Biochemical and molecular characterization of cellulase producing bacterial isolates from cattle dung samples

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Abstract

Recently production of bio fuel and other platform chemicals from lignocellulosic biomass is gaining constant attention to meet the demand of energy all over the Globe. Utilizing agricultural residues for bio ethanol generation using novel bacterial isolates will provide a solution for energy problem. This study has been conducted with the following objectives; i) to search the novel bacterial isolates that can efficiently degrade the cellulose and ii) their biochemical and molecular characterization by using 16S rRNA sequencing. Almost, 15 bacteria's have been isolated from cattle dung out of which 10 isolates showed cellulase activity on CMC (carboxymethyl cellulose) screening media. These bacterial isolates with high cellulase activity were further characterized on the basis of gram staining, morphology and also by various biochemical utilization tests (Citrate, lysine, ornithine, urease, phenylalanine, H_2S production, nitrate reduction, glucose, lactose, adonitol, sorbitol, arabinose, and 35 different carbohydrate sources). Results showed that out of three isolates (AKPCD108, AKPCD109 and AKPBD1), the isolate number AKPBD1 showed 58.33 % utilization of biochemical's which is followed by AKPCD108 and AKPCD109, respectively. Among all carbohydrates utilized, AKPBD1 showed 68.57% utilization and other isolates (AKPCD108 & AKPCD109) were at par. These three isolates were molecularly identified using 16S rDNA sequencing. The obtained sequences were blast and phylogenetic analysis of their sequences showed that AKPCD108 has 99% similarity with *Pseudomonas otitidis* strain MCC10330, AKPCD109 has 99% similarity with *Stenotrophomonas koreensis* strain TR6-01 (*Flavobacterium lutescens*) and AKPBD1 has 99% similar with *Serratia marcescens* subsp. sakuensis strain KRED. In this study, we conclude that, these isolates are highly promising and have the ability to produce cellulase enzyme and can be used for efficient biological pretreatment of lignocellulosic biomass following production of biofuels and bioproducts.

Keywords: Cellulolytic bacteria; Lignocelluloses; 16S rDNA; Biomass; Biofuel

Introduction

Depletion of fossil fuels and worldwide increases of energy demand and environmental concern has lead in search for an alternative renewable energy resource like lignocellulosic biomass [1,2]. Lignocelluloses are renewable, cost efficient, eco-friendly potential feedstock for the production of bio fuels. However, the bioconversion of this lignocellulosic biomass into sugars is not easy due to the cellulose crystallinity, degree of polymerization, presence of lignin which poses hurdle for enzymatic hydrolysis. Thus, utilizing different pre-treatment methods for bioconversion process increases the overall digestibility of cellulose and hemicelluloses, and therefore removal of lignin from lignocellulosic biomass. However, among the pre-treatment, use of chemical pre-treatment is hazardous for the environment. Therefore, the biological pre-treatment method on the other hand is environmental friendly and gaining popularity now a day because this method is less toxic, requires very low energy input, and much higher sugar yield. The biomass of the plant is mainly composed of cellulose, hemicelluloses and lignin, which constitute an important material to be converted into various type of liquid fuels [3–5].

Present trends to produce cellulosic ethanol has been focused mainly on sorghum, wheat straw, sunflower, maize, sugarcane, and non-edible oil seed plants; however, use of agricultural residues that are not exploited or which could be used more efficiently for ethanol production. The waste material generated from various agricultural fields like stalks, straw, husk, stover, and stems, etc. are highly rich in cellulosic content that had generated great interests to use them as an energy source [6–9]. Among all the component of biomass, cellulose is the principal constituent of the cell wall and mainly composed of units of D-glucose that are linked together to form a linear chain by ß-1,4-glycosidic linkages. Cellulose is generally found as micro fibrils that are "2–20nm" in diameter and "100–40,000 nm" long in the biomass. The enzyme named cellulase has great potential to degrade cellulose and also convert cellulose into simple sugars. Cellulase mainly composed of three major components namely, cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase), and beta-glucosidases [10]. Microorganism particularly bacteria and fungi produced this enzyme in nature and exploring different natural resources to isolate bacteria is imperative for higher production of cellulolytic enzymes [11,12].

Although, it is validated fact that proper degradation of lignocellulosic substrates requires a complex set of enzymes

such as laccases, exoglucanases, endoglucanases, peroxidases, fucosidases, xylanases and β-glucosidases [13]. Variety of microbes that are present in nature like many bacterial and fungal species secretes different types of lignocellulolytic enzymes which help in degradation of lignocellulosic biomass into simple sugars. These sugars can be further converted into bio fuels and other platform chemicals. The present work was conducted to isolate cellulolytic bacteria from cattle dung and to identify them biochemically and molecularly by using 16S rRNA gene.

Materials and Methods

Isolation and selection of bacterial isolates for cellulase activity

Cattle dung samples were collected in sterile plastic bottles from Sagar, Madhya Pradesh. One gram of dung sample was used to grow bacteria in Nutrient Agar media (Hi Media, India) and incubated at 28°C for 48hrs in incubator and maintained at nutrient broth for further use. Pure culture of bacterial isolates was maintained in separate plate before testing them on selective media. Then 0.1 ml of the bacterial suspension was adjusted to 0.05 cell density by sterile 0.85% NaCl solution. Then these bacterial inoculums was transferred to Carboxymethyl Cellulose (CMC) plates for screening CMC activity (Shankar et al., 2011) and incubated at 37°C for 2 days. These plates were then flooded with 0.1% Congo red/Gram's Iodine solution for about 20 min and then washed by using 1 M NaCl for 15 min. Formation of the clear halos zones by these bacterial isolates was indicated by their cellulose degrading activity or cellulase activity [14]

Biochemical characterization of cellulase producing bacterial isolates

A combination of 12 biochemical tests (HiAssortedTM KB002, HIMEDIA, INDIA) and 35 tests for utilization of carbohydrates (HiCarboTM, HIMEDIA, INDIA) were used for biochemical characterization of cellulase producing bacterial isolates by following the instruction provided by the manufacturer. Tests used in this kit are presented in table 1 and table 2. These tests are based on the principle of color change, change in pH, and utilization of substrate by bacterial isolates. The bacterial isolates used for these test were isolated and purified. Only pure cultures were used and kit was opened aseptically. 50µl of the prepared inoculums was added to each well by using surface inoculation method. The kit were also be inoculated by stabbing each individual well with a loopful of inoculums and incubation at 35°C temp for 18-24 hours and changes in color were observed and recorded.

DNA Extraction and molecular identification

Total genomic DNA of bacterial isolates was extracted by using the Insta Gene TM Matrix Genomic DNA isolation kit. DNA concentration was determined by nano drop. The isolated bacterial DNA was further amplified by using universal forward primer 27F with primer sequence (AGAGTTTGATCMTGGCTCAG) and reverse primer 1492R with primer sequence (TACGGTACCTTGTTACGACTT). The PCR mixture contained 1 µL of DNA template, and 20 µL of PCR reaction mixture. The PCR amplification were performed by using automated thermal cycler (PTC-200, Biorad, Hercules, CA, USA) with the following PCR conditions: an initial denaturation at 94°C for about 5 min followed by 30 cycles of amplification at 94°C for 30 s, annealing at 52°C for about a min and extension at 72°C for 2 min, final elongation at 72°C for 10 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Selected PCR products were then sequenced using an ABI Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Grand Island, NY, USA) and these sequences were read on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems). Sequence analysis was performed using Sequence Scanner v1.0 software. Amplified PCR products of 16S ribosomal gene were confirmed on 1% agarose gel, purified using QIAquick PCR purification kit (QIAGEN)

Bioinformatics protocol

These 16S rDNA sequences were then compared with other sequences available in Gene Bank databases using the NCBI BLAST at http://www.ncbi.n1m.nih.gov/blast/Blast.cgi.Sequences were submitted to NCBI GenBank data base for getting accession numbers. The phylogenetic analysis of sequences with the closely related sequence of NCBI blast results was performed by using the automated NCBI pipeline. A phylogenetic tree for these bacterial sequences were constructed by using iTOL (Interactive tree of life) after establishing relationship among the similar sequences analysis generated from Mega 5.05 software [15,16].

Results and Discussion

The large number of microorganisms from the diverse environment permits screening for more efficient lignocellulolytic enzymes to help overcome current challenges in bio fuel production [4,17–20]. Previous studies reported that cow/ buffalo dung is rich in cellulase producing bacterial isolates [21]. Therefore, in this study we have tried to isolate good cellulase producing bacteria from the cow dung collected from the Dr. Harisingh Gour University Campus, Sagar, Madhya Pradesh. Almost, 15 bacteria's have been isolated from cattle dung out of which 10 isolates showed cellulase activity on CMC (Carboxymethyl Cellulose) screening media (Figure 1). Cellulase enzyme activities were seen by the appearance of zones around the bacterial colonies on the screening media. Out of ten isolates we selected only three for further characterization; these were named as AKPCD108, AKPCD109 and AKPBD1. These isolates were found to be gram negative after gram staining test. These bacterial isolates with high cellulose activity were tested by gram staining, morphological ground and also by various biochemical utilization tests (Citrate, ornithine, lysine, phenylalanine, urease, H_2S production, nitrate reduction, glucose, lactose, adonitol, arabinose, sorbitol and 35 different carbon sources) (Table 1 & 2).

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Figure 1: Bacterial isolates from buffalo dung showing cellulase activity

Biochemical utilization test	Strain AKPCD108	Strain AKPCD109	Strain AKPBD1
Citrate utilization			
Lysine utilization			
Ornithine utilization			
Urease			
Phenylalanine deamination			
Nitrate reduction			
H₂S production			
Glucose			
Adonitol			
Lactose			
Arabinose			
Sorbitol			
Control			
Utilization (%)	41.66	33.33	58.33

Table 1: Heat map of Biochemical Utilization by selected bacterial isolates from cattle dung
Note: (Dark blue colors indicates positive test for the respective source and grey indicates negative test result.)

Carbohydrates Utilization Test (35 Carbon Source)	Strain AKPCD108	Strain AKPCD109	Strain AKPBD1
Lactose			
Xylose			
Maltose			
Fructose			
Dextrose			
Galactose			
Raffinose			
Trehalose			
Melibiose			
Sucrose			
L-Arabinose			
Mannose			
Inulin			
Sodium gluconate			
Glycerol			
Salicin			
Glucosamine			
Dulcitol			
Inositol			
Sorbitol			
Mannnitol			
Adonitol			
α-Methyl-D-Gluconate			
Ribose			
Rhamnose			
Cellobiose			
Melezitose			
α-Methyl- D-mannoside			
Xylitol			
ONPG			
Esculin			
D-Arabinose			
Citrate			
Malonate			
Sorbose			
Control			
Utilization (%)	40.00	42.86	68.57

Table 2: Heat map of Carbohydrate utilization tests of selected bacterial isolates from cattle dung
Note: (Black colors indicates positive test for the respective source and grey indicates negative test result)

Results showed that among these three isolates (AKPCD108, AKPCD109 and AKPBD1), the isolate number AKPBD1 showed 58.33 % utilization of biochemical's (12 different biochemical's and one control) which is followed by AKPCD108 and AKPCD109. Among all carbohydrates (35 different carbon source and one control) utilized, AKPBD1 showed 68.57% utilization and other isolates (AKPCD108 & AKPCD109) were at par for the utilization of these carbohydrates (Table 1 and Table 2).

Among the biochemical (ornithine, nitrate and glucose) were utilized by all isolates and phenylalanine and arabinose have not been used by any isolates. On the other hand dextrose, sodium gluconate and inositol were used by all the isolates and lactose, melezitose, xylitol have not been used by any isolate. Our results are supported by Khianngam et al. (2014), in which they have screening and identified cellulase producing bacteria from oil palm meal. They obtained 8 isolates belongs to Bacillus, and one to *Paenibacillus* and *Lysinibacillus* by using 16S rDNA sequencing. The obtained sequences were blast and phylogenetic analysis of their sequences showed that AKPCD108 has 99% similarity with *Pseudomonas otitidis* strain MCC10330, AKPCD109 has 99% similarity with *Stenotrophomonas koreensis* strain TR6-01 (*Flavobacterium lutescens*) and AKPBD1 has 99% similar with *Serratia marcescens* subsp. sakuensis strain KRED (Table 3). The phylogenetic relationship of these identified cellulase producer are shown in Figures 2a-c. Among the bacterial isolates most common cellulase producer are belong to the following genera; *Cellulomonas, Cellvibrio, Clostridium, Paenibacillus, Pseudomonas, Ruminococcus, B. subtilis, B. cereus, B. altitudinis, Paenibacillus, Acetivibrio, Bacillus, Sporocytophaga, Bacteroides, Micrococcus* etc [14, 22]. But the isolates screened in this study for cellulase production were not reported by other workers.

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Table 3: Different bacterial isolates from cattle dung identified using 16SrRNA sequencing

S.No.	Isolate number	Bacterial strain	Gram staining reaction	NCBI GENE BANK NO	NCBI database match	Percentage of identity /Accession
1.	AKPCD108	*Pseudomonas otitidis*	Gram negative	KX698105	*Pseudomonas otitidis* strain MCC10330	99%/NR_043289.1
2.	AKPCD109	*Flavobacterium lutescens*	Gram negative rod	KX698103	*Stenotrophomonas koreensis* strain TR6-01	99%/NR_041019.1
3.	AKPBD1	*Serratia marcescens*	Gram negative rod	KX698107	*Serratia marcescens* subsp. sakuensis strain KRED	99%/NR_036886.1

Figure2 (a): AKPCD108

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Figure2 (b): AKPCD 109

Figure2 (c): AKPBD1

Figure 2: Phylogenetic tree created by iTOL software to present relationship between closely related species

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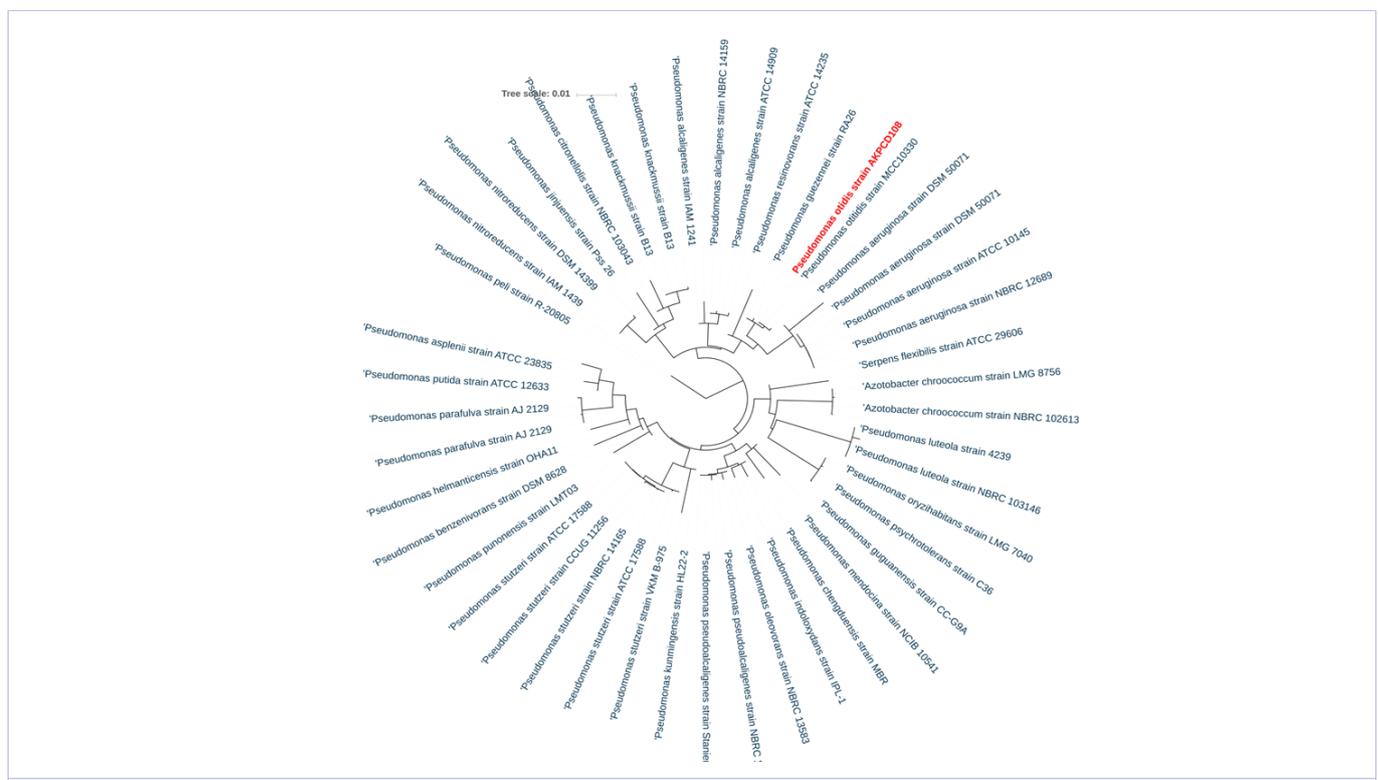
Conclusion

In conclusion, 10 cellulase producing bacteria were isolated from cattle dung samples from University Campus, Sagar Madhya Pradesh, India. They were screened for their cellulase activity by Congo red test and they showed hydrolysis capacity on plates. On the basis of their phenotypic characteristics and phylogenetic analyses, three isolates AKPCD108, AKPCD109 and AKPBD1 were closely related to *Pseudomonas, Stenotrophomonas* and *Serratia marcescens*, respectively. However, the further research on these isolates would be required utilize their full potential or to maximise the production of cellulase enzymes and degradation of agriculture residues. Finally based on the present research it can be concluded that cattle dung can be a very good source for the isolation of cellulase producing bacteria and their advance biotechnological application, mainly for biomass hydrolysis.

Contributory Statements

PV has conducted the experiment. AK proposed the experiments, prepared and edited the manuscript.

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