Research Article

Isolation, Identification, and Characterization of a Cellulolytic Bacillus amyloliquefaciens Strain SS35 from Rhinoceros Dung

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Received 30 March 2013; Accepted 8 May 2013

Academic Editors: L. Brusetti, J. Kreth, M. C. Lai, and J. Maupin-Furlow

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Cellulose hydrolyzing bacteria were isolated from rhinoceros dung and tested for clear zone formation around the colonies on the agar plates containing the medium amended with carboxymethylcellulose as a sole carbon source. Isolates were further screened on the basis of carboxymethylcellulase production in liquid medium. Out of 36 isolates, isolate no. 35 exhibited maximum enzyme activity of 0.079 U/mL and was selected for further identification by using conventional biochemical tests and phylogenetic analyses. This was a Gram-positive, spore forming bacterium with rod-shaped cells. The isolate was identified as Bacillus amyloliquefaciens SS35 based on nucleotide homology and phylogenetic analysis using 16S rDNA and gyrase A gene sequences.

1. Introduction

Cellulose, a structural carbohydrate of the plant cell wall, is an abundant and ubiquitous polymer. The use of cellulose for the second generation biofuel production involves the hydrolysis of cellulotic biomass, that is, saccharification, to form simple sugar monomers for the fermentation into bioethanol [1–3]. Cellulases are the group of enzymes involved in the conversion of cellulotic substrates to fermentable sugars. Main members of this group include endoglucanase (EC 3.2.1.4), exoglucanase or celllobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [4]. The endoglucanase hydrolyzes β-1,4 bonds in cellulose molecule, whereas exoglucanase cleaves the ends to release celllobiose, and β-glucosidase converts cellobiose to glucose [5]. Several cellulase producing fungi such as Aspergillus, Rhizopus, and Trichoderma species [6, 7] and bacteria such as Bacillus, Clostridium, Cellulomonas, Thermomonospora, Ruminococcus, Bacteroides, Erwinia, and Acetivibrio species [8–10] have been identified. However, the isolation and characterization of novel cellulose hydrolyzing enzymes from bacteria are still a highly active research area, because bacteria have a higher growth rate than fungi, leading to greater production of enzymes [11]. Also, the habitat of bacteria covers different environmental niches, which favors the existence of versatile strains such as thermophiles [12], psychrophiles, alkaliphiles, and aci-dophiles. The culturable cellulase producing bacteria have been isolated from the variety of sources such as composting heaps, decaying agricultural wastes, the feces of cow [13] and elephant, gastrointestinal tract of buffalo and horse [14], soil, and extreme environments like hot-springs [15]. Cellulose degrading bacteria play an important role in energy supply for forage animals. Wahyudi et al. [14] and Varga and Kovler [16] have reported that the feed fibers were not completely converted to animal product in intensive animal farming, and 20–70% undigested cellulose was carried out with feces. Some studies have explained that the crude fiber degradation in gut is not optimal, and the fiber content of feces is still high [17], which can be utilized efficiently by microbes present in the feces of the herbivores. Rhinoceros are presumed to have an efficient system for cellulose digestion, as its main food wild grass primarily consists of cellulose. In this study, the dung of the pachyderm from Kaziranga National Park, Assam, India, has been used as the source of cellulolytic bacteria.
2. Materials and Methods

2.1. Substrate and Chemicals. Carboxymethylcellulose (CMC) (low viscosity, 50–200 cP) was procured from Sigma Aldrich (St. Louis, Mo, USA). Medium components and congo red (analytical grade) were procured from Hi-Media Pvt. Ltd., India.

2.2. Sample Collection. The dung sample of one-horned Indian Rhinoceros (*Rhinoceros unicornis*) was collected from its natural habitat, Kaziranga National Park, Assam, India. Prestereilized spatula and plastic bags were used for sample collection, and before bacterial isolation the samples were stored at 4°C in ice box for approximately 12 h.

2.3. Isolation of Cellulolytic Bacteria. Dung sample (0.5 g) was suspended with 50 mL 0.85% (w/v) sterile NaCl solution in a 250 mL conical flask, which was shaken at 180 rpm for 1 h at 37°C. Serial dilutions from 10^(-1) to 10^(-7) were prepared using sterilized saline solution. An aliquot of 100 μL of each dilution was spread plated onto Bushnell Haas medium (BHM) [18] agar plates amended with carboxymethylcellulose (CMC) (pH 7.0) containing (g/L) CMC (10.0), K2HPO4 (1.0), KH2PO4 (1.0), MgSO4·7H2O (0.2), NH4NO3 (1.0), FeCl3·6H2O (0.05), CaCl2 (0.02), and agar (20.0) [19, 20]. The plates were incubated at 37°C for 96 h.

2.4. Qualitative Screening of Cellulolytic Bacteria by Plate Staining Method. Morphologically dissimilar and discrete colonies were picked from different dilution plates and streaked on separate BHM-CMC plate with grids drawn over it and incubated at 37°C for 96 h. The replica plates were also prepared separately for staining [21]. The replica plates were flooded with 0.3% congo red for 20 min. The stain was poured off, and the plates were washed with 1 M NaCl [22]. The isolates showing clear zone around the colonies were picked from master plate and further used for the enzyme production in liquid medium. The selected cultures were maintained on nutrient agar slants containing (g/L) peptone (5.0), beef extract (1.0), yeast extract (2.0), NaCl (5.0), and agar (20.0). The culture slants were stored at 4°C and subcultured every 10–15 days.

2.5. Quantitative Determination of Extracellular Carboxymethylcellulase (CMCase) Production. The isolates, selected on the basis of plate staining method, were grown in 50 mL enzyme production medium (at pH 7.0) containing the following components (g/L): CMC (10.0), K2HPO4 (1.0), KH2PO4 (1.0), MgSO4·7H2O (0.2), NH4NO3 (1.0), FeCl3·6H2O (0.05), CaCl2 (0.02), and yeast extract (5.0). This medium is the same as the previously used medium during isolation, with the only difference of addition of yeast extract. This addition is to provide additional nitrogen source and enhance the growth rate. 50 mL medium (containing 2% inoculum) was taken in 250 mL Erlenmeyer flask and incubated at 37°C at 180 rpm for 72 h. After every 6 h, the culture was centrifuged at 12000 g for 20 min at 4°C. The cell-free culture broth containing the crude enzyme was used for estimation of CMCase activity. Based on the higher CMCase activity (as described later), an isolate SS35 (named after its colony number) was selected for further characterization and identification. The enzyme production by the isolate SS35 was monitored with cell growth at 600 nm using UV-visible spectrophotometer (Perkin Elmer, Model lambda-45).

2.6. CMCase Activity Assay. The CMCase activity (U/mL) was measured by estimation of reducing sugars liberated from CMC. The enzyme assay was carried out by incubating the enzyme with CMC for 15 min at 37°C. The reaction mixture (100 μL) contained 50 μL of enzyme and 1.0% (w/v) final concentration of CMC in 50 mM phosphate buffer (pH 7.0). The reducing sugar was estimated by the method of Nelson and Somogyi [23, 24]. The absorbance was measured at 500 nm using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45) against a blank with n-glucose as standard. One unit (U) of cellulase activity is defined as the amount of enzyme that liberates 1 μmol of reducing sugar (glucose) in 1 min at 37°C and pH 7.0.

2.7. Morphological and Biochemical Characterizations of the Isolate SS35. Morphological and biochemical properties of the isolate were identified, evaluated, and compared, as described in Bergey’s Manual of Systematic Bacteriology [25]. The cell morphology of the selected isolate was observed under scanning electron microscope (Leo 1430 VP, Leo Electron Microscopy Ltd., Cambridge, UK) at 14 kV. Gram staining, endospore staining, and urease test were done as per standard protocol [26]. The catalase activity was determined adding few drops of 3% (v/v) H2O2 to 5 mL of 18 h grown culture [27]. The Nitrate Agar slants (M072, HiMedia) were used to test nitrate reducing property of the isolate SS35. BHM amended with starch was used for amylase activity determination. Triple Sugar Iron (TSI) slants (M0211, HiMedia) containing three sugars, namely, glucose, lactose, and sucrose, were used for acid and H2S production test. Acid production after carbohydrate fermentation was detected by the visible change in color from red to yellow. The temperature tolerance test was performed by growing the isolate in nutrient broth and incubating at the temperatures ranging 20°–50°C.

2.8. Analyses of 16S Ribosomal DNA (rDNA) and Partial Gyrase A (gyrA) Gene Sequences. 16S rDNA and partial gyrA gene sequencing of bacterial culture were done in Xcelris Labs Limited, Ahmedabad, India. The genomic DNA of the isolate SS35 was extracted using Qiagen DNA extraction kit and purified by QIAamp DNA Purification Kit (Qiagen) for nucleotide sequence analysis. The universal 16S rDNA primer 8F (5′- AGAGTTTGATCCTGCTCAG 3′) and 1492R (5′- ACCGCTACCTTGTTACGACTT 3′) were used for amplification of genomic DNA by polymerase chain reaction (PCR). The gyrA region was amplified using the primers, p-gyrA-F (5′- CAGTCGAGAAATGGTGACGCCAC 3′) and p-gyrA-R (5′- CAAGATTTCGCTAGCTGTCGTTGC 3′) [28]. The concentration of each primer in 25 μL PCR reaction mixture was 10 pmol and 1X PCR master mix (MBI Fermentas). The PCR reaction was
3. Results and Discussion

3.1. Isolation and Screening of Cellulolytic Bacteria by Plate Staining Method. Among 36 isolates, 9 cellulose hydrolyzing microorganisms were screened on the basis of plate staining method. The isolates (no. 21, 24, 25, 28, 31, 32, 34, 35, and 36) showed clear zone around colonies after staining the plates with Congo red and destaining with 1 M NaCl as shown in Figure 1. However, plate-screening method is not quantitative because of poor correlation between enzyme activity and colony to clear zone ratio [12]. The colonies showing yellow-colored halo zones were picked from replica plate and further screened on the basis of CMCase production in liquid medium. Out of 9 isolates, isolate no. 35 exhibited maximum CMCase activity of 0.079 U/mL (details given in Table 1). This value was higher than activity of CMCase produced from some known natural isolates (expressed per mL of cell-free culture broth), for example, *Cellulomonas* sp. (0.0336 U/mL, isolated from coir retting effluents), *Micrococcus* sp. (0.0152 U/mL, isolated from coir retting effluents), *Bacillus* sp. (0.0197 U/mL, isolated from coir retting effluents), *Brevibacillus* sp. JXL (0.02 U/mL, isolated from swine waste), *Brevibacillus* sp. DUSELG12 (0.02 U/mL, isolated from gold mine), *Geobacillus* sp. DUSELR7 (0.058 U/mL, isolated from gold mine), *Geobacillus* sp. (0.0113 U/mL, isolated from sugar refinery wastewater), and *Bacillus subtilis* AS3 (0.07 U/mL, isolated from cow dung) [33–37]. Ariffin et al. [38] have reported maximum CMCase activity of 0.079 U/mL by *Bacillus pumilus* EB3 produced in a 2 L stirred tank reactor, which was equal to the CMCase activity of the isolate in this study. Thus, the isolate no. 35 was revealed to be an efficient CMCase producer species and was designated as SS35. Further analysis of this species was done as described below. The growth curve of SS35 along with CMCase production profile (Figure 2) revealed that the enzyme production was associated with cell growth and reached maximum at late log phase. Slight reduction in enzyme production after 48 h could be a consequence of instability of the enzyme at 37°C or the activity of proteases present in the crude enzyme solution.

### Table 1: CMCase activity (U/mL cell-free culture broth) of cellulase producing isolates (after 48 h at 37°C, 180 rpm, and medium pH 7.0).

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>CMCase activity (U/mL)</th>
</tr>
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<tbody>
<tr>
<td>21</td>
<td>0.063 ± 0.008</td>
</tr>
<tr>
<td>24</td>
<td>0.059 ± 0.003</td>
</tr>
<tr>
<td>25</td>
<td>0.072 ± 0.010</td>
</tr>
<tr>
<td>28</td>
<td>0.073 ± 0.008</td>
</tr>
<tr>
<td>31</td>
<td>0.071 ± 0.006</td>
</tr>
<tr>
<td>32</td>
<td>0.067 ± 0.007</td>
</tr>
<tr>
<td>34</td>
<td>0.057 ± 0.004</td>
</tr>
<tr>
<td>35</td>
<td>0.079 ± 0.011</td>
</tr>
<tr>
<td>36</td>
<td>0.049 ± 0.003</td>
</tr>
</tbody>
</table>

*Values are mean ± SE (n = 3).*

3.2. Morphological and Biochemical Characterization of the Isolate SS35. The isolate SS35 was found to be rod-shaped cells with a width and length of 0.5–0.6 μm and 1.5–1.6 μm, respectively, as observed under scanning electron microscope (Figure 3). The isolate was found to be a Gram-positive, spore forming bacterium, and it gave positive test for catalase, nitrate reduction, and starch hydrolysis, whereas negative for urease and hydrogen sulfide production. The absence of black precipitate at the base of the tube indicated that hydrogen sulfide was not produced. The color of TSI agar slant was pink due to hydrolysis of lactose, whereas light yellow due to hydrolysis of glucose. The isolate SS35 was revealed to be a Gram-positive, spore forming bacterium, and it gave positive test for catalase, nitrate reduction, and starch hydrolysis, whereas negative for urease and hydrogen sulfide production. The absence of black precipitate at the base of the tube indicated that hydrogen sulfide was not produced. The color of TSI agar slant was pink due to hydrolysis of lactose, whereas light yellow due to hydrolysis of glucose.

3.3. Identification on the Basis of Phylogenetic Analyses. The phylogenetic tree generated using 16S rDNA gene sequences of the isolate SS35 showed that the bacterium has the highest homology with *Bacillus amyloliquefaciens* NBRC 3035 (GenBank accession no.: AB679994.1) (Figure 4). The bacterial identification using 16S rDNA gene sequence is a widely practiced technique, although with limitations for the members of closely related taxa [39]. To overcome this limitation, several studies have been done, which concluded that some
Figure 1: Petri plates containing 1% CMC agar incubated at 37°C for 96 h. (a1), (b1), and (c1) colonies before staining with 0.3% congo red; (a2), (b2), and (c2) colonies after staining.

Figure 2: Enzyme production profile and growth curve of strain SS35.

Figure 3: SEM micrograph for morphological characterization of isolate SS35 (inset: enlarged micrograph of a single cell).

protein-coding genes such as RNA polymerase (rpoB) gene [40], RNA polymerase sigma factor (rpoD) gene, Gyrase B (gyrB) gene [41], and gyrase A (gyrA) gene [28] can be used for the identification of closely related taxa, because the genetic variation in protein-coding genes are much higher. Chun and Bae [28] demonstrated that the gyrA sequences, code for DNA gyrase subunit A, can be used for accurate identification of Bacillus amyloliquefaciens and related taxa including Bacillus subtilis, Bacillus vallismortis, Bacillus mojavensis, Bacillus atrophaeus, and Bacillus licheniformis.
Therefore, in this study partial gyrA gene sequences have been used for the confirmation of the result obtained from 16S rDNA sequence analysis. The phylogenetic analysis using partial gyrA gene sequences also revealed that the isolate SS35 has the highest homology with Bacillus amyloliquefaciens FZB45 (GenBank accession no.: FN662840.1) [42], as shown in Figure 5. Numbers at nodes of the tree are indications of the levels of bootstrap support based on a neighbor-joining analysis of 1,500 resampled datasets. The isolate SS35 was identified as Bacillus amyloliquefaciens and designated as Bacillus amyloliquefaciens SS35. The 16S rDNA and gyrA gene sequences of the isolate B. amyloliquefaciens SS35 have been deposited in the NCBI nucleotide sequence database under the accession nos. JX674030 and KF019284, respectively.

### 4. Conclusions

Cellulose hydrolytic bacteria have been isolated from rhinoceros dung, and some potent cellulose degrading bacteria have been identified. Among all cellulolytic bacteria, isolate no. 35 exhibited maximum CMCase production and has been further characterized by biochemical methods. Bacterium has been identified as Bacillus amyloliquefaciens SS35 on the basis of 16S rDNA and partial gyrase A gene sequence analyses. The CMCase production has been observed to be associated

<table>
<thead>
<tr>
<th>Characteristic/biochemical test</th>
<th>Observation</th>
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<tr>
<td>Cell shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Cell size</td>
<td>(0.5 - 0.6 \mu m \times 1.5 - 1.6 \mu m),</td>
</tr>
<tr>
<td>Gram's reaction</td>
<td>+</td>
</tr>
<tr>
<td>Endospore formation</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+</td>
</tr>
<tr>
<td>Urease test</td>
<td>−</td>
</tr>
<tr>
<td>(\text{NO}_3) reduction into (\text{NO}_2)</td>
<td>+</td>
</tr>
<tr>
<td>(\text{H}_2\text{S}) production</td>
<td>−</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Growth between 20 and 50°C</td>
<td>+</td>
</tr>
</tbody>
</table>

+: positive reaction; −: negative reaction.
with cell growth and has maxima at the late exponential phase of growth. Optimization of medium composition and fermentation parameters can further increase the cellulase production from B. amyloliquefaciens SS35. These attributes also make B. amyloliquefaciens SS35 a potential candidate in solid state fermentation for CMCase production using cellulotic biomass.

**Acknowledgment**

Research funding from Department of Biotechnology, Government of India, for this work is gratefully acknowledged.

**References**


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