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Research article

Isolation, screening and optimization of β-glucosidase producing *Bacillus sp.* isolated from Egyptian environment

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Key words: Cellulase, Bacteria Optimization, β-glucosidase.

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Abstract

The aim of the present study is isolation, screening, identification and optimization of bacterial strains with high β-glucosidase activity from Egyptian soil environment. These strains were isolated and primary screened by growth in medium containing Carboxymethyl cellulose (CMC) for cellulases ability and the promising isolates were secondary analyzed for β-glucosidase and Endo-glucanase (Eg) activity. Among eight isolated strains, isolate (Eg 5) showed the highest β-glucosidase, Endo-glucanase activity and protein content 37.24 U ml^{-1}, 1.89 U ml^{-1} and 220 µg/ml, respectively. According to morphological characteristics, the highest β-glucosidase producing isolate (Eg 5) identified as *Bacillus sp.* strain Eg 5, it was gram positive, *bacilli*, motile, facultative anaerobe and spore former. Based on biochemical tests and physiological studies, it was positive for Catalase, Oxidase, Citrate, nitrate reduction, Gelatin, H_2S production and esculin test, and negative for starch and casein hydrolysis. Also, the physiological studies it was grown at wide range of pH (5-9), with tolerance against NaCl concentration (1-5) % at high temperature 40°C. Different cultural, physical, and nutritional factors were studied for optimal production of β-glucosidase. Optimized conditions for β-glucosidase production were characterized as 1% inoculum size and pH 7 at 45°C for 48 hours of incubation using cellobiose (1%w/v) as the best carbon source induced β-glucosidase production. Also, the nitrogen source Ammonium chloride at (1% w/v) was optimum for β-glucosidase production by this bacterium. This can contribute its β-glucosidase producing ability for industrial processes and biotechnological application.

Introduction

Cellulose is a polymer of cellobiose units, a disaccharide of two glucose molecules linked by a β-1,4 linkage [1]. It is considered as the most component biological compound on both terrestrial and aquatic ecosystems [2]. Because it is the main component of plant biomass including stalks, stems and husk, cellulose is the dominant waste material from agricultural industries. There has been great interest in utilizing cellulose as an energy resource through its utilization as animal feed [3]. Cellulose is hydrolyzed by an enzyme called cellulases which act synergistically towards the complete breakdown of cellulose. Cellulases system consists of three major enzymes; endoglucanase, exo-glucanase, and β-glucosidase. Endo-glucanases are responsible for degradation of cellulose to get their reducing and non-reducing ends. Cellobiohydrolases act on both chain ends to release cellobiose which serve as substrate for β-glucosidase to release glucose [1]. In the industrial processes, cellulolytic enzymes have many useful uses in textile, detergent and paper industries, and also they are used for recycling of plant materials into high energy animal feed [4]. cellulases are produced by various fungi, bacteria and actinomycetes. The cellulolytic potentials of bacterial species belonging to different genera such as *Acetivibrio, Bacillus*, *Erwinia*, and *Cellulomonas* 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 [5]. The main objective of this study was to investigate, isolate and screen of β-glucosidase producing bacteria from soil, and to identify isolated strains based on morphological, biochemical and physiological characteristics. In addition to optimize various cultural and nutritional factors, including incubation time, temperature, pH, nitrogen sources and carbon sources.

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Material and methods

Isolation of cellulolytic bacteria
Egyptian bacterial strains were isolated from soil by using serial dilutions and pour plate technique [6]. The purified colonies were preserved at 4°C for further identification and screening for β-glucosidase production.

Screening of cellulolytic bacteria
Pure strains of selected isolates were individually screened on CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1 % Congo red. hydrolysis zones were recorded around bacterial colonies indicating cellulose hydrolysis [7].

Identification of selected β-glucosidase producing bacterial isolates
Identification of the β-glucosidase producing bacterial isolates was performed based on their morphological, physiological, and biochemical characterizations, as described in Bergey's Manual of Systematic Bacteriology [8].

Qualitative determination of cellulolytic activity
The isolate that showed maximum zone of hydrolysis was inoculated in LB broth medium and incubated at 37ºC for 24h. Then, were centrifuged, and clear supernatant was used as a source of crude enzyme solution.

Endo-β-1, 4-glucanase activity assay by DNS
0.5 ml of crude enzyme was mixed with 0.5 ml of substrate (1% CMC) and incubated in a water bath at 60°C for 30 minutes. The quantity of glucose produced was assayed by Dinitro salicylic acid test (DNS) [9].

β-glucosidase activity assay by PNPG method
The assay of β-glucosidase activity was done according to Daroit *et al.* [10].

Protein determination
Protein content was determined by Bradford's method [11] using bovine serum albumin as standard.

Optimization of β-glucosidase Production
Effect of pH
pH of the broth is adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 in different broth and sterilized. Isolate was inoculated and incubated at 37°C for 24h. Then, β-glucosidase activity was determined.

Effect of temperature
The broth was incubated at different temperatures from 35, 40, 45, 50, 55, and 60°C for 24 h. then, β-glucosidase activity was determined [12].

Effect of incubation time
β-glucosidase activity was done at different incubation time ranging from 6, 12, 24, 48, 72, 96 and 120h [12].

Effect of inoculums size
The broth medium was inoculated with different inoculum size ranged from 0.5, 1, 2, 4, 6 and 10 % of overnight bacterial culture. All the broth culture was incubated at 45°C, pH 7 for 48h.

Effect of carbon sources
β-glucosidase activity by different carbon sources was recorded in the fermentation medium, all broth culture was incubated at 45°C, pH 7 for 48h with 1% inoculum size [12].

Effect of nitrogen sources
β-glucosidase activity by different nitrogen sources were determined in the broth medium. All broth culture was incubated at 45°C, pH 7 for 48h with 1% inoculum size [12].

Results and discussion

Isolation and screening of isolates
Cellulose is the main building block of plants and have major fraction of organic carbon in soil. Microorganisms, which live in soil, are accountable for recycling of this organic carbon to the environment [13]. In Primary screening of cellulose degrading bacteria, a total of 8 bacterial strains were isolated from the various soil from Egypt. The cellulolytic bacterial strains which produced strong haloes zone was selected for further study (Figure-1). Three (3) out of the 8 isolates showed positive potential for endo-glucanase secretion as shown in table 1. Isolate Eg 5 appeared the largest zone of inhibition (28.0 mm) with enzymatic index of 7.8, followed by isolate Eg 4 (20.0 mm) and enzymatic index of 9.5 while isolate Eg 7 had the lowest clear zone of inhibition (10.5 mm) with enzymatic index of 3.4. By contrast, isolates Eg 1, Eg 2, Eg 3, Eg 6 and Eg 8 were showed any hydrolysis zone around their colonies. In Secondary screening for cellulolytic bacteria by evaluating the CMCase and β-glucosidase activity, results were presented in table 2 the isolate Eg 5 had maximum β-glucosidase, Endo-glucanase activity and protein content of 37.24 U ml^{-1}, 1.89 U ml^{-1} and 220µg/ml, respectively. While, β-glucosidase and Endo-glucanase activity of isolates Eg 1, Eg 2, Eg 3, Eg 4, Eg 6, Eg 7 and Eg 8 were 0.84, 8.04, 12.48, 8.11, 5.02, 12.32, 6.88 U ml^{-1} and 0.01, 0.12, 0.08, 0.22, 0.04, 0.07, 0.11 U ml^{-1} respectively. Also, protein content (µg ml^{-1}) of isolates Eg 1, Eg 2, Eg 3, Eg 4, Eg 6, Eg 7 and Eg 8 were 184, 144, 174, 121, 134, 199, 258 µg ml^{-1} respectively. This was shared with Agarwal *et al.* [15]. Therefore, Isolate Eg 5 was selected for further

study after the screening process and identified as *Bacillus sp.* strain Eg 5 based on cultural, morphological and biochemical properties. The organism was used for β-glucosidase production in submerged fermentation process. This result was harmony with [14] was reported that cellulose producing microbes have been isolated from different sources such as soil, water, compost.

Identification of β-glucosidase producing bacteria

The morphological, biochemical and physiological properties of the isolate is shown in table 3. According to the morphological tests, the organism was found to be gram positive, bacilli in cell shape, white in colony color, smooth in colony shape, lobate in colony margin, motile, facultative anaerobe and spore former. As presented in figure 1. According to the biochemical tests, the results showed that the isolates are positive for catalase, oxidase, voges proskauer, citrate utilization, nitrate reduction, gelatin hydrolysis, h_2s production and esculin hydrolysis test, and negative for starch and casein hydrolysis test. these results confirm that the isolate was belongs to the genus *Bacillus* as compared with Berge's Manual of Systematic Bacteriology [8]. Also, according to the physiological tests the results showed that the isolate was grown at wide range of pH (5-9), with tolerance against NaCl concentration (1-5) % at high temperature 40°C. Mostly *Bacillus* spp. are used in industries because they are not pathogenic, grow and reproduce easily, do not produce foul odors or gases, some species can survive in alkaline condition and at high temperature, secrete proteins extracellular and are considered relatively safe to use with regard to health and environmental aspects [16]. Morphological, Biochemical and physiological characteristics were performed for the isolate Eg 5 and recorded as *Bacillus* spp and the results were presented in table 3 and this result were similar with Agarwal *et al.* [15].

The most potent of the cellulolytic bacterial strain was isolated and the production of â-glucosidase activity was optimized by studying different culture parameters, such as time incubation, temperature, pH, nitrogen sources and carbon sources.

Table 1. Primary screening indices of screened cellulose degrading bacteria isolates.

Isolate Code	Colony size (C)	Halo zone (mm) (H)	Enzymatic index (EI=H/C)
Eg 1	1.5	-	-
Eg 2	2.5	-	-
Eg 3	2.0	-	-
Eg 4*	2.1	20	9.5
Eg 5*	3.6	28	7.8
Eg 6	1.8	-	-
Eg 7*	3.1	10.5	3.4
Eg 8	2.9	-	-

**Isolates with EI values less than one (EI<1) or no visible halo.*

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

Isolate Code	Growth CFU X10^8	Protein Content (µg/ml)	β-glucosidase Activity U ml^{-1}	CMCas Activity U ml^{-1}
Eg 1	2.12	184	0.84	0.01
Eg 2	1.89	144	08.04	0.12
Eg 3	2.41	174	12.48	0.08
Eg 4	1.99	121	08.11	0.22
Eg 5*	1.74	220	37.24	1.89
Eg 6	2.22	134	05.02	0.04
Eg 7	1.87	199	12.32	0.07
Eg 8	1.99	258	06.88	0.11

**Isolates with high level of beta-glucosidase and CMC activity.*

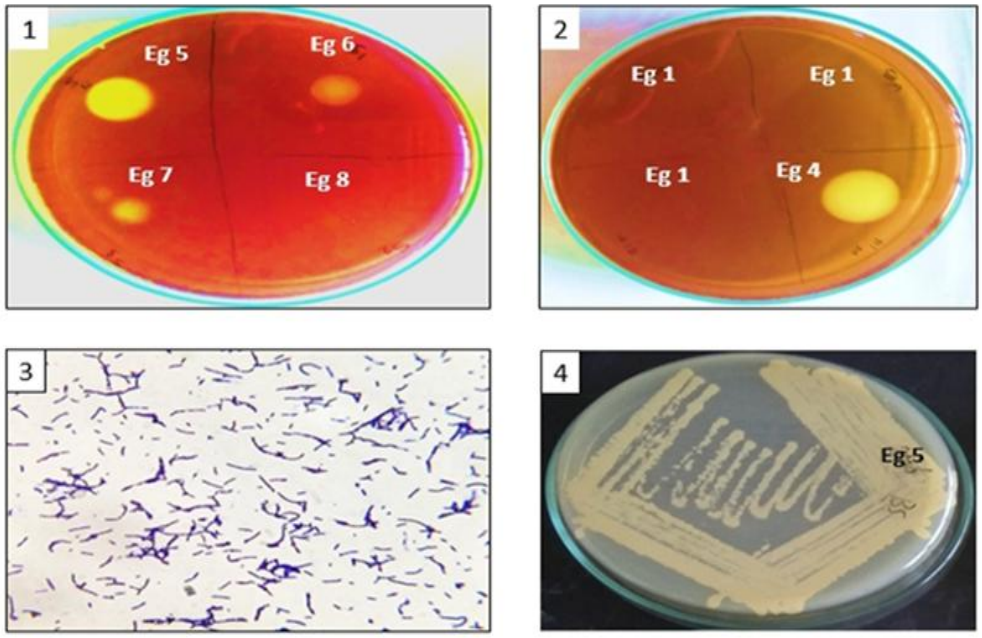


Figure 1. [1, 2] Primary screening of selected cellulose degrading bacteria isolates, [3] Microscopic examination of selected bacterial isolate Eg 5, [4] Colony characteristics of purified selected isolate.

Optimization of β-glucosidase production

Effect of incubation time on β-glucosidase activity

The effect of incubation time for enzyme activity by isolate Eg 5 was determined in the fermentation medium and cellobiose act as a substrate for enzyme production. β-glucosidase activity, protein content and specific activity were increased gradually from 6.11 U ml^{-1}, 55 µg/ml and 198 U/mg at 6 h to 27.96 IU/ml, 95 µg/ml and 250 U/mg at 48 h then decreased gradually to 11.24 U ml^{-1}, 66 µg/ml and 150 U/mg at 120 h as shown in figure 2. Our results revealed that, 48h was considered as the optimum incubation time for the production of β-glucosidase by *Bacillus sp* isolate Eg 5. Same optimum incubation time for maximum β-glucosidase production by *Bacillus sp.* was reported by Agarwal *et al.* [15]. It may be due to decrease in nutrients of media and respective cell death in the medium.

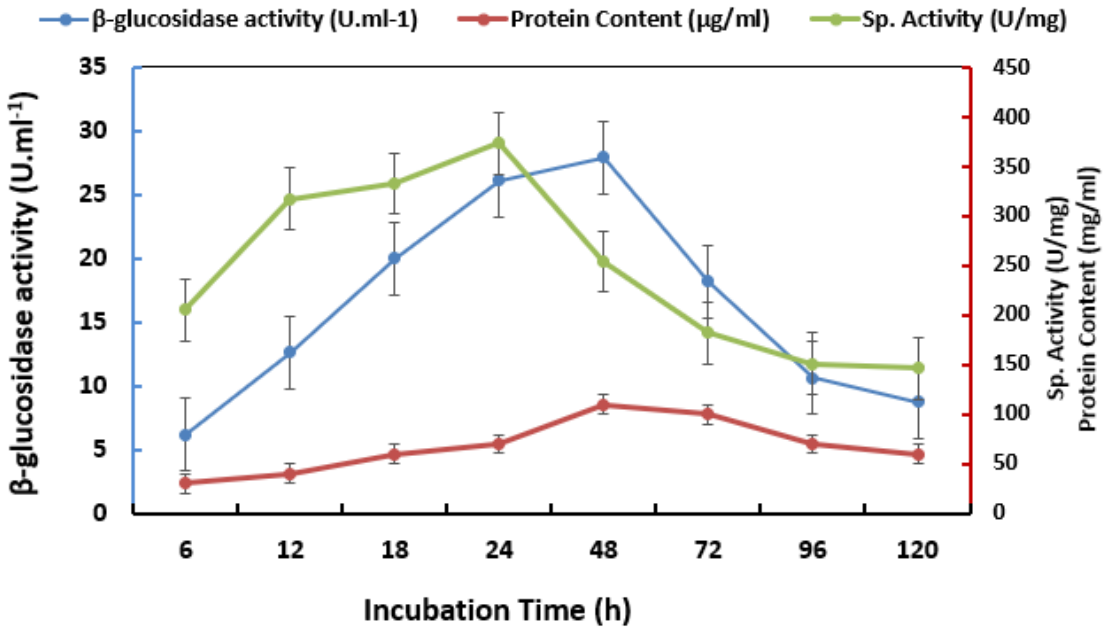


Figure 2. Effect of incubation time (h) on protein content and β-glucosidase production in *Bacillus sp.* strain Eg 5.

Effect of temperature on β-glucosidase activity

Temperature plays a critical role in enzyme activity. Effect of temperature from 25°C to 60°C was studied. β-glucosidase production, protein content and specific activity were increased gradually from 15.33 U ml^{-1}, 105 µg/ml and 150 U/mg at 25°C to 33.87 IU/ml, 112 µg/ml and 190 U/mg at 45°C then decreased gradually to 12.24 U ml^{-1}, 65 µg/ml and 122 U/mg at 60°C, as shown in figure 3. Our results revealed that, 45°C was considered as the optimum incubation temperature for the production of β-glucosidase by *Bacillus sp* isolate Eg 5. These results are close to those of [17] 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.

Effect of pH on β-glucosidase activity

The results obtained on effect of various pH on enzyme production by *Bacillus sp.* strain Eg 5 using cellobiose as carbon source shown in Figure 4. β-glucosidase production, protein content and specific activity were increased gradually from 17.24 U ml^{-1}, 75 µg/ml and 175 U/mg at pH 3 to 31.88 IU/ml, 77 µg/ml and 310 U/mg at pH 7 then decreased gradually to 18.87 U ml^{-1}, 61 µg/ml and 260 U/mg at pH 10, as shown in Figure 3. Our results revealed that, pH 7 was considered as the optimum incubation pH for the production of β-glucosidase by *Bacillus sp* isolate Eg 5. Similar observations was observed by [18] when isolated cellulase producing bacteria from different environment.

Effect of inoculum 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%. β-glucosidase production, protein content and specific activity were increased gradually from 32.11 U ml^{-1}, 75 µg/ml and 375 U/mg at 0.5 % inoculum size to 35.66 IU/ml, 77 µg/ml and 410 U/mg at 1 % inoculum size then decreased gradually to 24.89 U ml^{-1}, 61 µg/ml and 150 U/mg at 10 % inoculum size, as shown in Figure 5. Our results revealed that, 1 % inoculum size was considered as the optimum inoculum size for the production of β-glucosidase by *Bacillus sp* isolate Eg 5. This results was agreement with Shaikh *et al.* [18]. CMC concentration ranged from 0.2 % to 1.5 % used and got maximum activity by *Bacillus subtilis.*

Effect of Carbon source on β-glucosidase activity

Carbon source is one of the essential constituents of the microbial fermentation medium, which affects the overall cellular growth. The production of β-glucosidase in *Bacillus* Eg 5 was found to be maximum protein content 90 µg ml^{-1} and enzyme activity at 34.17 U ml^{-1} with Cellobioses at the higher 48 h of incubation, as shown in Figure 6. β-glucosidase activity (Uml^{-1}) of isolate *Bacillus sp.* Eg 5 with glucose, fructose, lactose, sucrose, cellobiose, CMC, avical, salicin, starch, mannitol and cellulose were 14.03, 18.44, 27.48, 15.62, 34.22, 29.88, 23.55, 32.54, 11.84, 16.55 and 19.84 U ml^{-1} respectively. Our study fined that optimum β-glucosidase production was observed with cellobiose (1%) as a carbon source. Similar results was observed by Samiullah *et al.* [19] studied β-glucosidase activity from *Acidothermus cellulolytic* when grown on a variety of carbon sources.

Table 3. Biochemical and physiological characteristics of Eg 5 bacterial isolate.

	No	Characteristics	Eg 5
Morphological	1	Gram Reaction	+
	2	Cell shape	Bacilli
	3	Colony color	White
	4	Colony shape	Smooth
	5	Colony margin	Lobate
	6	Mobility	+
Biochemical	7	Spore 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	H_2S 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. 5°C	-
		10°C	-
		20°C	+
		30°C	+
		40°C	+
		50°C	-
		55°C	-
		65°C	-
		Bacillus sp.	

(+) Positive **(-) Negative**

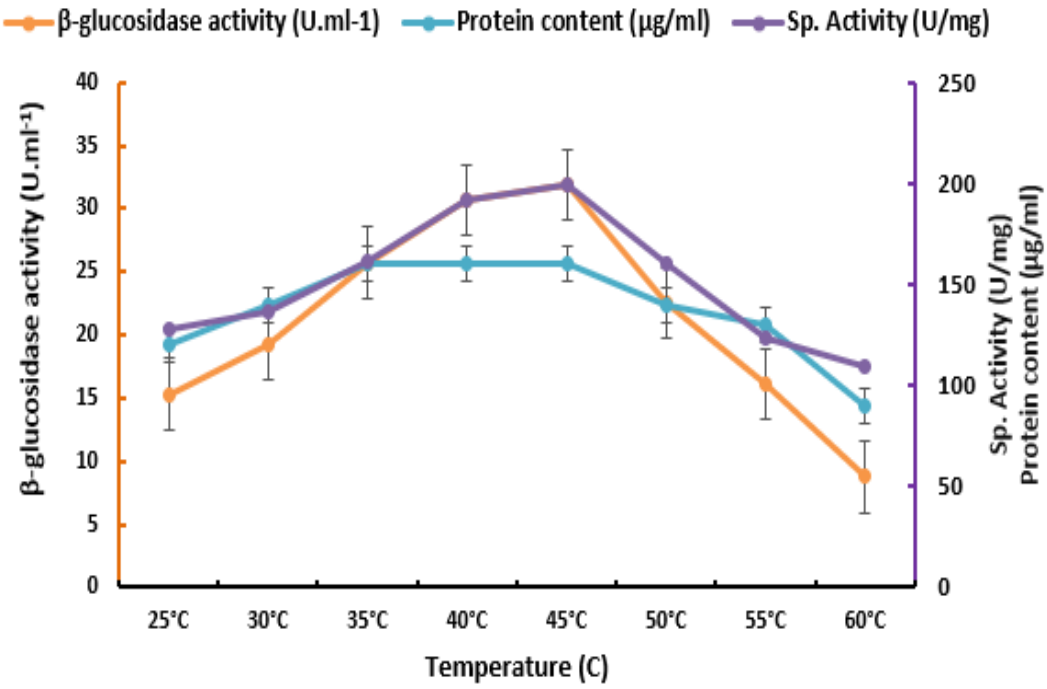


Figure 3. Effect of temperatures on protein content and β-glucosidase production in *Bacillus sp.* strain Eg 5.

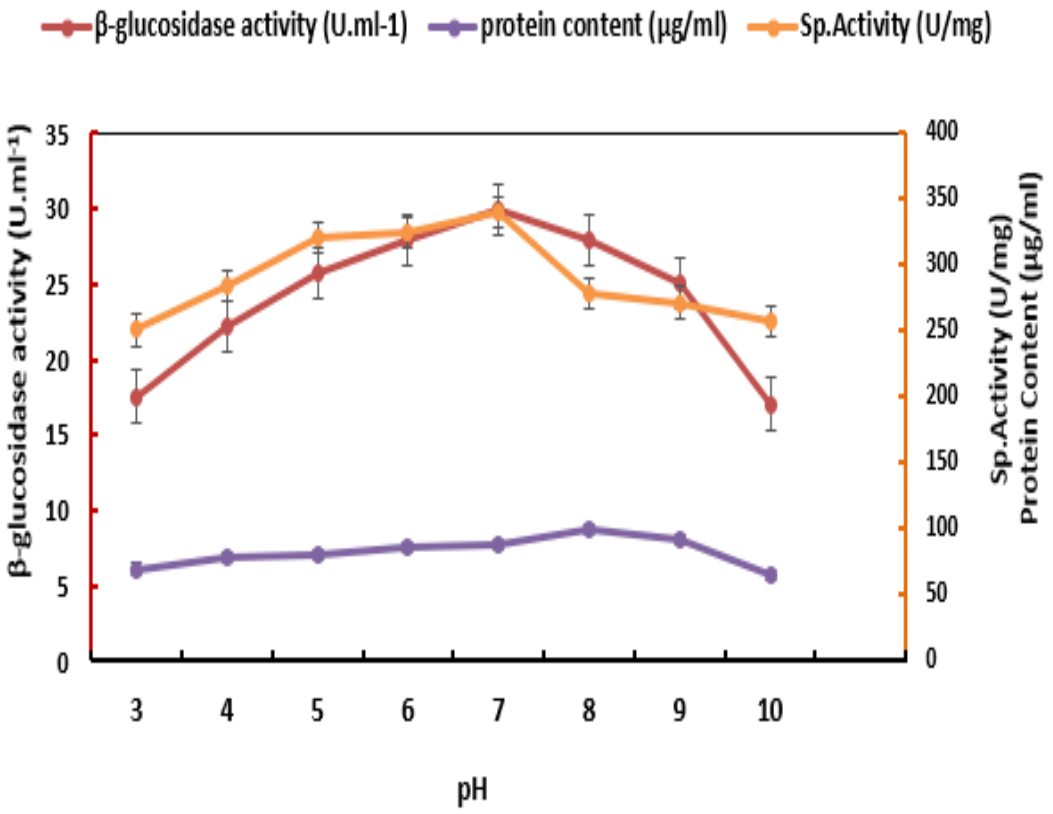


Figure 4. Effect of pH on protein content and β-glucosidase production in *Bacillus sp.* strain Eg 5.

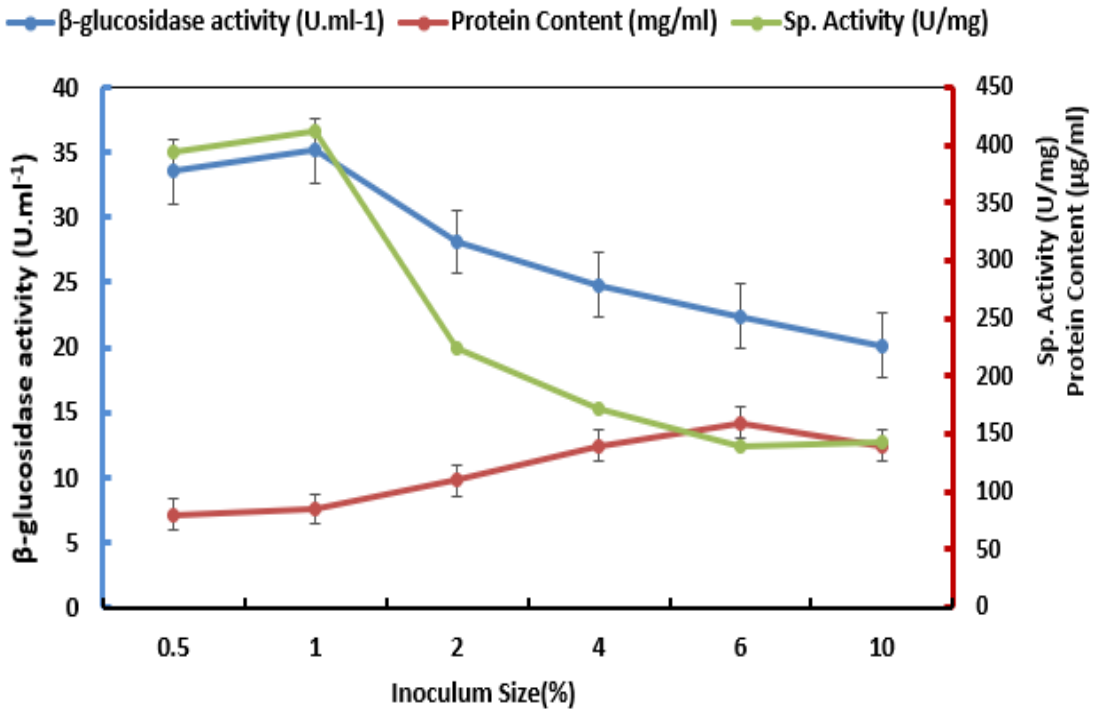


Figure 5. Effect of inoculum size on protein content and β-glucosidase production in *Bacillus sp.* Eg 5.

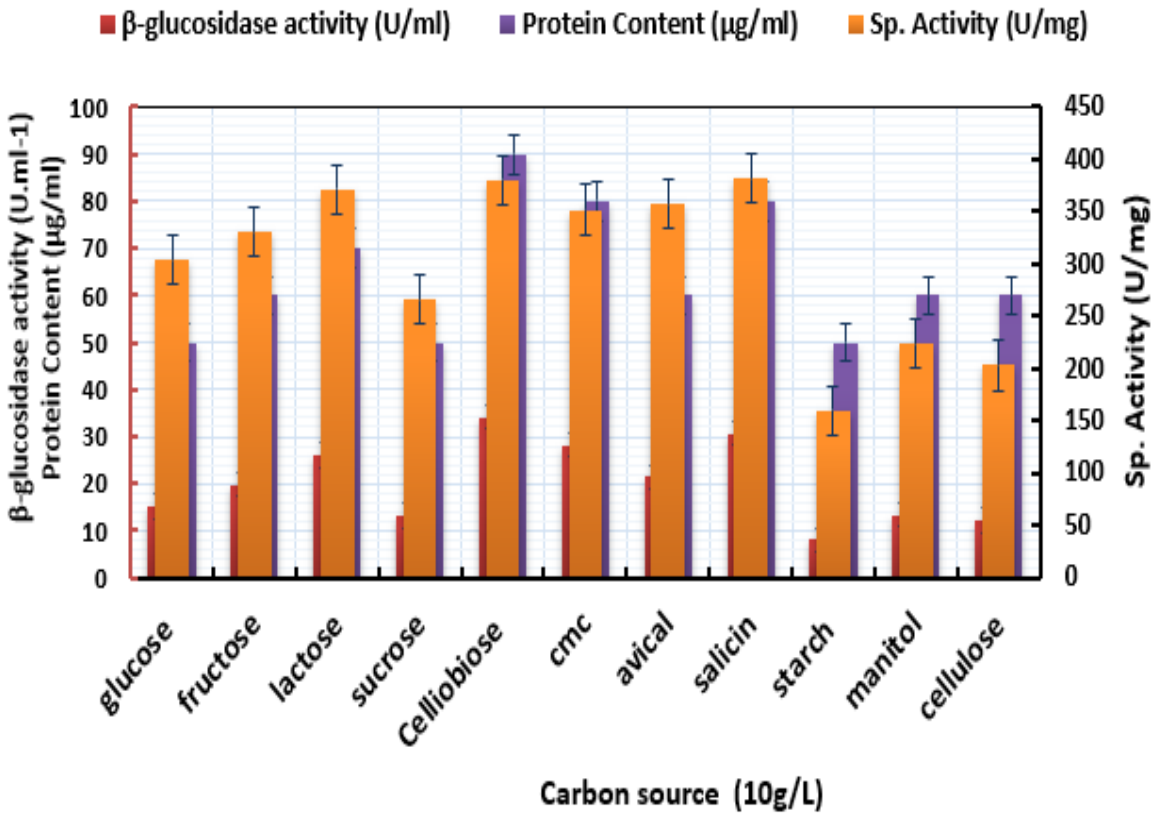


Figure 6. Effect of carbon sources on protein content and β-glucosidase production in *Bacillus sp.* Eg 5.

Effect of nitrogen source on β-glucosidase activity

The results showed that strain Eg 5 can utilize organic and inorganic nitrogen sources efficiently, and the maximum β-glucosidase activity (35.42 U/mL) was observed when 1% Ammonium chloride was used as the sole nitrogen source. However, Ammonium hydrogen phosphate, sodium nitrate, potassium nitrate and urea produced U ml^{-1} lower levels of β-glucosidase activity as shown in Figure 7. β-glucosidase activity (U ml^{-1}) of isolate *Bacillus sp.* Eg 5 with sodium nitrate, ammonium chloride, ammonium sulphate, potassium nitrate, yeast extract, soyameal, beef extract, NH_4NO_3, peptone and urea were 18.12, 35.42, 33.84, 19.22, 34.22, 14.87, 26.41, 30.21, 28.74 and 8.41 U ml^{-1} respectively. Our study fined that optimum β-glucosidase production was observed with ammonium chloride (1%) as nitrogen source. This could be because the hydrolysis of inorganic nitrogen contributes to medium acidification. Similar results were agreement with *Cellulomona s*sp. has been reported to grow well when 0.3-0.6% ammonium sulphate was used in the fermentation medium [20].

Conclusion

Numbers of reports on β-glucosidase production have been focusing on microorganisms. Bacteria have a vital role as highly promising producer of industrially important enzymes for the conversion of cellulosic biomass. For this reason, we have isolated, screened and identified β-glucosidase producing bacteria from different ecological niches in Egypt and identified as *Bacillus sp.* strain Eg 5. Results of this study indicate that among eight bacterial isolated strains, isolate Eg 5 was selected as promising and most potent β-glucosidase producing bacterial strain. According to morphological, biochemical and physiological characterization, the highest β-glucosidase production strain Eg 5 was identified as *Bacillus sp.* strain Eg 5. Also, β-glucosidase production from *Bacillus sp.* strain Eg 5 was optimized under physical, nutritional and chemical conditions. By physical condition, incubation time, inoculum size, incubation pH and temperature were optimized and showed maximum β-glucosidase activity at pH 7.0 and 45°C temperature on 48 h incubation period at 1% inoculum size. Also, nutritional factors such as carbon sources were optimized and showed maximum β-glucosidase production at cellobiose with 1% concentration as sole of carbon source. In addition, nitrogen sources were screened optimized and showed highest β-glucosidase production at ammonium chloride with 1% concentration as sole of nitrogen source.

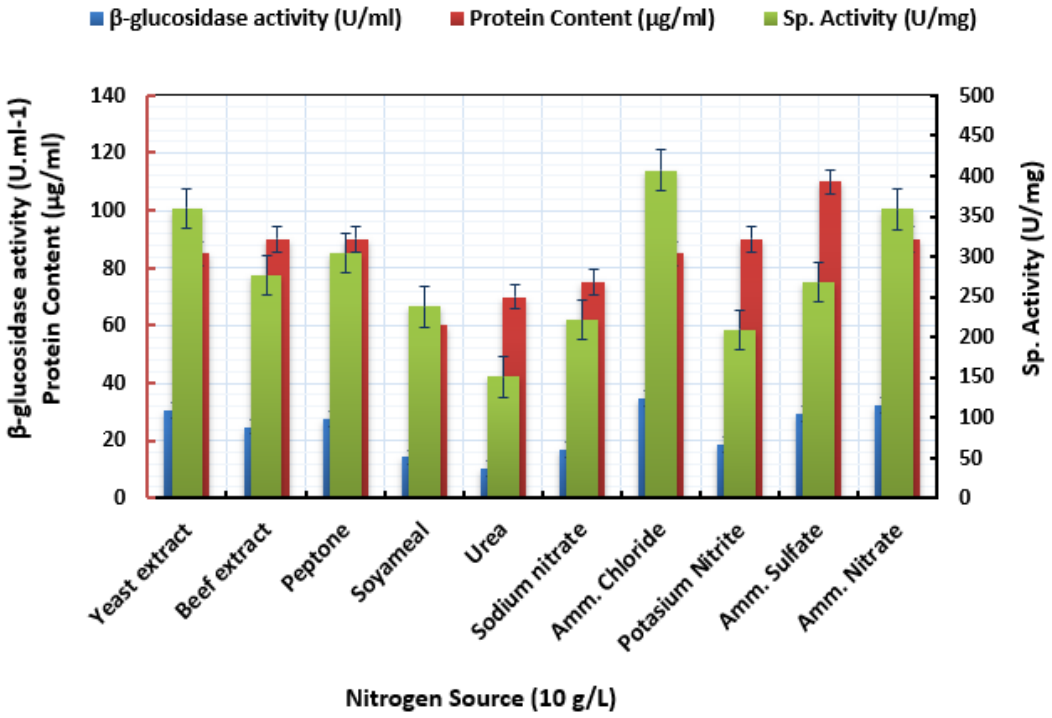


Figure 7. Effect of nitrogen sources on protein content and β-glucosidase production in *Bacillus sp.* Eg 5.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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