglucanase and cellobiohydrolase, β-glucosidase catalyzes the hydrolysis of the β-glucosidic linkages of aryl and alkyl β-glucosides, β-linked oligosaccharides and several other oligosaccharides with the release of glucose. It is involved in the last step of cellulose saccharification cleaving cellobiose to glucose (Yun *et al.,* 2001). β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi *et al.,* 2002). β-glucosidases have been the focus of much research recently because of

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their important roles in a variety of fundamental biological and biotechnological processes (Czjzek *et al.*, 2000). Bacteria serve as a novel source cellulases due to their higher growth rate, more complex glycoside hydrolases, thermo stability, acid and alkali stability, which makes them successful candidate for industrial applications (Maki *et al.*, 2009). The cellulolytic potentials of bacteria belonging to different genera such as *Acetivibrio*, *Bacillus*, *Bacteroides*, *Cellulomonas*, *Clostridium*, *Erwinia*, *Ruminococcus*, and *Thermomonospora* have been well studied. Among them *Bacillus* spp. are known to produce and secrete large quantities of extracellular enzymes and the strains of *B. sphaericus* and B. *subtilis* are excellent cellulase producers (Sajith *et al.*, 2016). Accountable studies were carried out to produce ethanol by a wide range of cellulase producing microorganisms using different types of lignocellulosic material as a substrate including bacteria such as *Pseudomonas sp.* from CMC (Bakare *et al.*, 2005). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed (Patel, 2001). The most accurate method of determining phylogenetic relationships is the comparison of DNA composition and sequence. In the last thirty years, since the discovery of restriction endonucleases, rapid DNA sequencing and polymerase chain reaction (PCR), the analysis of DNA sequence or DNA polymorphisms has become the standard for determining relationships among the bacteria (Zhou *et al.*, 2008). The aim of this research is screen, identify and characterize several efficient β-glucosidases producing bacteria were isolated from different ecological niches of Egypt, and displaying the greatest β-glucosidases activity for the possible use in the large scale bio-refining and bioconversion.

Material and Methods

Isolation and screening of β-glucosidase producing bacteria

Screening of cellulase producing isolates was carried out on CMC agar plates as mentioned by (Teather and Wood, 1982). Isolation method includes serial dilution method, by exposing CMC agar into environment which is rich in decayed cellulosic material and soil. The soil and decayed cellulosic samples were serially diluted and spread plated on a CMC agar. The plates were incubated for 48 h at 37ºC and observed for clear zone around colony. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1M NaCl. To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on CMC agar were measured.

Enzyme assay

Preparation of crude enzyme

The isolate that showed a maximum zone of hydrolysis was cultured in LB broth medium and incubated at 37 °C for overnight. Then the cultures were centrifuged and clear supernatant was used as a source of crude enzyme solution.

Endo β 1, 4 glucanase activity assay

Endo-β-1, 4-glucanase activity was measured by DNS (3, 5-dinitrosalicylic acid) method. 100 µl crude enzymes and 1 ml citrate buffer were added into the mixture of 1 ml CMC solution. The mixture was incubated at 37 °C for 30 min. Then DNS was added to the solution to stop the reaction, the treated samples were boiled for 10 min, cooled in water for color stabilization and the optical density was measured at 540 nm. One unit of endo-β-1, 4-glucanase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µmol of glucose within 1 min of reaction (Shanmugapriya *et al.*, 2012).

β-glucosidase assay

Activity of the β-glucosidases was determined using 5 mM, 4-Nitrophenyl, β-D-glucopyranoside (pNPG) as substrate. Reaction mixture contained 0.2 mL of crude enzyme sample, 0.2 mL of 5 mM pNPG and 1.0 mL of phosphate buffer (0.05 M, pH 7.0). It was incubated at 37°C for 15 min. This reaction was terminated with 2.0 mL of cold 0.2 M Na_2CO_3. The activity was observed by the liberation of p-nitro-phenol and was estimated in a spectrophotometer by reading the absorbance at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required for the hydrolysis of one µmole of pNP per minute under assay condition (Daroit *et al.*, 2007).

Protein determination

Soluble proteins in the culture supernatant were estimated by dye binding method of Bradford using bovine serum albumin (Sigma Chemical Co., USA) as a standard (Bradford, 1976).

Identification of β-glucosidase producing bacteria

Morphological and biochemical characteristics of selected strains

The promising β-glucosidase producing NRC-S4 isolate was subjected to various morphological,

biochemical and physiological characteristic studies using the methods listed in Bergey's Manual of Determinative Bacteriology (Van Berkum and Eardly, 1998).

Molecular Identification Using 16S rRNA Gene Sequence

The strain NRC-S4 which show maximum β-glucosidases activity was further subjected to molecular identification by analyzing 16S r RNA sequence.

Genomic DNA extraction from NRC-S4 strain

2 ml of overnight culture was centrifuged at 10,000 rpm at 4ºC for 3 minutes. The pellet was suspended in 100 µl of 10mM Tris-HCl, centrifuged at 10,000 rpm at 4ºC for 10 minutes. After discarding the supernatant, the pellet was re-suspended in 100 µl of TE buffer containing 20µl of lysozyme (50mg/ml) and incubated at 37ºC for 20 min, in that solution 1µl of RNase (10 mg/ml) was added and incubated at room temperature for 20 minutes. 2µl of Proteinase K (20mg/ml) was added and incubated at 55ºC for 30 min. The sample was extracted in same volume phenol, Chloroform and Iso-amyl alcohol (25:24:1) and DNA was precipitated with one volume of isopropanol. The pellet was washed with 70% Ethanol, dried and dissolved in 100 µl of TE buffer and stored at -20ºC for further use. (Picard *et al.,* 1992)**.**

Identification of NRC-S4 strain by sequencing of the 16s rRNA

PCR amplification of the 16S rRNA gene was carried out using universal primers 8f and 1492r. The PCR was performed in 25µl reaction mixture containing 4µl of 10X assay buffer, 2µl dNTP mix of 2.5 mM, 0.5µl of mgcl2, 1µl each of forward and reverse primer (5pmol), 0.5µl of Taq polymerase, 1µl of template DNA and 13.5µl of HPLC grade water with the following amplification for 16s rRNA initial denaturation at 95ºC for 4 min followed by 38 cycles of denaturation, annealing and extension (94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min) and final extension at 72ºC for 10 min followed by hold for infinity at 4ºC. Amplified PCR product was purified and sequenced as described by (Saha and Chakrabarti, 2006).

Molecular Phylogenetic and Bioinformatics Analysis

PCR product was directly sequenced using a DNA sequencer (ABI PRISM 3100). The data of 16S rRNA gene sequences were compared with database at GenBank using BLAST-N search program in National enter Biotechnology Information (http:// www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences were aligned and phylogenetic tree was constructed using MEGA 7.0 software with neighbor-joining method at 1000X bootstraps (Tamura *et al.,* 2013).

Optimization of β-glucosidase production for NRC-S4 strain

An attempt was also made to determine the optimum culture conditions such pH, temperature, incubation period, carbon and nitrogen sources requirement for maximum growth and production of β-glucosidases (U/ml), Protein content (µg/ml), and Specific activity (U/mg protein) (Yin *et al.,* 2011).

Incubation Period

The effect of Incubation Period on β-glucosidases production was studied by varying the time of incubation. To determine the optimum incubation period of the isolate NRC-S4 for maximum β-glucosidases production, the supernatant was collected after 6, 12, 18, 24, 48, 72, 96 and 120 h of incubation (Yin *et al.,* 2011).

Medium pH

The effect of pH of the medium on β-glucosidases production was studied by varying the pH of the culture medium, selected medium of different pH (such as 3, 4, 5, 6, 7, 8, 9 and 10) was inoculated with the isolate (Yin *et al.,* 2011).

Temperature

The effect of temperature on cell growth and β-glucosidases production was studied by incubating at various temperatures, the culture medium was incubated at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C temperature was inoculated with the NRC-S4 isolate (Yin *et al.,* 2011).

Substrate Concentration

The effect of substrate concentration on β-glucosidases production was studied by varying the concentration of cellobiose. To determine the optimum substrate Concentration of the isolate NRC-S4 for maximum β-glucosidases production, the supernatant was collected after 0.5%, 1%, 2%, 4%, 8% and 10% of incubation (Yin *et al.,* 2011).

Effect of Nitrogen Sources

The effect of various nitrogen sources on β-glucosidases production was studied by using number of nitrogen sources such as (Yeast extract, Beef extract, Peptone, Soymeal, Urea, Sodium nitrate, Ammonium Chloride, Potasium Nitrite, Ammonium Sulfate Ammonium Nitrate) Nitrogen sources were used at a concentration of 1% (Yin *et al.,* 2011).

Effect of Carbon Sources

The effect of various carbon sources on β-glucosidases production was studied by using number of

carbon sources such as CMC, Glucose, Sucrose, Fructose, Maltose, Cellulose, Lactose, Mannitol and Sorbitol cellobiose, sucrose, lactose, glucose, and Avicel (1%) on the production of β-glucosidases was studied after specific time intervals (Yin *et al.*, 2011).

Results and Discussion

Isolation and primary screening of cellulolytic bacteria

In this study, two samples were collected from soil and organic wastes in Egypt. The resulting 9 isolates were tested on CMC agar plate for cellulase activity. The isolated bacterial colonies forming clear-zones after application of Congo red were selected as cellulase producers. Zone of CMC hydrolysis of the isolates are given in the (Table 1) (Fig.1A). Singh and Kaur had studied the isolation of cellulase-production bacteria from soil (decomposing logs and composts). They had screened 30 bacteria to find which one had more cellulase production (Singh and Kaur, 2012). For confirming their cellulolytic activity all isolates were then analyzed for secondary screening by CMCase and β-glucosidase activity assay (Table 2). The isolate NRC-S4 had maximum β-glucosidase and Endo-glucanase activity of 35.12 and 1.78 U/ml respectively. This was shared with (Agarwal *et al.*, 2014).

Morphological examination showed the surface of the colonies to be smooth, opaque, and blue in color, indicating that the species is aerobic. Microscopically, the organism was found to be gram-negative, rod shape and motile (Fig. 1D). Biochemical tests were performed for the isolate NRC-S4 and the results were tabulated in Table (3) and this result were similar with (Agarwal *et al.*, 2014).

Biochemical examination was determined for the highest *β*-glucosidase producing isolate, the bacteria exhibited positive results for Nitrate reduction, H_2S Production, Esculin, catalase, oxidase and they hydrolyzed gelatin and not utilized Starch. This result was harmony with (Van Berkum and Eardly, 1998).

According to previously reported methods (Patagundi *et al.*, 2014), the physiological and biochemical characteristics of the species were identified as *Pseudomonas* species as presented in (Table 3). Also, the enzyme activity of this strain NRC-S4 (1.78 U/mL) was higher than that of other strains cultured for 24 h as reported by (Singh *et al.*, 2013) and (Immanuel *et al.*, 2006). Also, our result were similar with the cellulolytic bacteria *Bacillus megaterium* RU4 and *Paracoccus yeei* RA2 that isolated by (Ferbiyanto *et al.*, 2015).

According to previous study suggesting that, 16S

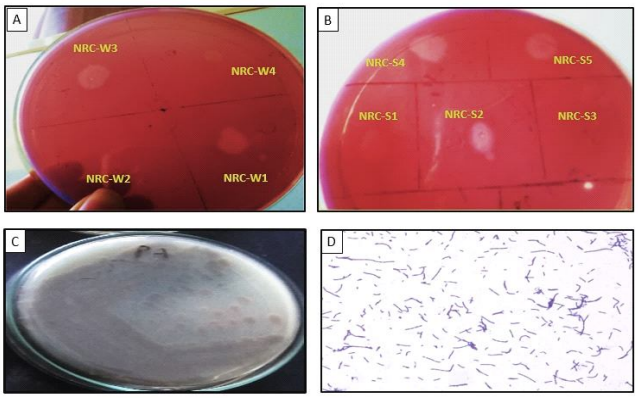

Fig. 1: Screening for cellulolytic bacteria by covering the petri dishes with Congo red solution from soil (B) and decayed wood (A) sample. (C, D) Morphological Characteristics of NRC-S4 bacterial isolate.

rDNA sequencing is an accurate method for species identification and distinguishing between closely related bacterial species (Clarridge, 2004), The 16s rRNA sequence of the *β*-glucosidase producing isolate NRC-S4 was 1500 bp long as shown in (Fig. 2B) and was submitted to GenBank under accession number LC455963.1. Homology analysis showed that the degree of sequence similarity of this strain to some *Pseudomonas* species exceeded 96%. As shown in (Fig. 2A), the phylogenetic tree constructed using MEGA7, the strain is related to *Pseudomonas aeruginosa* strain Pse6 (GenBank accession no: KR815843.1), with a similarity of 96.14%. Thus, all characterization methods showed that the isolated NRC-S4 was a *Pseudomonas aeruginosa* strain, (Fig. 2) (Table 3).

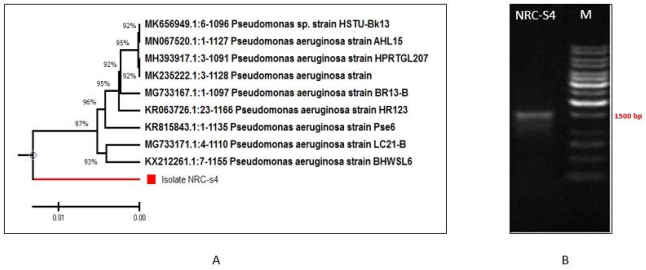

Fig. 2: A) Neighbor-joining phylogenetic tree showing relationship of strain NRC-S4 with in different type strain of the genus *Pseudomonas* sp. The tree was generated using MEGA 7 software. **B)** 1.5% agarose gel electrophoresis of PCR product of the isolated NRC-S4 -: Lane (1): DNA marker 1Kb. lane (2): isolate NRC-S4.

Effect of incubation time on β-glucosidase activity

The effect of incubation time for β-glucosidase production by *Pseudomonas aeruginosa* strain NRC-S4 was observed in the production medium Lb. and cellobiose act as a substrate for enzyme production. The

activity was determined from 6 to 120 h. The β-glucosidase activity and protein content determined ranged from 11.25 IU/ml and 80 µg/ml at 6 h. to maximum 33.21 IU/ml and 88 µg/ml at 48 h.

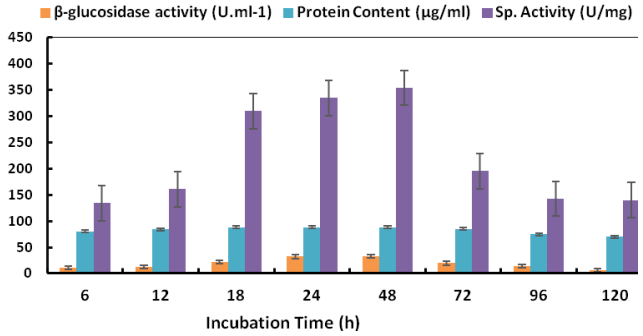

Fig. 3: Effect of incubation time on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4.

The optimum incubation time for the β-glucosidase production was found at 48 h with the higher 88 µg ml^{-1} of protein content and enzyme activity of 33.21 U ml-1, it was also observed that the enzyme activity and protein showed the decreasing pattern after 48 h. However, the minimum yield of the protein content of 70µg ml^{-1} and enzyme activity of 6.17 U ml^{-1} was observed at 120 h (Fig. 3). The maximum specific activity of enzyme was found between 24 and 48 hours. The major peak of specific activity was observed at 48 hours which was 35.39 U/mg. Same Optimum incubation time for maximum β-glucosidase production by *Bacillus sp.* was reported by (Agrawal *et al.*, 2013) It may be due to decrease in nutrients of media and respective cell death in the medium (Ariffin *et al.*, 2006). Results revealed that, the enzyme production was increased until 48 h and further increase in the incubation period, decreased enzyme activity was seen. Thus, 48 h was considered as the optimum incubation period for the production of β-glucosidase by *Pseudomonas aeruginosa* strain NRC-S4.

Effect of temperature on β-glucosidase activity

Activity of enzyme at higher temperature range is an advantageous factor for the scarification of biomass and can also prevent contamination to allow the reaction to proceed at higher range of temperature. Enzyme activity determined at different temperatures revealed that the maximum β-glucosidase production 35.12 U ml^{-1} at 40°C (Fig. 4). The temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane. β-glucosidase activity was recorded as 28.71, 33.21, 35.12 and 30.88 U ml^{-1} at 30, 35, 40 and 45°C respectively. The results showed that the β-glucosidase activity increased from 30 to 45°C after which decrease in activity was observed. According to (Daroit *et al.*, 2007) increase in activity was observed with increase in temperature. However, when the incubation temperature reached 80°C, the enzymatic activity diminished. Optimum temperature for maximum growth of *Bacillus subtilis* was 40°C (Jansová *et al.*, 1993) These results are close those of (Bakare *et al.*, 2005) who found that the β-glucosidase enzyme produced by *Pseudomonas fluorescence* was activated at 35 to 40°C showing the optimum temperature at 40°C. Results revealed that, 40°C was considered as the optimum temperature for the production of β-glucosidase by *Pseudomonas aeruginosa* strain NRC-S4.

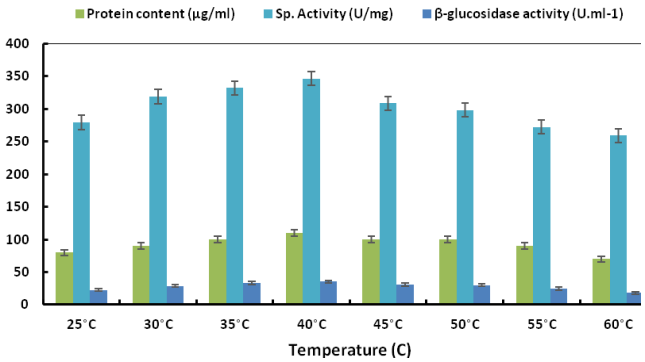

Fig. 4: Effect of temperature on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4 for 48h.

Effect of pH on β-glucosidase activity

Hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane, it has been shown that growth medium pH strongly influences many enzymatic reactions by affecting the transport of a number of chemical products and enzymes across the cell membrane (Liang *et al.*, 2010). Our results also confirmed that growth medium pH was an important factor affecting the β-glucosidase activity. The optimum growth medium pH was 7.0 for maximum production of β-glucosidase in NRC-S4. The enzyme activity at different pH values were shown in Fig.5. The β-glucosidase activity in culture supernatant (Crude enzyme) increased from 2.017 to 16.14 IU/ml with an increase of the pH of culture medium from pH 3.0 to 10.0. There was a decrease in β-glucosidase activity from 35.12 to 17.58 IU/ml on increasing the pH from 7 to 10. Similar observations was observed by (Shaikh *et al.*, 2013) when he isolated cellulase producing bacteria from different environment. In general, higher β-glucosidase activity was produced when *Bacillus subtilis* sp. was grown in the culture medium of neutral pH 7 (Agrawal *et al.*, 2013).

Results revealed that, pH 7.0 was considered as th optimum pH for the production of β-glucosidase b *Pseudomonas aeruginosa* strain NRC-S4.

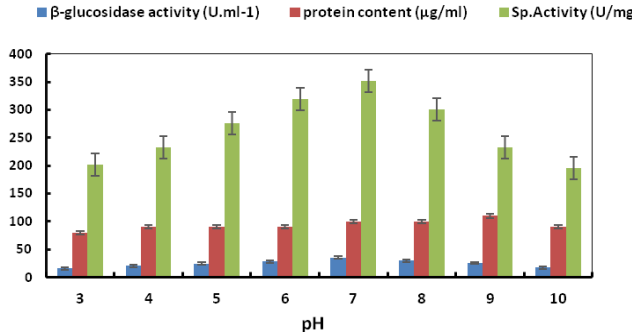

Fig. 5: Effect of pH on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4 for 48h. at 40°C.

Effect of inoculums size on β-glucosidase activity

The effect of inoculum size on optimum β-glucosidase production was observed at the inoculum load of 0.5, 1, 2, 4, 6 and 10%. The enzyme activity was found to be highest with 38.12 U ml^{-1}. with 90 µg ml^{-1} of protein at 1% inoculum size of incubation. However, lower protein concentration of 81 µg ml^{-1} was found at the inoculum load of 0.5 % with enzyme activity 26.13 U ml^{-1} after 48 h of incubation period as shown in (Fig. 6). β-glucosidase production by *Pseudomonas aeruginosa* strain NRC-S4 was studied by testing β-glucosidase secretion in the culture medium using different substrate cellobiose concentrations at 40°C. Cellobiose is used as a substrate for β-glucosidase production due to its less complexity and easy digestion by the microbes (Shanmugapriya *et al.*, 2012). Highest level of β-glucosidase activity and protein 38.13 IU/ml and 90 µg/ml respectively were produced when strain NRC-S4 was grown on Lb. along with Cellobiose at 1% for 48 hours at 40°C. this results was agreement with (Shaikh *et al.*, 2013) CMC concentration ranged from 0.2 % to 1.5 % used and got maximum activity by *Bacillus subtilis*. Therefore, results revealed that, Cellobiose was considered as the optimum carbon sources with concentration 1% for the production of β-glucosidase by *Pseudomonas aeruginosa* strain NRC-S4.

Effect of carbon sources on β-glucosidase activity

In the present study, different carbon sources were examined to study their effects on NRC-S4. β-glucosidase production under identical conditions (temperature, 40°C; 1% inoculum size; incubation time, 48 h). The production of β-glucosidase in *Pseudomonas aeruginosa* strain NRC-S4 was found to be maximum protein content 90 µg ml^{-1} and enzyme activity at 34.17 U ml^{-1} with Cellobiose

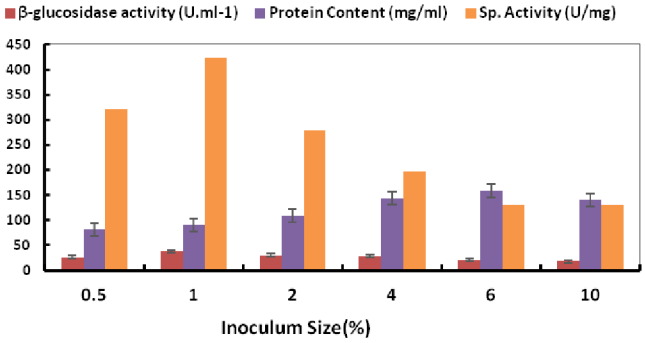

Fig. 6: Effect of inoculums size on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4 for 48h. at 40°C, pH.7.

at 48 h of incubation. Starch and cellulose yielded the least enzyme activity of 8.03 and 12.23 U ml^{-1} with protein content of 50 and 40µg ml^{-1} respectively after 48 h of incubation period. The present results suggested that optimum β-glucosidase production was observed with cellobiose as a carbon source (Fig. 7).

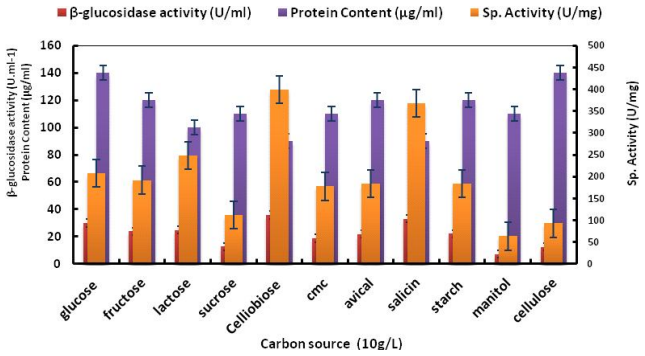

Fig. 7: Effect of carbon sources on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4 for 48h. at 40°C, pH.7.

The production of β-glucosidase in *Pseudomonas aeruginosa* strain NRC-S4 was found to be maximum protein content 120 µg ml^{-1} and enzyme activity at 35.88 U ml^{-1} with cellobiose at 48 h. of incubation. Mannitol yielded the least enzyme activity of 7.12 U ml^{-1} with protein content of 110 µg ml^{-1} after 48 h. of incubation period. The present results suggested that optimum β-glucosidase production was observed with Cellobiose as a carbon source (Fig. 7).

Effect of Cellobiose concentration on β-glucosidase production.

β-glucosidase production by *Pseudomonas aeruginosa* was studied by testing β-glucosidase secretion in the culture medium using different substrate cellobiose concentrations at 40°C for 48h at pH.7. Cellobiose is used as a substrate for β-glucosidase production due to its less complexity and easy digestion by the microbes (Shanmugapriya *et al.*, 2012). Highest

level of β-glucosidase activity and protein 35.44 IU/ml and 140 µg/ml respectively were produced when culture was grown on Lb. along with cellobiose at 2 % for 48 hours at 40°C**.** Cellobiose concentration ranged from 0.5 % to 10 % used and got maximum activity by *Pseudomonas aeruginosa* at cellobiose concentration of 2 %. The different concentrations of cellobiose were tested for β-glucosidase production, among which 2% cellobiose was optimum for this strain (Fig. 8). Above this concentration β-glucosidase production was inhibited. Similarly, cellulase production was inhibited by 1% cellulose in *Thermomonospora curvata* isolated from municipal solid waste compost (Shaikh *et al.*, 2013).

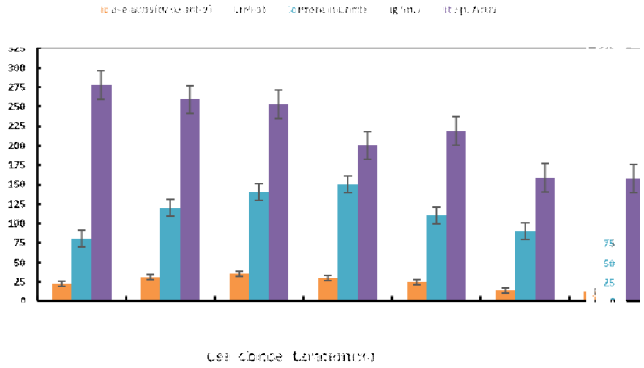

Fig. 8: Effect of Celliobiose concentration on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4 for 48h. at 40˚C, pH. 7.

Effect of Nitrogen sources on β-glucosidase activity

The enzyme production is affected significantly under different concentration of the organic and inorganic nitrogen sources. Many nitrogen sources 1% (Yeast extract, Beef extract, Peptone, Soymeal, Urea, Sodium nitrate, Ammonium Chloride, Potasium Nitrite, Ammonium Sulfate Ammonium Nitrate) were examined with other conditions identical (as mentioned above). The results showed that strain NRC-S4 can utilize inorganic nitrogen sources efficiently, and the maximum β-glucosidase activity (27.44 U/ml) was observed when ammonium chloride was used as the sole nitrogen source. However, the β-glucosidase activity was almost zero when organic nitrogen sources (Soymeal or urea) were used as the sole nitrogen sources (Fig. 9).

This could be because the metabolism of inorganic nitrogen contributes to medium acidification, which in turn affects β-glucosidase production. Similar observations were observed by (Rajoka and Malik, 1997). Also, KNO_3 and NH_4NO_3 were used as the best N sources for cellulose production in *Cellulomonas flavigena*. NH_4 compounds was considered as most favorable N sources for cellulase synthesis as noted in *Thermomonospora*

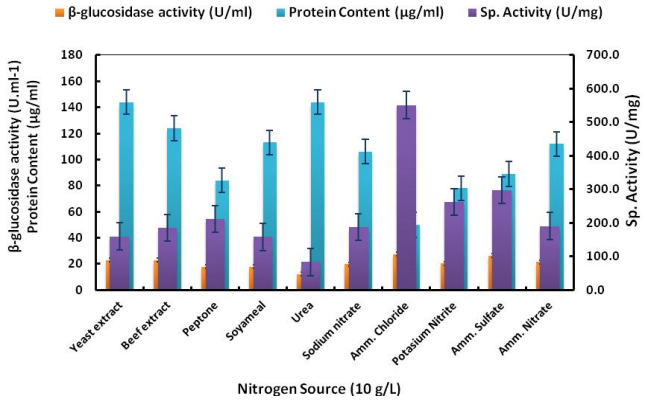

Fig. 9: Effect of nitrogen sources on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4.

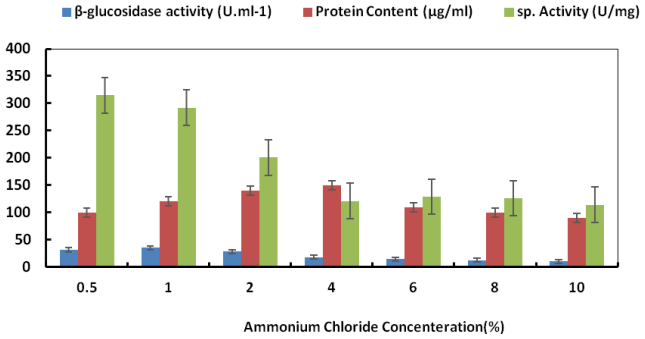

Fig. 10: Effect of Ammonium Chloride concentration on protein content and β-glucosidase production in *Pseudomonas aeruginosa* strain NRC-S4.

fusca (Spiridonov and Wilson, 1998).

Conclusion

Majority of studies on β-glucosidase production have been focused on fungi and bacteria. However, bacteria may serve as highly potent sources of industrially important enzymes for the conversion of cellulosic biomass due to their higher growth rate; more complex

Table 1: Primary screening for cellulolytic bacteria by zone of clearance surrounding the colonies is indicative hydrolysis by secreted CMCase.

Isolate Code	Source	Colony size	clear zone (mm)
NRC-S1	Soil	2.0	-
NRC-S2	Soil	3.0	22
NRC-S3	Soil	4.0	-
NRC-S4	Soil	0.9	28
NRC-S5	Soil	1.5	20
NRC-W1	Wood	1.0	20.5
NRC-W2	Wood	1.5	10.5
NRC-W3	Wood	3.0	-
NRC-W4	Wood	4.0	-

Table 2: Secondary screening for cellulolytic bacteria by evaluating the Endo-glucanase and β-glucosidase activity.

Isolate Code	Growth at OD_{600}	Protein Concentration (mg/ml)	β-glucosidase Activity		Endo-glucanase Activity	
			U/ml	Sp. Activity (U/mg)	U/ml	Sp. Activity (U/mg)
NRC-S1	0.841	0.221	5.15	23.30	0.02	0.09
NRC-S2	0.822	0.284	18.45	64.96	0.41	1.443
NRC-S3	0.894	0.174	22.12	127.12	0.08	0.459
NRC-S4	0.902	0.121	35.12	290.24	1.78	14.71
NRC-S5	1.023	0.220	7.23	32.86	0.84	3.81
NRC-W1	0.915	0.221	6.02	27.23	0.07	0.31
NRC-W2	0.888	0.299	11.32	37.85	0.04	0.133
NRC-W3	0.874	0.245	08.12	33.14	0.91	3.71
NRC-W4	0.856	0.155	13.12	84.64	0.13	0.83

glycoside hydrolases with providing synergy and their extremely high natural diversity have the capability to produce thermostable, alkali stable enzymes. For this reason, we have screened β-glucosidase producing bacteria from different ecological niches and obtained this strain of *Pseudomonas aeruginosa*. Results of this study indicate that β-glucosidase producing bacterial strains can be grown at different optimized conditions. The isolated strain *Pseudomonas aeruginosa* NRC-S4 showed maximum β-glucosidase activity at pH 7.0 and 40 °C temperature on 48 h incubation period at 2% cellobiose substrate concentration.

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Table 3: Biochemical and physiological characteristics of NRC-S4 bacterial isolate.

	No.	Characteristics		NRC-S4
Morphological	1	Gram Reaction		-
	2	Cell shape		bacilli
	3	Colony color		Blue
	4	Colony shape		Smooth
	5	Colony margin		Lobate
	6	Mobility		+
Biochemical	7	Spor formation		-
	8	Oxidases test		+
	9	Catalase test		+
	10	Aerobic Growth		+
	11	Anaerobic growth		-
	12	Voges-Proskauer		+
	13	Casein		-
	14	Starch		-
	15	Gelatin		+
	16	Citrate		-
	17	Nitrate reduction		+
	18	H2S Production		+
	19	Esculin		+
Physiological	20	Growth at pH	5	+
			6	+
			7	+
			8	+
			9	+
			10	-
	21	Growth in NaCl	1%	+
			2%	+
			5%	+
			7%	-
			10%	-
	22	Growth at Temp.	5ºC	-
			10ºC	-
			20ºC	+
			30ºC	+
			40ºC	+
			50ºC	-
			55ºC	-
			65ºC	-

(+) Positive (-) Negative

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