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# Isolation, Screening and Identification of Cellulolytic Bacteria from Soil

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## Authors' contributions

This work was carried out in collaboration between both authors. Author PM carried the experimental work, performed literature searches and wrote the first draft of the manuscript. Author AK designed the study, managed the literature searches, corrected the entire manuscript and performed the analyses of the study. Both authors read and approved the final manuscript.

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## ABSTRACT

**Background:** Cellulose is the most abundant carbohydrate on earth and is considered as a good candidate for production of second generation biofuel (ethanol) and many other products of routine use. For degradation, cellulases are used which are mostly secreted by microbes such as fungi. Cellulases also play an important role in senescence of plants and in host-parasite relationship for invading the plant cell wall. However, comparatively lesser studies have been carried out on cellulase producing bacteria. Therefore, present study was aimed to isolate cellulase (Endo- $\beta$ -1,4-D-glucanase; EC. 3.2.1.4.) from bacterial sources.

**Methodology:** To isolate thermophilic/ mesophilic cellulase producing bacteria, soil samples were collected from wood furnishing area and agricultural farm around Indore. Besides, soil sample was also collected from the vicinity of Amlai Paper Mill in Budhar district, Madhya Pradesh. These soil samples after suitable dilutions were streaked on different nutrients agar petri-dishes having carboxymethyl cellulose (CMC) as an inducer. After screening, four colonies were isolated capable of producing good amount of cellulase. Screening was done using Congo red staining and confirmation was done after growth of the bacteria in liquid nutrient medium having CMC. These colonies individually were grown in suitable nutrient media having CMC as an inducer and enzyme activity was determined in the nutrient media after harvesting bacterial cells by centrifugation.

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**Results:** The highest enzyme producing bacteria were identified as *Bacillus licheniformis* and *Ochrobactrum anthropi* after biochemical analyses, 16S rRNA sequencing and subsequently phylogenetic tree analysis.

**Keywords:** Cellulase; isolation; bacteria; screening; *Bacillus licheniformis*; *Ochrobactrum anthropi*.

## 1. INTRODUCTION

Cellulose is considered to be the most abundant carbohydrate on earth and it along with hemicelluloses and lignin constitutes plant cell wall. For degradation of cellulose, cellulases are used which are mostly secreted by microbes such as fungi [1]. Cellulases also play important role in senescence of plants and in host-parasite relationship for invading the plant. However, comparatively lesser studies have been carried out on cellulose producing bacteria. To explore more sources for cellulase production, nowadays, efforts are being made to identify cellulase producing bacteria because of their lesser generation time, adaptation to extreme environment, easy to modify their gene(s) using genetic engineering and secretion in large quantities as extracellular cellulolytic enzymes [2-4]. Cellulolytic enzymes are the backbone of various industries including food, animal feed, brewing, wine, agricultural biomass refining, pulp, paper, textile and ethanol production [5]. The crisis of non-renewable energy resources such as petroleum products further increased the demand of biofuel production through the use of cellulose [6]. The cost of cellulase production and optimization intensely influences the economics of the entire production process. Therefore, the cost of cellulase production is one of the major factors that decide the economic viability of cellulose based industries.

Soil is the hub of different types of microbes, and especially bacteria are present in large number. *Bacilli* are more dominant in soil after *Cocci* and *Spirilla*. Soil bacteria are profoundly capable of degrading cellulose, hemicelluloses and proteins. Bacteria secrete a broader range of enzymes in the surrounding culture media and therefore, these enzymes are easy to isolate and are called as extracellular enzymes [7]. Among cellulase producing bacteria, *Bacillus* sp. predominantly secrete maximum number of extracellular enzymes [8]. Other cellulase producing bacterial species include *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Ruminococcus*, *Erwinia* and *Acetivibri*. Among *Bacilli*, *Bacillus cellulosilyticus*, *Bacillus subtilis* and *Bacillus sphaericus* which are reported to produce higher amount of

extracellular cellulase enzyme [9,10]. In the present study, screening of various bacteria from different soil samples for cellulase production was carried out. Highest cellulase producing bacteria were identified using various biochemical analyses and 16S rRNA sequence analyses.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection

Soil samples from five different areas namely agricultural farm, paper mill, fodder storage house, agricultural waste dumping area and vegetable wholesale market were collected and brought up to the laboratory. In the laboratory, samples were put in separate sterile polybags and stored in a refrigerator at nearly 4 to 8°C temperature, if not used immediately.

### 2.2 Inoculation of the Petri-dishes for Isolation of Cellulase Producing Bacteria

All the soil samples individually were diluted using 0.90% saline solution. For that, one gm of soil sample was suspended in 10 mL of saline solution and this suspension was further diluted 10x with the saline. The diluted sample was used as inoculum to inoculate the sterile petri-dishes containing carboxymethyl cellulose (CMC) agar medium. The CMC agar medium was consisted of 0.5% CMC, 0.1% NaNO<sub>3</sub>, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.1% KCl, 0.05% MgSO<sub>4</sub>, 0.05% yeast extract and 1.5% agar-agar, pH 7.0. After inoculation, these petri-dishes were incubated at 37°C for 48 h in a bacteriological incubator. The colonies obtained were grown individually in liquid CMC medium for 24 h and cultures were used to streak the plates. The streaked plates were incubated at 37°C for 48 h in a bacteriological incubator where much dispersed colonies were visible indicating pure bacterial colonies [11].

### 2.3 Enzyme Screening Using Indicator Dyes

The replica plates were prepared and one plate was flooded with 0.1% Congo red solution

followed by washing using 1M sodium chloride. The colonies having clear zones were considered to secrete cellulase enzyme. Bacterial colonies having larger clear zones were further used considering them as potential positive isolates [11].

## 2.4 Cellulase Production

Potential isolates were grown in CMC liquid media having 1% CMC at 37°C for 24 h and 150 rpm shaking speed using an environmental shaker. These bacterial broths were used as inoculums to inoculate further. For enzyme production, bacteria were grown for 72 h using environmental shaker. The cultures were centrifuged at 5000 x g for 15 minutes at 0 to 4°C in a cooling centrifuge to collect the supernatants which were used for determining the presence of cellulase. These supernatants were stored at 0 to 4°C for further use [12].

## 2.5 Enzyme Assay

Cellulase enzyme was assayed according to the procedure as described by Sethi et al. [13] with some modifications. The enzyme assay was consisted of 1 mL of 0.5% CMC in 0.05M sodium phosphate buffer, pH 7.0 and to it up to 100 µl of enzyme (supernatant) and 0.05M sodium phosphate buffer were added to make the volume 2 mL. This reaction mixture was incubated at 50°C for 30 minutes in a water bath. After 30 minutes, reaction was stopped by adding 3 mL of dinitrosalicylic acid (DNS) reagent [14] and thereafter, tubes kept in a boiling water bath for 5 minutes and cooled to room temperature. The absorbance was measured at 575 nm in a spectrophotometer. In the control, enzyme was added after adding DNS reagent. One unit of the enzyme activity was taken as the amount of the enzyme that liberates 1µmol of reducing sugar as glucose equivalent per minute under the conditions of enzyme assay.

Cellulase activity was calculated using the formula [15]:

$$\Delta E \times V_f / \Delta t \times \Sigma \times V_s \times d$$

Where,  $\Delta E$  is absorbance at 575 nm,  $V_f$  is final volume of reaction mixture including DNS,  $V_s$  is the volume of enzyme in mL,  $\Delta t$  is incubation time in minutes,  $\Sigma$  = extinction coefficient whose value was taken as 0.0026 (for glucose equivalent), and  $d$  = length of light path.

## 2.6 Identification of Isolates

Various morphological and biochemical tests were carried out to identify the isolates as described by Cullimore [16]. Besides, 16S rRNA sequencing was also carried out to identify the bacteria. Phylogenetic tree analysis was also used for further identification.

## 2.7 Morphological Characterization

Gram staining was carried out to test whether Gram-positive or Gram negative bacteria.

## 2.8 Biochemical Characterization

Different biochemical tests such as catalase enzyme test, indole production, starch hydrolysis, urease test, methyl red test, Voges proskauer (MR-VP) test and sugar fermentation test were performed [16].

## 2.9 16S rRNA Gene Sequencing

The 16S rRNA sequencing was carried out at Yaazh Xenomics, Coimbatore, India. The individual isolates were grown in monolayer on nutrient agar media at 37°C for 24 h. Isolation of DNA was done using EXpure Microbial DNA isolation kit from Bogar Bio Bee stores Pvt Ltd., Coimbatore, Tamil Nadu, India. For that, 1 to 3 colonies were suspended aseptically in 450 µl lysis buffer to lyse the colonies by repeated pipetting. Thereafter, to the suspension, 4 µl of RNase A was added followed by 250 µl neutralization buffer and vortexed. The suspension was incubated at 65°C in a water bath for 30 minutes. Care was taken to minimize shearing of DNA molecules. Thereafter, it was centrifuged for 20 minutes at 14000 x g at 8 to 10°C temperature. The viscous supernatant was transferred into a fresh 2 mL size micro-centrifuge tube without disturbing the pellet. Afterwards, 600 µl binding buffer was added to supernatant and mixed well, and incubated the mixture for 5 minutes at the room temperature. A 600 µl aliquot of reaction mixture was transferred to a spin column and put in a 2 mL size collection tube and centrifuged at 14000 x g for 2 minutes to discharge the flow through. A 500 µl of washing buffer was added to the spin column and centrifuged at 14000 x g for 2 minutes. The step of spin column was repeated to transfer the remaining 600 µl aliquot. The spin column was transferred into a sterile 1.5 mL size micro-centrifuge tube and 100 µl of elution buffer was added at the middle of spin column, and incubated for 5 minutes at room temperature and

again was subjected to centrifugation at  $6000 \times g$  for 1 minute. The above process was repeated once more to elute maximum. The presence of DNA in the eluted sample was tested using miniagarose gel electrophoresis. The DNA samples were stored at  $-20^{\circ}\text{C}$  for further use [17].

For PCR, 5  $\mu\text{l}$  of isolated DNA was added to the PCR reaction solution (1.5  $\mu\text{l}$  of Forward Primer and Reverse Primer each, 5  $\mu\text{l}$  of deionized water, and 12  $\mu\text{l}$  of Taq Master Mix having Taq DNA polymerase, Taq reaction buffer, all the four dNTPs,  $\text{MgCl}_2$  and bromophenol blue). Denaturation was carried out by heating at  $94^{\circ}\text{C}$  for 3 minutes followed by annealing of the primers at  $60^{\circ}\text{C}$ , and amplification was done at  $72^{\circ}\text{C}$  using 30 cycles [18].

### 2.10 Phylogenetic Analysis

The 16S rRNA query sequence was blast using NCBI blast similarity search tool. Thereafter, MUSCLE 3.7 program was used for multiple sequence alignment [19]. The resulting aligned sequences were cured using the program Gblocks 0.91b which resulted in elimination of alignment noise [20]. Thereafter, PhyML 3.0 aLRT program was used for phylogeny analysis and HKY85 as Substitution model. The program Tree Dyn 198.3 was used for tree rendering [21,22].

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation and Screening of Cellulase Producing Bacteria

A total of 14 positive isolates of cellulase producing bacteria were obtained from five different soil samples. Out of these, 4 isolates showed maximum zone of clearance after staining with Congo red dye. A list of all the isolates along with area of clearance zone is given in Table 1. As shown in Table 1, isolates from the soil of fodder storage house Vijay Nagar, Indore were coded as CD01, FSVN-1, FSVN-2, FSVN-14, FSVN-15 and isolates from the soil of Amlai Paper Mill were coded as CD02, PM-1, PM-9. The bacteria isolated from the soil of agricultural farm, Vijay Nagar, Indore were coded as AgF-1, AgF-5 and AgF-6. The isolates from the soil of Choithram vegetable wholesale market, Indore were named as CSM-5 and CSM-6. The only isolate from the soil of agricultural waste dumping area, Satwas was named as SW-1. The size of zones of clearance with these 14 isolates ranged from 2.1 mm to 30 mm, whereas, hydrolytic capacity of these isolates varied from 5 to 29. On the basis of zone of clearance area and hydrolytic capacity, four isolates namely CD01, CD02, AgF-6 and CSM-6 were marked as maximum cellulase producing bacteria (Fig. 1).

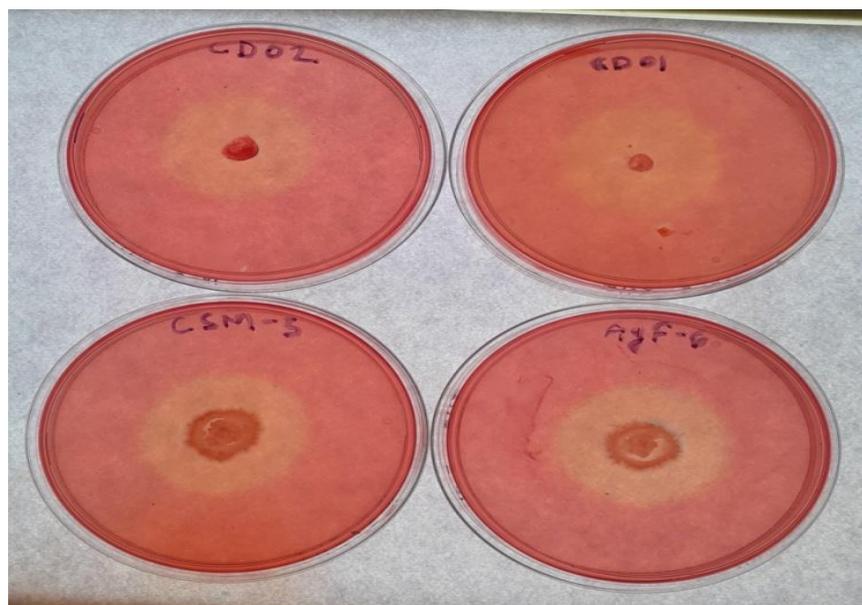


Fig. 1. CMC agar plate having four bacterial isolates namely CD01, CD02, AgF-6 and CSM-6 stained by Congo red dye showing zone of clearance

**Table 1. Various isolates along with their size of zones of clearance and hydrolytic capacity**

| Source organism   | Sample code | Maximum zone of clearance (mm) | Average HC value | Maximum HC value |
|---|-------------|--------------------------------|------------------|------------------|
| Choithram Vegetable wholesale market, Indore            | CSM5        | 2.1                            | 8.07±0.31        | 8.4              |
|   | CSM6        | 1.7                            | 10.33±1.53       | 12               |
| Agricultural farm Vijay Nagar, Indore                   | AGF1        | 10                             | 6.97±0.21        | 7.2              |
|   | AGF6        | 13                             | 12±1.00          | 13               |
|   | AGF5        | 5                              | 7.17±0.76        | 8                |
| Fodder storage house, Vijay Nagar, Indore               | FSVN1       | 2.5                            | 6.47±0.35        | 6.8              |
|   | FSVN2       | 4                              | 6.43±0.25        | 6.7              |
|   | CD01        | 30                             | 29.03±0.95       | 30               |
|   | FSVN14      | 7                              | 6.90±0.20        | 7.1              |
|   | FSVN15      | 7                              | 7.20±0.20        | 7.4              |
| Amlai Paper Mill District Budhar, Madhya Pradesh        | CD02        | 22                             | 22±1.00          | 22               |
|   | PM1         | 7                              | 7.20±0.26        | 7.4              |
|   | PM9         | 7.1                            | 7.0±0.20         | 7.2              |
| Agricultural waste dumping area, Satwas, Madhya Pradesh | SW1         | 6                              | 6.23±0.25        | 6.5              |

Similar results of hydrolytic capacity have been reported earlier for *Bacillus sp.* In earlier studies also, maximum cellulase production has been shown to occur on incubation at 37°C for 48 h using CMC agar medium [23-25].

### 3.2 Cellulolytic Potential of Bacterial Isolates

A total 14 bacterial isolates capable of producing extra-cellular cellulase were screened and isolated. All the 14 isolates were grown in liquid CMC media at 37°C for 72 h for maximum cellulase production. The amount of cellulase in terms of enzyme activity has been shown in Table 2. Among them, 4 bacterial isolates showed higher cellulase activity as shown in Table 2. The four isolates namely CD01, CD02, AgF-6 and CSM-6 produced 4.52, 3.16, 2.81 and 2.71 units/mL cellulase activity. The cellulase activity of these isolates is comparable with earlier reports [12,26,27]. Okeke et al. [28] reported higher cellulase production, 20.69 units per mg protein using bacterial consortium. However, this value can't be compared directly since they did not report activity per mL medium.

### 3.3 Morphological and Biochemical Characterization

Out of 14 isolates, only two isolates namely CD01 and CD02 which were found to produce maximum cellulase enzyme were characterized to identify them. The morphological and biochemical characterization of these two isolates has been given in Table 3. Upon Gram

staining, CD01 was found to be Gram positive whereas CD02 was Gram negative.

**Table 2. Various cellulase producing isolates with enzyme activity**

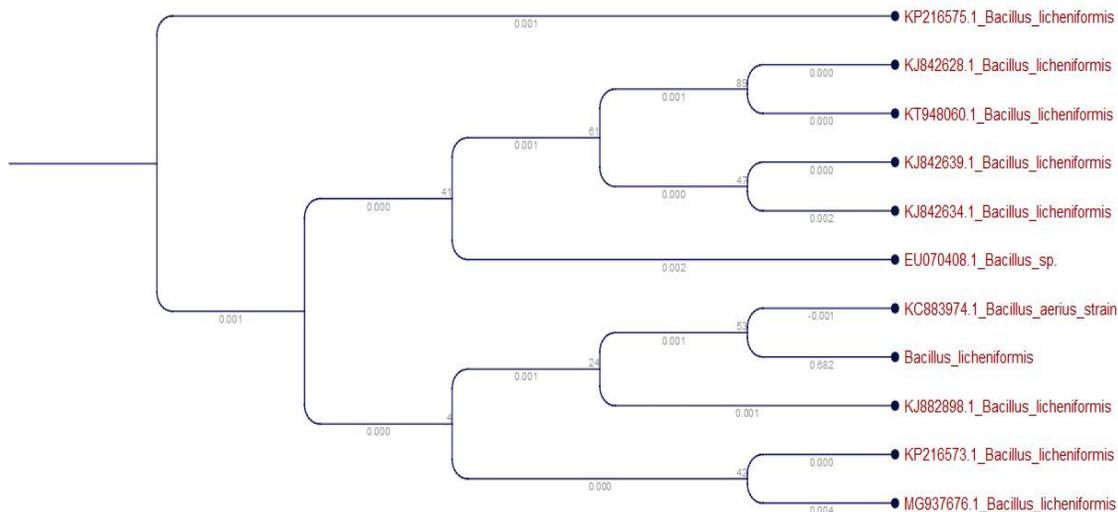
| Isolates code | Enzyme activity (IU/mL) |
|---------------|-------------------------|
| 1 CD01        | 4.52 ± 0.25             |
| 2 CD02        | 3.16±0.12               |
| 3 FSVN-1      | 0.50±0.06               |
| 4 AgF-6       | 2.81±0.10               |
| 5 AgF-5       | 0.95±0.04               |
| 6 AgF-1       | 1.11±0.04               |
| 7 FSVN-14     | 0.76±0.02               |
| 8 FSVN-15     | 2.05±0.02               |
| 9 SW-1        | 0.44±0.08               |
| 10 PM-1       | 1.06±0.03               |
| 11 PM-9       | 0.82±0.06               |
| 12 CSM-6      | 2.71±0.03               |
| 13 FSVN-2     | 0.35±0.06               |
| 14 CSM-5      | 2.41±0.34               |

16S rRNA sequences of both the isolates have been submitted to NCBI and these have been assigned the Accession No. MK29027 for CD01 and MK29032 for CD02. NCBI BLAST analysis revealed that CD01 has 99% similarity to *Bacillus licheniformis*. However, CD02 isolate sequence has nearly 99% similarity with *Ochrobactrum anthropi*. Phylogenetic analysis of both the isolates with closely related sequences was performed followed by multiple sequence alignment. For CD01, phylogenetic tree was constructed using sequences having Accession Numbers KP216575.1, KJ842628.1, KT948060.1, KJ842639.1, KJ842634.1,

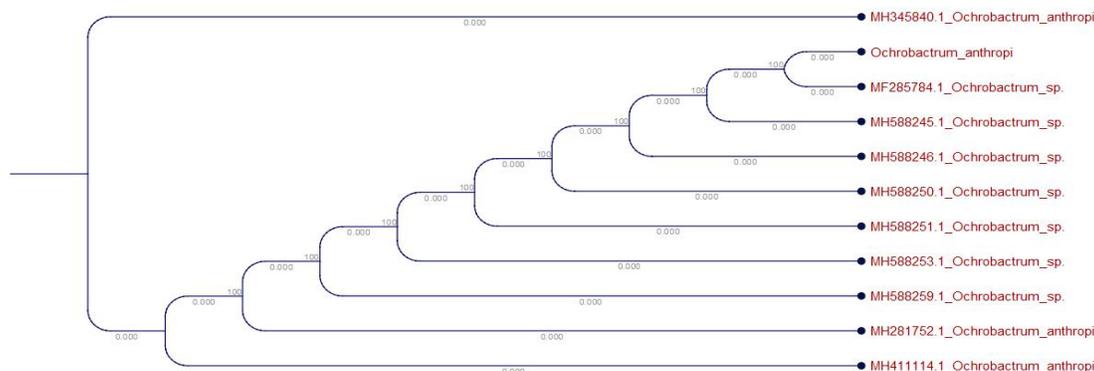
EU070408.1, KC883974.1, KJ882898.1, MH588246.1, MH588250.1, MH88251.1, KP216573.1 and MG937676.1 (Fig. 2). Similarly, for CD02, phylogenetic tree was constructed using sequences having Accession Numbers MH345840.1, MF285784.1, MH588245.1, MH588253, MH588259.1, MH281752.1 and MH411114.1 (Fig. 3). These trees were generated by using the neighbor joining method at 1000X bootstraps.

**Table 3. Morphological and biochemical characteristics of CD01 and CD02 isolates**

| S. no. | Test                             | CD01 | CD02 |
|--------|----------------------------------|------|------|
| 1.     | Gram staining                    | +ve  | -ive |
| 2.     | Catalase                         | +ve  | +ve  |
| 3.     | Citrate utilization              | +ve  | +ve  |
| 4.     | Methyl Red test                  | -ve  | +ve  |
| 5.     | Voges Proskeur test              | +ve  | -ve  |
| 6.     | H <sub>2</sub> S Production test | +ve  | +ve  |
| 7.     | Indole test                      | -ve  | -ve  |
| 8.     | Urease                           | +ve  | +ve  |
| 9.     | Starch Hydrolysis test           | +ve  | +ve  |



**Fig. 2. The phylogenetic tree of 16S rRNA of CD01 isolate with the closely related sequences**



**Fig. 3. The phylogenetic tree of 16S rRNA of CD02 isolate with the closely related sequences**

#### 4. CONCLUSION

Cellulase is a commercially important enzyme which is used in various food, paper, biofuel industries. In the present study, cellulase producing bacteria were from soils collected from different places having chances of more cellulase production. A total 14 isolates showed cellulase production on primary screening. Out of those, 04 isolates were selected for secondary screening. Ultimately two isolates showing more cellulase production were identified as *Bacillus licheniformis* and *Ochrobactrum anthropi* using various tests including 16S rRNA sequencing and phylogenetic tree analyses. Cellulase secreted by these bacteria, although is not thermophilic but still can be used for industrial purposes.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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