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Isolation and Identification of a Potential Cellulose Degrading Bacterium *Bacillus* sp. Strain BCD1 from Cow Dung Sample

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ABSTRACT

Cellulose polysaccharide is comprised with β-1,4 glycosidic linked D-glucose molecules and it became a most abundant organic substance in the environment. These resistant and water insoluble materials are majorly found in cell wall of plant system. It is gradually accumulated in environment as a dominant organic waste. We can utilize these cellulosic waste materials as an amenable energy source in form of easy sugars. The current article reveals on a potential cellulose degrading bacterium. The bacterium, BCD1 is isolated from a cow dung sample of Natunpally (23.17170°N, 87.52487°E) village of Mirzapur area, Burdwan, West Bengal, India. The mesophilic bacterium is a gram-positive in nature and grown optimally at 32°C temperature. The bacterial isolate is identified as *Bacillus* sp. strain BCD1 based on 16S rRNA gene based molecular phylogenetic approach. The BCD1 bacterium expresses 0.67 Cellulolytic index in Congo red screening technique. The article also discusses about the quantitative estimation of cellulose degradation by performing extracellular protein concentration and dinitrosalicylic assay. The efficiency of the enzyme liberated by *Bacillus* sp. strain BCD1 is 16.9780 mg glucose liberated/mg protein / 30 minutes at 32˚C temperature.

Keywords *Bacillus* sp. strain BCD1, Cellulose degradation, CMC-agar, Screening, 16S rRNA gene.

INTRODUCTION

Cellulose biopolymer is comprised with β-1,4-glycosidic linked D-glucose molecules. It is the most abundant and resistant organic matter on the earth and an inexhaustible renewable resource (Zhang *et al.* 2008). Cellulosic biomass is comprised with cellulose, lignin and hemi-cellulose molecules and it is considered as a potential and promising raw material for future renewable energy (Limayem and Ricke 2012). Cellulose can be converted into

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various important products like ethanol, glucose, hexitols, 5-hydroxymethylfurfural, levulinic acid, methane, butanol, alkanes, hexane, succinic acid, ethyl lactate (Ching *et al*. 2017 and Mcintosh *et al.* 2016). Cellulose contributes approximately 35–50% of plant dry weight. Both of them are present in plant dry weight about 20–35% and 5–30% respectively. The cellulases enzyme can breaks this insoluble cellulose into soluble and easy sugars (Behera *et al.* 2017). Cellulases enzyme are used extensively in various industries like paper, textiles, laundry, food and (Gaur and Tiwari 2015). Cellulase is belongs to glycosyl hydrolase family that cleaves β-1-4 glycosidic bonds of cellulose molecule. Cellulase enzymes can be classified into three groups: Endoglucanase (EC 3.2.1.4), exoglucanase, cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) based on their specific hydrolytic sites (Sharma *et al.* 2016). This group of enzymes work together like endoglucanases hydrolyzed the polymeric chain of cellulose at internal position, which is further degraded by cellobiohydrolase and β-glucosidase (Brás *et al*. 2011). Due to incomplete combustion of fossil fuels and enhancement of green-house gases, now an attention has been arising in conversion of lignocellulosic waste to bioethanol production and sugar is formed through enzymatic action of cellulases and bioethanol conversion by fermenting the sugar (Zaldivar *et al.* 2001 & Sun and Cheng 2002). Few fungal genera like *Trichoderma*, *Aspergillus*, *Melanoporia* exhibit a strong ability to secret multi-cellulases (do Rêgo de Oliveira *et al.* 2016). Some bacteria like *Pseudomonas fluorescens*, *Bacillus subtilis*, *E. coli* and *Serratia marscens* produce cellulase enzymes that can hydrolyze cellulose biopolymer (Sethi *et al.* 2013). The major industrial applications of cellulases are fabrics bio-polishing, creating stonewashed look in textile industry and the improvement of household laundry detergents and also as an animal fodder for improving the nutritional quality and digestibility, in processing of fruit juice and in baking, while de-inking of paper is yet another emerging application (Hill *et al.* 2006). This study reports about a potential cellulose-degrading bacterium from cow-dung sample. This report will be carried a better commercial market through cellulose bioconversion via the bacterial metabolism and it will create a safe management system of cellulose-rich waste.

MATERIALS AND METHODS

Collection of sample

The cow-dung sample was collected from Natunpally (23.17170˚N, 87.52487˚E) village of Mirzapur area, Burdwan, West Bengal, India. It was collected in sterile container and stored in refrigerator at 4˚C temperature until used.

Medium for isolation

Isolation was carried out by serial dilution of the cow-dung sample followed by spread plate technique on Carboxymethyl cellulose (CMC) agar. The media comprised with 10 g carboxymethylcellulose, 2 g tryptone, 4 g KH_2PO_4, 4 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.001 g $CaCl_2$ $.2H_2O$, 0.004 g $FeSO_4.7H_2O$, 15 g Agar and pH 7 for 1 liter (Ray *et al.* 2007). The plates were incubated at 32°C temperature in BOD incubator for 24-48 hr. For purification, the selected bacterial isolates were streaked on CMC agar plate for several times.

Screening for cellulose degrading bacteria and measurement of cellulolytic index

The confirmation test was performed by using Congo red reagent. The selected bacterial isolate was grown on CMC agar. The media comprised with 10 g carboxymethylcellulose, 2 g tryptone, 4 g KH_2PO_4, 4 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.001 g $CaCl_2$ $.2H_2O$, 0.004 g $FeSO_47H_2O$, 15 g Agar and pH 7 for 1 liter (Ray *et al.* 2007). The bacterial isolate was incubated for 24–48 hrs growth. Then, the culture plate was flooded with 0.1% (w/v) Congo red reagent and allowed it for 15 min at room temperature. Finally, it was washed with 1M NaCl (Apun *et al*. 2000) and waited for 5–10 minutes to visualize a light halo zone around the culture.

Cellulose hydrolyzation was detected through the appearance of a clear zone around the bacterial culture. The diameter of halo zone and colony were measured to calculate the Cellulolytic index (CI). The following formula was used to calculate the Cellulolytic index (Cellulolytic index (CI) = (Diameter of zone - Diameter of colony) / Diameter of bacterial colony) (Choi *et al*. 2005). The cellulolytic efficiency of any microorganism is depended upon their CI value.

Phenotypic characterization

The selected bacterial culture was maintained in both CMC agar plates and CMC broth media by periodic sub-culturing at regular 7–10 days interval. The culture was stored in refrigerator at 4°C temperature and used as inoculum for various biochemical assays like Gram's reaction, citrate utilization, gelatinase activity, catalase test, caseinase activity, amylaze production test.

Identification and phylogenetic tree construction

In order to identification, genomic DNA was isolated from the isolate according to Murmur's protocol (Johnson 1994) followed by agarose gel electrophoresis. Amplification of 16S rRNA gene was carried out from genomic DNA using two universal primers namely 27f (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492r (5′ TAC GGT TAC CTT GTT ACG ACT T 3′). Then, 16S rRNA gene sequence was amplified with an automated, programmable thermal cycler (Bio-Rad). Finally, the amplified product was sent to Barcode Biosciences for sequencing.

Manually the forward and reverse complement sequences of 16S rRNA gene amplicon were aligned and joined to obtain a contig gene sequence. 16S rRNA gene sequence was analyzed through nucleotide BLAST tool in NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi ? PROGRAM=blastn & PAGE_TYPE=Blast Search & LINK_LOC=blasthome) Some closely related sequences were retrieved from the NCBI core nucleotide database by BLAST program. A phylogenetic tree was constructed with the closest phylogeny groups using MEGA 11 software version by bootstrap method with 1000 bootstrap replications.

Extraction and estimation of bacterial extracellular protein

The bacterium was grown in CMC broth in BOD incubator shaker at 120 rpm and 32°C temperature for 7 days. In extraction of extracellular protein sample, the entire suspension was filtrated through Whatman filter No.1 filter paper. Then, this filtrated sample was used for quantification of extracellular protein and cellulases activity (Lone *et al*. 2012).

Firstly, Bovine Serum Albumin (BSA) standard curve was prepared against 10 different concentrated solutions (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/ml). One blank set was also prepared and used as reference. Here, 2 ml of Bradford reagent was added in 1 ml of each concentrated solution. Then, the cuvettes were covered with plastic paraffin film and mixed by gentle inversion. These were kept at room temperature for about 10 minutes and measured absorbance spectrophotometrically at 595 nm (Bradford 1976).

The extracellular protein content of the bacterial isolate was estimated by using the Bradford's method (Bradford 1976). Then, the quantification of cellulases of extracellular proteinaceous supernatant sample was calculated by comparing the absorbance of the extracted protein sample to the BSA standard curve.

Assay of cellulases estimation by dinitrosalicylic acid (DNS)

Firstly, glucose standard curve was prepared against 8 different concentrated (0.71, 0.8, 1, 1.25, 1.65, 2, 2.5 and 3.35 mg/0.5 ml) glucose solution in citrate buffer. In preparation of 0.8 mg/0.5 ml concentrated glucose solution, 5 ml of 0.05 M citrate buffer (pH 4.8) was added with 1 ml of glucose stock solution (10 mg/ml). In each standard set, 1 ml of standard solution was taken first and next 3 ml of DNS reagent was mixed and boiled it for 5 min. Then, diluted this mixture solution with 20 ml of distilled water and taken the absorbance at 540 nm (Ghose 1987).

In case of bacterial cellulases assay, 0.5 ml of CMC solution (0.45 ml CMC broth and 0.05 ml of 0.05 M Citrate buffer (pH 4.8)) was taken in reaction tube and then, 0.5 ml of culture supernatant was added. Then the reaction was incubated at 32°C temperature for 30 min. Later, 3 ml of DNS reagent was added to halt the reaction. Lastly, it was boiled for 5 min and diluted this mixture solution with 20 ml of distilled water and taken the absorbance at 540 nm with blank reference (Zhang Jing Qiang *et al*. 2008). The blank mixture contains 0.5 ml citrate buffer, 0.5 ml distilled water and 3.0 ml of DNS solution. Similarly, it was boiled and diluted as previous way.

The quantity of extracellular protein sample of bacterial isolate *Bacillus* sp. strain BCD1 was calculated in 1ml of the supernatant (mg/ml), but 0.5 ml supernatant was used. Cellulases enzymatic activity was determined through the following formula:

Cellulases Enzymatic activity = Amount of glucose liberated / mg protein / 30 minutes (Zhang Jing Qiang *et al.* 2008).

RESULTS AND DISCUSSION

Isolation and screening of cellulose degrading bacterium

The isolated bacterium, BCD1 was grown on CMC agar medium Fig. 1 (A) at 32°C temperature and it was purified through periodic subculturing in fresh CMC agar media. The bacterium is rod shaped and Gram-positive in nature Fig. 1 (B). The isolate was screened through Congo red confirmation test with control plate Fig. 1 (C). The isolate BCD1 developed a prominent halo zone Fig. 1 (D) around the culture that indicates cellulase production.

Table 1. Cellulolytic index of BCD1 isolate.

Isolate	Zone diameter (cm)	Colony diameter (cm)	CI value
Bacillus sp. strain BCD1	3.5	2.1	0.67

Calculation of cellulolytic index

CMC decomposition was identified by the appearance of a halo zone Fig. 1 (D) surrounding the bacterial colony. The bacterium, BCD1 exhibited 0.67 Cellulolytic index (CI) that was calculated in the Table (Table 1).

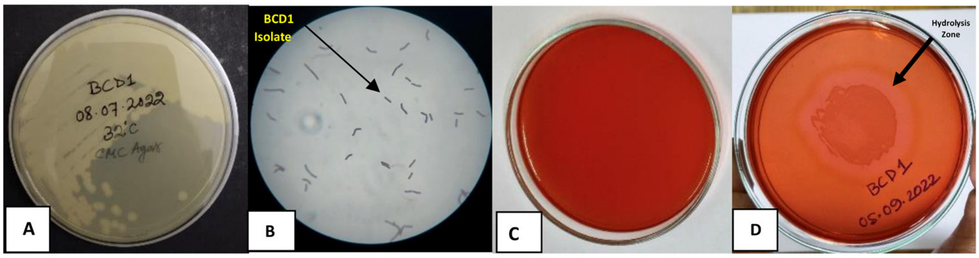

Fig. 1. (A) Growth on CMC agar plate, (B) Gram's staining (Oil immersion view), (C) Congo red staining in CMC agar (control plate) and (D) BCD1 isolate in CMC agar with congo red staining.

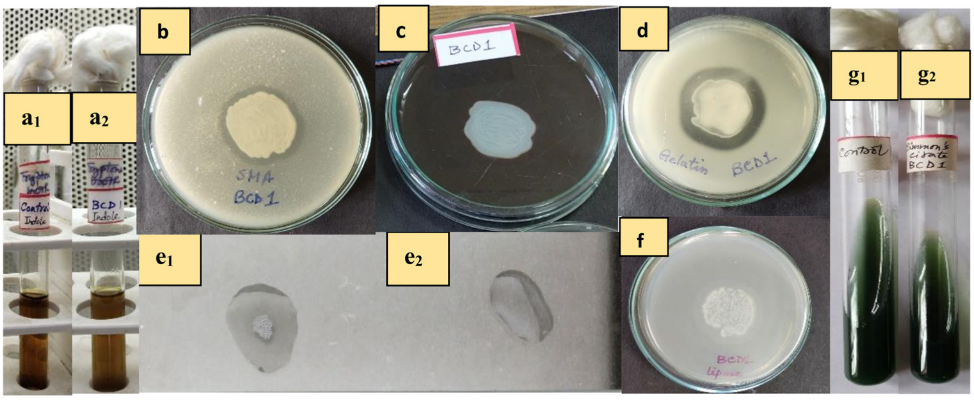

Fig. 2. (a) Indole production test (a_1) control and (a_2) BCD1, (b) Caseinase test in Skim milk Agar (SMA), (c) Amylase test, (d) Gelatinase test, (e) Catalase test (e_1) BCD1 and (e_2) control, (f) Lipase activity, (g) Citrate utilization test (g_1) control and (g_2) BCD1 in simmon citrate agar.

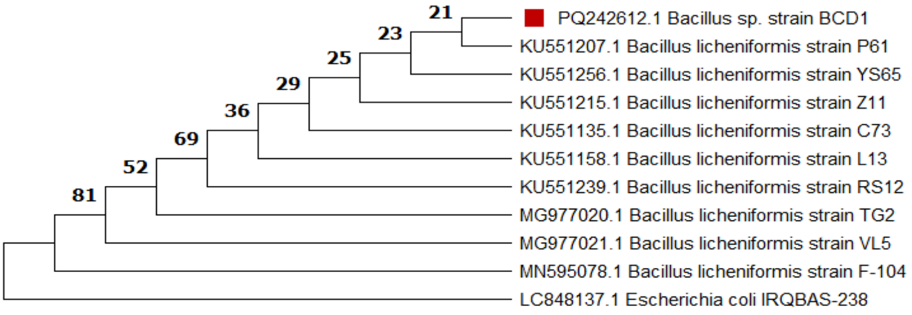

Fig. 3. Phylogenetic tree of the *Bacillus* sp. strain BCD1 with its closest phylogeny groups.

Table 2. Phenotypic characters of BCD1 isolate. '+' sign indicates positive result and '–' sign indicates negative result.

Phenotypic characters	Results
Colony color	White
Colony shape	Round
Colony elevation	Flat
Colony surface	Entire
Gram staining	+
KOH test	-
Indole production	-
Citrate utilization	-
Gelatinase activity	+
Catalase test	+
Caseinase activity	+
Amylaze activity	-
Lipase activity	+
Urease test	-

Table 3. Extracellular protein sample from BCD1 strain.

Protein sample	Absorbance at 595 nm	Protein concentration of BCD1 sample (mg/ml) ((OD-intercept)/slope)
Blank	0	0
BCD1	0.192	0.3667

Morphological and phenotypic characterization

Several phenotypic characters of BCD1 isolate were examined. The results were enlisted (Table 2) and documented (Fig. 2 (a–g).

Identification through 16S rRNA gene sequence analysis

Manually the forward and reverse complement sequences of 16S rRNA gene amplicon were aligned and joined to obtain 1558 nucleotides long contig gene sequence. The processed 16S rRNA gene was submitted in the GenBank database of NCBI with an accession no. PQ242612.1. We retrieved closely 9 sequences which showed up to 99.28% sequence identity with BCD1 isolate and one *E. coli* strain IRQ-BAS-238 used here as an out group. A phylogenetic tree (Fig. 3) was constructed using MEGA 11 software version by bootstrap method with 1000 bootstrap replications. As the isolate BCD1 shows highest sequence similarity with *Bacillus licheniformis* strain P61. Thus, the authors designated the isolate BCD1 as *Bacillus* sp. strain BCD1.

Extracellular protein estimation

The extracellular protein content of the bacterial isolate was estimated by using the Bradford's method. Then, the optical density of the extracted protein sample was compared with the BSA standard curve (Fig. 4). The extracted extracellular proteins were

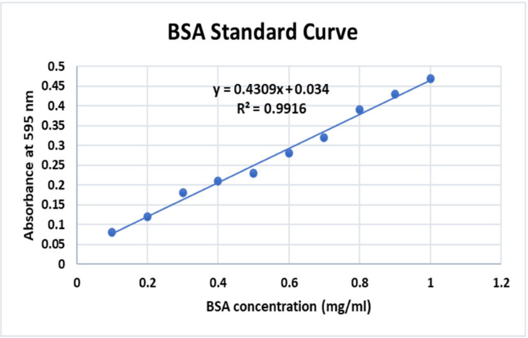

Fig. 4. BSA standard curve.

Table 4. Cellulase enzymatic activity and OD_{540}.

Sample	Absorbance at 540 nm	Glucose concentration of BCD1 sample (mg/0.5 ml) (OD-intercept)/slope)
Blank	0	0
BCD1	2.237	3.1242

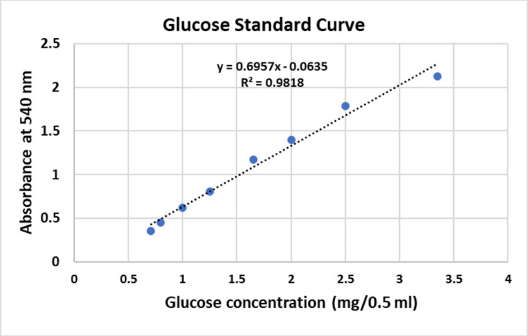

Fig. 5. Glucose standard curve (DNS method)

present in the supernatant at 0.3667 mg/ml concentration (Table 3).

Assay of cellulases by DNS method at 32˚C temperature

The cellulase activity of BCD1 isolate was mathematically calculated by using the data of protein content (0.3667 mg/ml) present in the supernatant of isolate which was estimated by Bradford's method. The quantification of liberated glucoses in cellulases assay was estimated with glucose standard curve (Fig. 5) and according to reaction set up, the bacterium BCD1 showed glucose liberation at 3.1242 mg per 0.5 ml culture supernatant (Table 4). In calculation, the efficiency of the enzyme liberated by *Bacillus* sp. strain BCD1 was found to be 16.9780 mg glucose liberated / mg protein / 30 minutes at 32°C.

CONCLUSION

In this current article, author also concluded that the strain *Bacillus* sp. strain BCD1 may be a potential candidate as an industrially important microorganism for cellulase production. With the increasing advancement of biotechnological application, the market value for industrial cellulase will be grew up in very next future. Presently, industries are competing for cellulase production for industrial and pharmaceutical purpose. We can use this dominant cellulosic waste as a source of sugar (D-glucose) in industrial beverage production, pharmaceutical syrup production. Thus, these vast amounts of cellulosic waste can be reduced from nature and create a beneficial approach towards society. In this regard, pharmaceutical as well as large scale industries were giving a huge emphasis on isolation of cellulase producing microorganisms from environment. This will play a crucial role in minimizing the costs of production. Now a day's researchers expressed more curiosity about bacterial cellulase than fungal cellulase due to their higher growth along with thermostable and alkali stable properties.

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