Journal of Ecology of Health & Environment
An International Journal

http://dx.doi.org/10.18576/jehe/060103

Isolation and Identification of Cellulose Degrading Bacteria from Different Sources at Assiut Governorate (Upper Egypt)

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Received: 23 Jul. 2017, Revised: 23 Aug. 2017, Accepted: 27 Aug. 2017.
Published online: 1 Jan. 2018.

Abstract: The cellulolytic bacteria were screened out from five different sources on two selective media, cellulose agar and carboxymethyl cellulose agar. Ten bacterial isolates characterized by high potent ability to produce cellulases enzymes. These isolates were characterized and identified. The extracellular cellulases activities ranged from 4.389 to 7.724 U/ml for filter paper cellulase (FPCase) and 6.175 to 12.152 U/ml for carboxymethylcellulase (CMCase) assay. Two isolates (CDB6 & CDB10) were found to be highest productivity of cellulases enzymes, 7.268 and 7.724 U/ml respectively, on FPCase, whereas on CMCase assay maximum activity was11.077 U/ml by CDB6 and 12.152U/ml by CDB10. Ten cellulose degrading bacterial isolates were identified as *Bacillus subtilis*(four isolates: CDB6, CDB8, CDB9 and CDB10), *Bacillus thuringeinsis*(two isolates: CDB1 and CDB5), *Brevibacillusbrevis*(two isolates: CDB2 and CDB4), *Brevibacillusparabrevis*(one isolate: CDB7) and *Bacillus pumilus*(one isolate: CDB3).

Keywords: FPCase: Filter Paper Cellulase, CMCase: carboxymethylcellulase, CDB: cellulose degrading bacteria.

1 Introduction

Plant biomass contains cellulose as the major component of the cell walls. Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes [1]. Cellulose is a strong fibrous, crystalline polysaccharide, resistant to hydrolysis and is water insoluble [2]. Cellulose is a polysaccharide composed of repeating D-glucose units that are linked together with β-1, 4-glycosidic bonds[3, 4]. Cellulases enzymes can hydrolyze the β (1-4) glycosidic linkages to smaller oligosaccharides and eventually glucose. For years, cellulases have been a target for academic and industrial research and are currently being applied in many industries [5]. Microbial cellulases have many potential industrial and biotechnological applications, and hence are in high demand [6].

Bacteria are now being widely explored for cellulases production because of their rapid growth, expression of multi-enzyme complexes, stability at extremes of temperature and pH, lesser feedback inhibition, capacity to colonize a wide variety of environmental niches, and ability to withstand varieties of environmental stress [7, 8]. *Bacillus subtilis* continues to be a dominant workhorse due to its capacity to secrete large quantities of extracellular cellulolytic enzymes [9, 10].

2 Materials and Methods

2.1 Samples Collection.

The samples for isolation of cellulose degrading bacteria were collected from five different sources, where the natural process of cellulose degradation is taking place, such as garden soil, agricultural soil, gut of ruminants, sediment of River Nile and compost, at Assiut Governorate (Upper Egypt). All samples were collected in sterile containers and transported under aseptic conditions to laboratory for further investigations.

2.2 Isolation of Cellulose Degrading Bacteria.

Cellulolytic bacterial strains were isolated from all samples by using serial dilutions and spread plate technique. For each sample, several sub-samples were taken, homogenized in sterile physiological saline solution 0.85% NaCl (w/v) and serially diluted to suspend the cells and spread plate technique was done using two selective media.The first medium was cellulose agar containing (g/l): cellulose 2.0, gelatin 2.0, $MgSO_4$ 0.25, KH_2PO_4 0.5 and agar 15.The second medium was carboxymethyl cellulose agar (CMC agar) with the following composition (g/l): peptone 10.0, carboxymethyl cellulose (CMC) 10.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.3, $(NH_4)_2SO_4$2.5, gelatin 2.0 and agar 15. pH was adjusted at 6.8-7.2, and the plates were incubated at

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37 °C for 72 hours [11].All different bacterial colonies that appeared on the plates of the two selective media were selected and subjected to the purification process. The purified colonies were preserved at 4°C for further identification and screening for cellulases enzymes production.

2.3 Screening for Cellulose Degrading Bacteria.

All purified bacterial isolates were screened for cellulases production as described by Lisdiyanti*et al*.,[12]. Each isolate was individually streaked on the plates of CMC agar and incubated at 37 °C for 72 hours. After incubation, CMC agar plates were flooded with 0.1% (w/v) Congo red reagent and allowed to stand for 15 min. at room temperature, then washed with 1 M NaCl[13].The formation of a clear zone of hydrolysis around bacterial colonies indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulases activity producer. The largest ratio was assumed to contain the highest activity [14,15].Hydrolysis capacity (HC) value was calculated as described by Sreeja*et al*.,[16].

2.4 Secondary Screening For Cellulose Degrading Bacteria Using Iodine Solution Indicator.

All bacterial isolates were screened for cellulases production as described by Kasana *et al*., **[**6**]**. The hydrolysis capacity was calculated from the ratio between the diameter of the cellulolytic zone and the diameter of the bacterial colony [17].

2.5 Confirmation test for Cellulolytic Activity of Bacterial Isolates By Cellulose Congo Red Agar.

Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking the isolates on the cellulose Congo-red agar [18]. Colonies showing discoloration of Congo red were selected as positive cellulose degrading bacterial colonies [19], and only these were taken for further study.

2.6 Production of Cellulases Enzymes.

For the quantitative estimation of cellulases enzymes, the selected cellulose degrading bacterial isolates were grown in carboxymethyl cellulose broth medium (enzymes production medium) contain (g/l): CMC 10.0, peptone 10.0, $MgSO_4.7H_2O$ 0.3, K_2HPO_4 2.0, $(NH_4)_2SO_4$ 2.5, and gelatin 2.0. pH was adjusted at 6.8-7.2. The medium was incubated in a shaking incubator at 37ºC for 72 hours, with agitation speed of 160 rpm **[**11**]**. After three days of incubation, inoculated broth was subjected to centrifugation at 5000 rpm for 20 min at 4°C. Supernatant was collected and preserved as a crude enzyme for further enzymes assay.

2.7Cellulases Activity Assay by Spectrophotometric Method.

The carboxymethylcellulase (CMCase) and filter paper cellulase (FPCase) enzymes activities were determined according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission on biotechnology [20].

2.8 CMCase Activity.

CMCase activity wasdetermined and estimated according to the method described by Wang *et al*.,[21].One unit of CMCase activity is expressed as the quantity of enzyme, which is required to release 1µg of glucose per minute under standard assay conditions. The values obtained are compared with glucose standard curve [22].

2.9 FPCase Activity.

Filter paper cellulase (FPCase) activity was determined as described by Gadgil*et al*.,[23].One unit of FPCase activity is expressed as the quantity of enzyme, which is required to release 1µg of glucose per ml per minute under standard assay conditions. The values obtained are compared with glucose standard curve [22].

2.10 Phenotyping of The Highest CMCase And FPCase Producing Bacterial Isolates.

Morphological and biochemical identification of the selected ten bacterial isolates were carried out according to the standard methods described in Bergey's Manual of Determinative Bacteria [24].

2.11 Molecular Characterization of Selected Ten Bacterial Isolates.

Molecular characterization of selected ten bacterial isolates was done with the help of Solgent Company, Daejeon South korea. Cultures were sent to the Solgent Company for rRNA gene sequencing. Bacterial DNA was extracted and isolated using Solgent purification bead. Prior to sequencing, the ribosomal RNA gene was amplified using the polymerase chain reaction (PCR) technique in which two universal primers were used for amplification: Forward primer:27F(AGAGTTTGATCCTGGCTCAG).Reverse primer:1492R(GGTTACCTTGTTACGACTT).PCR products were purified and sequenced using a PCR purification kit. The purified PCR products were reconfirmed by gel electrophoreses with 1% agarose gel. Bands were eluted and sequenced with the incorporation of

di-deoxy nucleotides (dd NTPs) in the reaction mixture **[**25**]**. Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done help of MegAlign (DNA star) software version 5.05. The percentages of sequence matching were also analyzed and the sequence was submitted to NCB1-GeneBank to obtain accession numbers.

3 Results and Discussion

3.1 Isolation And Purification of Cellulose Degrading Bacteria.

In this study, 120 bacterial isolates were isolated from five different sources (garden soil, agricultural soil, gut of ruminant, sediment of River Nile and compost). Garden soil was the richest source in isolates number with 49 isolates (40.83%) followed by gut of ruminant with 32 isolates (26.67%), compost with 19 isolates (15.83%), agricultural soil with 13 isolates (10.83%) and River Nile sediment with 7 isolates (5.83%).These results are in agreement with those reported in a previous studies, which the cellulolytic microorganisms have been isolated from diverse environments such as, soil [26, 27],organic waste [28,29],gut [30, 31],animal waste [32],marine sediments [33, 34, 35]and seaweeds [36].One hundred and sixteen isolates exhibited Gram positive bacteria while four isolates only were Gram negative. From these isolates, 98 isolates were bacilli, 10 isolates were cocci and 12 isolates were coccobacilli (Table1).

3.2 Screening for cellulases producing Bacteria (Qualitative Assay of CMCase And FPCase Activity)

All 120 purified bacterial isolates were screened for cellulases production. One hundred and seven isolates (89.16 %) exhibited cellulases positive, whereas thirteen isolates (10.83 %) were cellulases negative. Ten Gram positive isolates were not exhibited cellulolytic activity whereas only one Gram negative isolate was exhibited cellulolytic activity (Table 1). Agar media containing cellulose or CMC for the screening of cellulases producing bacteria through the formation of zone of hydrolysis have been reported by many researchers **[**37, 38, 39, 40**]**.Among 107 isolates that showed cellulases activity, 10 isolates exhibited the largest clear zones, were selected and named as cellulose degrading bacteria (CDB1-10). Clear zones ranged from 23 to 57 mm, and the average of hydrolysis capacity (HC) values (ratio of zone size to colony diameter) ranged from 1.33 to 2.87 (Table 2). These results are very similar to the findings reported by Hatami*et al*.,[41] who also found the hydrolytic value between 1.38 to 2.33 and 0.15 to 1.37 cm of cellulolytic aerobic bacterial isolates from farming and forest soil, respectively. **Lu *et al*., [**42**]** observed maximum clearing zones ranged between 25 to 64 mm with maximum HC value of 4.85-13.11 cm. Nevertheless, the observed HC value is lower than the value observed by **Gupta *et al*., [**43**]** who observed maximum HC value of 9 to 9.8 cm. The maximum clearing zone of 57mm was estimated for isolate CDB6 as shown in Figure 1.

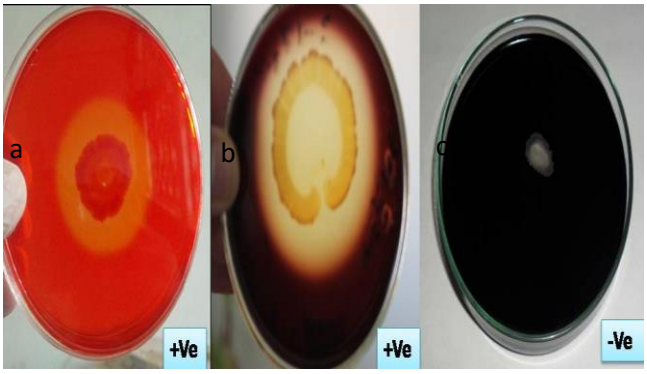

Figure 1.CDB6 isolate exhibited the largest clear zone on CMC agar medium by using Congo red reagent **(a)**, and by using Gram's iodine solution **(b)**; negative result for cellulases activity**(c)**.

3.3 Quantitative Assay For CMCase And FPCase Activity By Spectrophotometric Method.

Because the diameter of the clearing zone may be not accurately reflect the true cellulases activity **[**44**]**, therefore, all the bacterial colonies having the large clear zones were screened again by colorimetric method to determine the most potent isolates for FPCase and CMCase production. Ten bacterial isolates (CDB1-10) that showed the largest clear zones were selected for cellulases production and their respective cellulolytic activity was estimated. The activities ranged from 4.389 to 7.724 U/ml for FPCase and 6.175 to 12.152 U/ml for CMCase assay. Enzyme assay for cellulase activity on filter paper was found to be highest for CDB6 and CDB10 with 7.268 and 7.724 U/ml, respectively, while for CMCase assay maximum activity was determined to be 11.077 U/ml by CDB6 and 12.152U/ml by CDB10 (Table3).

These results are in agreement with previous studies, which also recorded a CMCase activity greater than FPCase and β-glucosidase**[**45, 46**]**.Generally, FPCase activity is found to be lower than CMCase and β-glucosidase**[**47, 48**]**.In a study carried out by **Soares*et al*.,[**26**]**46.9% of the isolates displayed the presence of endoglucolytic activity, 9.1% showed exoglucolytic activity, while only a minority (4.72%) could degrade both the substrates on plate assay.

Table 1. Summery of samples collection, isolation, Gram staining and cellulases activity of isolates from different sources.

Source of samples	No. of isolates	Cellulases activity		Gram stain		%
		+Ve	-Ve	+Ve	-Ve	
Garden soil	49	41	8	48	1	40.83
Gut of ruminant	32	30	2	31	1	26.67
Compost	19	18	1	17	2	15.83
Agricultural soil	13	11	2	13	0	10.83
River Nile sediments	7	7	0	7	0	5.83
Grand total	120	107	13	116	4	100%

Table 2. Evaluation of cellulases activity of some cellulose degrading bacterial in CMC agar plate through halo zone formation.

Isolates code	Isolation source	Mean clear zone diameter (ZD) (mm)	Mean colony diameter (CD) (mm)	HC Value (ZD/CD)
CDB1	Garden soil	42	19	2.21
CDB2	Garden soil	23	8	2.87
CDB3	River Nile sediments	28	21	1.33
CDB4	Garden soil	35	16	2.19
CDB5	Garden soil	28	13	2.15
CDB6	Garden soil	57	34	1.68
CDB7	Agricultural soil	33	20	1.65
CDB8	Agricultural soil	39	19	2.05
CDB9	Gut of ruminant	47	26	1.81
CDB10	Gut of ruminant	41	19	2.16

Table 3. Extracellular cellulases activity of two enzymes (FPCaseandCMCase) for isolates (CDB1-10).

Isolates code	Enzymes activity (U/ml)	
	FPCase	CMCase
CDB1	4.844	7.669
CDB2	5.391	6.229
CDB3	4.389	6.175
CDB4	4.826	7.687
CDB5	4.717	7.469
CDB6	7.268	11.077
CDB7	6.065	9.419
CDB8	6.503	10.148
CDB9	6.156	9.637
CDB10	7.724	12.152

CMCase activity obtained in this study was higher than that exhibited by some known natural isolates. For example, *Bacillus subtilis* AS3, isolated from cow dung, produced CMCase with productivity of 0.07U/ml **[**49**]**, *Bacillus amyloliquefaciens* SS35, isolated from rhinoceros dung (0.079 U/ml) [32],and *Brevibacillus* sp. DUSELG12 and *Geobacillus* sp. DUSELR7, isolated from gold mine (0.02 U/ml and 0.058 U/ml, respectively)[50].On the other hand, the FPCase activity obtained in the present study was higher than that exhibited by some known natural isolates, for example, lower FPCase activities were obtained from *Brevibacillus* sp. DUSELG12 and *Geobacillus* sp. DUSELR7 studied by Rastogi*et al*.,[50].Also, **Ariffin *et al*.**,[15] recorded lower FPCase activity (0.011 U/ml) by *Bacillus pumilus* EB3.

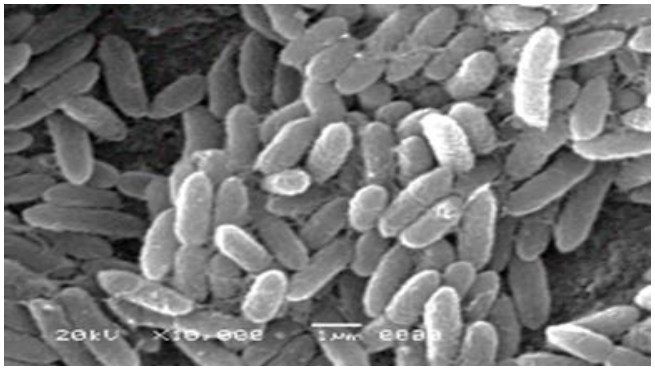

Figure2. Isolate CDB6 under scanning electron microscope showing diplo-bacilli.

3.4 Identification of The Highest CMCase And FPCase Producing Bacterial Isolates.

3.4.1 Morphological And Biochemical Characteristics (Phenotypic).

Ten selected bacterial isolates (CDB1-10) were subjected to various morphological and biochemical characterization to identify them. The colony characteristics of the isolates were found variable. The colonies were undulate, convex, flat, filamentous and circular having gummy and sticky consistency. Microscopic observation of the isolates revealed that, all isolates were Gram positive, rod shaped (mono, diplo and streptobacilli), spore formation and motile.

These isolates were examined for starch hydrolysis, gelatin hydrolysis, casein hydrolysis, urea hydrolysis, catalase production, nitrate reduction, indole production, methyl red test, voges-proskauer test, citrate utilization and sugars fermentation. Results of all these tests were presented in Table (4, 5).

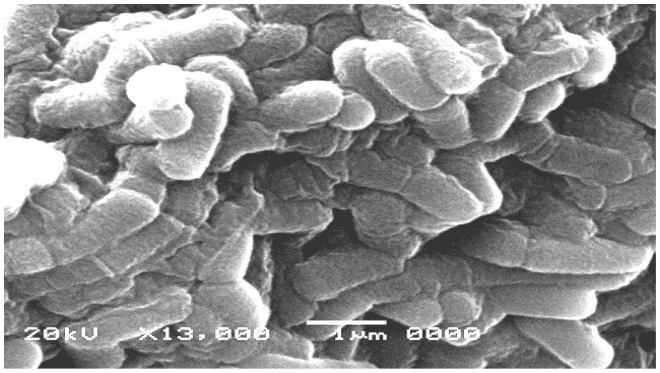

Figure 3. Isolate CDB10 under scanning electron microscope showing diplo-bacilli.

3.4.2 Scanning Electron Microscopy.

Scanning electron microscopy illustrates the shapes and sizes of bacterial cells for CDB6 and CDB10.

Figure (2) shows that, the average of cell size for CDB6 was 1.6 µm in length and 0.7 µm in width. On the other hand, Figure (3) shows that, the average of cell size for CDB10 was 1.4 µm in length and 0.7 µm in width. Bacterial cells appear as diplo-bacilli.

3.4.3 Genotyping Identification Using 16S Ribosomal RNA Gene Sequencing:

Based on 16S rRNA gene sequence and morphological and biochemical characteristics, ten cellulose degrading bacterial isolates (CDB1-10) were identified as *Bacillus subtilis*(CDB6, CDB8, CDB9 and CDB10), *Bacillus thuringeinsis*(CDB1and CDB5), *Brevibacillusbrevis*(CDB2 and CDB4), *Brevibacillus parabrevis*(CDB7) and *Bacillus pumilus*(CDB3). Isolates CDB6 and CDB10 were designated as *Bacillus subtilis*strainASB11, Gene Bank accession No. (KY007073) with identity (99%), and *Bacillus subtilis*strainASB1, Gene Bank accession No. (KU533851) with identity (98%), respectively, **(**Table 6).

These results are in agreement with those reported in previous studies, which many *Bacillus* sp. including *Brevibacillus brevis*[51],*Bacillus pumilus*[52],*Brevibacillus* sp. **[**50**]**,and *Bacillus subtilis* YJ1 [53],have been exploited for cellulases production.

A phylogenetic tree based on the comparison of 16S rRNA sequences of reference strains was constructed. The phylogenetic analysis was performed with (950 bp - 1500 bp) sequences for isolates (CDB1-10) using the software MEGA 6[54], using the neighbor-joining method and based on Jukes-Cantor distances.

3.4.3.1 Bacterial Isolates (CDB6):

DNA nucleotide sequence of 16S rRNA gene of CDB6 strain was amplified by PCR and partially sequenced for *Bacillus subtilis* strain ASB11. (KY007073.1).

Table 4 . Summary of the morphological and biochemical characteristics of the best cellulose degrading bacterial isolates (CDB1-10).

Code of isolates	Gram staining	Shape of bacterial cells	Spore forming	Pigmentation	Motility	Oxygen requirement
CDB1	+Ve	Streptobacilli	+Ve	None	Motile	Facultative
CDB2	+Ve	Diplobacilli	+Ve	None	Motile	Facultative
CDB3	+Ve	Monobacilli	+Ve	Endo-pigment (Yellow)	Motile	Facultative
CDB4	+Ve	Monobacilli	+Ve	Endo-pigment (Orange)	Motile	Facultative
CDB5	+Ve	Streptobacilli	+Ve	Endo-pigment (Yellow)	Motile	Facultative
CDB6	+Ve	Diplobacilli	+Ve	Endo-pigment (Brown)	Motile	Facultative
CDB7	+Ve	Diplobacilli	+Ve	None	Motile	Facultative
CDB8	+Ve	Diplobacilli	+Ve	None	Motile	Facultative
CDB9	+Ve	Diplobacilli	+Ve	None	Motile	Facultative
CDB10	+Ve	Diplobacilli	+Ve	None	Motile	Facultative

Table 5. Biochemical characteristics of cellulose degrading bacterial isolates (CDB1-10).

code of isolates	Indole test	M. R. test	V. P. test	Citrate test	Starch hydrolysis	Gelatin hydrolysis	Casein hydrolysis	Urea hydrolysis	Nitrate reduction
CDB1	-	+	+	-	+	+	+	+	+
CDB2	-	-	-	-	-	+	+	-	+
CDB3	-	-	+	-	+	+	+	-	+
CDB4	-	-	-	+	+	+	+	-	+
CDB5	-	-	-	+	+	+	+	-	+
CDB6	-	-	+	-	+	+	+	-	+
CDB7	-	-	+	-	-	+	+	-	+
CDB8	-	-	+	-	+	+	+	-	+
CDB9	-	-	+	-	+	+	+	-	+
CDB10	-	-	+	-	+	+	+	-	+

Table 5 continued. Sugars fermentation.

Isolate code	Glucose	Lactose	Fructose	Dextrose	Galactose	Xylose	Ribose	Mannose	Rhaffinose	Rhaminose
CDB1	+	+	+	+	+	+	+	+	+	+
CDB2	-	-	-	-	-	+	-	-	-	-
CDB3	-	-	+	-	+	-	+	-	+	-
CDB4	-	-	-	-	-	-	+	-	-	-
CDB5	-	-	-	-	-	+	-	-	-	-
CDB6	+	+	+	+	+	+	-	+	-	+
CDB7	-	-	-	-	-	-	-	-	-	-
CDB8	+	+	+	+	+	+	-	+	-	+
CDB9	+	+	+	+	+	+	-	+	-	+
CDB10	+	+	+	+	+	+	-	+	-	+

Table 6. Name, similarity and Gene Bank accession number of the selected cellulose degrading bacterial isolates(CDB1-10).

Isolates code	Name of isolates	Similarity	Gene Bank accession number
CDB1	*Bacillus thuringiensis*	98%	KU550946.1
CDB2	*Brevibacillusbrevis*	99%	KU973528.1
CDB3	*Bacillus pumilus*	98%	KU973527.1
CDB4	*Brevibacillusbrevis*	96%	KU973526.1
CDB5	*Bacillus thuringiensis*	97%	KU973525.1
CDB6	*Bacillus subtilis*	99%	KY007073.1
CDB7	*Brevibacillusparabrevis*	98%	KU555938.1
CDB8	*Bacillus subtilis*	98%	KU973529.1
CDB9	*Bacillus subtilis*	97%	KU533850.1
CDB10	*Bacillus subtilis*	99%	KU533851.1

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LOCUS       6f         986 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
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       61 GGTGAGTAAC ACGTGGGTAA CCTGCCTGTA AGACTGGGAT AACTCCGGGA AACCGGGGCT
      121 AATACCGGAT GCTTATTTGA ACCGCATGGT TCAAATATAA AAGGTGGCTT CGGCTACCAC
      181 TTACAGATGG ACCCGCGGCG CATTAGCTAG TTGGTGAGGT AACGGCTCAC CAAGGCAACG
      241 ATGCGTAGCC GACCTGAGAG GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC
      301 CTACGGGAGG CAGCAGTAGG GAATCTTCCG CAATGGACGA AAGTCTGACG GAGCAACGCC
      361 GCGTGAGTGA TGAAGGTTTT CGGATCGTAA AGCTCTGTTG TTAGGGAAGA ACAAGTACCG
      421 TTCGAATAGG GCGGTACCTT GACGGTACCT AACCAGAAAG CCACGGCTAA CTACGTGCCA
      481 GCAGCCGCGG TAATACGTAG GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC
      541 GCAGGCGGTT TCTTAAGTCT GATGTGAAAG CCCCCGGCTC AACCGGGGAG GGTCATTGGA
      601 AACTGGGGAA CTTGAGTGCA GAAGAGGAGA GTGGAATTCC ACGTGTAGCG GTGAAATGCG
      661 TAGAGATGTG GAGGAACACC AGTGGCGAAG GCGACTCTCT GGTCTGTAAC TGACGCTGAG
      721 GAGCGAAAGC GTGGGGAGCG AACAGGATTA GATACCCTGG TAGTCCACGC CGTAACGATG
      781 AGTGCTAAGT GTTAGGGGGT TTCCGCCCCT TAGTGCTGCA GCTAACGCAT TAAGCACTCC
      841 GCCTGGGGAG TACGGTCGCA AGACTGAAAC TCAAGGAATT GACGGGGGCC CGCACAAGCG
      901 GTGGAGCATG TGGTTTATTC GAAGCAACGC GAGACCTTAC CAGGTCTTGA CATCCTCTGA
      961 CATCCTAGAG ATAGGACGTC CCCTTC
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Figure 4. Partial sequence of 16S rRNA product gene sequence of isolate CDB6.

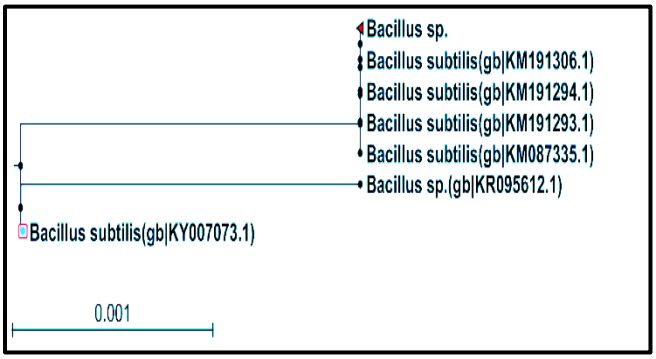

Figure 5. Phylogenetic tree for bacterial isolate CDB6.

3.4.3.2 Bacterial Isolate (CDB10):

DNA nucleotide sequence of 16S rRNA gene of CDB10 strain was amplified by PCR and partially sequenced for *Bacillus subtilis* strain ASB1. (KU533851.1).

```
LOCUS       10f        1149 bp    DNA     circular UNA
FEATURES             Location/Qualifiers
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       61 CGGACGGGTG AGTAACACGT GGGTAACCTG CCTGTAAGAC TGGGATAACT CCGGGAAACC
      121 GGGGCTAATA CCGGATGGTT GTTTGAACCG CATGGTTCAA ACATAAAAGG TGGCTTCGGC
      181 TACCACTTAC AGATGGACCC GCGGCGCATT AGCTAGTTGG TGAGGTAACG GCTCACCAAG
      241 GCAACGATGC GTAGCCGACC TGAGAGGGTG ATCGGCCACA CTGGGACTGA GACACGGCCC
      301 AGACTCCTAC GGGAGGCAGC AGTAGGGAAT CTTCCGCAAT GGACGAAAGT CTGACGGAGC
      361 AACGCCGCGT GAGTGATGAA GGTTTTCGGA TCGTAAAGCT CTGTTGTTAG GGAAGAACAA
      421 GTACCGTTCG AATAGGGCGG TACCTTGACG GTACCTAACC AGAAAGCCAC GGCTAACTAC
      481 GTGCCAGCAG CCGCGGTAAT ACGTAGGTGG CAAGCGTTGT CCGGAATTAT TGGGCGTAAA
      541 GGGCTCGCAG GCGGTTTCTT AAGTCTGATG TGAAAGCCCC CGGCTCAACC GGGGAGGGTC
      601 ATTGGAAACT GGGGAACTTG AGTGCAGAAG AGGAGAGTGG AATTCCACGT GTAGCGGTGA
      661 AATGCGTAGA GATGTGGAGG AACACCAGTG GCGAAGGCGA CTCTCTGGTC TGTAACTGAC
      721 GCTGAGGAGC GAAAGCGTGG GGAGCGAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA
      781 AACGATGAGT GCTAAGTGTT AGGGGGGTTT CCGCCCCTTA GTGCTGCAGC TAACGCATTA
      841 AGCACTCCGC CTGGGGAGTA CGGTCGCAAG ACTGAAACTC AAAGGAATTG ACGGGGGCCC
      901 GCACAAGCGG TGGAGCATGT GGTTTAATTC GAAGCAACGC GAGAACTTAC CAGGTCTTGA
      961 CATCCTCTGA AATCCTAGAG ATAGGACGTC CCCTTCGGGG GCAGAGTGAA CAGGTGGTGC
     1021 ATGTTGTCGT CAGCTCGTGT TCGTGAGATG TTGGTTAGTC CCGCACGAGC GCACCCTTTG
     1081 ATCTTANTTG CCAGCATTCA GTTGGGCACC TCTAAGGTGA CTGCCGGGTG ACCAACCGGA
     1141 GGAANNNNNN
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Figure 6. Partial sequence of 16S rRNA product gene sequence of isolate CDB10.

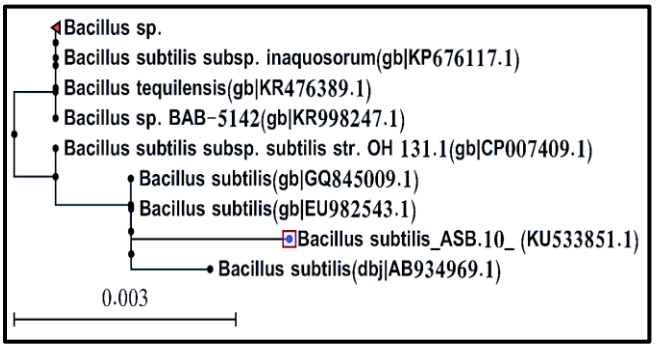

Figure 7. Phylogenetic tree for bacterial isolate CDB10.

3.4.3.3 Aggregated Phylogenetic Tree for Ten Bacterial Isolates (CDB1-10).

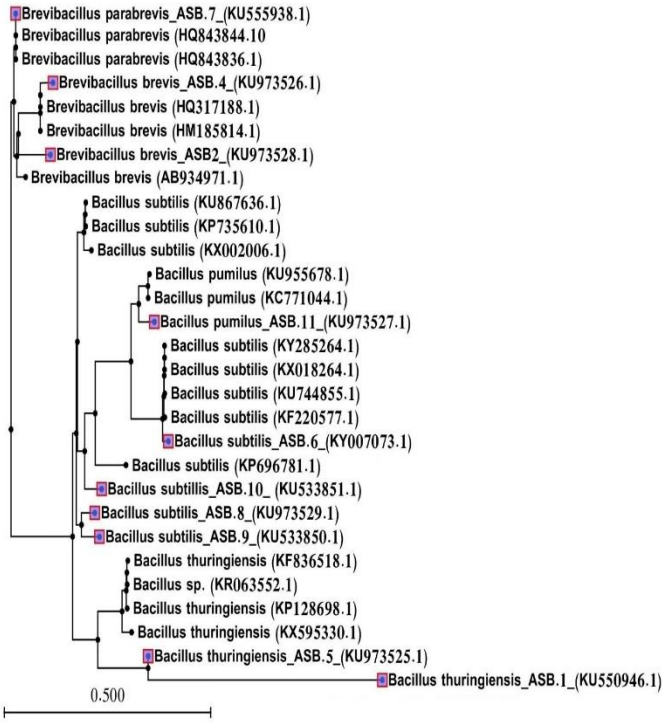

Figure 8. Phylogenetic tree for ten bacterial isolates (CDB1-10).

4 Conclusions

Based on the results of the current study, One hundred and seven isolates (89.16 %) exhibited cellulases positive, whereas thirteen isolates (10.83 %) were cellulases negative. Among 107 isolates that showed cellulases activity, ten bacterial isolates characterized by high potent ability to produce cellulases enzymes. These isolates were characterized and identified. Two isolates (CDB6 & CDB10) were found to be highest productivity of cellulases enzymes, 7.268 and 7.724 U/ml on FPCase, respectively, whereas on CMCase assay maximum activity was 11.077 U/ml by isolate CDB6 and 12.152U/ml by CDB10. Ten cellulose degrading bacterial isolates were identified as *Bacillus subtilis* (four isolates: CDB6, CDB8, CDB9 and CDB10), *Bacillus thuringeinsis* (two isolates: CDB1 and CDB5), *Brevibacillus brevis* (two isolates: CDB2 and CDB4), *Brevibacillus parabrevis* (one isolate: CDB7) and *Bacillus pumilus* (one isolate: CDB3).

References

[1] S. Haruta, S. Kato, Z. Cui, M. Ishii, and Y. Igarashi: *Cellulose degrading microbial community*, In Proc. JSPSNRCT/ DOST/LIPI/VCC Multilateral Cooperative Research Program in the Field of Biotechnology, 287-291, (2003).

[2] Y. Habibi, L. A. Lucia, and O. J. Rojas: Cellulose nanocrystals, chemistry, self- assembly and applications.

Chem. Rev.,**110 (6)**, 3479-3500,(2010).

[3] S. Jagtap, and M. Rao: Purification and properties of a low molecular weight 1,4-beta-D-glucan glucohydrolase having one active site for carboxymethyl cellulose and xylan from an alkalothermophilic*Thermomonospora* sp. *Biochem. Biophys. Res. Commun*., **329(1)**, 111-116,(2005).

[4] S. Koizumi, Z.Yue, Y. Tomita, T. Kondo, H. Iwase, D. Yamaguchi, and T. Hashimoto: Bacterium organizes hierarchical amorphous structure in microbial cellulose. *Eur. Phys. J. E*., **26 (1)**, 137-142,(2008).

[5] A. Singh, R. C.Kuhad, and O. P. Ward: Industrial application of microbial cellulases. In R. C.Kuhad, and A. Singh, Lignocellulose Biotechnologgy: Future Prospects,New Delhi, India: *I. K. International Publishing House*,345-358, (2007).

[6] R. C. Kasana, R.Salwan, H.Dhar, S.Dutt, and A. Gulati: A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. *Current Microbiology*, 57, 503-507, (2008).

[7] L. R. Lynd, P. J. Weimer, W. H. van-Zyl, and I. S. Pretorius: Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiol. Mol. Biol. Rev*., **66(3)**, 506-577, (2002).

[8] M. Maki, K. T. Leung, and W. Qin: The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int. J. Biol. Sci*., 5, 500-516, (2009).

[9] R. Rawat, and L. Tewari: Purification and characterization of an acidothermophiliccellulase enzyme produced by *Bacillus subtilis* strain LFS3. Extremophiles, **16(4)**, 637-644, (2012).

[10] B. K.Kim, B. H.Lee, Y. J. Lee, I. H.Jin, C. H. Chung, and J. W. Lee: Purification and characterization of carboxymethylcellulase isolated from a marine bacterium, *Bacillus subtilis* subsp. subtilis A-53. *Enzym. Microb. Technol*., 44, 411-416, (2009).

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

[12] P. Lisdiyanti, E.Suyanto, N. F.Gusmawati, and W. Rahayu: Isolation and characterization of cellulose produced by cellulolytic bacteria from peat soil of OganKomeringIlir, South Sumatera. *Int. J. Environ. Bioener*., 3, 145-153, (2012).

[13] K. Apun, B. C. Jong, and M. A. Salleh: Screening and isolation of a cellulolytic and amylolytic Bacillus from sago pith waste. *Journal of Genneral and Applied Microbilogy*, 46, 263-267, (2000).

[14] R. L.Howard, P. Masoko, and E. Abotsi: Enzyme activity of a Phanerochaetechrysosporiumcellobiohydrolase (CBHI. 1) expressed as a heterologous protein from *Escherichia coli*. *African Journal of Biotechnology*, **2 (9)**, 296-300,(2003).

[15] H. Ariffin, N. Abdullah, M. S.UmiKalsom, Y.Shirai, and M. A. Hassan: Production and characterization of cellulase by *Bacillus pumilus* EB3. *International Journal of Engineering and Technology*, **3(1)**, 47-53,(2006).

[16] S. J.Sreeja, P. W.Jeba-Malar, F. R. Sharmila Joseph, T. Steffi, G. Immanuel, and A. Palavesam: Optimization of cellulase production by *Bacillus altitudinis* APS MSU and *Bacillus licheniformis* APS2 MSU, gut isolates of fish Etroplussuratensis. *IJOART*., 2, 401-406,(2013).

[17]A. Chantarasiri: Novel halotolerant cellulolytic *Bacillus methylotrophicus* RYC01101, isolated from ruminant feces in Thailand and its application for bioethanol production. KMUTNB *Int. J. Appl. Sci. Technol*., **7 (3)**, 63-68, (2014).

[18] C. W. Hendricks, J. D. Doyle, and B. Hugley: A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Applied and Environmental Microbiology*, **61(5)**, 2016-2019,(1995).

[19] W. J. Lu, H. T. Wang, and Y. F. Nie: Effect of inoculating flower stalks and vegetable waste with ligno-cellulolytic microorganisms on the composting process. *Journal of Environmental Science and Health*, Part B., **39 (5-6)**, 871-887,(2004).

[20] T. K. Ghose: Measurement of cellulase activity. *Pure and Applied Chemistry*, 59, 257-268, (1987).

[21] C. Wang, T.Hseu, and C. Huang: Introduction of cellulase by cello-oligosaccharides in *Trichodermakonigii*G-39. *J. Biotechnol*., 9, 47-60,(1988).

[22] G. L. Miller: Use of di-nitrosalicylic acid reagent for determination of reducing sugar. *Biotechnol. Bioeng. Symp*., 5, 193-219,(1972).

[23] N. J. GadgilH. F.Daginawala, T.Chakarabarti, and P. Khanna: Enhanced cellulase production by a mutant of *Trichodermareesei*.*Enzyme Microb Technol*., 17, 942-946,(1995).

[24] R. E. Buchanan, and N. E. Gibbons:Bergey's manual of determinative bacteriology. (Eighth edition), The Williams and Wilkins Co., Baltimore, 747-842,(1974).

[25] D. J. Lane: 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics, Eds, Stackebrandt E, Goodfellow M (Wiley, New York), 115-175,(1991).

[26] F. L. Soares, I. S. Melo, A. C. Dias, and F. D. Andreote: Cellulolytic bacteria from soils in harsh environments. *World Journal of Microbiology and Biotechnology*, **28(5)**, 2195-2203,(2012).

[27] S. Sethi, A.Datta, B. L. Gupta, and S. Gupta: Optimization of cellulase production from bacteria isolated from soil. *ISRN Biotechnol*., 2013: 985685. 10.5402/2013/985685 [PMC free article].

[28] M. F., Eida, T. Nagaoka, J.Wasaki, and K. Kouno: Isolation and characterization of cellulose-decomposing bacteria inhabiting sawdust and coffee residue composts.*Microbes Environ*., 27, 226-233,(2012).

[29] S. Ghio, G. S. D. Lorenzo, V.Lia, P. Talia, A.Cataldi, and D. Grasso: Isolation of *Paenibacillus*sp. and *Variovorax* sp. strains from decaying woods and characterization of their potential for cellulose deconstruction. *Int. J. Biochem. Mol. Biol*., 3, 352-364,(2012).

[30] K. P. P. Jyotsna, K.Vijayalakshmi, N. D.Prasanna, and S. K. Shaheen: Isolation and characterization of cellulose producing *Lysinibacillussphaericus* MTCC No 9468 from gut of EiseniaFoetida. *Bioscan*., 6, 325-327,(2010).

[31] K. I. Dantur, R. Enrique, B.Welin, and A. P.

Castagnaro:Isolation of cellulolytic bacteria from the intestine of Diatraeasaccharalis larvae and evaluation of their capacity to degrade sugarcane biomass. *AMB Exp*., 5: 15,(2015).

[32] S. Singh, V. S.Moholkar, and A. Goyal: Isolation, identification, and characterization of a cellulolytic *Bacillus amyloliquefaciens* strain SS35 from rhinoceros dung. *ISRN Microbiology*, 1-7,(2013).

[33] S. Ji, S. Wang, Y. Tan, X. Chen, W. Schwarz, and F. Li: An untapped bacterial cellulolytic community enriched from coastal marine sediment under anaerobic and thermophilic conditions. *FEMS Microbiol. Lett*., 335, 39-46,(2012).

[34] K. Harshvardhan, A. Mishra, and B. Jha: Purification and characterization of cellulase from a marine *Bacillus* sp. H1666: a potential agent for single step saccharification of seaweed biomass. *J. Mol. Catal. B Enzy*., 93, 51-56,(2013).

[35] V. S. Santhi, A. Gupta, S.Saranya, and S. R. D. Jebakumar: A novel marine bacterium *Isoptericola* sp. JS-C42 with the ability to saccharifying the plant biomasses for the aid in cellulosic ethanol production. *Biotechnol. Rep*., 1-2, 8-14,(2014).

[36] V. S. Santhi, A. K.Bhagat, S.Saranya, G.Govindarajan, and S. R. D.Jebakumar: Seaweed (*Eucheumacottonii*) associated microorganisms, a versatile enzyme source for the lignocellulosic biomass processing. *Int. Biodeterior. Biodegradation*, 96, 144-151,(2014).

[37] A. S. Baharuddin, M. N. A.Razak, L.S. Hock, M. N. Ahmad, S.Abd-Aziz, N. A. A. Rahman, U. K. M. Shah, M. A. Hassan, K. Sakai, and Y. Shirai: Isolation and characterization of thermophiliccellulase producing bacteria from empty fruit bunches palm oil mill effluent compost. *The American Journal of Applied Sciences*, **7(1)**, 56-62,(2010).

[38] A. V. Gomashe, P. A.Gulhane, and P. M. Bezalwar: Isolation and screening of cellulose degrading microbes from nagpur region soil. Int. J. of Life Scie., 1, 291-293,(2013).

[39] P. Das, R. Solanki, and M. Khanna: Isolation and screening of cellulolytic actinomycetes from diverse habitats. *International Journal of Advanced Biotechnology and Research*, 5, 438-451,(2014).

[40] B. I. Patagundi, C. T. Shivasaran, and B. Kaliwal: Isolation and characterization of cellulase producing bacteria from soil. *International Journal of Current Microbiology and Applied Sciences*, 3, 59-69,(2014).

[41] S. Hatami, H. A.Alikhani, H.Besharati, N.Salehrastin, M.Afrousheh, and Z. Y. Jahromi: Investigation on aerobic cellulolytic bacteria in some of north forest and farming soils. *The American-Eurasian Journal of Agricultural and Environmental Sciences*, 5, 713-716,(2008).

[42] W. J. Lu, H. T. Wang, S. J. Yang, Z. C. Wang, and Y. F. Nie: Isolation and characterisation of mesophilic cellulose degrading bacteria from flower stalks-vegetable waste co-composting system. *Journal of General Applied Microbiology*, **51 (6)**, 353-360,(2006).

[43] P. Gupta, K.Samant, and A. Sahu: Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *International Journal of Microbiology*, Oct. 2012.

[44] S. Sadhu, P.Saha, S. K.Sen, S.Mayilraj, and T. K. Maiti:Production, purification and characterization of a novel thermotolerantendoglucanase (CMCase) from Bacillus strain isolated from cow dung. *Springer Plus*.,**2(1)**, 1-10,(2013).

[45] P. Pason, K. L.Kyu, and K. Ratanakhanokchai:*Paenibacilluscurdlanolyticus* strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. *Applied and Environmental Microbiology*, **72 (4)**. 2483-2490,(2006).

[46] G. D.Saratale, R. G. Saratale, and S. E. Oh: Production and characterization of multiple cellulolytic enzymes by isolated *Streptomyces* sp. MDS. *Biomass and Bioenergy*, 47, 302-315,(2012).

[47] A. K. Kumar, and B. S. Parikh: Cellulose-degrading enzymes from *Aspergillusterreus* D34 and enzymatic saccharification of mild-alkali and dilute acid pretreated lignocellulosic biomass residues. *Bioresources and Bioprocessing*, **2 (1)**, 1-13,(2015).

[48] P. R.Waghmare, S. D.Kshirsagar, R. G.Saratale, S. P. Govindwar, and G. D. Saratale: Production and characterization of cellulolytic enzymes by isolated *Klebsiella* sp. PRW-1 using agricultural waste biomass. *Emirates Journal of Food and Agriculture*, **26(1),** 44-59, (2014).

[49] D. Deka, P.Bhargavi, A. Sharma, D.Goyal, M. Jawed, and A. Goyal: Enhancement of cellulase activity from a new strain of *Bacillus subtilis* by medium optimization and analysis with various cellulosic substrates. *Enzyme Research*, 1-8,(2011).

[50] G. Rastogi, G. L.Muppidi, R. N.Gurram, A.Adhikari, K. M. Bischoff, S. R. Hughes, and R. K. Sani: Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. *Journal of Industrial Microbiology and Biotechnology*, **36(4)**, 585-598,(2009).

[51] V. K. Singh, and A. Kumar: Production and purification of an extracellular cellulase from *Bacillus brevis* VS-1. *Biochem. Mol. Biol. Int*., 45, 443-452,(1998).

[52] S. O. Kotchoni, E. W.Gachomo, B. O.Omafuvbe, and O. O. Shonukan: Purification and Biochemical Characterization of CarboxymethylCellulase (CMCase) from a Catabolite Repression Insensitive Mutant of *Bacillus pumilus*. *Int. J. Agri. Biol*., 8, 286-292,(2006).

[53] L. J. Yin, H. H. Lin, and Z. R. Xiao: Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. *J. Marine Sci. Technol*., 18, 466-471,(2010).

[54] K. Tamura, D. Peterson, N. Peterson, G.Stecher, M.Nei, and S. Kumar: MEGA 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Molecular Biology and Evolution*, 28, 2731-1739,(2011).