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

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Abstract

Recently production of biofuel 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, H2S production, nitrate reduction, glucose, lactose, adonitol, sorbitol, amnibose, and 35 different carbohydrate sources). Results showed that out of three isolates (AKPBD108, AKPBD109 and AKPBD1), the isolate number AKPBD1 showed 58.33 % utilization of biochemical’s which is followed by AKPBD108 and AKPBD109, respectively. Among all carbohydrates utilized, AKPBD1 showed 68.57% utilization and other isolates (AKPBD108 & AKPBD109) 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 AKPBD108 has 99% similarity with Pseudomonas otitidis strain MCC10330, AKPBD109 has 99% similarity with Stenotrophomonas koreensis strain TR6-01 (Flavobacterium luteum) and AKPBD1 has 99% similar with Serratia marcescens subsp. sakuenensis 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, hemicellulloses 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 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 cellulytic 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 48 hrs 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 cellullose 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 plate surface inoculation method. The kit were also be inoculated by stabbing the bacterial isolates within the inoculum well. The bacterial colonies on the screening media. Out of ten isolates, enzyme activities were seen by the appearance of zones around 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, H₂S production, nitrate reduction, glucose, lactose, adonitol, arabinose, sorbitol and 35 different carbon sources) (Table 1 & 2).
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.
Table 3: Different bacterial isolates from cattle dung identified using 16SrRNA sequencing

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

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

Figure 2 (b): AKPCD 109

Figure 2 (c): AKPBD1
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 AKPB1 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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