



Cellulolytic *Bacillus* May or May Not Produce β -Glucosidase Due to Their Environmental Origin – A Case Study

Lutfun Neesa*, Nasrin Jahan, Md. Abdullah Al Noman Khan, Mohammad Shahedur Rahman

Department of Biotechnology and Genetic Engineering, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh

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ABSTRACT

Microbial cellulases have been drawing attention worldwide because of their massive capacity to process the most abundant cellulosic biomass into sustainable biofuels and other valuable products. Profitable biomass conversion processes are highly dependent on the use of efficient enzymes for lignocellulose degradation. Among the cellulose degrading enzymes, β -glucosidases are essential for efficient hydrolysis of cellulosic biomass as they relieve the inhibition of the cellobiohydrolases and endoglucanases by reducing cellobiose accumulation. In this study cellulolytic bacteria with potential β -glucosidases activity were isolated and screened from biogas plant effluent and dairy effluent near Jahangirnagar University campus. From initial screening a total of 16 isolates were found to have cellulolytic activity, among them three isolates (B_1 , B_5 , D_4) were selected based on their superior results. All the three bacterial isolates were identified as *B. subtilis* (B_1), *Bacillus amyloliquefaciens* (B_5) and *B. subtilis* (D_4) respectively based on their morphological, biochemical and molecular characteristics. The β -glucosidases activity of these three potential cellulolytic bacteria was performed by measuring the release of PNP using pNPG as a substrate and interestingly D_4 strain was resulted with β -glucosidases negative where B_1 strain was found to have efficient for β -glucosidases activity.

Key words: Cellulosic biomass, Cellulase, β -glucosidase, *Bacillus subtilis*, Fermentation.

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Corresponding author: Lutfun Neesa

e-mail: l.neesa@bgeju.edu.bd

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INTRODUCTION

Large quantities of lignocellulosic wastes are generated through forestry, agricultural practices and industrial processes, particularly from agro-allied industries such as breweries, paper-pulp, textile and timber industries [1]. These lignocellulosic wastes are causes of environmental pollution otherwise lignocellulosic wastes are major natural resources of the world and major resource of renewable organic matter. As lignocellulosic biomass is an abundant and inexpensive renewable energy resource [2], attention has been drawn towards production of platform molecules for new bioproducts by microbial fermentation from glucose or other simple carbohydrates. The plant biomass

regarded as wastes are biodegradable and in natural course they are degraded by microorganisms. They can be converted into valuable bioproducts such as biofuels, chemicals, cheap energy sources for fermentations, improved animal feeds and human nutrients [3]. Thus, profitable biomass conversion processes are highly dependent on the use of efficient enzymes for lignocellulose degradation. Microorganisms bring about most of the cellulose degradation occurring in nature. They meet this challenge with the aid of a multi-enzyme system [4]. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials [5]. They are composed of independently folding, structurally and functionally discrete units called domains or modules, making cellulases [6]. Cellulase is a family of at least 3 groups of enzymes, endo-(1, 4)- β -D-glucanase, exo-(1, 4)- β -D-glucanase and β -

glucosidases. Their action is considered to be synergistic as all three classes of cellulase can yield much more sugar than the addition of all three separately. Among the cellulolytic enzyme complex, β -glucosidase plays a key role and is the most critical that catalyze the final step in cellulose and hemicellulose into glucose [7]. It is generally responsible for the regulation of the whole cellulolytic process as it not only produces glucose from cellobiose, but also reduces cellobiose inhibition, allowing endocellulase and exocellulase enzymes to function more efficiently [8]. Thus, β -glucosidases allow the cellulolytic enzymes to function more efficiently by producing glucose from cellobiose and reducing cellobiose inhibition [9].

There has been much research aimed at obtaining new microorganisms capable of producing cellulolytic enzymes with higher specific activities and greater efficiency [10]. Cellulolytic enzymes play important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes, and protozoa. The conversion of cellulose to glucose is regarded as the rate limiting step in the production of biofuels and various valuable bioproducts from lignocellulosic materials, due to high cost of cellulases and their low efficiencies. So, identification, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research, biodegradation and bioremediation. The present research work is focused on presumptive identification of most efficient cellulase producing bacteria from biogas production plant effluent and dairy effluent based on morphological and biochemical properties and subsequent molecular characterization of the selected cellulase producing strains. Furthermore, identification of potential cellulolytic bacteria producing efficient β -glucosidase by determining β -glucosidase activity.

MATERIALS AND METHODS

This study was mainly conducted at the Bio-resource Technology and Industrial Biotechnology Laboratory, Department of Biotechnology and Genetic Engineering, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh and Invent Technologies Ltd, Banani, Dhaka- 1213, Bangladesh.

Isolation, screening and identification of cellulolytic bacterial isolates

Isolation of cellulolytic bacteria

For isolating cellulolytic bacterial isolates, samples were collected from biogas production plant effluent and dairy effluent of dairy farm near Jahangirnagar University campus area by the standard sample collection procedure in a clean sterile plastic container and stored at 4°C until the analysis was carried out. For isolating cellulolytic bacteria 1 g of samples were first mixed with 10 ml of distilled water in a test tube and heated for 15 minutes at 85°C in hot water bath. After ten-fold serial dilution with saline water (0.85% NaCl), 0.1ml of each dilution was spread on carboxyl methyl cellulose (CMC) agar plates under laminar airflow. Then the plates were incubated at 37°C for 24-48 hour in the incubator. Morphologically different bacterial colonies were purified by repeated streaking. The purified isolated single cells were preserved and aliquoted as a stock ampoule in 40% glycerol solution and kept at -80°C for further identification and screening for cellulase production.

Primary Screening of cellulolytic bacteria

For screening cellulolytic bacteria, purified bacterial isolates were inoculated into 5 ml of modified No.3 medium (3% Peptone, 1% CMC, 0.1% KH_2PO_4 , 0.05% MgSO_4 , pH 6.8) and incubated at 37°C and 120 rpm for 24 hour in shaking water Bath. Then 5 μ l of overnight grown culture of isolated bacterial cells were individually transferred in CMC agar plates and incubated at 37°C for 24-48 hour. After proper incubation, CMC agar plates were flooded with Grams iodine solution (2g of KI and 1g iodine in 300 ml distilled water) for 3-5 minutes and subsequently washed with 70% alcohol solution. After washing, only the cell producing an inhibition zones are distinct, clear, and prominent zones of clearance around the colonies indicating cellulase production were selected.

Secondary screening and production of cellulase enzyme

The potential isolates selected by primary screening were then evaluated for enzyme production. Those isolates showing maximum CMCase activity were then considered for further studies.

Crude cellulase production

Newly isolated isolates were screened for cellulase enzyme production in submerged

fermentation process. A basal media containing (2% Tryptone, 1% CMC, 0.1% KH_2PO_4 , 0.1% K_2HPO_4 , 0.04% MgSO_4 , 0.005% NaCl, and 0.000125% FeSO_4 , pH 7.0) was used for production of cellulase. For seed culture, a fresh isolated colony was inoculated in 5 ml basal media and incubated at 37°C and 120 rpm for 24 hour. The seed culture (5%) was then inoculated in 50 ml production media in a 250 ml conical flask and incubated at 37°C and 120 rpm for 24 hour in shaking water bath.

Preparation of crude enzyme

The cell free supernatant obtained by centrifugation served as crude enzyme source was used for determining the cellulase activity or further investigations. After 24 hour of incubation in shaking water bath, the cultures were centrifuged at 10000 rpm for 10 min using centrifuge to remove the unwanted material and supernatant was used as a source of crude enzyme. The obtained crude enzyme solution was utilized for determination of enzyme activities and stored at 4°C for subsequent use.

Determination of CMCase activity

The carboxy methyl cellulase (CMCase) activity was assayed using a method described by [11], with some modifications [12]. For this assay, 500 μL of crude enzyme was added to 500 μL of substrate (1% CMC prepared in 50 mM sodium citrate buffer pH 5) in a test tube and incubated at 60°C for 30 min in the incubator. Two controls were run with the test sample. In the negative control, 500 μL of substrate was added with 500 μL of citrate buffer & in positive control 500 μL of standard cellulase enzyme was added to 500 μL substrate. The reaction was terminated by adding 2.0 ml of dinitrosalicylic acid (DNS) reagent and subsequently placing the reaction tubes in a water bath at 90°C for 15 minutes. One ml of 40% Rochelle salt (sodium potassium tartrate) solution was then added to the test tubes and kept in ice for 2 minutes to stabilize the color. The absorbance was recorded at 575 nm wave length using UV/Vis Spectrophotometer (OPTIZEN POP, Mecasys, 5U4603-124019-00, Korea) against the negative control. One unit of CMCase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar (glucose) in 1 min at 37°C and pH 7.0 [13]. Those isolates showing maximum CMCase activity were then selected for identification.

Identification of most efficient cellulase producing bacteria

Bacterial isolates producing significant clear zone on CMC agar as well as higher CMCase activity were identified based on cultural, morphological and biochemical characteristics. For this, a series of conventional biochemical tests were carried out for the identification of the genus of bacterial strains according to the Bergey's manual of systemic bacteriology [14].

The molecular identification was also done by 16S rRNA gene sequencing. Two universal primer for 16s rRNA gene amplification were used for the purpose of PCR. 27F (AGAGTTTGATCMTGGCTCAG) was used as the forward primer while U1492R (GGTTACCTTGTTACGACTT) was used as the reverse primer. The 16s rRNA gene was sequenced using ABI 3700 Genetic Analyzer in the 1st Base Laboratory SdnBhd, Malaysia. The 16s rRNA gene sequences were aligned using BioEdit7.2 software. The sample sequences were analyzed using BLAST-NCBI.

Determination of β -glucosidase activity

Three bacterial isolates showing higher CMCase activity were used for assaying β -Glucosidase activity. β -Glucosidase activity was assayed using (pNPG) p-nitrophenyl- β -D glucopyranoside as a substrate. A reaction mixture 500 μl containing 375 μl of culture supernatant (enzyme), 50 μl of pNPG (10 mM) as substrate and 75 μl sodium acetate buffer (2M, pH 5.0) was incubated at 50°C for 10 min in hot water bath. The reaction was terminated by addition of 1M Na_2CO_3 . The developed yellow color was measured at 410 nm using spectrophotometer. The amount of p-nitrophenol (PNP) released was quantified using the PNP standard curve. One unit of β -glucosidase activity was expressed as the amount of enzyme required to release 1 μmole of PNP per minute under the assay conditions.

RESULTS AND DISCUSSION

Isolation and primary screening of cellulolytic bacteria

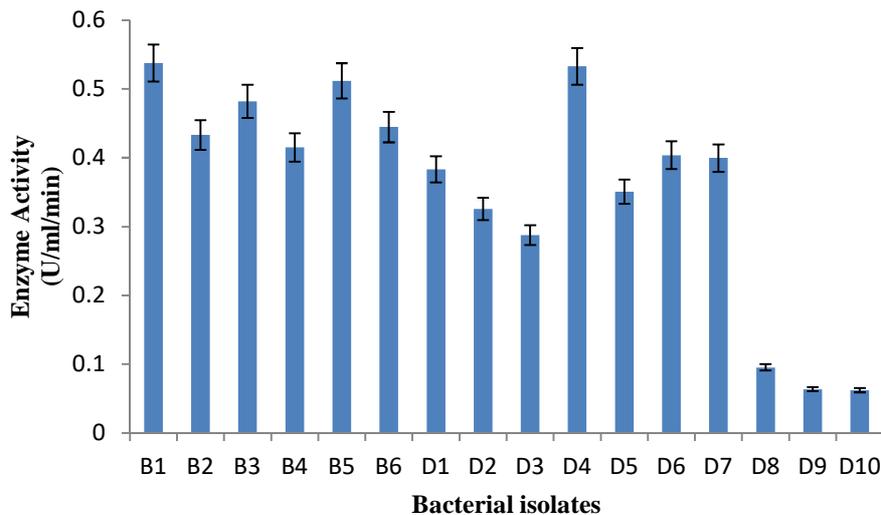
In this study, two samples were collected from two different sites and total 16 isolates were obtained as shown in (Table 1). The resulting 16 isolates were then tested on CMC agar plate for cellulase activity.

Table 1: Different sites selected for sample collection to identify cellulase producers

Sites	Sample no.	Total no. of isolates	Labeled as
Biogas plant effluent	1	6	B ₁ -B ₆
Dairy effluent	2	10	D ₁ -D ₁₀

Table 2: Zone of CMC hydrolysis of different isolates(n≥3)

Serial no	Cell name	Colony diameter n (mm)	Zone diameter z (mm)	Ratio (z/n) (mm)
1.	B ₁	4	10	2.5
2.	B ₂	5	9	1.8
3.	B ₃	6	10	1.6
4.	B ₄	6	15	1.83
5.	B ₅	5	13	2.6
6.	B ₆	7	15	2.14
7.	D ₁	4	9	2.25
8.	D ₂	7	15	2.10
9.	D ₃	4	8	2.00
10.	D ₄	6	14	2.33
11.	D ₅	5	9	1.80
12.	D ₆	6	10	1.60
13.	D ₇	4	8	2.00
14.	D ₈	8	11	1.37
15.	D ₉	4	5	1.25
16.	D ₁₀	5	6	1.20

**Figure 1: Screening of potential cellulolytic bacteria based on higher CMCase activity. Number of independent experiments, n≥3.**

Bacterial isolates (B₁, B₅, D₄) producing significant clear zone on CMC agar as well as higher CMCase activity were selected and identified based on biochemical and molecular characteristics for further studies.

The isolated bacterial colonies forming clear-zones after application of Gram's iodine solution were selected as cellulase producers. Zone of CMC hydrolysis of the isolates are given in the (Table 2). Among them 6 isolates gave the maximum ratio of clear zone diameter to colony diameter on

the CMC agar plate as compared to plates cultured with the other strains. So these 6 isolates are efficient cellulase producers. For confirming their cellulolytic activity all these 16 isolates were then analyzed for secondary screening by CMCase activity assay.

Secondary screening and production of cellulase enzyme

On the basis of primary screening the potential isolates were then evaluated for their enzyme productivity in submerge fermentation process. CMCase activity assay for all the 16 bacterial isolates were carried out for secondary screening.

Determination of CMCase activity

For this enzyme activity study, crude enzyme samples were assayed using 1% CMC as a substrate by DNS method. Three bacterial isolates giving higher absorbance at 575nm in (Figure 1) CMCase assay were selected as efficient cellulase producer.

Identification of most efficient cellulase producing bacteria

Observation of biochemical characteristics

The biochemical identification of the selected isolates was performed according to the Bergey's

manual of systemic bacteriology [14] and all of the isolates were presumptively identified as *Bacillus sp.* The biochemical test results are presented in Table 3.

Observation of molecular characteristics:

DNA quantification and PCR

From B₁, B₅ and D₄ bacterial isolates genomic DNA was extracted and quantified by Nanodrop Spectrophotometer and PCR was done by Takara PCR thermal cycler. The result of the extracted DNA quantification is presented in Table 4.

Agarose Gel Analysis for Genomic DNA

After amplification of the extracted genomic DNA from the three cellulolytic bacterial strain agarose gel electrophoresis was performed using 1Kb DNA ladder as a marker DNA for gel analysis. Three bands were found for our isolated bacterial DNA in the gel which were nearly 1.5Kb (1500bp) in comparing with 1Kb DNA Ladder (Marker DNA).

Table 3. Results of different morphological and biochemical tests on the isolates.

Biochemical test	B ₁	B ₅	D ₄
Gram staining	+	+	+
Shape	Rod	Rod	Rod
Methyl Red (MR) Test	-Ve	-Ve	-Ve
Voges-Proskaur (VP) Test	+Ve	+Ve	+Ve
Catalase Test	+Ve	+Ve	+Ve
Urease Test	+Ve	+Ve	+Ve
Oxidase Test	-Ve	-Ve	-Ve
Indole Test	-Ve	-Ve	-Ve
Citrate Test	-Ve	-Ve	-Ve
Gelatin Test	+Ve	+Ve	+Ve
Carbohydrate Fermentation Test			
Glucose	+Ve	+Ve	+Ve
Fructose	+Ve	+Ve	+Ve
Sucrose	+Ve	+Ve	+Ve
Maltose	+Ve	-Ve	+Ve
Mannitol	+Ve	+Ve	+Ve
Finally identified bacteria	<i>Bacillus sp.</i>	<i>Bacillus sp.</i>	<i>Bacillus sp.</i>

Table 4: DNA quantification result

Cell	B ₁	B ₅	D ₄
Concentration of DNA (ng/μl)	82.6	183.1	29.5
A 260	1.652	3.661	0.59
A 280	0.883	1.987	0.316
A 260/280	1.87	1.84	1.87
A 260/230	1.07	1.49	0.91

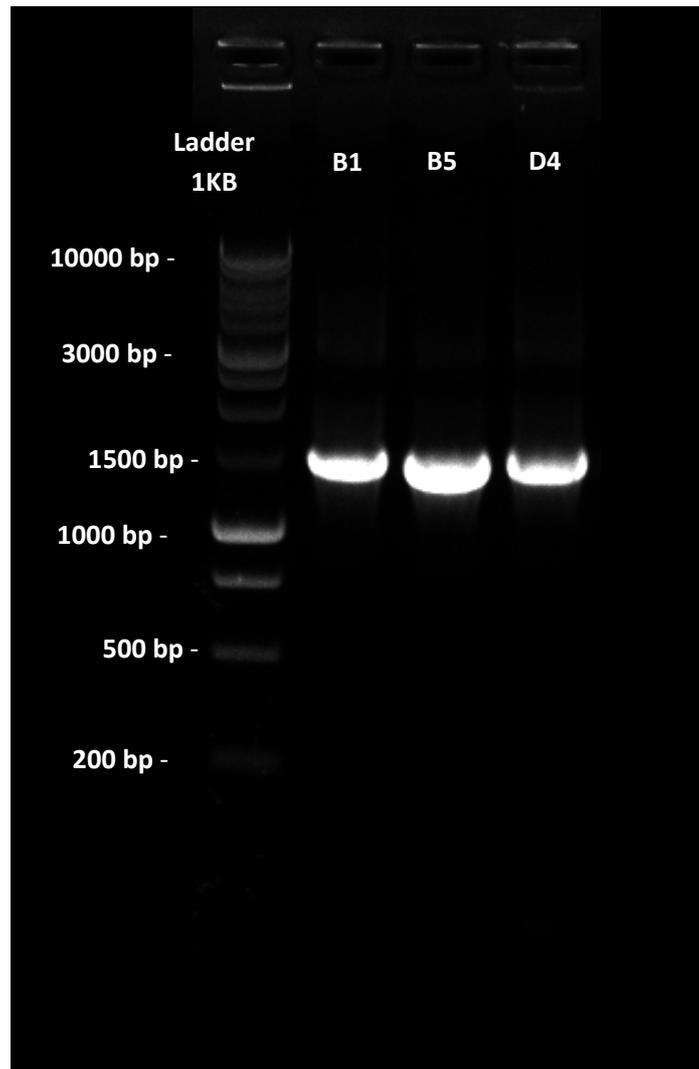


Figure 2: Agarose gel analysis of PCR product for molecular identification of three cellulolytic bacteria. Lane 1: B₁, Lane 2: B₅, Lane 3: D₄

16S rRNA gene sequences analysis

For all the three strains, the forward and the reverse sequence were analyzed. The nucleotide sequences coding for the 16S rRNA gene after BLAST query revealed that this gene is 100 % homologous to *Bacillus subtilis* (B₁), 99% homologous to *Bacillus amyloliquefaciens* (B₅) and 100% homologous to *Bacillus subtilis* (D₄).

Phylogenetic analysis

A phylogenetic tree based on the blast search was constructed. Figure 3 shows the phylogenetic tree for 16S rDNA gene sequences.

Evolutionary analyses were conducted in MEGA7. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches.

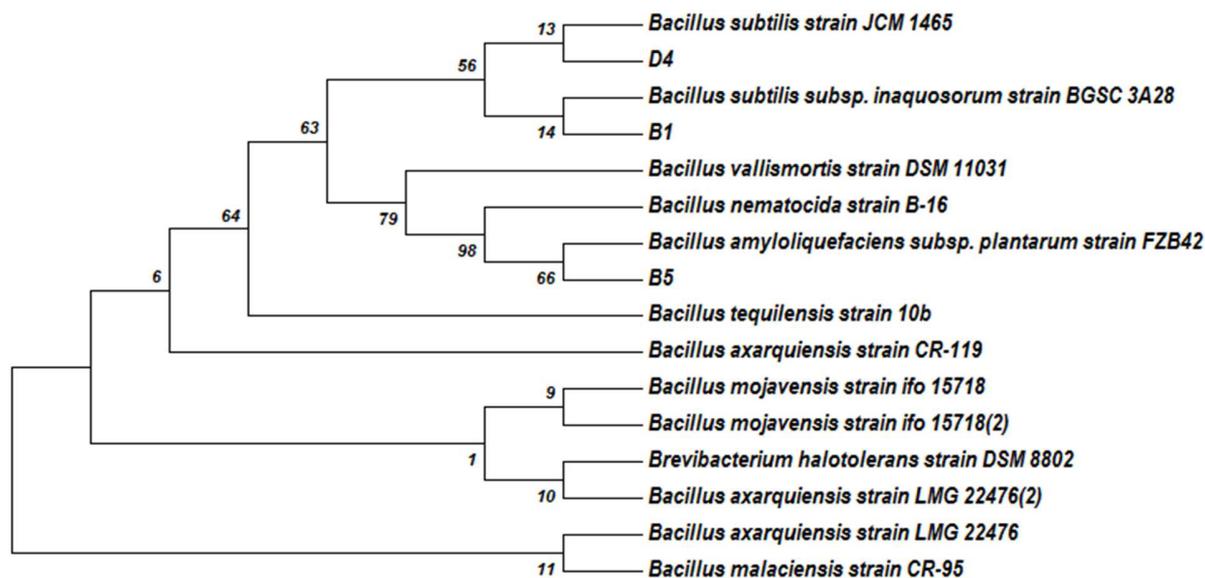


Figure 3: Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between the isolated strains B₁, B₅, D₄ with their related bacteria in BLAST analysis.

The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. Three cellulolytic bacteria isolated in this study were, *Bacillus subtilis* (B₁), *Bacillus amyloliquifaciens* (B₅) and *Bacillus subtilis* (D₄), based on phylogenetic analysis of 16S rDNA sequence.

β -glucosidase activity assay was carried out with culture supernatant of three bacterial isolates having higher CMCase activity by using 10mM pNPG as substrate. After terminating the reaction, absorbance was taken at 410nm using spectrophotometer. Among the three selected cellulolytic bacterial strains, B₁ showed higher absorbance thus it can produce β -glucosidase where D₄ showed negative result thus it does not have β -glucosidase activity showing in (Figure 4).

Determination of β -glucosidase activity

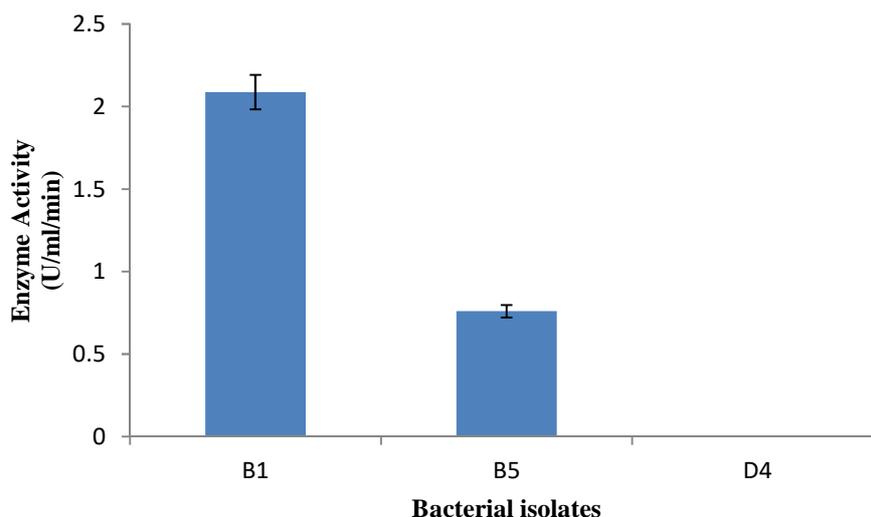


Figure 4: Comparison of β -glucosidase activity of B₁, B₅ and D₄ isolates. Number of independent experiments (n \geq 3). Within these two strain B₁ produce higher amount of β -glucosidase than B₅ according to (Figure 4).

The identified bacterial isolates B₁, B₅ and D₄ all are *Bacillus*, due to their different environmental origin of sources they produce cellulolytic enzymes depending on their own needs. Both B₁ and D₄ bacterial isolates are *Bacillus subtilis*, where B₁ was isolated from biogas effluent plant and these sources is enriched with municipal cellulolytic waste materials. So these microbes produce higher amount of β -glucosidase due to some signal induction provided from their surrounding environment to fulfill their requirement by utilizing cellulolytic wastes as their nutrients. On the other hand, D₄ bacterial isolate was isolated from dairy effluent which is enriched with dairy waste materials. These microbes cannot produce β -glucosidase because they are not induced from their surroundings to produce this enzyme. Instead of being *Bacillus subtilis*, both B₁ and D₄ cannot produce β -glucosidase due to their different environmental origin of sources. As B₁ bacterial isolate can produce significant amount of β -glucosidase, therefore further studies will be carried out with this B₁.

CONCLUSION

Cellulase research has been concentrated mostly in fungi but there is increasing interest in cellulase production by bacteria due to their higher growth rate, thermo stable and alkali stable properties. The greatest potential importance is the ease with which bacteria can be genetically engineered. So development of rapid and reliable methods for the screening of cellulases from microorganisms within inhospitable environments will allow a greater number of novel bacterial cellulases to be isolated with purpose for industrial use. Moreover, most of the bioconversion processes used today does not allow complete saccharification of biomass. Hydrolysis of biomass can be enhanced by several approaches, one of which is by supplementation of cellulase complex with accessory enzymes like β -glucosidase. Our current knowledge of the production, purification, characterization, biochemistry, molecular biology of these enzymes and of their producer bacteria may help in this regard. Therefore, more advance research is required for a deeper understanding about these bacterial strains to improve their enzymatic production, purification and proper application of the enzyme from the isolate.

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