Genetic identification and optimization of novel β-glucosidase-producing *Lysinibacillus sphaericus* QS6 strain isolated from the Egyptian environment

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Background and objective
β-Glucosidase-producing bacteria are potential sources for biotransformation of lignocellulose biomass and agricultural wastes into biofuels. The aim was the isolation, screening, molecular identification, and optimization of highly efficient β-glucosidase-producing bacteria under different growth conditions.
Materials and methods
Cellulose-degrading bacteria were isolated and screened for β-glucosidase enzymes. Then, they were identified by phenotypic and genotypic identification. Optimization for β-glucosidase production was studied under different culture conditions.
Results and conclusion
Highly efficient β-glucosidase-producing strain QS6 was selected and identified morphologically and biochemically as Lysinibacillus sp. using 16 s rDNA gene sequencing approach and bioinformatics analysis. Strain QS6 was most similar to *Lysinibacillus sphaericus*, with similarity of 98%. Phylogenetic analysis was done to determine the relationship of strain QS6 with different strains of genus Lysinibacillus sp. It indicated that the suitable culture conditions of producing β-glucosidase were the culture temperature of 35°C, the initial pH of 7.0, the incubation time of 24 h, and 1% inoculum size. While studying the effect of carbon sources on β-glucosidase production, it was found that cellobiose (1%w/v) was the best carbon source for inducing β-glucosidase production. Moreover, the nitrogen source peptone at 0.5% w/v was optimum for β-glucosidase production by this bacterium. *L. sphaericus* QS6 was found to be sensitive to antibiotics (amoxicillin, streptomycin, tetracycline, cefadroxil, kanamycin, chloramphenicol, ampicillin, erythromycin, and tobramycin). Moreover, in-vitro antibacterial bioassay of the most potent β-glucosidase-producing strain (QS6) showed high antimicrobial activity against *Escherichia coli* (1.9 cm) and *Pseudomonas aeruginosa* 1.0 cm). A promising Lysinibacillus sp. completely identified as *L. sphaericus* QS6 (GenBank MN493725.1) is an efficient source of β-glucosidase production.

Keywords:
16S rDNA gene, celluloses, *Lysinibacillus sphaericus*, optimization, β-glucosidase

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Introduction

Lignocellulose biomass (all plants and plant-derived materials) is the Earth's most abundant and renewable organic material with great potential for production of bioenergy and commodity chemicals [1]. Certain microbes are categorized as cellulolytic owing to their capability of growing on cellulose as sole carbon and energy source and secreting cellulases [2]. Three kinds of cellulases are required to carry out cellulose hydrolysis: first, endoglucanases locate surface sites randomly along the cellodextrin creating a new reducing and nonreducing chain end; second, exoglucanases cleave cellulose chains at the ends to release cellobiose; and third, β-glucosidases convert cellobiose to glucose, and thus relieve the system from end-product inhibition [3]. Cellulases are widely produced by various microorganisms such as bacteria, fungi, molds, and microbes present in the animal gut. Microbial cellulases are used in various industries such as food and animal feed, laundry and detergents, pulp and paper, textiles, and biofuel. Moreover, they have some applications in the pharmaceutical industry, genetic engineering, waste treatment, and protoplast production [4]. Therefore, worldwide research has been focused on isolation and exploitation of new microbial resources for the extraction of cellulolytic enzymes with desirable

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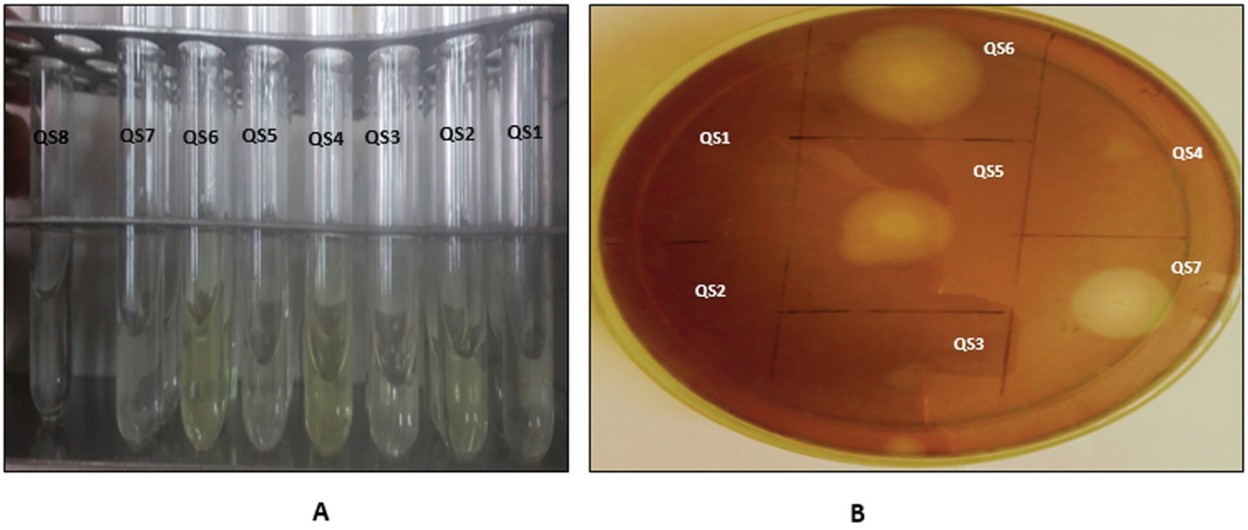
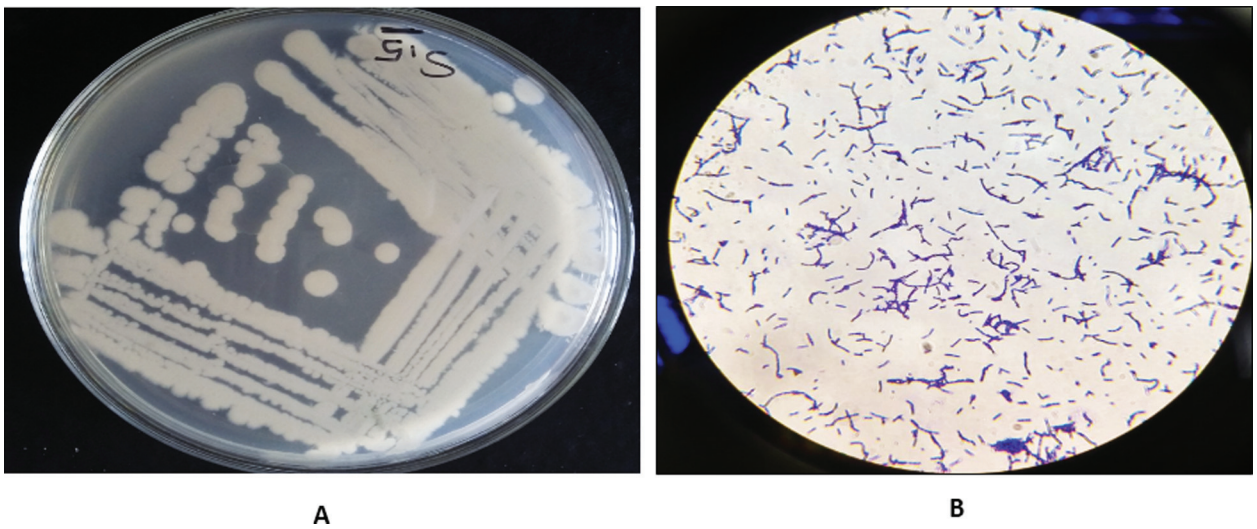
Figure 1



(a) Secondary screening (quantitative) for cellulolytic bacteria by evaluating glucosidase activity; (b) primary screening (qualitative) for cellulolytic bacteria by covering the petri dishes with Congo red solution.

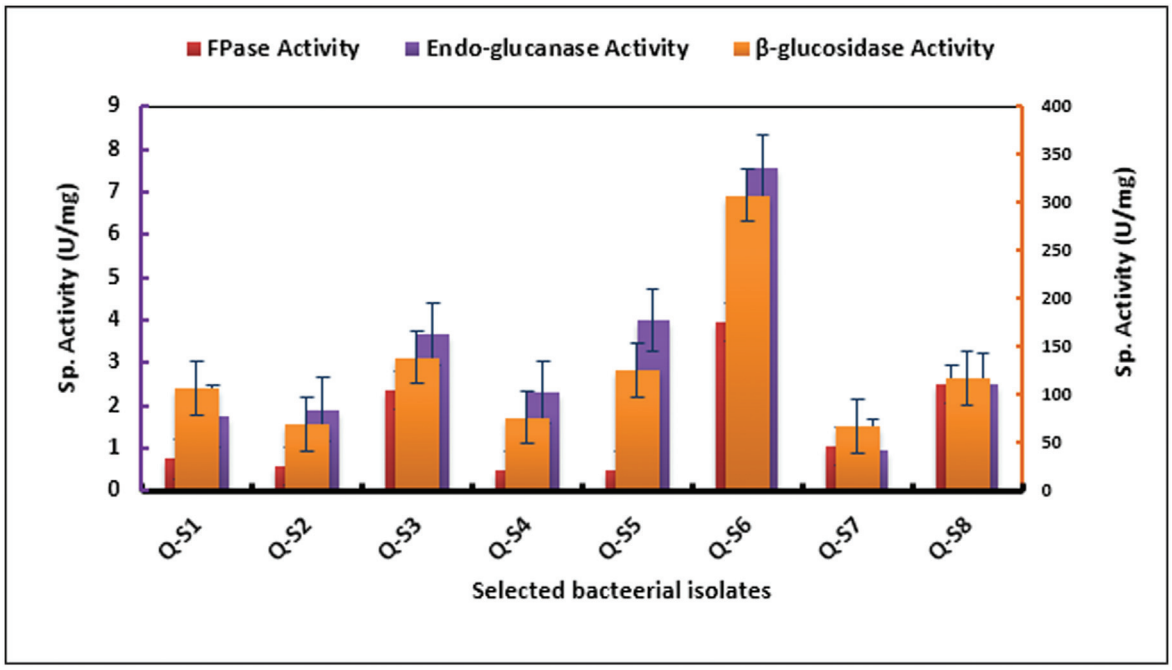
Figure 2



Morphological characteristics of QS6 bacterial isolate: (a) morphological characteristics of purified colony; and (b) microscopic characterization of microorganism.

catalytic potential [5]. Cellulase production is inducible in bacteria and is significantly influenced by nutritional composition and physical process parameters such as incubation period, temperature, pH and agitation speed [6]. Optimization of both nutritional and production parameters can considerably improve cellulase production in bacteria and plays a significant role in development of industrial bioprocess for enzyme production [7]. An ideal practice to optimize such parameters is response surface methods (RSM). This method employs a statistical experimental design and provides statistically validated predictions to ease the optimization process [7]. The 16S ribosomal RNA (rRNA) genes have been the most predominantly used molecular markers for bacterial classification. The bacterial 16S rRNA gene is ~1500bp long and contains both conserved and variable regions that evolve at different rates. The slow evolution rates of the former regions enable the design of universal primers that amplify genes across different taxa, whereas fast-evolving regions reflect differences between species and are useful for taxonomic classification [8]. The relatedness between bacterial species can be studied by the construction of phylogenetic tree or dendrogram using freely available tree-making software such as MEGA7.0. The phylogenetic tree confirms the genus to which

Figure 3



Secondary screening (quantitative) for cellulolytic bacteria by evaluating the endoglucanase, FPase activity, and glucosidase activity.

Table 1 Primary screening (qualitative) for cellulolytic bacteria by zone of clearance surrounding the colonies is indicative of hydrolysis by secreted CMCase

Isolate code	Source	Colony size	Clear zone (mm)
Q-S1	Soil	2.0	–
Q-S2	Soil	3.0	–
Q-S3	Soil	4.0	–
Q-S4	Soil	0.9	–
Q-S5	Soil	1.5	22±0.02
Q-S6	Soil	1.0	33±0.11
Q-S7	Soil	1.5	24±0.13
Q-S8	Soil	3.0	–

the query sequence strain belongs and its closest neighbors by comparing with other sequences from database followed by which further genotypic, chemotaxonomic, and phenotypic analysis platforms are designed [9]. The objective of this work is to isolate and provide molecular identification of some high β-glucosidase-producing microorganisms from soil in the Egypt, and to experimentally find out the favorable conditions required in one of them to produce maximum amount of β-glucosidase, so that it could be used on an industrial scale.

Materials and methods

Screening of cellulolytic producing strains

For isolation of cellulolytic microorganisms, 1 g of different soil samples was collected from the Qalubia governorate, Egypt, and was transferred to the fresh 100 ml salt medium [(g/l): CMC, 10; $NaNO_3$, 0.5; K_2HPO_4, 1.0; $MgSO_4$. $7H_2O$, 0.5; KCl, 0.5; $FeSO_4$; $7H_2O$, 0.001], containing CMC as the sole carbon source in 500 ml sealed bottles for incubation at 37°C for 48 h. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (0.1% w/v) for 15 min. The excess Congo red solution was poured off, and the plates were further treated by flooding with 1 mol/l NaCl for 15 min. The ratio of the clear zone diameter was measured to select the highest cellulase-producing bacterium. Isolates were maintained on CMC plates for additional experiments and also stored in 15% glycerol at −80°C for future use [10].

Identification of selected cellulolytic producing strains

The isolated microorganism was further identified based on morphological, biochemical, and physiological characteristics as described in Bergey's Manual of Systematic Bacteriology [11].

Preparation of crude enzyme

The highest cellulase-producing strain was inoculated in production medium at pH 7 and incubated at 37°C for 48 h. After incubation, the supernatant obtained after centrifugation served as a crude enzyme source for cellulases assay [12].

Determination of filter paper assay

Filter paper activity of the culture filtrates was determined according to the method of Miller [13]. Whatman filter paper containing 50 mg weight was

Table 2 Secondary screening (quantitative) for cellulolytic bacteria by evaluating the endoglucanase, FPase activity, and β-glucosidase activity

Isolate code	Growth at OD_{600}	Protein content (mg/ml)	β-glucosidase activity		Endoglucanase		FPase activity	
			U/ml	Sp. activity (U/mg)	U/ml	Sp. activity (U/mg)	U/ml	Sp. activity (U/mg)
Q-S1	1.120	0.121±0.05	12.68±0.14	106.62±0.43	0.21±0.02	1.73±0.12	0.09±0.00	0.74±0.02
Q-S2	1.110	0.214±0.04	14.18±0.33	70.33±0.88	0.41±0.11	1.91±0.24	0.12±0.02	0.56±0.03
Q-S3	0.999	0.174±0.01	24.29±0.18	139.33±0.35	0.64±0.08	3.67±0.13	0.41±0.03	2.35±0.04
Q-S4	1.120	0.221±0.03	16.35±0.26	76.28±0.21	0.51±0.09	2.30±0.22	0.11±0.01	0.49±0.11
Q-S5	1.023	0.184±0.05	28.52±0.25	125.65±0.41	0.84±0.03	4.56±0.08	0.09±0.02	0.48±0.10
Q-S6	1.022	0.124±0.03	33.79±0.33	307.58±0.21	0.94±0.03	7.58±0.07	0.49±0.01	3.95±0.81
Q-S7	1.099	0.233±0.01	15.28±0.16	66.73±0.11	0.22±0.01	0.95±0.11	0.24±0.05	1.03±0.12
Q-S8	1.231	0.189±0.02	22.37±0.11	117.14±0.08	0.47±0.02	2.48±0.13	0.47±0.07	2.48±0.21

Table 3 Morphological, biochemical, and physiological characteristics of QS6 bacterial isolate

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

+, positive; –, negative.

suspended in 1 ml of 0.05 mol/l sodium citrate buffer (pH 4.8) at 50°C in a water bath. Suitable aliquots of enzyme source were added to the aforementioned mixture and incubated for 60 min at 37°C. After incubation, the liberated reducing sugars were estimated by the addition of 3, 5-dinitrosalicylic acid. One unit of filter paper unit was defined as the amount of enzyme releasing 1 μmol of reducing sugar from filter paper/ml/h.

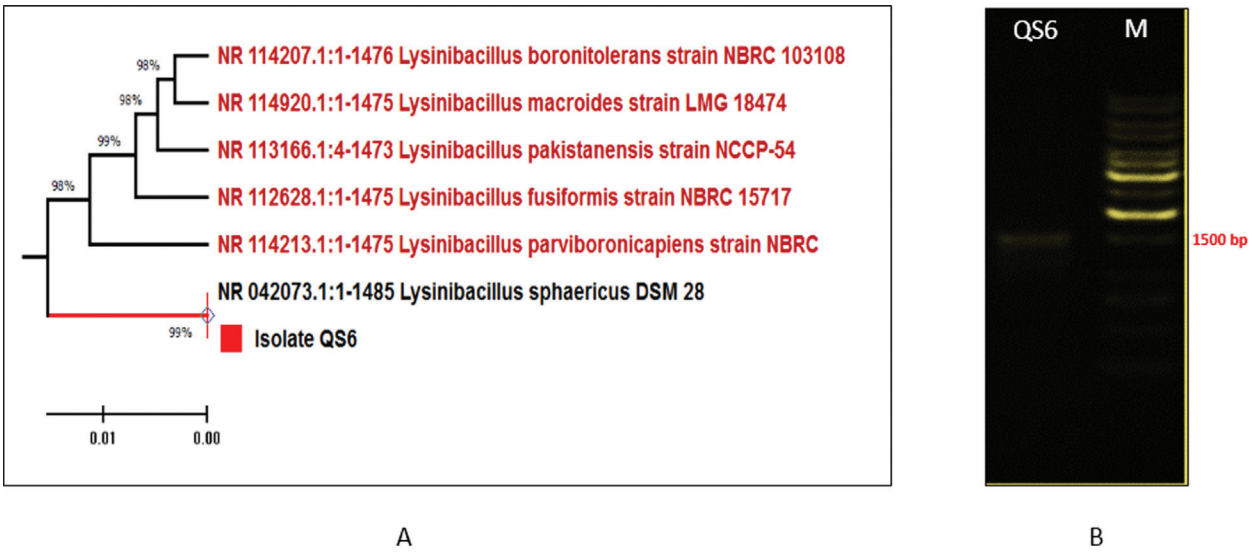
Determination of endoglucanase activity

Endoglucanase activity was quantified by carboxymethylcellulose method [14]. The reaction mixture with 1.0 ml of 1% carboxymethylcellulose in 0.2 mol/l acetate buffer (pH 5.0) was preincubated at 37°C in a water bath for 20 min. An aliquot of 0.5 ml of culture filtrate was added to the reaction mixture and incubated at 37°C for an hour. The reducing sugar produced in the reaction mixture was determined by 3, 5-dinitrosalicylic acid method. One unit of enzyme was defined as the amount releasing 1 μmol of reducing sugar/ml/h.

Determination of β-glucosidase activity

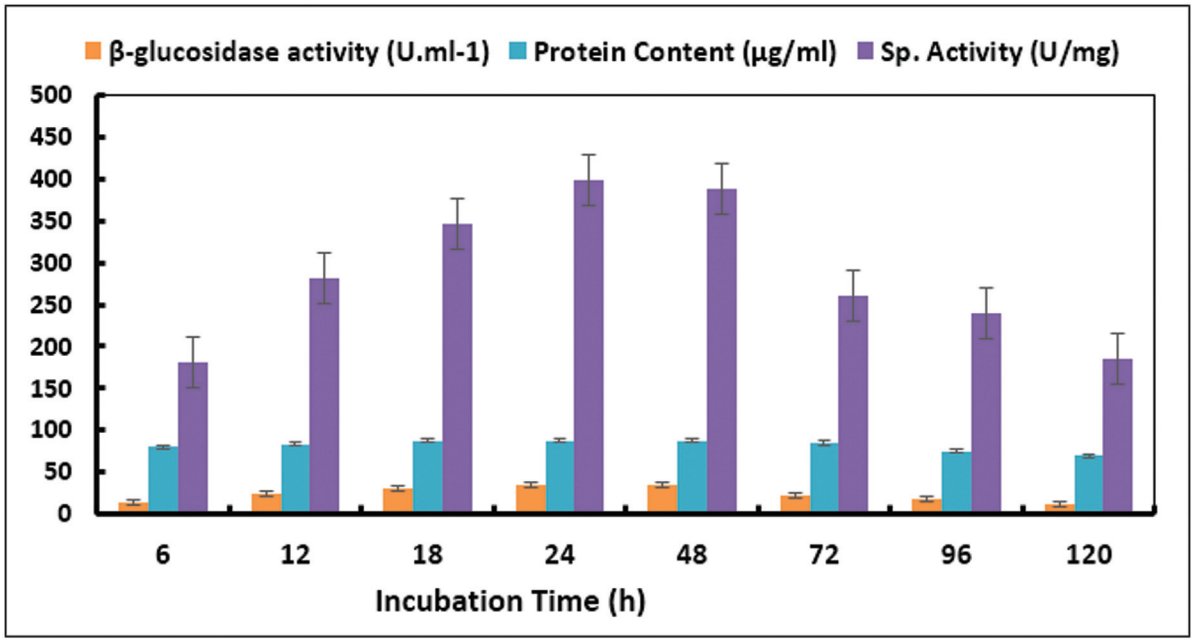
The β-glucosidase activity was determined with 100 μl of enzymatic extract, 250 μl of sodium acetate buffer (0.1 mol/l, pH 6.5), and 250 μl of p-nitro phenyl-β-D-glucopyranoside (5 mmol/l, pNPβG; Sigma Chemical Co., USA) during 30 min reaction at 37°C. The reaction was stopped with 2 ml of sodium carbonate (2 mol/l), and the liberated product was spectrophotometrically quantified at 410 nm. One unit of enzyme was defined as the amount of

Figure 4



(a) Neighbor-joining phylogenetic tree showing relationship of strain QS6 with different types of strain of the genus *Lysinibacillus* spp. The tree was generated using MEGA X software; (b) 1.5% agarose gel electrophoresis of PCR product of the isolated QS6. Lane 1: DNA marker 1 kb, and lane 2: isolate QS6.

Figure 5



Effect of incubation time on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6.

enzyme required to release 1 µmol of nitro phenol per minute of reaction [15].

Determination of total protein

Soluble proteins in the culture supernatant were estimated by dye-binding method of Bradford using bovine serum albumin (Sigma Chemical Co.) as a standard [16].

Molecular identification of QS6 strain using 16s rDNA gene sequencing

Isolation of bacterial genomic DNA

For molecular identification of most potent β-glucosidase-producing strain (QS6), DNA extraction was performed using Promega wizard Genomic DNA purification Kit (Promega Company, WI, USA) according to the manufacturer's protocol.

Table 4 Effect of physical factor on β-glucosidase production

Physical factor	β-glucosidase activity (IU/ml^{-1})	Protein content (μg/ml)	Specific activity (U/mg)
Incubation time			
6 h	14.48±0.087	81.67±0.881	184.43±1.770
12 h	23.66±0.038	84.33±0.333	283.31±1.234
18 h	30.38±0.078	87.66±0.331	343.01±1.732
24 h	35.17±0.026	86.33±0.881	399.93±0.635
48 h	34.25±0.041	88.33±0.332	389.52±0.742
72 h	22.17±0.014	85.01±0.577	263.33±1.763
96 h	18.17±0.029	74.67±1.452	241.35±0.881
120 h	12.91±0.043	74.01±2.081	184.01±1.154
Temperature			
25°C	22.29±0.416	82.66±1.451	274.02±2.516
30°C	27.49±0.690	91.33±0.881	317.33±1.201
35°C	37.25±0.161	101.0±0.577	373.00±2.081
40°C	32.79±0.588	109.0±0.577	306.66±0.881
45°C	30.54±0.632	100.0±1.732	299.00±0.577
50°C	28.34±0.454	102.3±1.763	281.66±1.201
55°C	22.45±0.297	91.33±0.881	247.33±0.881
60°C	19.50±0.329	75.33±0.881	211.66±1.201
pH			
3	17.82± 0.318	80.33±0.881	215.36± 1.329
4	22.88± 0.502	90.12±1.154	246.02± 1.151
5	26.59± 0.202	89.38±1.201	290.16± 1.301
6	27.64± 0.112	90.33±0.333	300.33± 0.333
7	36.38± 0.281	100.6±0.881	366.33± 0.881
8	33.66± 0.276	99.67±0.333	331.40± 0.871
9	24.40± 0.290	109.3±0.881	227.66± 0.726
10	12.54± 0.313	90.33±1.201	136.01± 0.577
Inoculum size			
0.5%	32.86±0.204	80.33±0.881	415.38±0.898
1%	37.32±0.308	89.66±0.881	422.97±1.301
2%	27.58±0.207	90.66±0.666	305.09±0.230
4%	26.24±0.298	92.01±0.577	291.66±1.201
6%	22.33±0.271	100.2±0.577	220.01±0.577
10%	18.28±0.232	101.1±0.962	182.66±0.881

PCR amplification of 16s rRNA gene

Amplification was done using forward primer 8F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse primer 1492R (5′-GGG CGG GGT GTA CAA GGC-3′). The PCR mixture was carried out in a volume of 50 μl, contained 22 μl of MQ, 25 μl of DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, USA), 1 μl of each forward and reverse primer (10 μmol/l each, IDT synthesized), and 1 μl of template. The PCR amplification conditions were 4 min of preheating at 95°C, 30 s denaturation at 95°C, 45 s of primer annealing at 50°C, 1 min extension step at 72°C, and postcycling extension of 10 min at 72°C for 35 cycles. The reactions were carried out in a thermal cycler (Applied Biosystem Thermal Cycler, USA) [17].

Sequence alignment, phylogenetic analysis, and bioinformatics analysis

Amplified PCR product was purified and sequenced at Macro gene, Korea. Raw data of sequencing were edited (contig and peak chromatogram verification) using the Finch T.V 1.4.0 program. Analysis of 16 s rRNA sequences of QS6 strain was performed using the BLAST (N) program of the National Center of Biotechnology Information (NCBI) (Rockville Pike, Bethesda MD, USA). Multiple sequence alignment was done using the ClustalX 2.1 program. The phylogenetic trees were constructed using neighbor-joining method by MEGA. X [18].

Nucleotide sequence accession number

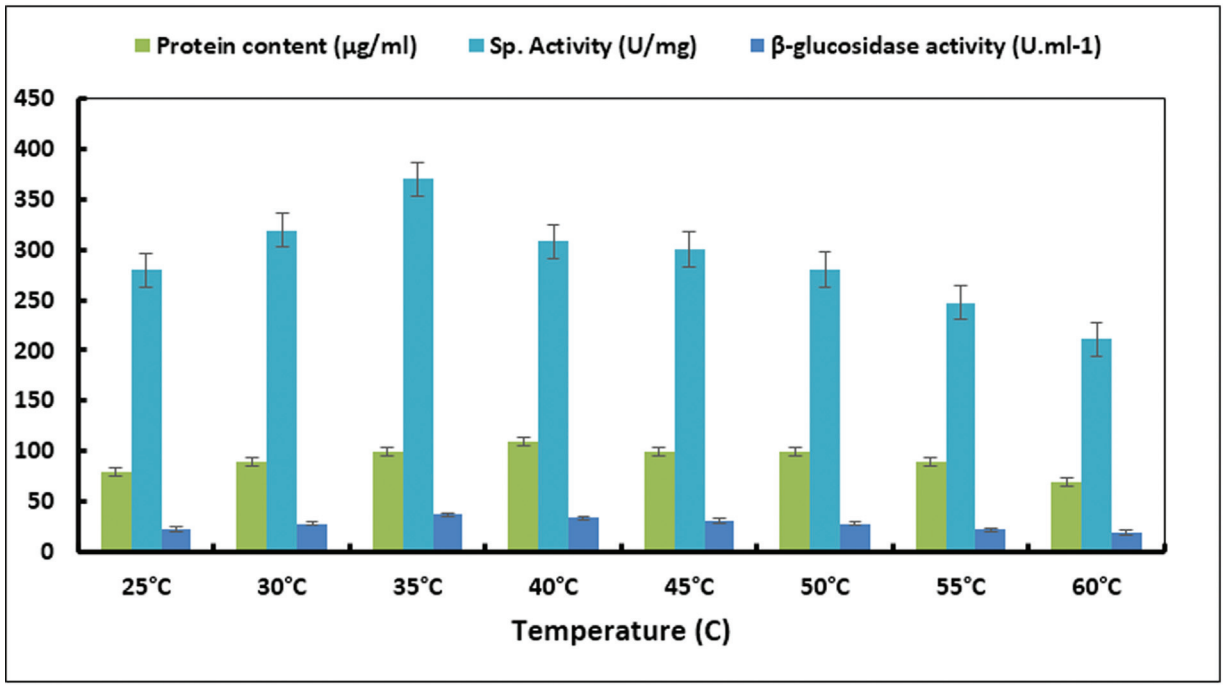
The 16s rRNA gene sequence of the isolated *Ly*sinibacillus *sphaericus* QS6 was determined, edited. and submitted to GenBank database under accession number MN493725.1.

Optimization of cultural condition for β-glucosidase production

Effect of incubation time

Incubation period was an important parameter for enzyme production. To determine the optimum

Figure 6



Effect of temperature on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6 for 24 h.

incubation period of the isolate *L. sphaericus* QS6 for maximum β-glucosidases production, the supernatant was collected after 6, 12, 18, 24, 48, 72, 96, and 120 h of incubation.

Effect of temperature

To determine the effective temperature for β-glucosidase production by *L. sphaericus* QS6, the experiment was carried out at 25, 35, 40, 45, 50, 55, and 60°C for 24 h.

Inoculum size

To determine the effective inoculum size for β-glucosidase production by *L. sphaericus* QS6, aliquots (50 ml) of the production medium were inoculated with different inoculum size of the selected strain (0.5, 1, 2, 4, 6, 10% v/v) at 35°C for 24 h.

Effect of pH

The effect of pH on β-glucosidases production was studied by varying pH levels (3, 4, 5, 6, 7, 8, 9, and 10). Selected medium of different pH was inoculated with the *L. sphaericus* QS6 isolate at 35°C for 24 h. with 1% inoculum size [19].

Different carbon sources

Different carbon sources, such as glucose, fructose, mannitol, sucrose, lactose, cellobiose, salicin, CMC, cellulose, starch, and avice (1 g/l), were separately added as a sole carbon source. At the end of incubation, the β-glucosidase activity was assayed. Carbon sources were autoclaved separately and added to the medium under aseptic conditions [19].

Different of nitrogen source

The liquid medium was separately supplemented with different nitrogen sources, such as yeast extract, beef extract, peptone, soymeal, urea, sodium nitrate, ammonium chloride, potassium nitrite, ammonium sulfate, and ammonium nitrate (1 g/l), at the end of incubation, the β-glucosidase activity was assayed as studied by Jahangeer [19].

Antibacterial activity

The examination of antimicrobial activity was based on the in-vitro agar well diffusion method according to Hindler *et al.* [20]. The prepared crude was tested against standard microorganisms of gram-positive bacteria (*Enterococcus faecalis*, *Bacillus cereus*, *Bacillus. lichniforms*, *Bacillus thuringiensis*, *Enterobacter ludwigii*, and *Bacillus subtilis*) and gram-negative bacteria (*Pseudomonas aeruginosa*, *Pseudomonas putida*, *Klebsiella pneumonia*, and *Escherichia coli*) at a concentration of 100 µl per well. The diameter of the inhibition zones was measured (mm) at 37°C after 24 h.

Table 5 Effect of nutritional factor on β-glucosidase production

Nutritional factor	β-glucosidase activity (IU/ml)	Protein content (μg/ml)	Specific activity (U/mg)
Nitrogen sources			
Yeast extract	23.03±0.095	80.33±0.881	289.71±1.131
Beef extract	26.31±0.071	99.01±0.577	263.96±0.956
Peptone	27.71±0.202	85.22±0.577	322.33±0.883
Soyameal	17.59±0.120	111.2±1.154	158.46±0.573
Urea	12.69±0.312	143.1±0.577	88.81±2.539
Sodium nitrate	19.66±0.279	105.4±0.577	185.33±0.882
Ammonium Chloride	17.88±0.361	57.33±1.452	244.45±0.714
Potassium nitrite	20.45±0.259	78.33±0.881	259.66±0.881
Ammonium sulfate	23.63±0.300	124.6±1.452	187.31±0.681
Ammonium nitrate	21.22±0.455	112.8±0.577	185.16±1.301
Peptone (%)			
0.5	37.00±0.103	94.41±0.429	392.66±1.201
1	34.28±0.116	100.01±0.577	344.21±0.577
2	30.76±0.321	104.66±0.881	290.33±0.881
4	26.21±0.166	111.12±0.577	233.32±1.458
6	22.54±0.135	102.22±0.577	125.31±0.566
8	14.95±0.223	123.31±0.881	119.28±0.676
10	10.576±0.245	114.62±0.881	92.87±0.425
Carbon sources			
Glucose	29.69±0.380	138.6±0.881	208.27±0.546
Fructose	23.80±0.138	120.3±0.881	193.44±0.496
Lactose	24.66±0.336	100.1±0.577	248.01±0.577
Sucrose	13.47±0.454	109.2±0.577	113.43±0.808
Cellobiose	35.18±0.527	89.23±0.577	399.56±0.721
CMC	19.08±0.156	111.3±0.745	178.33±0.674
Avical	21.92±0.139	121.6±0.881	184.16±0.440
Salicin	33.66±0.274	101.3±0.331	366.38±0.881
Starch	23.05±0.504	120.3±0.321	185.16±0.440
Mannitol	8.52±0.7092	110.4±0.231	64.89±0.757
Cellulose	13.17±0.172	140.5±0.577	92.65±0.333
Cellobiose (%)			
0.5	22.59±0.224	82.25±1.154	271.33±0.881
1	35.25±0.044	85.66±1.201	413.91±1.069
2	30.76±0.321	138.3±0.881	222.33±0.881
4	29.11±0.287	152.3±1.452	191.01±0.577
6	24.74±0.315	108.3±1.201	181.67±0.881
8	15.26±0.407	96.68±0.577	154.62±1.452
10	14.03±0.096	97.76±1.154	140.01±0.577

CMC, carboxy methyl cellulose.

Antibiotic susceptibility

Strain *L. sphaericus*. QS6 was tested for its susceptibility to some antibiotic. The test was carried out by diffusion in Müller-Hinton agar. Antibiotic discs included amoxicillin (AX, 25 μg), streptomycin (S, 10 μg), tetracycline (TE, 30 μg), cefadroxil (CFR, 30 μg), kanamycin (K, 10 μg), chloramphenicol (C, 30 μg), ampicillin (AM, 10 μg), erythromycin (E, 10 μg), nalidixic (NA, 30 μg), and tobramycin (TOB, 10 μg) [20].

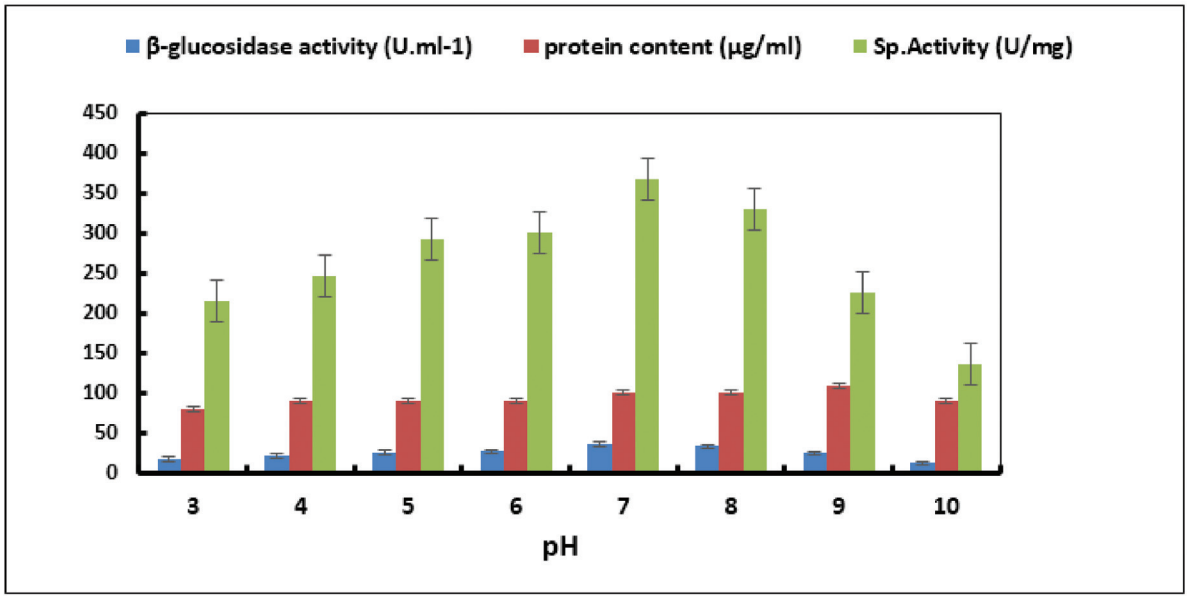
Results and discussion

Identification of producer organism

For isolation of cellulose-degrading bacteria, samples were collected from agricultural soil in Egypt, as it contains cellulose-degrading bacteria in it, because of the soil being rich with lignocellulose biomass. The colony morphology was studied in detail, and the results are presented in Fig 2 and Table 3, followed by biochemical tests performed for the isolated microorganisms (Table 3). The collected samples were then subjected to serial dilution technique and were inoculated onto a plate containing CMC agar medium and incubated at 37°c for 24–48 h (Fig. 1).

After incubation, Cong red test was performed for screening of the cellulolytic organisms. The strains showing maximum clear zones around the colonies were selected for further studies. A total of eight bacterial isolates were found to be positive on screening media (cellulose Cong red agar) by

Figure 7



Effect of pH on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6 for 24 h. at 35°C.

producing clear zone around their colonies (Figs 2 and 3 and Table 1). Furthermore, QS6 was selected for enzyme production and their respective cellulolytic activity was estimated.

The β-glucosidase activity was measured by PNPG method. Among the potent β-glucosidase producers, strain QS6 showed the highest β-glucosidase activity (33.79±0.33 U/ml) at 24 h after incubation. Therefore, strain QS6 was used for future study and identification through 16 s rRNA sequence analysis and phenotypic characterization. Based on the results of primary screening and secondary screening, QS6 isolate was selected for further study because it had the highest cellulase endoglucanase activity and β-glucosidase activity as presented in Table 2.

Morphological, physiological, and biochemical characterization

The isolated QS6 strain was initially identified and tested for primary characterization. According to morphological characteristics, the cells were rod shaped, single, gram positive, motile, and strictly aerobic, and the colonies were pale white colored, spore forming, as shown in Fig. 2, catalase negative, and produced ammonia from arginine hydrolysis, which is the main characteristic feature, as observed with all bacilli. The culture could grow at different temperatures 30–37°C, pH (5.0–8.0), and NaCl concentrations (1–10%) (Table 3).

To identify the isolated strains, we used the microscopic examination techniques, and biochemical test. Considering a previous report [14], the stain QS6 showed positive results in catalase test, oxidase test, gelatin test, nitrate reduction, and esculin test.

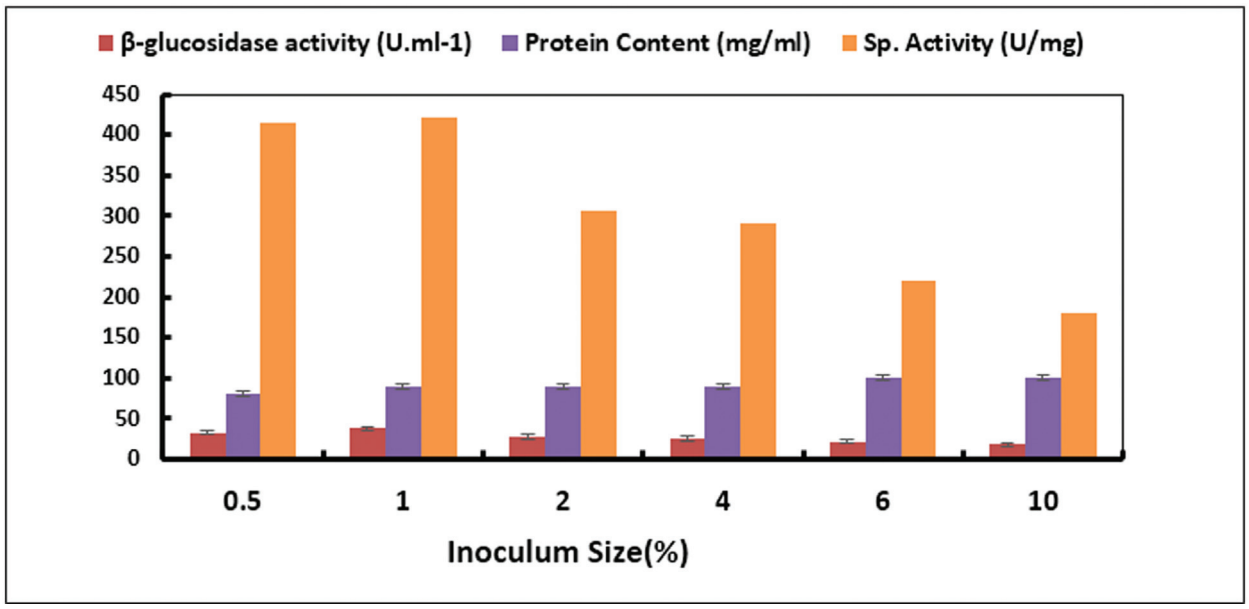
These results correlate that the organisms resemble *L. sphaericus* strain QS6. The isolates were identified as belonging to member of the genus Bacillus sp. QS6 strain was identified as *Lysinibacillus* spp. (Table 3). All the biochemical and physiological tests were found to be *Lysinibacillus* spp. The result is in consistent with previous reports [21].

Phylogenetic analysis of the producer microorganism

To identify the QS6, a fragment of 16S rDNA was generated and sequenced. The sequencing result was aligned online in the nucleotide BLAST tool through the (NCBI) database to identify the possible genera of the isolates base on homology. From BLAST search results, QS6 isolate has 97% homology to 16S rDNA fragment of *Lysinibacillus* spp. CMJ2-5 (accession number NR 042073.1). Then, 16S rDNA sequences of QS6 strain was deposited in NCBI database with an accession number MN493725.1. Evolutionary analyses were conducted in MEGA X [18]. Based on the homology and phylogenetic analysis (Fig. 4), it was concluded that the isolated QS6 culture was *L. sphaericus.*

The alignment of the 16S rRNA nucleotide sequence of QS6 bacterial isolate comprised 1440 bp. The 16SrRNA stated gene sequence was coordinated in the gene bank database through the NCBI BLAST

Figure 8



Effect of inoculums size on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6 for 24 h at 35°C, pH 7.

(http://www.ncbi.nlm.nih.gov). A correlation between the 16S rRNA sequence of this strain and those individuals in the genomic database bank was accomplished. This correlation demonstrated an extraordinary level of sequence similarity (98%) with *Lysinibacillus sp*.

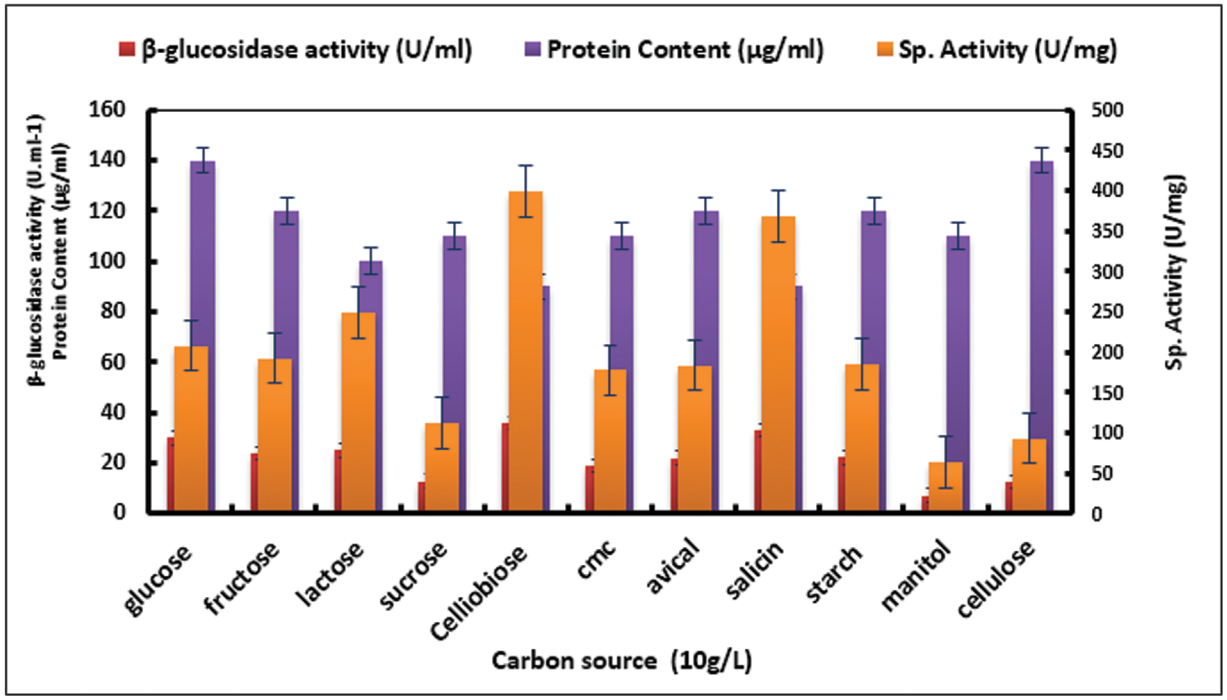
Effect of incubation period on β-glucosidase production

The effect of incubation time on enzyme production was studied from 6 to 120 h. using cellobiose as substrate. The production increased with increase in incubation period, and for *L. sphaericus* QS6, β-glucosidase activity reached maximum (35.17 ±0.026 IU) at 24 h after incubation. The β-glucosidase activity and protein content determined ranged from 14.48±0.087 IU/ml and 81.67±0.881 µg/ml at 6 h. to maximum 35.17±0.026 IU/ml and 86.33±0.881 µg/ml at 24 h. The optimum incubation time for the β-glucosidase production was found at 24 h with the higher 88 µg/ml of protein content and enzyme activity of 35.12 U/ml. It was also observed that the enzyme activity and protein showed decreasing pattern after 24 h. However, the minimum yield of the protein content of 74.01 ±2.081 µg/ml and enzyme activity of 12.91±0.043 IU/ml was observed at 120 h (Fig. 5 and Table 4). At the same incubation period (24 h) [22], much lesser FPase (0.011 IU/ml) and CMCase (0.079 IU/ml) activities by *Bacillus pumilus* EB3 were recorded. Decrease in enzymatic activity with time might be owing to the depletion of nutrients and production of other by-products in the fermentation medium [23]. Our results revealed that, the enzyme production was increased until 24 h, and with further increase in the incubation period, decreased enzyme activity was seen. Thus, 24 h was considered as the optimum incubation period for the production of β-glucosidase by *L. sphaericus* strain QS6.

Effect of temperature on β-glucosidase production

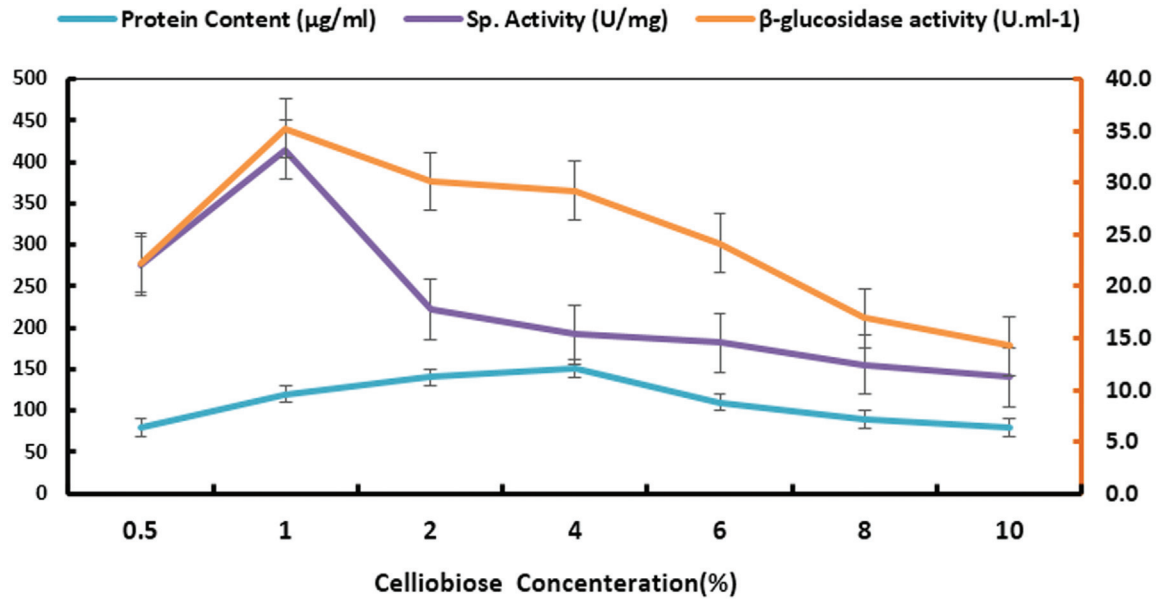
Temperature is one of the most important parameters essential for the success of a fermentation process. For β-glucosidase production by *L. sphaericus* strain QS6, 35°C was the most effective. The production started declining after further increase in temperature. As shown in Fig. 6 and Tables 4 and 5, β-glucosidase activity was recorded as 27.49±0.690, 37.25±0.161, 32.79±0.588, and 32.79±0.588 IU/ml at 30, 35, 40, and 45°C, respectively. The activity of β-glucosidase changes slightly with temperature, but there is marked increase in the activities of β-glucosidase at incubation temperature of 35°C. Our results revealed that 35°C was considered as the optimum temperature for the production of β-glucosidase by *L. sphaericus* strain QS6. Similar observations was observed by Acharya and Chaudhary [14], who reported similar kinds of results for *Bacillus licheniformis* and *Bacillus* spp. Moreover, Gondalez *et al*. [24] reported that higher β-glucosidase activities was obtained from *Trichoderma reesei* GM9414 on wheat straw as carbon source. Kato [25] reported optimum temperature for growth and cellulose degradation of *C. straminisolvens* at 50 to 55°C, and Immanuel [26] recorded maximum

Figure 9



Effect of carbon sources on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6 for 24 h at 35°C, pH 7.

Figure 10



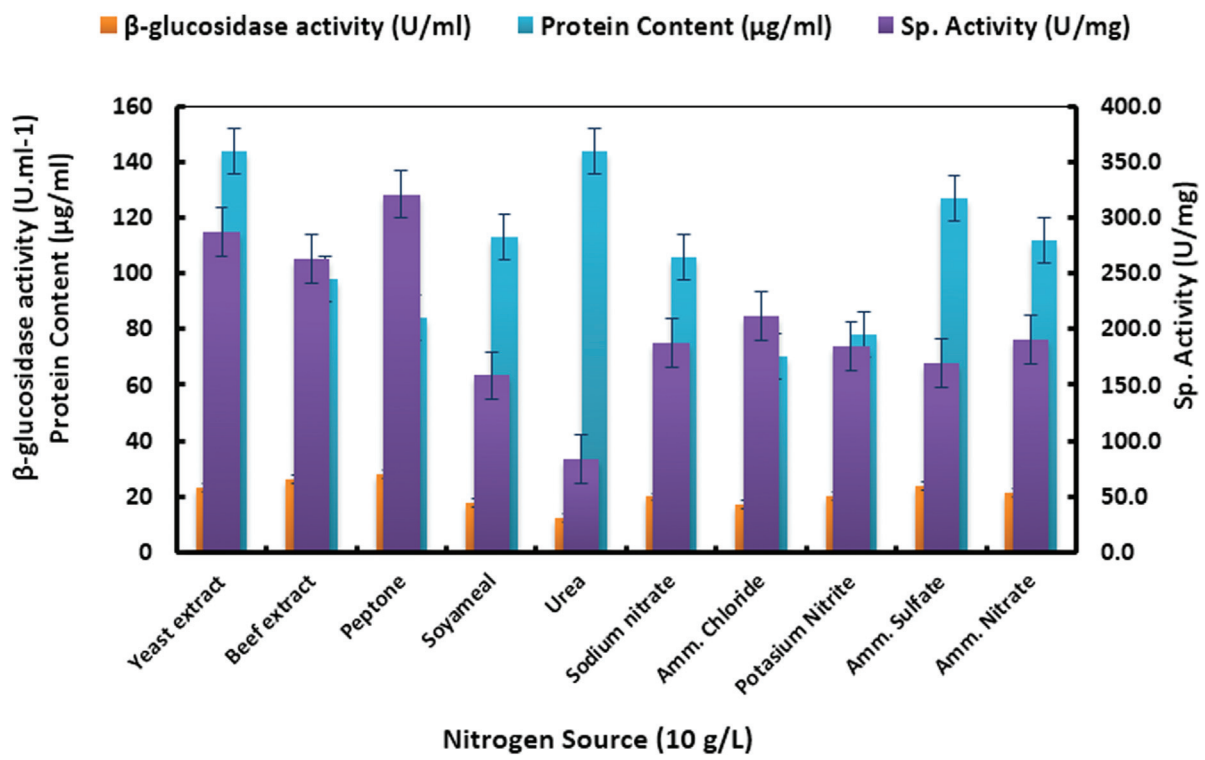
Effect of cellobiose concentration on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6 for 24 h at 35°C, pH 7.

endoglucanase activity in *Cellulomonas*, *Bacillus*, and *Micrococcus* spp. at 40°C at neutral pH.

Effect of pH on β-glucosidase production

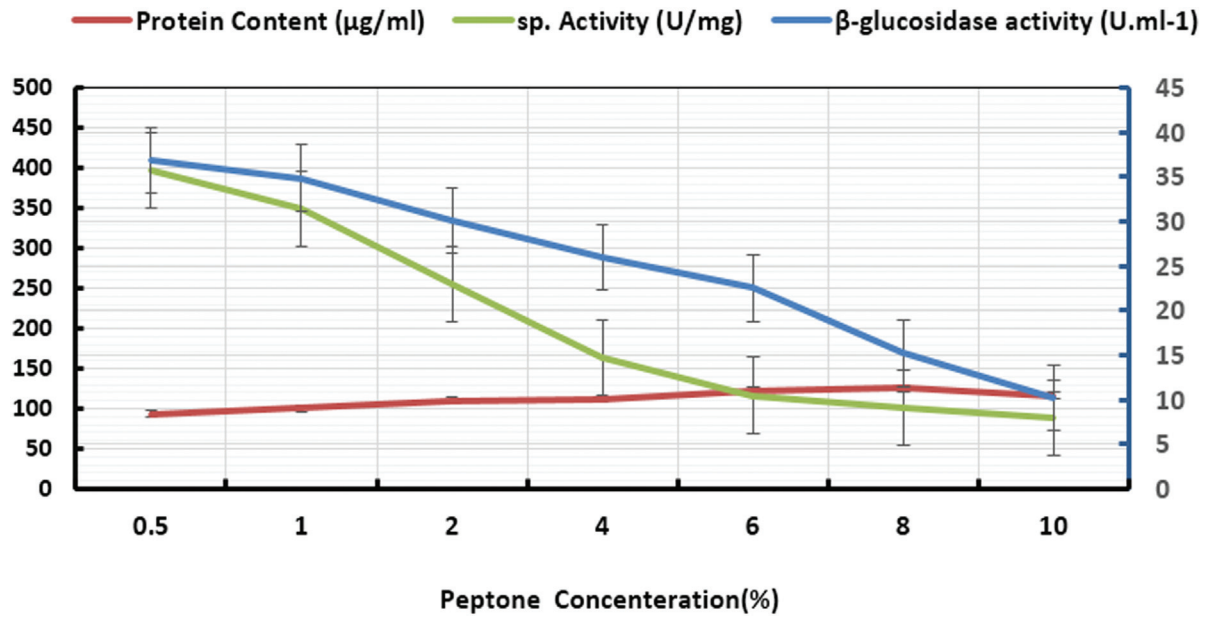
Like temperature, hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. It has been shown that growth medium pH strongly influences many enzymatic reactions by affecting the transport of a number of chemical products and enzymes across the cell membrane [27]. The effect of pH on β-glucosidase production by *L. sphaericus* strain QS6 was investigated by growing culture at different initial pH between 3 and 10. A high level of β-glucosidase activity was obtained in the culture medium with pH 7 with β-glucosidase

Figure 11



Effect of nitrogen sources on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6 for 24 h at 35°C, pH 7.

Figure 12



Effect of Peptone concentration on protein content and glucosidase production in *Lysinibacillus sphaericus* strain QS6 for 24 h at 35°C, pH 7.

production at 36.38±0.281 IU/ml and specific activity at 366.33±0.881 U/mg. As presented in Fig. 7, there was a decrease in β-glucosidase activity from 36.38 ±0.28 to 12.54±0.313 IU/ml on increasing the pH from 7 to 10. The obtained results are inconsistence with several researchers, such as Immanuel [26], who reported that the cellulolytic enzyme, endoglucanase, from *Cellulomonas*, *Bacillus*, and *Micrococcus* spp., isolated from the estuarine coir netting effluents hydrolyzes substrate in the pH range of 4.0–9.0, with maximum activity at pH 7.0. Contrary to that, Lee et al [28] observed optimal cellulase production at

Table 6 Antibiotic susceptibility and antibacterial activity on β-glucosidase-producing *Lysinibacillus sphaericus* QS6 strain

No.	Antibiotic susceptibility				Antibacterial activity	
	Antibiotic	Abbreviation	Concentration (μg/disc)	Inhibition (mm)	Microorganism	Inhibition (mm)
1	Amoxicillin	AX	25	**S** (33)	*Escherichia Coli*	**+ (19)**
2	Streptomycin	S	10	**S** (13)	*Bacillus Subtilis*	–
3	Tetracycline	TE	30	**S** (38)	*Enterococcus. faecalis*	–
4	Chloramphenicol	C	30	**S**(16)	*Pseudomonas Aeruginosa*	**+ (10)**
5	Cefadroxil	CFR	30	**R** (0.0)	*Klebsiella pneumonia*	–
6	Nalidixic	NA	30	**S** (10)	*Bacillus Cereus*	–
7	Kanamycin	K	10	**S** (18)	*Bacillus Lichniforms*	–
8	Ampicillin	AM	10	**I** (06)	*Bacillus Thuringiensis*	–
9	Erythromycin	E	10	**S** (24)	*Pseudomonas putida*	–
10	Tobramycin	TOB	10	**S** (12)	*Enterobacter ludwigii*	

pH 9.0 by *Clostridium acetobutylium*. Our results revealed that pH 7.0 was considered as the optimum pH for the production of β-glucosidase by *L. sphaericus* strain QS6.

Effect of inoculum size on β-glucosidase production

For optimum enzyme production in fermentation medium, inoculum size was optimized, and 1% inoculum size was found to be better for maximum enzyme production (Fig. 8). Highest levels of β-glucosidase activity and protein (38.13 IU/ml and 90 μg/ml, respectively) were produced when strain QS6 was grown on Lb. along with cellobiose at 1% for 48 h at 40°C. For production of raw starch-hydrolyzing amylase by *Bacillus spp.*, 2% inoculum was recommended [29] Inoculum size (2–3%) was also found to be optimum for cellulases produced by *Bacillus subtilis CY5* and *Bacillus circulans TP3* [30].

Effect of different carbon sources on β-glucosidase production

The results of the effect of various carbon sources on β-glucosidase production by *L. sphaericus* QS6 are illustrated in Fig. 9. Among all the carbon sources tested, cellobiose gave maximum β-glucosidase activity when used as a sole carbon source.

In our results, different carbon sources were examined to study their effects on *L. sphaericus* strain QS6 β-glucosidase production under identical conditions (temperature, 35°C; 1% inoculum size; incubation time, 24 h). The production of β-glucosidase in *L. sphaericus* strain QS6 was found to be maximum protein content of 89.23±0.577 μg/ml and enzyme activity at 35.18±0.527 U/ml with cellobiose at 24 h of incubation. Starch and cellulose yielded the least enzyme activity of 23.05±0.504 and 13.17±0.172 U/ml, respectively, with protein content of 50 and 40 μg/ml, 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. 10).

β-glucosidase production by *L. sphaericus* strain QS6 was studied by testing β-glucosidase secretion in the culture medium using different substrate cellobiose concentrations at 35°C for 24 h at pH.7. Cellobiose is used as a substrate for β-glucosidase production owing to its less complexity and easy digestion by the microbes [31]. The different concentrations of cellobiose were tested for β-glucosidase production, among which 1% cellobiose was optimum for this strain (Fig. 10). 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 [12].

Effect of different nitrogen sources on β-glucosidase production

The nitrogen source in the control was better over other treatments for both CMCase and FPase production for both wheat and rice straw. The present results showed lower β-glucosidase activity with inorganic nitrogen sources, which suggested the reduced utilization of inorganic nitrogen by aerobic bacteria. These data were in accordance with the results of Ray *et al.* [30] who reported that organic nitrogen sources were more suitable for optimizing the cellulase production by *B. subtilis* and *B. circulans* than inorganic sources. On the contrary, Spiridonov and Wilson found that NH_4 compounds were the most favorable nitrogen sources for cellulase synthesis

The results showed that strain QS6 can utilize organic nitrogen sources efficiently, and the maximum β-glucosidase activity (27.71±0.202) was observed when peptone 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 (Figs 11 and 12). 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 *et al*. [32]. Moreover, KNO3 and NH4NO3 were used as the best N sources for cellulose production in *Cellulomonas flavigena*. NH4 compounds were considered as the most favorable N sources for cellulase synthesis as noted in *Thermomonospora fusca* [33].

Antibiotic susceptibility and antibacterial activity

The antibiotic susceptibility of the *L. sphaericus* strain QS6 strain was assessed by disk diffusion using 10 antimicrobial agents (Table 6). Our data evidenced that *L. sphaericus* strain QS6 was sensitive to amoxicillin, streptomycin, tetracycline, cefadroxil, kanamycin, chloramphenicol, erythromycin, and tobramycin, but was resistant to nalidixic antibiotics, with the exception of ampicillin, where the strain showed intermediate resistance. Moreover, in-vitro antibacterial bioassay of the most potent β-glucosidase-producing strain (QS6) showed high antimicrobial activity against *Escherichia coli* (1.9 cm) and *Pseudomonas aeruginosa* (1.0 cm).

Conclusion

β-glucosidase is commercially produced by several industries globally and is widely being used in food, animal feed, fermentation, agriculture, pulp and paper, and textile applications. For this reason, we have screened β-glucosidase-producing bacteria from different ecological niches and obtained this strain of *L. sphaericus* strain QS6. The culture conditions like pH, temperature, carbon sources, and nitrogen sources were optimized. The optimum conditions found for β-glucosidase production are 35°C at pH 7 on 24-h incubation period with cellobiose (1%) as the carbon source and peptone (0.5) as the nitrogen source.

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Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Somerville C. The billion-ton biofuels vision. Am Assoc Adv Sci 2006; 312:1277.

2 Schwarz W. The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol 2001; 56:634–649.

3 Himmel ME. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 2007; 315:804–807.

4 Yin L-J, Huang P-S, Lin H-H. Isolation of cellulase-producing bacteria and characterization of the cellulase from the isolated bacterium *Cellulomonas sp*.YJ5. J Agric Food Chem 2010; 58:9833–9837.

5 Harshvardhan K, Mishra A, Jha B. Purification and characterization of cellulase from a marine *Bacillus* sp.H1666: a potential agent for single step saccharification of seaweed biomass. J Mol Catalysis B 2013; 93:51–56.

6 Yang W. Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production. Electron J Biotechnol 2014; 17:262–267.

7 Singh S, Moholkar VS, Goyal A. Optimization of carboxymethylcellulase production from *Bacillus amyloliquefaciens* SS35. 3 Biotech 2014; 4:411–424.

8 Srinivasan R. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. PLoS One 2015; 10:e0117617.

9 Ciccarelli FD. Toward automatic reconstruction of a highly resolved tree of life. Science 2006; 311:1283–1287.

10 Lo YC. Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. Enzyme Microbial Technol 2009; 44:417–425.

11 Brown JH. Bergey's manual of determinative bacteriology. Washington: American Public Health Association; 1939.

12 Shaikh N. Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulase production. Univ J Environ Res Technol 2013; 3:39–49.

13 Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959; 31:426–428.

14 Acharya S, Chaudhary A. Optimization of fermentation conditions for cellulases production by *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3 isolated from Indian hot spring. Brazil Arch Biol Technol 2012; 55:497–503.

15 de Palma-Fernandez E, Gomes E, Da Silva R. Purification and characterization of two β-glucosidases from the thermophilic fungus*Thermoascus aurantiacus*. Folia microbiologica 2002; 47:685–690.

16 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–254.

17 Rastogi G. Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. J Ind Microbiol Biotechnol 2009; 36:585.

18 Kumar S. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 2018; 35:1547–1549.

19 Jahangeer S. Screening and characterization of fungal cellulases isolated from the native environmental source. Pak J Botany 2005; 37:739.

20 Hindler J, Howard B, Keiser J. Antimicrobial agents and antimicrobial susceptibility testing. In: Howard BJ (Ed). Clinical and Pathogenic Microbiology. 2nd ed. St. Louis: Mosby 1994.

21 Tuomela M. Biodegradation of lignin in a compost environment: a review. Bioresource Technol 2000; 72:169–183.

22 Ariffin H. Production and characterization of cellulase by *Bacillus pumilus* EB3. Int J Eng Technol 2006; 3:47–53.

23 Ikram-ul-Haq UH. Cotton Saccharifying activity of cellulases by *Trichoderma harzianum* UM-11 in shake flask. J Bot 2005; 1:19–22.

24 Gonzalez G, Caminal G, Lopez-Santin J. Cellulase production on lignocellulosic materials and performance of a packed-bed reactor for enzymatic hydrolysis. J Bot 1986; 3:120–128.

25 Kato S. Clostridium straminisolvens sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. Int J System Evol Microbiol 2004; 54:2043–2047.

26 Immanuel G. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int J Environ Sci Technol 2006; 3:25–34.

27 Liang Y. Optimization of growth medium and enzyme assay conditions for crude cellulases produced by a novel thermophilic and cellulolytic bacterium, Anoxybacillus sp. 527. Appl Biochem Biotechnol 2010; 160:1841–1852.

28 Lee SF, Forsberg CW, Gibbins L. Cellulolytic activity of *Clostridium acetobutylicum*. Appl Environ Microbiol 1985; 50:220–228.

29 Avendano M, Cornejo I. Formation of a raw starch-hydrolyzing alpha-amylase by *Clostridium* 2021: effect of carbon sources. Biotechnol Lett 1987; 9:123–124.

30 Ray A. Optimization of fermentation conditions for cellulase production by B*acillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acta Ichthyologica Piscatoria 2007; 1:47–53.

31 Shanmugapriya K. Isolation, screening and partial purification of cellulase from cellulase producing bacteria. Int J Adv Biotechnol Res 2012; 3:509–514.

32 Rajoka MI, Malik KA. Cellulase production by *Cellulomonas biazotea* cultured in media containing different cellulosic substrates. Bioresource Technol 1997; 59:21–27.

33 Spiridonov NA, Wilson DB. Regulation of biosynthesis of individual cellulases in Thermomonospora fusca. J Bacteriol 1998; 180:3529–3532.