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Isolation of Cellulose-Degrading Bacteria from the Gut of the Earthworm *Eisenia Fetida*

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cellulose activity;
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Congo-red;
earthworm gut;
Eisenia fetida;

Abstract

Cellulose is the most abundant biopolymer in nature. Cellulose provides a carbon source for the microorganisms responsible for the decomposition of organic matter in soil. Despite the vast number of cellulase producers, there is a deficiency of microorganisms that can produce a significant amount of cellulase enzyme to efficiently degrade cellulose to fermentable products. Earthworms influence this decomposition by enhancing the structure and dynamics of the microbial population inside their gut, as any efficient bioreactor. The present study explored the gut flora of the earthworm Eisenia fetida to isolate potential cellulolytic bacteria from their gut. The bacterial isolates were aseptically isolated from the gut of this earthworm and screened on CMC agar medium by using CMC as a sole carbon source. Their cellulolytic potential was checked by using the Congo-red overlay method. The latter method was found to be more efficient with a rapid and distinctly visible zone of hydrolysis. Basic identification of the bacterial genera was conventionally done by Bergey's Manual of Determinative Bacteriology based on their biochemical properties.

Contents

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1 Introduction

Cellulose is a linear polysaccharide of glucose residues with β-1.4-glycosidic linkages. [Gupta et al., 2012] Cellulose can be changed into glucose & other soluble sugars by the process called cellulolysis. For cellulolysis, a set of enzymes named cellulase is required, which includes endoglucanase (endo 1, 4-Beta-D glycanase); cellobiohydrolase or exoglucanase (exo-1, 4-Beta-D gluconase), and B-glucosidase (1, 4-Beta-D glucosidase) [Bahotkar et al., 2022].

Cellulase is the most abundant polymer in nature and constitutes large pool of carbon for microorganisms, the main agents responsible for soil organic matter decomposition [Manuel Aira et al., 2006] with the help of cellulolytic system, cellulose can be converted to glucose, which is multiutility product, in a much cheaper & biologically favorable process, source of cellulose system extraction is best suitable from microbial system found in the gut of organisms thriving on cellulosic biomass as their major feed [Gupta et al., 2012].

Different microorganisms, mostly fungi & bacteria, produce this inducible bioactive Compound during their growth & development of cellulosic matter. However, there is a deficiency of microorganisms that can produce a significant amount of cellulose enzyme to efficiently convert cellulose into fermentable products [Bahotkar et al., 2022]. Insects like termites (Isoptera), bookworms (Lepidoptera), and so forth, are found to have symbiotic microflora in their gut responsible for cellulosic feed digestion. Cellulase, due to its massive applicability, has been used in various industrial processes. Such as biofuel like bioethanol, triphasic biomethanation, agricultural & plant waste management, chiral separation & ligand binding studies. The culturing of cellulase-degrading bacteria & yeast was also carried out for simultaneous saccharification & fermentation of cellulose into ethanol [Gupta et al., 2012].

Earthworms act in the soil as aerators, grinders, Crushers, chemical degraders & biological stimulators. [Sinha et al., 2002]. Earthworms are often referred to as ecosystem engineers because they make up the biggest Component of the animal biomass found in the soil [Yang et al., 2023]. They secrete enzymes, proteases, lipases, amylase, cellulases & chitinase. This brings about rapid biochemical Conversion of the cellulosic & the proteinaceous materials in a variety of organic wastes, which originate from homes, gardens, dairies & farms. The process is odor-free because earthworms release coelomic fluid in decaying waste biomass, which has antibacterial properties that kill pathogens [Sinha et al., 2002]. Earthworms have developed sophisticated gut systems. To digest a variety of food, predominantly plant matter, this is considered to be an important food source for earthworms. Cellulose is regarded as one of the most basic carbon sources for earthworms to produce energy

Carbon dioxide & water account for 40-50% of the dry weight of plant cell walls [Yang et al., 2023]. Earthworm action was shown to enhance natural biodegradation & decomposition of waste (60-80% under optimum Conditions). Thus, significantly reducing the Compositing time by several weeks, within 5-6 weeks. 95-100%, degradation of all cellulosic materials was achieved. Even hard fruit and egg shells & bones can be degraded, although these may take longer [Sinha et al., 2002].

Diverse types of solid waste, including waste of municipalities, agriculture, & Farm animals, pose both problems & prospects in terms of disposal & Conversion to useful bioinputs in agriculture. Organic materials of biological origin in the municipal solid waste (MSW) are also an important source of compost, biogas & biofuel. It is estimated that MSW is composed of 40-50% cellulase. 9-12% hemicellulose & 10-15% lignin on a dry weight basis [Devi et al., 2018].

In topographical regions, earthworms are exposed to large temperature fluctuations and food resource variation, which are the major variables that limit earthworm growth & productivity [Yang et al., 2023]. The carboxy Methyl Cellulose producing potential of the earthworm gut bacterial isolates, which can be further optimized for the maximum cellulase production, & it can be used for further industrial applications [Shankar et al., 2011]. Municipality biowaste (MSW) can also be converted to nutrient-rich compost. Use of an inoculum of cellulose-degrading microorganisms (CDMs) can convert cellulose-rich MBW to compost rapidly and increase nutrient content in the final compost by reducing its loss during composting. In general, MBW is alkaline in nature & under Conditions of high pH, loss of nitrogen may occur by the process of volatilization during composting [Devi et al., 2018].

Earthworms form the dominant component in the animal biomass [Sims & Gerard et al., 2023]. Earthworms can be used in the vermicomposting process, and they belong to the phylum Annelida. To determine the full potential of the compost warm *Eisenia fetida* is used as a waste processor and as a source of protein [Venter et al., 1988]. The earthworm (Eisenia fetida) is considered a representative of soil fauna. This is characterized by its increased sensitivity to chemical agents compared with other species of earthworms. (Ifemeje et al., 2015).

Earthworms are one of the most abundant terrestrial species, and play an important role in maintaining the ecological function of soil [Wang et al 2015]. The development, growth & reproduction of Eisenia fetida were studied on cattle manure under favorable conditions of moisture, temperature & nutrients. Each cocoon produced a mean of 2.7 hatchlings after a mean incubation period of 23 days. These worms attained sexual maturity, offering 40-60 days & produced their cocoons within 4 days after mating took place [Venter et al., 1988].

The growth & reproduction of *Eisenia fetida* were measured by studying parameters such as gain in body weight no. of cocoons produced, and number of hatchlings emerged. These worms were grown in this organic manure for about six months [Siddique et al., 2005]. *Eisenia fetida* reared in vermibeds containing landfill leachate. These bacteria could be further exploited not only in the degradation of waste, but also as beneficial biofertilizers to enhance plant growth [Shaarani et al., 2019]. Earthworms influence decomposition indirectly by affecting microbial population structure & dynamics & also directly because the guts of the same species possess cellulolytic activity. Eisenia fetida digests cellulose directly (i.e., with its associated gut microbiota). The activity of Eisenia Fetida triggered fungal growth during vermicomposting [Aira, et al., 2006].

Earthworms make up the majority of the biomass of soil invertebrates & their activities enhance decomposition & mineralization of organic matter. It is cellulases produced by termites themselves & by symbiotic microorganisms contributes to the digestion of cellulose. It has developed a mutualistic earthworm-microorganisms digestive system with the microorganisms to digest soil organic matter [Nozaki et al., 2009]. This is supported by In Situ Tissue culture experiments of earthworm gut walls, showing that some species could not produce cellulases that can break down cellulose.

The roles of gut microbiota in the environmental adaptation of earthworms at a large scale remain obscure. The earthworm gut harbors millions of decomposer microbes, providing the host with physiological & ecological advantages in the digestion of cellulose-rich diets (Sharma & Hobson, 1985). The complex cellulases, produced by gut micro biota, are responsible for breaking down the cellulose contained in the plant matter & converting it into a simple sugar that can be used as a source of energy for both hosts & microbes, some bacteria in the gut of - earthworms, such as *Pseudomonas, Bacillus* & *Acinetobacter* - are characterized by strong cellulase activity & were found to significantly improve the digestion ability to cellulose [Yang et al.. 2023].

2 Materials and Methods

Screening of Cellulose-Degrading Bacteria:

Fig.1: **Eisenia fetida**

Fig.2: **Gut content of Earthworm**

Earthworm *(Eisenia fetida)* collected from the vermicompost Unit of Dr. Ghali College, Gadhinglaj and washed with distilled water. Sterilized externally with 70% ethanol and anesthetized by placing in crushed ice. Gut of earthworm was excised and content was suspended in 10 ml sterile 0.85%NaCl solution. Suspension was diluted serially and 0.1 ml of each dilution was inoculated on CMC agar medium (Peptone 10 gm/L, CMC 10 gm/L, $MgSO_4$ 0.2 gm/L, $NaCl_2$ 0.5 gm/L, $CaCl_2$ 0.1 gm/L, Agar-agar 15 gm/L, pH 6.5). The plates were incubated at room temperature for 48 hours. Colony characteristics were observed after incubation. The isolated bacterial colonies were further characterized for their morphological and biochemical characters by following standard keys of Bergey's Manual of Determinative Bacteriology (Ueda et al., 2010).

Screening of amylase activity:

100µL suspension of all isolates was streaked on separate Starch Agar medium plate, Starch Agar containing (g/L): Nutrient Agar (NA) 28 and starch 10, and incubated at R. T. for 48 hours. Determination of starch hydrolysis was done by iodine test. Bacterial isolates that produce amylase enzymes will show clear zone around the colony and this is visible after rinsing with iodine solution.

Screening of cellulase activity:

Pure cultures of cdb-A, B and C were transferred on CMC agar plates and incubated at room temperature for 48 hours. After incubation the plates were flooded with 1 % Congo red and the allowed to stand for 20 minutes at room temperature. Then the plates were thoroughly washed with 1M NaCl solution. A clear zone is visible around the growing colonies of cellulase positive cultures against the dark red background. This is indication of cellulase positive activity.

Screening Of Endoglucanase Activity:

The supernatant of the culture broth centrifuged at 5000X g for 20 minutes at 4^0C served as the enzyme source. This enzyme solution 0.5 ml was added to 0.5 ml of 1 % substrate (CMC) taken in 0.2 M Citrate Phosphate buffer (pH-7) and incubated at 45^0C for 30 minutes. The reaction was stopped by the addition of 2 ml dinitrosalicylic acid reagent by keeping for 5 minutes in boiling water bath and quick cooling to room temperature. The degree of enzymatic hydrolysis of the cellulase was determined spectrophotometrically (UV-Vis Spectrophotometer) by measuring the absorbance at 540 nm.

3 Results and Discussions

3.1 Results

Cellulase-degrading bacteria isolated from *Eisenia fetida* shows following morphological & biochemical characteristics.

Cultural Characteristics:

Fig. 3, 4 & 5: **Isolate cdb-A, B & C**

Table No. 1
Cultural characteristics

Characteristics	cdb-A	cdb -B	cdb -C
size	1.5 mm	1mm	1mm
shape	Circular	Circular	Circular
color	Off white	Off white	Creamy white
margin	Entire	Entire	Entire
surface	Smooth	Smooth	Smooth
opacity	Opaque	Opaque	Opaque
elevation	Convex	Convex	Convex
consistency	Sticky	Sticky	Sticky

Morphological characteristics:

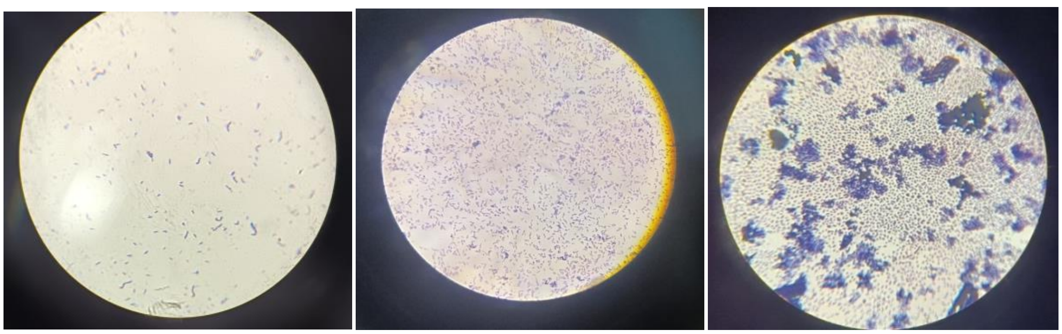

Fig. 6, 7 & 8: **Gram-positive short rods of cdb-A, B & C**

Table 1
Morphological characteristics

Characteristics	**cdb-A**	**cdb-B**	**cdb-C**
Gram Nature	Gram-positive, short rods	Gram-positive, short rods	Gram-positive, short rods
Motility	Motile in nature	Motile in nature	Motile in nature

Biochemical characteristics:

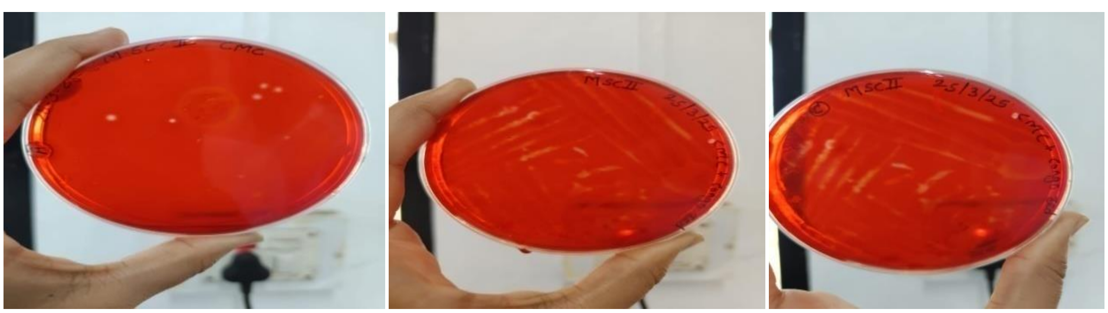

Fig. 9, 10 & 11. Cellulase Production by cdb-A, B & C

Table 3
Biochemical characteristics

Test	cdb-A	Cdb-B	cdb-C
Dextrose	+ -	- -	- -
Lactose	+ -	- -	- -
Maltose	+ -	- -	- -
Arabinose	+ -	- -	- -
Galactose	+ -	- -	- -
Mannitol	+ -	- -	- -
Sucrose	- -	- -	+ +
Oxidase	-	-	+
Catalase	-	+	-
Amylase	+	+	+
Cellulase	+	+	+
Endoglucanase	+	+	+

Note:
+ = Positive
- = Negative
++ = Acid and Gas Production

3.2 Discussion

Earthworms that had been placed on sterile sands for 5 days, cellulolytic microorganisms could not be isolated from the worm's gut, suggesting that the isolates are not symbionts of earthworms but are derived from ingested foods, such as litter fragments (Fujii et al., 2012). Earthworms can be viewed as ecological engineers that contribute to the digestion of lignocellulose. Efficient lignocellulose digestion in the earthworm gut requires the synergistic action of a suite of enzymes, including exocellulase, hemicellulase (e.g., xylanase), and lignin peroxidase, as well as endocellulase (Lynd et al., 2002).

A total of 3 isolates were screened from the gut of *Eisenia fetida*. All isolates are morphologically Gram-positive short rods and motile in nature. Isolates (cdb-A, cdb-B, cdb-C) were selected for enzyme production, and their respective cellulolytic activity was estimated. All the isolates showed positive cellolytic activity on CMC agar plates when stained with Congo-red solution.

As per morphological, cultural, and biochemical tests, isolate cdb-A is a Gram-positive short rod, motile in nature. It shows the positive carbohydrate fermentation test, such as Dextrose, Lactose, Maltose, Arabinose, Galactose, Mannitol, Sucrose, and positive enzyme activity such as Amylase, Cellulose, Endoglucanase. Isolate cdb - B is Gram-positive, short rods, motile in nature. It shows the positive Catalase, Amylase, Cellulose, and Endoglucanase activity (Fujii, 2012).

As per morphological, cultural, and biochemical tests, isolate cdb-C is Gram-positive, short rods, and motile in nature. It shows positive Oxidase, Amylase, Cellulase, and Endoglucanase activity.

4 Conclusion

The data gathered in this study give evidence that the bacterial isolates of earthworm gut have ability to produce cellulase.

Table no. 1 shows growth morphology of bacteria.
Table no. 2 shows morphological characteristics of bacteria.
Table no. 3 shows biochemical properties of bacteria.

On the basis of cultural, morphological, and biochemical characteristics tests by following standard keys of Bergey's Manual of Determinative Bacteriology, we could conclude that bacterial isolate cdb-A may belong to the genus *Cellulomonas.*

Based on cultural, morphological, and biochemical characteristics tests by following standard keys of Bergey's Manual of Determinative Bacteriology, we could conclude that bacterial isolate cdb-B may belong to the genus *Microbacterium*

On the basis of cultural, morphological, and biochemical characteristics tests by following standard keys of Bergey's Manual of Determinative Bacteriology, we could conclude that bacterial isolate cdb-C may belong to the genus *Aureobacterium.*

This study gives us a hint that the microbial wealth of cellulase-producing bacteria isolated from the earthworm gut can be harnessed for biochemical processes. Also, the study might be potential useful candidate for efficient cellulosic biomass conversion and can be used as inoculants for microbial composting to enhance the cellulose degradation, of which the agricultural waste is composed.

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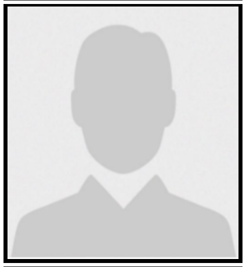

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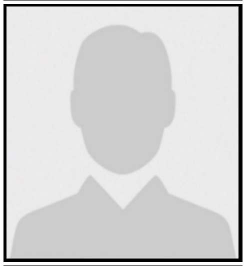

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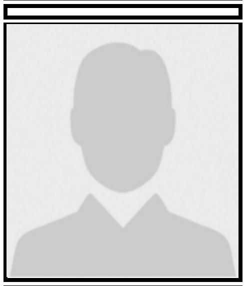

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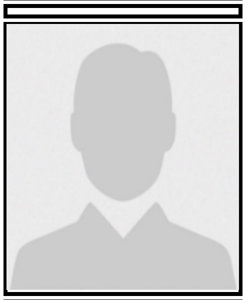

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