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### Isolation and Screening of Cellulose Degrading Microorganisms from the Gut of Composting Earthworms and Its Industrial Applications.

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#### ABSTRACT

Our present study aims on the isolation and screening of cellulose degrading bacteria from the gut of composting earth worms. The bacterial colonies isolated from the midgut of earthworms were grown on nutrient agar medium. Four different strains were isolated on nutrient agar medium among them, two strains showed cellulolytic activity on CMC media and was selected for further activities. These colonies were further screened for their cellulolytic activity on Carboxy Methyl Cellulase (CMC) plates. The isolated bacterial colonies were screened for their extracellular cellulase (endoglucanase) and intracellular cellulase (exoglucanase) enzyme production.

**Keywords:** Carboxy Methyl Cellulase (CMC), Endoglucanase, Exoglucanase.

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## INTRODUCTION

Cellulose is a plentiful organic compound in the atmosphere. It is found in the plant cell wall and is a polymer of glucose linked by  $\beta$ -1, 4 glycosidic bonds. It acts as a carbon source for the microorganisms responsible for the decomposition of organic matter in the soil. For several years of studying, cellulose degrading bacteria have been isolated for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, organic matters, faeces of ruminants and composts. The conversion of cellulosic mass to fermentable sugars from cellulolytic microorganisms has been suggested as a possible process that possesses potential to reduce the use of fossil fuels and reduce environmental pollution [6]. Earthworms are the most important organisms in organic matter decomposition on soil environment. Much like human engineers, they are organisms responsible for soil physical, chemical and biological properties. Earthworms have different role in an agro ecosystem. Their burrowing capacity can lead to the humus formation, organic matter decomposition, litter etc. and improving the soil structure, and enhancing the nutrient availability to plants[1]. Nevertheless there is not much studies were carried out on the interaction between earthworm, soil microorganisms and the gut microbial flora. The burrowing and feeding habits of earthworm are signified by the presence of ecological group-specific gut wall bacterial communities. The gut of earthworms is the residence for the production of the beneficial microorganisms and their products to defecate thousand times more to augment the surrounding soil. Hence to gain more information about the role of earthworms in the ecosystem, the intestinal micro flora must be identified [5].

The present study pay an attention to the carboxyl methyl cellulose producing capability of the earthworm gut bacterial isolates and their industrial applications.

## MATERIALS AND METHODS

### Collection of earthworm and processing

The earthworms were collected from the vermicomposting unit of KrishiVighyan Kendra, Virinjipuram, Vellore. After washing it with sterile tap water, the earthworms were placed in a sterile petriplate containing moistened filter paper for 24 hours. After cleaning them externally with 70% ethanol they were dissected and the midgut portion alone was collected.

### Isolation of bacteria from the mid gut of the earthworm

The isolation was carried out using the plate dilution method of Cappucino and Sherman, 2008 with slight modifications. 1 gram of the midgut was collected in 10 ml of 85% NaCl and homogenised in a vortex mixture for 5 minutes. The sample was then serially diluted ( $10^{-1}$  to  $10^{-7}$ ) triplicates of each dilution was plated on to nutrient agar plates and were incubated at  $30^{\circ}\text{C}$  for 24 hours.

## Screening for cellulose degrading bacteria

The isolated colonies obtained in the nutrient agar plates were inoculated on to CMC agar plates (Carboxy Methyl Cellulose agar) and incubated at 30°C for 24 hours. The colonies produced on plates were streaked on to CMC agar slant and was kept at 4°C for further studies. The cellulolytic bacteria were identified using the Congo red overlay method. Then after 72 hours of incubation of the pure culture on CMC agar, the plates were flooded with 1% Congo red (pH 8 to 7.2) for 20 minutes. A clear zone of lysis around the colonies, on washing the plates with 1 M NaCl solution indicates the presence of cellulose degrading bacteria. The contrast of the zone formed is enhanced by overlaying the plates with acetic acid for 1 to 3 minutes followed by washing with distilled water. The colonies showing the zone of lysis was selected and used for further studies.

## Morphological and Biochemical Characters

The morphological and biochemical characteristics of the isolated colonies were determined by following standard keys of Bergey's Manual of Determinative Bacteriology. The biochemical analysis was performed for indole, methyl red, Voges Proskauer, citrate utilization, catalase, nitrate reduction, glucose, fructose, maltose and starch utilization.

## Assays for enzyme activities

### Endoglucanase activity

The enzyme source was obtained as the supernatant on centrifuging the culture broth at 5000 rpm for 20 minutes at 4°C. To 0.5 ml of this supernatant equal amount of 1% substrate (CMC) in 0.2 M Citrate Phosphate buffer (pH -7) was added and incubated at 45°C for 30 minutes. 2 ml dinitrosalicylic acid reagent was added to the above tube and was kept in boiling water bath for 5 minutes. It was then cooled quickly to room temperature. This was done to stop the reaction. The absorbance was measured at 540 nm using a UV-Visible spectroscopy. The enzyme activity was determined according to the method by the International Union of Pure and Applied Chemistry (IUPAC) commission on biotechnology. The endoglucanase activity was measured as the amount of enzyme required to release the reducing sugar from the substrate in terms of glucose equivalent min/g of the enzyme sample.

$$\text{CFU/ ml} = \text{Concentration of the glucose} / 0.18 \times 0.5 \times 30$$

### Exoglucanase activity

The culture supernatant centrifuged at 5000 rpm for 10 minutes at 4°C was taken as the enzyme substrate. 0.5 ml of enzyme substrate and 1 ml of sodium citrate buffer with a pH of 5.8 and was incubated in a water bath at 50°C. To each of the tube 1.0 x 6.0 cm (~ 50mg) of Whatman no.1 filter paper was added and vortexed to settle the filterpaper. This served as the substrate. After 1 hour incubation 3 ml of dinitrosalicylic acid was added. The enzyme and

spectro zero were prepared with the enzyme and DNSA in sodium citrate buffer respectively. The glucose standards were prepared by dissolving 0.2- 5.0 mg of glucose per ml. All the tubes were boiled for 5 minutes and immediately cooled by placing in cold water bath [7]. The optical densities were measured using UV Visible spectrometer at 540nm. Exoglucanase unit was calculated in terms of filter paper units using the formula,

$$\text{FPU/ml units ml}^{-1} = \text{mg glucose released} \times 0.185.$$

### Bioethanol production

Two sets on the basal salt medium ( $\text{NaNO}_3$  2.5 g;  $\text{K}_2\text{HPO}_4$  2 g,  $\text{MgSO}_4$  0.2 g,  $\text{NaCl}$  0.2 g,  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  0.1 g in a litre) was prepared, one containing filter paper and the other containing cellulose powder as substrate. The two cellulose degrading bacterial isolates were inoculated as mixed culture in it and was incubated at  $37^\circ\text{C}$  for 3 days in a rotary shaker at 100 rpm. This would result in the release of the cellulolytic enzymes and initiate the saccharification process. After the incubation the above broth was cocultured with *Saccharomyces cerevisiae* and was incubated at  $27^\circ\text{C}$  for 5 days in stationary condition for the further saccharification and fermentation to occur [2]. The alcohol production was tested quantitatively using the  $\text{K}_2\text{Cr}_2\text{O}_7$  reagent test [10].

## RESULTS AND DISCUSSION

### Isolation of bacteria from the mid gut of the earthworm

After 24 hours of incubation, the nutrient agar plates inoculated with the homogenised contents of the mid gut of the earthworms developed colonies. Using standard plate count technique the number of cultivable bacteria was calculated and found to be  $3.2 \times 10^7$  CFU/g.

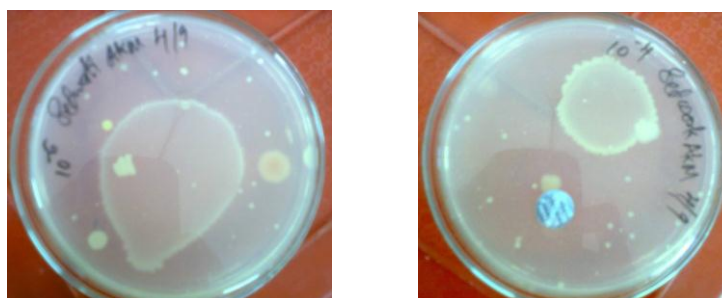


Figure 1: Earthworm gut isolates on nutrient agar

### Screening of cellulolytic bacteria

Further screening was done on CMC agar plates. The cellulose when produced it causes the hydrolysis of the polysaccharide CMC [8]. The congo red when added interacts with the 1, 4- $\beta$  D glucans. Thus clearing around two of the inoculated colonies were produced when it was

flooded with congo red. The zones of clearing were highlighted on addition of 1% acetic acid. This indicated the cellulolytic activity of the isolates.

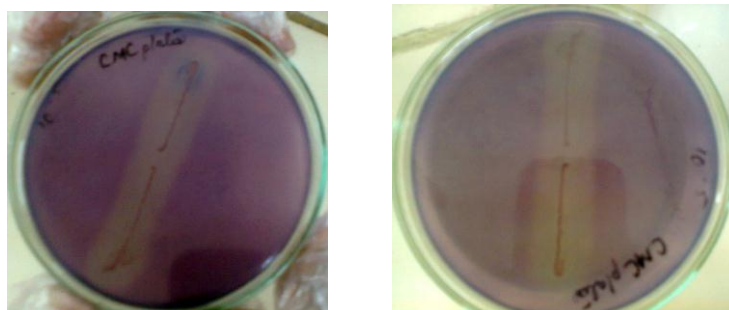


Figure 2: Clearance shown on CMC plates

### Morphological and biochemical characters

The characterisation of the isolates was done according to the Bergey's Manual of Determinative Bacteriology. The results of which is summarised in the table 1.

Table 1: Biochemical Characterization of the isolated strains

Biochemical Characteristics	Strain1	Strain2
Cell Shape	Rods	Rods
Grams reaction	—	—
Indole Production	—	+
Citrate Utilization	—	+
Voges Proskauer	—	—
Catalase	+	—
Nitrate Reduction	+	+
Glucose	+	+
Fructose	+	+
Maltose	+	+
Starch	+	+

### Exogluconase activity

Exogluconase activity for the two isolated cellulolytic strains was determined using Whatmann No.1 filter paper as substrate. The production of exogluconase enzyme by Strain A isolated from earthworm has a maximum of 0.213 FPU /ml and Strain B showed minimum activity of 0.138 FPU/ml which is shown in fig.3.

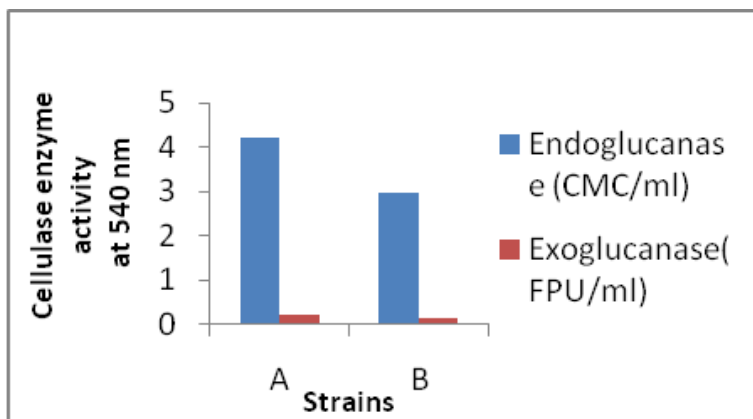


Figure 3: Total cellulose activity of isolated bacteria

### Endoglucanase activity

Two cellulolytic strains produced endoglucanase enzyme using CMC as a substrate. A comparison of enzyme activities among the strains revealed that the endoglucanase activity of Strain A (maximum of 4.21 CMC/ml) was greater than Strain B which showed a limited endoglucanase activity of (2.97 CMC/ml) which is depicted in fig.3.

### Bioethanol production

The coculturing of the two cellulose degrading isolates of bacteria and *Saccharomyces cerevisiae* resulted in the saccharification followed by the fermentation of the sugars to produce ethanol. The test shows the synergetic ability of the cellulolytic isolates to degrade the sugar and the utilisation of the so formed reducing sugars by the non-cellulolytic yeast *Saccharomyces cerevisiae*. These studies have been reported by many workers [9].

### CONCLUSION

The present study shows the ability of certain bacterial isolates present in the mid gut of composting earthworms to degrade cellulose. The ability of these bacterial isolates to form reducing sugars, which can be used as substrates for the production organic acids like ethanol, has the potential to be an environmentally sustainable alternative. Because of their low yield and lack of robustness they find limited application in the industrial levels. However, the study of these cellulolytic microorganisms could help in improving our knowledge of cellulolytic enzymes and how they work synergetically. This may eventually help in the recombinant strategy.

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