

Original Research Article

Isolation and Molecular characterization of Cellulase Producing Bacteria from Soil of Sacred Grove, Puducherry, India

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ABSTRACT

Keywords

Cellulose, Cellulase activity, 16S rRNA sequence, *P. aeruginosa*

Today the crisis of environment and energy has been a huge obstacle for the progress of human civilization. The most predominant energy source which is the fossil fuel supply is limited and it also causes environmental effects such as green house gases. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intensive research and industrial subject. The aim of the present study is to isolate the efficient cellulase producing organisms from the soil of sacred grove. The bacterial strain SG21 had the maximum cellulase activity of 2.9U/ml. 16S rRNA of the strain was sequenced and identified as *P. aeruginosa*.

Introduction

Cellulose is the primary structural component of the plant cell wall and the main product of photosynthesis in terrestrial plants (Zhang and Lynd, 2004). Cellulose is the most abundant and common natural renewable biopolymer available on Earth (Klemm, 2004). Therefore making use of them effectively through biological process will be a milestone in overcoming the increasing demand and rising cost of fossil fuels. The shortage of foods, feeds and fuels have shifted global efforts to utilize renewable resources for the production of 'greener' energy. As a result, cellulose prevails as an inexpensive resource for the production of biofuel and bio-based

products. Even though plants offer energy and carbon source for microorganisms, cellulose by itself cannot be taken up by the microorganisms. Cellulose is degraded by the hydrolytic action of a multicomponent enzyme system - the cellulase; which is predominantly produced by bacteria and fungi during their growth on cellulosic materials.

Cellulose, being the predominant carbohydrate in nature is a polymer of glucose residues connected by β -1, 4 glycosidic linkages. Cellulase hydrolyzes this β -1, 4 glycosidic linkages to glucose units. Complete hydrolysis of the enzyme is

accomplished by the synergistic action of three types of enzymes namely-endoglucanase (Carboxymethylcellulase-CMCase), exoglucanase (cellobiohydrolase) and beta-glucosidases. Endoglucanase hydrolyses β -1, 4 bonds in cellulose molecule, whereas exoglucanase cleaves the ends to release cellobiose and beta-glucosidases converts cellobiose to glucose (E.A Bayer, 1994). In nature, fungi tend to produce more cellulase than bacteria. But cellulase produced by bacteria is thermostable and can act as more effective catalyst. Bacteria also have higher growth rate than fungi. Since most industrial processes are carried out at higher temperatures and require less time, bacterial cellulase can be considered more beneficial. Several workers have isolated cellulose producing bacteria from variety of sources such as composting heaps, decaying agricultural wastes, soil, animal excreta, saw dust, etc. These resources are quite valueless and even offensive to our environment. But making such wastes as raw materials for cellulase production is valuable. Cellulase is used in textile industry for the biopolishing of fabrics for making it more soft and bright. Cellulase can be employed to improve the nutritional quality and digestibility in animal feeds. It is also used in the processing of fruit juices, in developing washing powder, in the paper and pulp industries, etc. Most importantly, cellulase is employed in biofuel production to compete the depleting energy source. Cellulolytic microorganisms can be found in all biota where cellulose biomass accumulates. The cellulolytic and the non-cellulolytic organisms act synergistically which leads to complete degradation of cellulose, giving rise to CO_2 and H_2O under aerobic condition and CO_2 , H_2O and CH_4 under anaerobic condition.

Sacred grove areas of Puducherry, India, hardly have any human influence. Hence this area provides the ample soil sample for

the discovery of novel cellulase-producing bacteria. In this study the effective cellulase producing bacteria was isolated, characterized, and identified by 16S rRNA sequencing.

Materials and Methods

Collection of Soil Sample

The soil sample was collected at Nallavadu, a sacred grove in Puducherry. The soil was collected at different areas within the sacred grove and pooled together. The samples were collected in sterile container and stored at 4°C until used.

Isolation of Cellulolytic Bacteria

Cellulase producing bacteria were isolated from the sample by serial dilution followed by plating them on a Cellulose Congo Red agar medium. Serial dilution was done by mixing 1gm of soil sample in 100ml of sterile distilled water and further proceeding up to 5 dilutions. 0.1ml of each dilution was plated on Cellulose Congo red agar (Hendricks and Doyle, 1995) containing the following composition: 2g Carboxymethyl cellulose, 0.25g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g K_2HPO_4 , 2g agar, 2g gelatin, 0.2g Congo red in 100ml at pH 7. The plates were incubated at 37° for 5 days.

Screening of Cellulase Producing Bacteria

The appearance of clear hydrolysis zones around the developing bacterial colony indicated cellulose hydrolysis (Wood, 1975). The bacterial colonies were selected depending upon the diameter of the zone. The zone of the bacterial colony having the largest diameter was selected for further study. Purification of selected colonies were done by repeated streaking and stored at 4°C in CMC slants.

Estimation of Cellulase Activity by DNS Method

The selected bacterial strains were inoculated in enzyme production medium containing the following composition: 20g Carboxymethyl cellulose, 5g yeast extract 0.2g MgSO₄.7H₂O, 5g K₂HPO₄, 10g NaCl in 1000ml at pH7 and incubated overnight at 37 °C in a shaker. After incubation, the culture was centrifuged and the supernatant was used for cellulase assay.

Cellulase Assay

The activity of cellulase was assayed using Dinitrosalicylic acid (DNS) reagent, by estimating the reducing sugars released from CMC (Miller *et al*, 1959 with modifications). 0.1ml of crude enzyme was mixed with 0.9ml of acetate buffer [25mM, pH 5.0].1ml of 0.1% CMC was also added and incubated at 50 °C for 10 minutes. After incubation, the reaction was halted by adding 1 ml of DNS reagent. This was followed by the incubation of the tube at 100 °C for 10 minutes in a water bath. The absorbance was read at 550 nm with glucose as standard. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1µmole of glucose/minute.

Molecular Characterization of Cellulolytic Bacteria

The bacterial strain SG21 was subjected to molecular identification by analyzing their 16S rRNA sequence.

Extraction of Genomic DNA (Sambrook *et al.*, 1989)

The Bacteria was grown in nutrient broth overnight at 37°C. The cells were pelleted at

10,000 rpm for 5 minutes in a microcentrifuge tube. The pellet was resuspended in 330µl of lysis solution (Glucose 50mM, EDTA 10mM, Tris 25mM, pH 8) and incubated for 10 minutes at RT. 10 µl lysozyme (10 mg/ml) was added and incubated at 37°C for 1 hour. To the mixture, 8.5µl of 20% SDS was added and incubated at 50°C for 10 minutes. 13µl of RNase was further added and incubated for 1 hour at 37°C. Then 17µl of 0.5 M EDTA was added and incubated at 50°C for 10 minutes. To the lysate, 10µl of Pronase was added and incubated at 37°C for 3 hours. This was extracted twice with equal volume of buffered phenol by centrifuging at 10,000 rpm for 10 minutes at RT. The aqueous layer obtained was extracted with equal volume of chloroform: isoamyl alcohol by centrifuging at 10,000 rpm for 10 minutes. To the resultant aqueous layer, 50 µl of 3M ammonium acetate and 1000 µl of 95% ice cold ethanol was added and mixed well to precipitate the DNA. The DNA was pelleted at 10,000 rpm for 5 minutes and the pellet was washed in 70% ice cold ethanol. Air dry the pellet and dissolve in 50 µl of 1X TAE and store at -20°C.

16S rRNA Gene Amplification and DNA Sequencing

16S rRNA gene of the genomic DNA was amplified by PCR using the universal primer set (Weisberg *et al* 1991):

Forward primer f D1 :(5'-GAGTTTGATC CTGGCTCA-3')

Reverse primer r R2: (5'-ACGGCTACCT TGTTACGACTT-3')

The PCR was performed in 50µl reaction mixture which contained 50pM of primer, 50 ng of genomic DNA, 1X Taq DNA polymerase, 1U of Taq DNA polymerase,

0.2mM of each dNTPs and 1.5mM MgCl₂. The PCR program for 16S rRNA gene amplification consisted of initial denaturation at 94 °C for 1 minute, annealing at 46°C for 30 seconds and extension at 72 °C for 4 minutes, with a final extension at 72 °C for 10 minutes. The presence of PCR product was detected by 0.7% agarose gel. The amplified product was purified and sent for sequencing to Eurofins Genome Sequencing Pvt. Ltd., Bangalore-Karnataka.

Molecular Phylogenetic Analysis

The 16S rRNA gene sequence of the isolate was compared with the sequences retrieved from NCBI database using BLAST N algorithm. The compared sequences were used for constructing molecular phylogenetic tree using Neighborhood Joining method available at MEGA 6.0 software (Tamura *et al*, 2013)

Results and Discussion

Screening of Cellulolytic Bacteria

A total of 32 cellulolytic bacterial strains were isolated from soil sample collected from sacred groove. Among them, 5 colonies which produced clearest and largest zone with respect to the other colonies on the Cellulose Congo red agar were selected. Hendricks *et al* (1995) reported that the incorporation of Congo red as one of the component in media would be a rapid method for detecting cellulase producing organisms. Teather and Wood (1982) proved that Congo red have specific interaction with the cellulosic substrates. When cellulose is broken down by the action of cellulase, the Congo red no longer has the affinity to the substrate and a halo will appear on the medium. The screening results reveal that each bacterium has diversified activity for the breakdown of cellulosic substrates which indicates that each isolate has the ability to

produce varying amount of cellulase.

Cellulase Assay

Since the cellulase activity and halo zone cannot be positively correlated by plating methods, the colony with bigger and clear zones were further picked up and quantitatively tested for enzyme production in a broth medium. Out of the five bacterial strains, the isolate SG21 had maximum cellulase activity of 2.9U/ml.

16S rRNA Gene Amplification and Sequencing

The PCR amplification of 16S rRNA of the isolate SG21 produced 1500bp band on agarose gel. The PCR product was sequenced and its 16S rRNA sequence (Fig.1) was deposited in NCBI with Accession number KR811027

Phylogenetic Analysis of 16S rRNA Sequence

The 16S rRNA nucleotide sequence of the strain SG21 isolated from sacred groove showed 97% similarity with *P. aeruginosa*. By comparing the phylogenetic tree analysis of 16S rRNA (Fig.2) along with type strains, the taxonomic affiliation of the strain was confirmed as *P. aeruginosa*.

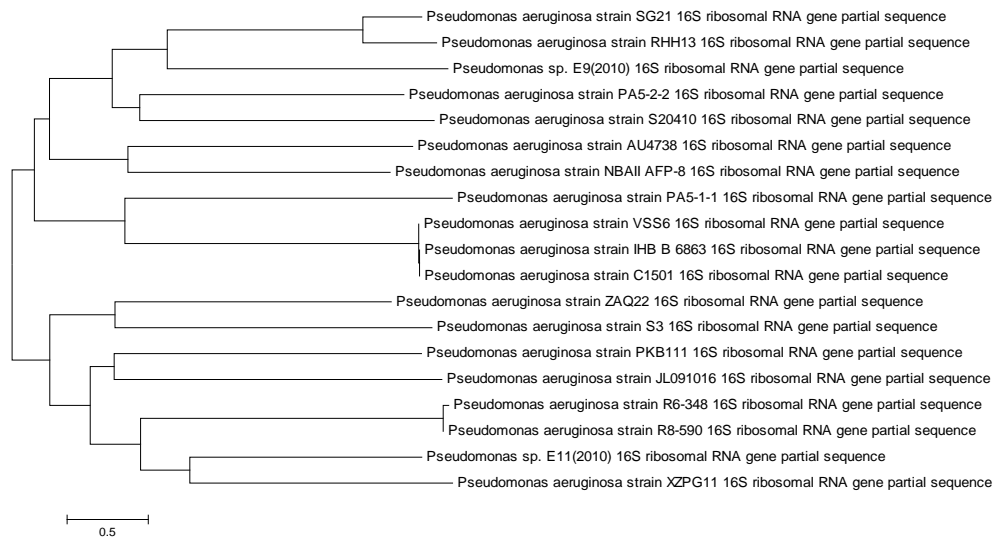
Using water and sediment samples Kalaiselvi *et al* (2013) identified that *P. aeruginosa* preferred cellulose as the most preferable substrate for cellulase production. Recently Taniya agarwal *et al* (2014) concluded that *P. aeruginosa* is capable of producing cellulase from sawdust with a high cellulase activity of 2.08U/ml.

Machiavelli Singh *et al* (2010) suggested that Pseudomonas could be mass produced as a biological agent towards magnification of microbial diversity and carbon source.

Fig.1 16S rRNA Sequence of *P. aeruginosa* SG21

TAGACCATTGCAACTACCTACACATGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTACAGCGGGACGGG
 TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCGGAAACGGGGCGTAATACCGCATAACGTCCTG
 AGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCCGATTAGCTAGTTGGTGGGGTAA
 AGGCCTACCAAGGCGACGATCCGTAACCTGGTCTGAGAGGATGATCAGTCACACTGGAAGTGAACACGGTCCAGA
 CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGCAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
 AAGGTTCTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACC
 AACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTAC
 TGGGCGTAAAGCGCGCTAGGTGGTTTCAGCAAGTTGGATGTGAAATCCCCCGGGGGTTCAAACCCTGGGGAAAC
 TTGCATTCCCAAAAACCTTACTGAGCTTAGAAGTACCGGTAAGAGGGGTGGGTGGAAATTTTCCTGGTGTAGCCGGT
 GAAAATGCGGTAGATATTGGGAAGGGAACACCCAGTGGGCGAAGGGCGACCCACCTGGAAGTACCTGACACT
 TGAGGTGCGAAAAGCGTGGGGAGCAAACAGGATTTAGATACCCCTGGTAGTCCACCGCCGTAACTGATGTGCGA
 CTTAGCCGTTGGGATCCTTGGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCA
 AGGTTAAAACCTCAAATGAATTGACGGGGGGCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGGCGAA
 GAACCTTACCTGGCCTTGACATGCTGAGAACTTCCAGAGATGGATTGGTGCCTTCGGGAACCTCAGACACAGGTGC
 TGCATGGCTGTGTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCTTAGTTAC
 CAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAAACGGAGGAAGGTGGGGATGACGTCAAGTCATC
 ATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCCGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCT
 AATCCATAAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGT
 GAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCGTCACACCATGGGAGTGGGTTGCTCC
 AGAAGTAGCTAGTCTAACCGCAAGGGGACGGTTACCATCGGAGGAGCACGTGGGA

Fig.2 Neighborhood Joining Tree Based on 16SrRNA Sequence Showing the Phylogenetic Relationship Between KR811027 and other Related Species of Genus *Pseudomonas*



Abdul Kadhim *et al* (2013) isolated *P. fluorescence* from agricultural soil and reported high cellulase activity ranging from 1.717-4.574 U/ml. Gautam *et al* (2010) also isolated *Pseudomonas* from municipal solid waste and found its cellulase activity as 1.733U/ml. Similar enzyme activity of 3.6U/ml was reported by Ulajeivo and Aluji (2010) who isolated *Pseudomonas*

from cow dung. Sonia Sethi *et al* (2013) found that *P. fluorescence* was the best cellulase producer among the other isolates from soil. Shengwei Huang *et al* (2013) isolated *Pseudomonas* species among varied bacterial flora from the gut of *Holotrichia parallela* larvae. Mannisto *et al* (2006) found that 60% of their isolates belonged to *Pseudomonas* species isolated from the

Alpine Tundra soils of Finnish Lapland. Behra *et al* (2014) isolated *Pseudomonas* sp from the soil of Mahanadhi river delta, India

In conclusion, microorganisms from various environments provides potential source of novel cellulase. Naturally occurring bacterial enzymes from the environment is important in many industrial application to help overcome costly hurdles. Isolation and characterization may provide a good starting point for the discovery of such beneficial enzymes. The industrial production of cellulase is however very expensive but the cost reduction may be possible by exploring the ways of cellulose degradation using cellulase producing organisms.

The Cellulolytic bacteria in the present investigation have several future applications either to develop an effective novel cellulase with distinctive characteristics or to develop microbial consortia for effectively degrading lignocelluloses.

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