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Original Research Article

IDENTIFICATION OF CELLULOLYTIC BACTERIA FROM THESOIL AND SCREENING OF ITS ENZYMATIC ACTIVITY

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Abstract

Cellulase synthesizing bacteria were identified from the five different sources of the soil *viz.*, garden soil, forest soil, municipal waste soil, kitchen waste soil, mangrove soil. Soil samples wereaseptically collected from different areas of Chidambaram. Efficacy of cellolase production were screened with all the ten isolates of cellulolytic bacteria using CMC agar (Carboxymethylcellulose agar) plates. Among the ten isolates, CPG-3 and CPMW-7 isolates showed high cellulolytic activity. These efficient two strains were selected for further studies. This two strainswere further identified based on morphological and biochemical test. The promising isolates were confirmed by 16s rRNA sequence. The isolate CPG-3 was confirmed as *Bacillus cereus*and the isolate CPMW-7 was confirmed as *Stenotrophomonas maltophilia*. The cellulase assay was performed for the two efficient isolates. The maximum enzyme activity (4.2 U/ml) was observed in *Bacillus cereus* (CPG-3) isolate.

Keywords: Cellulases, Soil samples, *Bacillus cereus*, *Stenotrophomonas maltophilia,* Enzyme assay

INTRODUCTION

Enzymes are the kind of protein which act as a biological catalyst and control all the process of the living cell. All the living cells requires enzymes to perform their life process[1]. Use of enzyme was increased in industry for the past two decades.[2]Substrates and many different enzymes are highly specifically required to bring about the sequence of metabolic of living cell which was performed. Most of all enzymes could be purified and areactually produced by living cells including the microorganisms. Even the single celled organisms produce an enormous quantity of enzymes with most promising activities[3].One of the most important sources of carbon on this planet is cellulose and the annual biosynthesis by land plants and marine occurs at rate of 0.85 x 1011 tonnes per an. Degradation of the cellulose by the cellulase enzyme is important for the its secondary metabolism at the global level.Cellulose hydrolysis in the subject of intense research and industrial interest has made the value of cellulose as a renewable source of energy[5].Industrial wastes and agricultural wastes are main cause of environmental pollution.Their conversion into useful products may reduce the intensity of the problems caused by them. These wastes

include green gram husk, black gram husk, rice bran, wheat bran etc., are underutilized in India. In most parts of India these materials are mainly used as animal feeds. A large quantity is left in farmlands to be decomposed by microorganisms such as bacteria and fungi[7].

Proper utilization of these wastes in the environment will eliminate pollution and convert them into useful by products[8].However, costs of cellulase for hydrolysis of pre-treated lignocellulose materials need to be reduced, and their catalytic efficiency should be further increased in order to make the process economically feasible[6].

Large demand of cellulases has been increased because of their prices which has large elevated, and the major reason is the cost of substrate. Since fermentation procedure is important to identify cheaper substrates and reduce fermentation costs. The present investigation also focuses on the same to search for substrates with zero cost.

Materials and methods

***Sample collection*:** Five different soil samples *viz*., Garden soil, Forest soil, Municipal waste soil, Kitchen waste soil, and Mangrove soil samples were collected from different places of Chidambaram. The soil samples were aseptically collected into sterile polythene bags and were brought to the laboratory. The soil samples were stored at 4°C for further studies.

***Isolation of cellulolytic bacteria*:** Cellulase-producing bacteria were isolated from soils by the dilution pour plate method using CMC agar (carboxymethyl cellulose) media. The Plates were incubated for 48 hours at 37°C[10].

***Screening of cellulolytic bacteria*:** The incubated CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. 1M NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having clear zone were selected for identification and cellulase production. Further bacterial strains were purified by repeated streaking. The purified colonies were preserved at 4°C[10].Sequencing of 16S rRNA region using universal primers, Genomic DNA Isolation from Bacteria Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel).

***Agarose Gel Electrophoresis for DNA Quality and Quantity check*:** The quality of the isolated DNA is verified using agarose gel electrophoresis. 1µl of 6X gel loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8,0) was added to 5µl OF DNA. The samples were loaded up to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) containing 0.5 µg/ml of edium bromide. Electrophoresis was performed with 0,5X TBE as an electrophoresis buffer at 75 V, while the bromophenol dye on the front did not migrate to the bottom of the gel. Gels are visualized in UV transilluminator (Genei) and the image is captured under UV light using gel documentation system (Bio-Rad).

***PCR Analysis*:** PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM TrisHCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Go+ld DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO(Dimethyl Sulfoxide), 5pM of forward and reverse primers and template DNA The PCR amplification was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Bio systems).

***Sequence Analysis*:** The sequence quality was checked using Sequence Scanner Software v1 (Applied Bio systems). Sequence alignment and required editing

of the obtained sequences were carried out using Geneious Pro v5.6[11].

***Phylogenetic analysis*:** The ITS rDNA region of the different celluloytic bacterial strains were sequenced and the sequence result obtained was matched with existing sequences available in NCBI (National Centre for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool). Phylogenetic analyses were carried out by using MEGA 6.0 software[12]. Sequences were aligned by ClustalW Multiple Sequence Alignment Program and analysed. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model[13]. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed[14]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.

Cellulase Assay

Preparation of Crude Enzyme: *After* incubation, the cultures were centrifuged, and supernatants were used as source of crude enzyme[15]. The crude enzyme solution was utilized for determination of enzyme activity.

DNS Method: *Cellulase* activity was measured by the DNS (3, 5 - dinitro salicylic acid) method[16]. 1959), through the determination of the amount of reducing sugars liberated from carboxymethyl cellulose (CMC) solubilised in 50 mMTris – HCL buffer, pH 7.0. This mixture was incubated for 20 min at 70˚C. For crystalline cellulose substrates, incubation times were extended to 2h and reaction was stopped by the addition of DNS (dinitro salicylic acid) solution. The treated samples were boiled for 10 min, cooled in water for colour stabilization, and the optical density was measured at 550 nm. 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 1µmol of glucose per minute.

Purification of cellulase by Ammonium sulphate precipitation: About 20ml of the crude enzyme prepared was brought to 80% saturation with solid ammonium sulphate[17]. The mixture was left overnight at 4ºC in a magnetic stirrer. Centrifuge the mixture and the pellet was dissolved in 10ml of 50mM sodium acetate buffer (pH-5.5) for further purification.

Results

***Authentication of the soil samples collected from different places of Chidambaram*:** The authentication of five different soil samples collected from various places was represented in Table–I and Figure 1. The sample name for the Garden soil, Forest soil, Municipal waste soil, Kitchen waste soil and Mangrove soil were designated *viz*., SG-1, SF-2, SMW-3, SK-4 and SM-5.

***Isolation of cellulase producing bacteria from different soil samples*:** The cellulase producing isolates were obtained from different soil samples were presented in Table-II. Five different source of soil samples were collected and used for the isolation of cellulase producing bacteria. From the SG-1 soil four cellulase producing isolates *viz*., CPG-1, CPG-2, CPG-3 and CPG-4 were isolated. The isolates CPF-5 and CPF-6 were isolated from SF-2 soil. The isolates CPMW-7 was isolated from SMW-3 soil. The isolates CPK-8 and CPK-9 were isolated from SK-4 soil. CPM-10 isolate was isolated from SM-5 soil.

***Screening of isolates for cellulase production*:** Cellulase producing isolates

obtained from various soil samples were primarily screened for cellulase production on solid CMC medium and the results were represented in Table–III and Figure 2&3. All the ten isolates exhibited an inhibition zone on CMC plates. The isolates CPG -13 (14mm) and CPMW-7 (13mm) showed the greatest zone of inhibition. Data of CPG-1 isolate showed a zone of inhibition of about 11mm followed by the isolate CPF-5 (10mm). The isolates CPG-2 and CPF-6 revealed a size of 9mm for the inhibition Zone. The minimum zone of inhibition was registered for the isolate CPK-8 (8mm) followed by CPM-10 (7.3mm) and CPK-9 one was (7mm). Among the ten isolates the two isolates CPG-3 and CPMW-7 have the maximum zone of inhibition. These two promising strains were selected for further studies. Molecular analysis is proved that the isolate (CPMW-7) >SR737-CS2-16S *Stenotrophomonas maltophilia.* The sequencing of 16S rRNA regions of the two isolates were shown in Figure 6.

***Phylogenitic tree analysis and Evolutionary relationships of the promising isolates*:** The phylogenetic tree in which the associated taxa clustered together in the bootstrap test was shown next to the branches in figure. Initial tree(s) for the heuristic search were obtained automatically. As the number of common sites were <100, the maximum parsimony method was used. The analysis involved 51 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated.

The phylogenetic analyses confirmed the systematic positions of the yeast species. The ITS and D1/D2 tree structures are not fully resolved as few clades showed a strong support, which conduct to insufficient evidence for nomenclatural decisions, particularly at the generic level. Consequently, clades have informal names and consist of species of different genera. However, the discovery of cellulolytic strains is in a primordial phase as possibly only 1% of the species in nature have been collected and described. These phylogenetic trees are dynamic, therefore lineages and clades will split, and as new phylogenetic groups in the branching arrangements will change and generic identities and relationships should become evident. At the present point of discovery, the trees provided by ITS and other genes

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 24.56718242 was shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon position included was the 1st. All positions containing gaps and missing data were eliminated. There was a total of 421 positions in the final dataset. Data of evolutionary analyses conducted in MEGA6 were represented in Figure 7.

The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 24.52571176 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st. All positions containing gaps and missing data were eliminated. There was a total of 421 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 and were shown in Figure 8.

***Cellulase assay*:** Cellulase activity results were summarized in Table–4. The enzyme activity was higher in the *Bacillus cereus*

(CPG-3) isolate (4.2 U/ml) when compared to the isolate (CPMW-7) *Stenotrophomonas maltophilia* (3.3 U/ml).

Purification of cellulase: The cellulase enzyme was partially purified by APS and the results were presented in Table–5. Purified enzyme exhibited the maximum cellulase activity in *Bacillus cereus* (CPG-3) 5.2 U/ml followed by *Stenotrophomonas maltophilia* (CPMW-7) 4.1 U/ml.

Discussion

Enzymes are responsible for the breakdown of β-1-4 glycosidic bonding to cellulose. They belong to the glycoside hydrolase families of the enzymes that make hydrolyse oligosaccharides and or polysaccharides. Cellulosic degradation is a complex process and requires the participation of microbial cellulolytic enzymes. Areas where these substrates exist are the best sources for the differentiation of cellulolytic microorganisms. About one-fifth of the water and soil samples produce degrading cellulose microorganisms[18].

Cellulose is one of the most common precursors in the world. It is well-known that plants are the most common source of renewable carbon and energy in the world. Cellulose is tasteless, odourless and hydrophilic[19]. Cellulose is found in D-glucose units, thickening with β (1 → 4) - glycosidic bonds[20]. oomycetes. Some types of bacteria hide it to make biofilms. Bacterial systems have also been investigated for biomass extraction and may be possible due to the rapid growth rate of bacteria. It has also been reported that enzyme modification from cellulolytic bacteria can effectively excrete different cellulosic substrates.

The present study is designed to evaluate the cellulase activity of cellulolytic bacteria isolated from different soil samples. To understand the biochemistry of cellulose degrading germs, it is necessary to grow under various physical and chemical boundaries. Shaikh et al[21] reported that 34 isolates from different soil samples using CMC media as a selective media. There were 11 isolates showed maximum zone of inhibition in the primary screening technique more than cellulase activity. These isolates were then evaluated by secondary screening for enzyme production.

Screening and identification of bacteria that produce cellulase from wet soil off were done on the Thai coast. Eighty-seven species of bacteria that exhibit cellulase-producing bacteria were investigated by cellulolytic properties. Bacteria identified as *Bacillus cereus* strain BR0302 have shown high hydrolysis activity in carboxymethyl cellulose agar plates[22].At the same time the *Bacillus cereus* alone in the garden showed high cellulase activity on carboxymethyl cellulose agar plates.

Muhammad Irfanet al[23] isolated cellulose stains from a soil sample using a serial dilution method and a pouring method. Bacteria were also identified by morphological and biochemical tests and were incorporated into cellulase production in a 250 ml Erlenmeyer flask using a potato disposal facility for 48 hours of fermentation at 35 ° C and a vibrational speed of 140rpm. Seven different types of bacteria were isolated and tested for cellulase production in an underwater fermentation process. Among these seven types of viruses have been tested; ASN2 has shown high yields for cellulase production.

In the present study, cellulases producing bacterial species were isolated from different soil samples. All isolated isolates were tested for cellulase production using Carboxymethyl cellulose plates. A total of 10 isolates were separated from different soil samples. Among them, one of the two *Bacillus cereus* (CPG-3) isolates exhibited 14mm high cellulolytic activity followed by *Stenotrophomonas maltophilia* (CPMW-7) 13mm blockade.

Joseph Vimal *et al*.,[24]reported that the cells that produce cellulase are isolated from the industrial and agricultural areas of Kerala. The potential isolate by cellulase production has been identified by Grams' iodine dye contamination. Distinguished are temporarily identified as Bacillus species based on cultural analysis, morphological, biochemical and labelled CB3, CB4 and CB8. In addition, genomic DNA was classified and amplified by 27F global primers and 1492 16S rRNA-specific primers. An enhanced 16S rRNA PCR product of 1500bp was tracked and an unknown object was identified using the high specific sequence of 16SrRNA found in the NCBI GenBank by BLAST search. A sample of CB3 and CB4 showed (100% and 99% respectively) homology in the *Bacillus subtilis* genus. The CB8 sample showed 98% homology in the *Bacillus cereus* genus. To test the evolutionary relationship, phylogenetic analysis was performed with the MEGA 6.0 system using 16S rRNA sequences.

Phylogenetic purposesconsider, each region separately and the choice of a given region depends on the taxonomic level. Therefore, rapidly evolving regions are used for phylogenetic assumptions of related species or generation. Its regions have been used for phylogenetic analysis in species depending on the standard. There are many methods available to build phylogenetic trees that predict the relationship between microbial species [25]and the possible emergence of their genetic traits (Two commonly used methods of calculating phylogenetic trees are matrix distance methods (e.g. UPGMA, neighbour joining) and methods of different data (e.g. parsimony, multiple probability or Bayesian methods) These methods use data obtained from multiple sequences and integrate them into a tree. - Two promising isolate producing genes that are genetically identified by 16ot rRNA nucleotide cells. CPG-3 isolate is identified as *Bacillus cereus* and CPMW-7 isolate as *Stenotrophomonas maltophilia*.

Fernandoet al[26] reported that cellulose activity was assessed using the Dinitrosalicylic acid (DNS) reagent, by measuring the reduced sugar extracted from CMC[16]. The cellulase enzyme produced was extracted and purified using ethanol precipitation and chromatography.

Enzyme activity is measured in the form of Dinitrosilsalic acid (DNS). Of the 26 divisions six were selected on the basis of a clear area produced 7mm ≥. These six potential isolated areas were also tested for cellulolytic activity where one SM3-M8 showed promising cellulase activity. This bacterium was then identified by morphological and biochemical experiments and identified as *Bacillus sp*. SM3-M8 provided high cellulase production and activity at a temperature of 45 ° C, pH 7, CMC concentration of 0.5% after 48 hours of incubation. The waste industry has provided an excellent source of cellulase-releasing bacteria. Separation and testing and cellulase-induced cell proliferation provided important and novel enzymes for the conversion of lignocellulosic waste into ethanol [27].

In our study the function of cellulase was tested using the Dinitrosilsalic acid method. Enzyme activity was higher in isolate *Bacillus cereus* (CPG-3) compared with *Stenotrophomonas maltophilia* (CPMW-7).

Electrophoretic analysis of extracellular Cellulase from a single site has been performed. The results of the SDS-PAGE have shown the presence of many

bands as well as cellulase some of the proteins can be produced by organisms. But the presence of a protein band near the molecular weight 60 Kda confirms the presence of the enzyme. Cellulose Weight Cellulases Separated from *Bacillus Sp*. identified using the SDS-PAGE analysis [28].

Conclusion

Microbial cellulases have shown their potential application in various industries including pulp, paper, textile, laundry, food and feed industry, brewing and agriculture. The third but the most significant group of lingo cellulolytic enzyme is cellulases, the key enzymes for the conversion of cellulose into simple sugars. Over whelming demand for natural products has elevated the significance of industrial enzymes, among which, cellulases occupy a pivotal position. The present work was carried out for isolation of potential cellulase producing bacteria.

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