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Isolation and Screening of Cellulolytic Bacteria Inhabiting Gut of Eisenia Fetida Fed on Municipal Solid Waste

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Abstract: Cellulose is the most abundant biopolymer in nature and constitutes a large pool of carbon source for the microorganisms responsible for the decomposition of organic matter in soil. Earthworms influence this decomposition by enhancing the structure and dynamics of the microbial population inside their gut as an efficient bioreactor. The assessment of cellulolytic activity in the microbes isolated from the mid-gut of the popular composting earthworm, Eisenia fetida (Redworm), revealed the bacterial community is responsible for the breakdown of cellulose and thereby decomposition of organic matter by this earthworm. The bacterial counts for viable microorganisms were made in the mid-gut section of Eisenia fetida collected from vermicomposting unit. The number of bacteria present in the mid gut identified by standard plate count technique. Based on the morphological characteristics of colony, 3 cultures were raised. They were further screened for their cellulolytic activity in modified czapex-dox agar. Out of 3 cultures, only 2 cultures proved to be cellulolytic. Carboxy methyl cellulase assay was then performed to find out the most efficient cellulase producers. Accordingly, the culture designated as Cdb1 produced 26.041 ± 14.062 IU/ml and the culture designated as Cdb2 produced 47.800 ± 25.812 IU/ml of CMCase 0.1% substrate in 72hours at 37°C and at pH 7.

Key words: Bacterial enzyme, Cellulose, Carboxy methyl cellulose, Earthworm Eisenia fetida.

I. INTRODUCTION

Earthworms are hermaphroditic, meaning each individual possesses both male and female reproductive organs. (Sherman, 2003) Eisenia fetida (older spelling foetida) known under various common names such as redworm, brandling worm, panfish worm, trout worm, tiger worm, red wiggler worm, red californian earth worm, etc., is a species of earthworm adapted to decaying organic material. These worms thrive in rotting vegetation, compost, and manure. They are epigean, rarely found in soil. In this trait they resemble Lumbricus rubellus. Eisenia fetida worms are used for vermin-composting. Earthworm's varieties influence this decomposition by enhancing the structure and dynamics of the microbial population inside their gut as an efficient bioreactor. The assessment of cellulolytic activity in the microbes isolated from the mid-gut of the different variety of earthworms revealed that there are bacterial communities which are responsible for the breakdown of cellulose by releasing of cellulose enzyme and thereby decomposition of organic waste by earthworms. (Shankar et al., 2011)

Earthworms are the crucial drivers of the process, as they aerate condition and fragment the substrate and thereby drastically alter the microbial activity and their biodegradation potential Several enzymes, intestinal mucus and antibiotics in earthworm's intestinal tract play an important role in the breakdown of organic macromolecules. Cellulose a biopolymer is the most abundant biopolymer in nature and constitutes a large pool of carbon source for the microorganisms responsible for the decomposition of organic municipal solid waste in soil. The degradation of the cellulose to glucose requires a combined or cooperative action of at least three enzymes namely an endo-1,4-β-glucanase (also referred to as carboxy methyl cellulase or CMCase; EC 3.2.1.4), an exo-1,4-β-glucanase (EC 3.2.1.91) and a β-glucosidase (EC 3.2.1.21) (Shankar et al., 2011).

Different types of bacteria have been isolated from the gut of the earth worm, Mainly bacteria, fungi and actinomycetes achieve bioconversion of these materials.

Several studies were carried out to produce cellulolytic enzymes in bio-waste degradation process by several microorganisms including fungi such as Trichoderma sp. Penicillium sp. Aspergillus sp. respectively by (Mathur et al., 2006). Similarly cellulolytic property of bacterial species like Pseudomonas, Cellulomonas, Cellulovibrio and Sporocytophaga sp. was also reported by (Nakamura and Kappamura, 1982)

II. MATERIALS AND METHODS

1. Collection and rearing of earthworm

The sample of earth worm was collected from Govt. Agriculture College of the town and was reared in suitable mud pots for increasing the population. The Municipal sewage waste (MSW) was collected from the Environmental Control Board, Health Office of the city and field survey. Cow dung was collected nearby the university.

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2. Experimental set up
Different ratio of MSW and cow dung was mixed and earthworm was allowed to feed the sample along with positive (Only Waste) and negative control (Cow dung). After certain time of feeding the gut of the earth worm was dissected for the isolation of Bacteria.

3. Isolation of bacteria from earthworm gut
The Isolation of enzymatic hydrolysis of the cellulase was determined bacteria was done by serial dilution from 10-1 to 10-7 was prepared.

4. Isolation of cellulose degrading bacteria
The colonies was isolated and streaked on Carboxy Methyl Cellulose (CMC) agar plates. Then plate was incubated for 24 hours at 37°C. The isolated colony on these plates was maintained on CMC agar slants at 4°C for further analysis.

5. Screening of cellulase activity
The purified colony was further screened for their cellulase activity. For this, a pure culture of bacterial colonies was transformed individually on CMC agar plates. After 72 hours of incubation, the plate was flooded with 1% Congo red and then a plate was allowed to stand for 20 minutes at room temperature. Then the plate was thoroughly washed with 1M NaCl solution. A clear zone formed around the growing colonies of cellulase positive cultures against the dark red background was taken as the indication of cellulose activity. The contrast was further enhanced by treating the plates with 5% acetic acid 1 to 2 minutes and then the bacteria that showed good clearance beyond the area of growth was selected for further studies as potential cellulose secretors.

6. Determination of cellulase activity
The supernatant of the culture broth centrifuged at 5000 rpm for 20 minutes at 4°C served as the enzyme source. Then enzyme solution 0.5 ml was added to 0.5 ml of 1% substrate (CMC) was taken in 0.2 M Citrate Phosphate buffer (pH-7) and incubated at 45°C for 30 minutes. Then reaction was stopped by the addition of 2 ml dinitrosalicylic acid reagent by keeping for 3 minutes in boiling water bath and quick cooling to room temperature. Then the degree of enzymatic hydrolysis of the cellulase was determined spectrophotometrically by measuring the absorbance at 540 nm.
Enzyme activity (IU/ml) = conc. of glucose × 1000 / molar mass × incubation time in min.

7. The growth kinetics of isolated bacteria
The Microbial growth kinetics is an important indicator of microbial activity to further understand the decomposition of organic substrate, effect of substrate concentration & time period studied on bacterial growth was needed. The experiment was based on CMC broth medium for bacterial culture. The bacterium Cdb1 and Cdb2 was incubated in 150rpm at 37℃ (±2) & at every 16 hours growth was measured by analyzing the turbidity at 600 nm using spectrophotometer.

8. Cultural characterization
The pure cultures of individual isolates were further characterized, on the basis of their Biochemical characterization and Morphological characterization.

8.1 Morphological characterization
For morphological characterization colonies were stained by Gram's staining technique and for suspected isolates special staining was also performed included acid -fast staining and endospore staining.

8.2 Biochemical characterization
The biochemical analysis was performed for Fermentation of sugar, (acid and gas production) catalase activity test, citrate utilization, and starch utilization.

III. RESULTAND DISCUSSION

Cellulose degrading bacteria was isolated in NAM medium at 37℃ & pH 6.8 to 7 and screened for cellulose degrading ability on in CMC medium at 37℃ & pH 7.0. Screening of Cellulase Activity:-The purified colonies were further screened for their cellulase activity. Bacteria enumerated from earthworm gut: The number of cultivable aerobic bacteria present in the mid gut of earthworm, Eisenia fetida, (redworm) as identified by standard plate count technique. (Fig. 1) Cellulase Producing Bacterial Strains: Over the last forty years, research on the degradation of cellulose and lignocellulosic biomass by microorganisms has received a considerable amount of attention. The potential use of cellulose as a renewable resource for the production of important industrial chemicals such as glucose, alcohol, solvents and goods such as paper, rayon and cellophane has increased attraction. Cellulases have been utilized for the preparation of plant protoplast in genetic research and improvement of nutritional values of animal feed and as microbial proteins (SCP) remains a very attractive prospect in the long term. In the present study, two different colonies were selected based on colony morphology and they was screened for their cellulase producing ability. Of these, 2 isolates namely Cdb1 &Cdb2 showed positive reactions indicated by a clearing zone of more than 10 mm in diameter (Fig 2 a & b). Attempts to increase the production of enzyme cellulase from bacteria include several processes like mutation, protoplast fission, optimization of medium composition and environmental factors. The bioconversion of various complex cellulosic waste materials such as bagasse, saw dust, corncob and coir retting effluents, have been reported.

The assay for enzyme activity is based on the ability of cellulase produced by the strains to hydrolyze CMC to reducing sugars which could be measured using 3,5-dinitrosalicyclic acid (DNS) is shown in (table 1) The activities of the two strains, screened as cellulose producers in the preliminary screening. At 72 hours of incubation, Cdb1 produced 26.041 ± 14.062 IU/ml the highest cellulose activity, which is significantly different from the rest of the strains.

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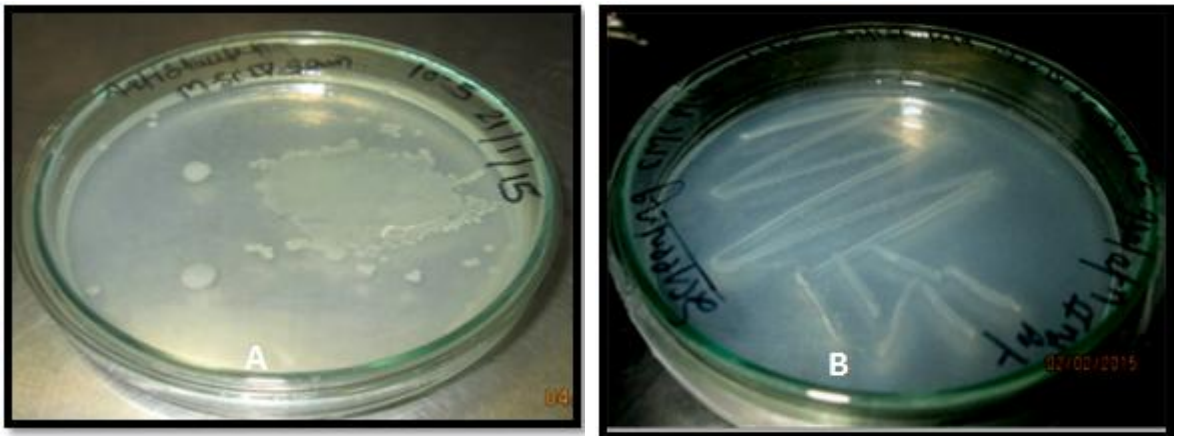

Fig. 1:- (A).Isolation and (B) purification- cellulose degrading bacteria

The next best cellulase activities was exhibited by Cdb2 and produced 47.800 ± 25.812 IU/ml. Maximum amount of enzyme production was observed in strain Cdb2.

The growths of bacteria were highly dependent on concentration of CMC. In present work the growth of bacterial isolate Cdb1 & Cdb2 was observed at 1000mg CMC/L of culture medium. In present work growth of isolates CdB1 in selective medium was observed between 64-96 hour. Hence, Culture was used for further Morphological & Biochemical characterization. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size (carbon source and cellulose quality), pH value, temperature, presence of inducers, medium additives, aeration and growth time etc. The particle size of cellulose can affect cellulase production by microorganisms. In the present study, based on the morphological Physiological and biochemical characteristics of the suspected colony was identified as Streptococcus, Staphylococcus, & Diplococcus by the following standard keys of Bergey's Manual of Determinative Bacteriology. (Table: - 2)

Cellulose is probably the most abundant biological compound on terrestrial and aquatic ecosystem. It is the dominant waste material from agricultural industry in the form of stalks, stems and husk and is one of the most abundant renewable sources.

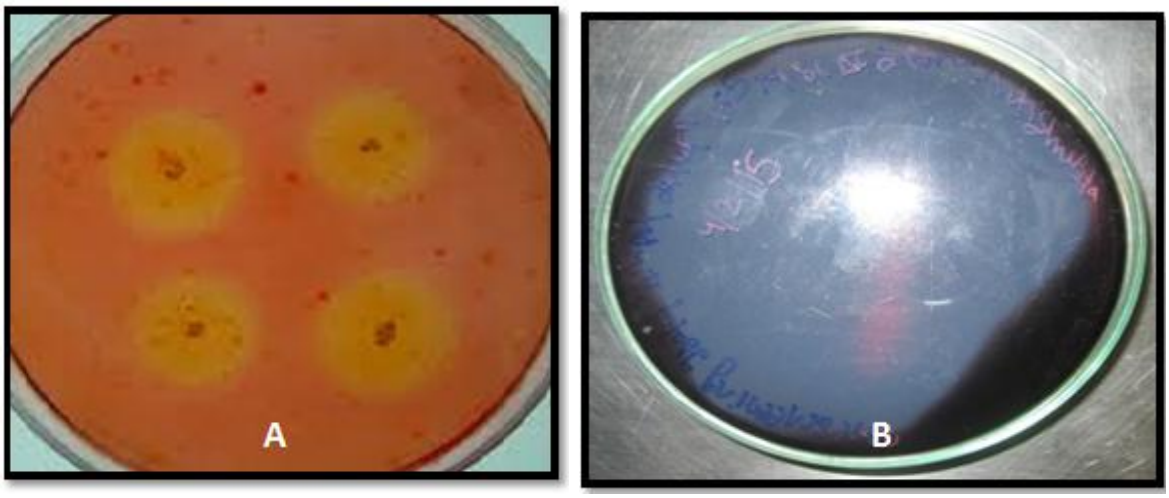

Fig2 a. Screening of cellulose activity with (A) Congo red and (B) Acetic acid

By means of chemical or bioconversion methods, it is possible to transform this insoluble polymer into glucose, an excellent substrate for industrial fermentation. Mainly bacteria, fungi and actinomycetes achieve bioconversion of these materials. Several studies were carried out to produce cellulolytic enzymes in biowaste degradation process by several microorganisms including fungi such as Trichoderma sp. Penicillium sp. Aspergillus sp. respectively by (Mathur, et al. 2006) Similarly cellulolytic property of bacterial species like Pseudomonas, Cellulomonas, Cellulovibrio and Sporocytophaga sp. was also reported by (Nakamura and Kappamura,1982). Cellulomonas fimiis one of the first bacterial cellulase systems. Many spore forming bacteria have been isolated from factors that have a feed stock from cattle waste, cow manure, woody biomass and municipal solid waste.

Table 1:- Determination of cellulase activity

S. No.	Sample	Enzyme activity
1	Cdb1	26.04166 u /ml.
2	Cdb2	47.8009 u/ml.

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Similarly many strains of cellulolytic anaerobic bacteria have been reported from various sources as human colon, estuarine sediments, fresh water sediments and decomposing vegetation. Because of the common occurrence of these bacteria in various natural environments, they are responsible for vast amount of cellulose degradation.

Table 02:- Characterization of bacteria for morphological & biochemical Test.

S.No.	**Characteristics**	**isolate's description**	
Morphological Test		Cdb1	Cdb2
01.	Colony shapes & colour	Cocci (spherical), white.	Cocci (spherical), yellow.
02.	Gram's staining	Negative.	Positive.
03.	Endospore staining	Positive.	Positive.
04.	Acid fast staining	Positive.	Positive.
Biochemical Test			
01.	Fermentation of sugars	Positive.	Positive.
02.	Citrate utilization	Positive	Positive
03.	Hydrolysis of starch	Positive	Positive.
04.	Catalase activity test	Positive (produces enzyme)	Positive (produces enzyme)

Reports of cellulolytic activity in the gut of some species of earthworms, especially in epigenic earthworms such as Eisenia fetida, indicate their ability to digest cellulose, although the effects exerted by earthworms on cellulolysis lie fundamentally in their interactions with microorganisms. These interactions are the subject of a certain amount of controversy, mainly because of the variety of species, substrate and experimental conditions assayed. It is generally agreed that microorganisms, for fungi, are part of the diet of earthworms; moreover, earthworms have been shown to graze selectively on fungal species. Although earthworms can digest fungi and bacteria, an increase in the number of microorganisms during gut transit has also been reported.

Vermi composting involves the bio-oxidation and stabilization of organic matter through the joint action of earthworms and microorganisms. The transformations in physicochemical and biochemical properties and the short time in which they occur make them a suitable system for studying microbe-earthworm interactions. The actions of earthworms during vermicomposting include not only digestion and release of easily assimilable substances, such as mucus for micro biota, but also the transport and dispersal of microorganisms through casting. Earthworm casts play an important role in decomposition because they have a different nutrient and micro biota composition to the material prior to ingestion, which makes possible a better exploitation of resources because of either the appearance of microbial species in fresh substrate or the pool of easily assimilable compounds of casts.

Degradation of cellulose in soils is a slow process that is limited by several factors involving cellulases, such as concentration, location and mobility of the enzymes.As organic waste contain huge amount of the cellulose containing material and earth worm used to feed on that it means earth worm contain large amount of cellulose digesting enzyme. (Cellulolytic microbes) This microbial approach helps a lot in reducing unwanted cellulose (Wastes) from the biosphere. This is economically affordable & doesn't generate any wastes.

IV. CONCLUSION

The data gathered in this study provides evidence for cellulase producing ability of the earthworm gut bacterial isolates. As organic waste contain huge amount of the cellulose containing material and earth worm used to feed on that it means earth worm contain large amount of cellulose digesting enzyme. The production of cellulase and lignocellulosic substrate interactions of bacterial strains in the earthworm gut was also evident in this study. This study gives us a hint that the microbial wealth of cellulase producing bacteria isolated from the earthworm gut can be harnessed for biotechnological processes.

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