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Microbial profiling of earthworm gut and its biotechnological potential

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Abstract
The present study focuses on the Isolation of Microorganisms from the Earthworm's Gut to explore its microbial diversity and ecological significance. Earthworms, often referred to as the "intestines of the earth," play a crucial role in soil fertility and organic matter decomposition. Their gut provides a favorable microenvironment for diverse bacterial and fungal communities that aid in nutrient cycling and bioremediation. In this study, healthy earthworms were collected from agricultural soil, and gut tissues were aseptically dissected, homogenized, and serially diluted for microbial isolation using nutrient agar, Luria-Bertani agar, and MacConkey agar. The isolated colonies were subjected to Gram staining and a series of biochemical tests including catalase, oxidase, indole, methyl red, Voges–Proskauer, citrate utilization, and starch hydrolysis to identify bacterial genera. The analysis revealed a diverse microbial population consisting of *Bacillus subtilis, Bacillus cereus, Clostridium spp., Pseudomonas spp., E. coli, Proteus spp., Staphylococcus, Micrococcus, Actinobacter, Acinetobacter, Aeromonas, and Nocardia. Fungal isolates such as Aspergillus, Rhizopus stolonifer, Penicillium chrysogenum, and Mucor* were also identified, with several rare species noted. These microorganisms play significant roles in polysaccharide degradation, nitrogen fixation, and detoxification of organic compounds within the soil ecosystem. The findings confirm that the earthworm gut harbors metabolically active and beneficial microbes that contribute to soil health and nutrient enrichment. This study supports the importance of the earthworm gut microbiome as a natural bioreactor and highlights its potential applications in sustainable agriculture and soil bioremediation.

Keywords: Earthworm gut microbiota, Microbial isolation, Biochemical characterization, Soil bioreactor, Bioremediation, Nutrient cycling

Introduction
The earthworm, famously called the "intestines of the earth" by Aristotle, plays a fundamental role in soil turnover and fertility. Charles Darwin's work in 1881 further demonstrated the significance of the earthworm gut in soil fertility and decomposition. The gut itself is a straight tube extending from the mouth to the anus, acting as a natural bioreactor. Earthworm (phylum-Annelida, class-Oligochaeta) and comprise approximately 800 genera and 8000 species that account for up to 90% of invertebrate biomass present in soior [1]. The Earthworm's gut plays a vital role in enriching the soil fertility. It is a tubular structure extending from mouth to the anus .It's different regions are the muscular pharynx, oesophagus, intestine and associative digestive glands. The analysis of gut contents in earthworms revealed the occurrence of different kinds of symbionts like micro fungi, bacteria, protozoa etc [2].

The microbial community within the earthworm gut is not merely incidental but is an essential component of the earthworm's ecology and the broader soil ecosystem. This symbiosis is defined by the earthworm providing a habitat while the microbes facilitate digestion and nutrient processing. It is well established that the earthworm gut provides suitable condition for the development of bacterial colonies since earthworm castings contain significantly higher counts of bacteria than in surrounding soil [3]. A number of researches have observed increased proliferation of a variety of microorganisms in the gut of earthworms, fungi in *P. millardi*, *L. mauritii* and *E.euginiae*, bacteria in *A.caliginosa*, *L.terrestris*, *L. mauritii* and actinomycetes in L. terrestris, *A. longaand A.caliginosa* and *L. rubellus.* Similar to the occurrence of greater number of microbes in the gut of earthworms than the surrounding soil. The cast also contains more microorganisms particularly a greater number of fungi, actinomycetes, bacteria and higher enzyme activity were reported in the cast [4]. A significant increase of microbial population in the casts of different species of earthworms was reported by Parthasarathi *et al.* [5].

The total microbial population has enhanced more than 397% of microbial activity reflected by the enhanced activity of dehydrogenase. Among the microbes, bacteria have abundantly multiplied (386%) and actinomycetes though low in population, have also multiplied (151%) more or less equally with fungi (169%).

Materials and Methods
Sample Collection and Preparation
Healthy Earthworms were collected from agricultural land. To minimize surface contamination, the earthworms were disinfected with ethanol (50-70%). The earthworms were then placed under sterile conditions for the surgical removal of the gut from the tissues. A longitudinal incision was made along the worm's body using a sterile blade. The gut was then removed and transferred into a sterile saline solution of 0.85% NaCl.

Microbial Isolation
1 gram of the earthworm's gut tissue is homogenized in 9 ml of distilled water, and 1 ml was transferred into a test tube containing 9 ml of sterile distilled water. It is labelled

as 10^{-1}. Like this 9 ml of distilled water was filled in 5 other test tubes and labelled as 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and10^{-6}. Then 1 ml of sample is taken from 10^{-1} using micro pipette and added into 10^{-2} test tube. From that 1 ml is taken and added into 10^{-3} test tube and serially diluted up to 10^{-6} Plating was performed on two types of media for the growth of microorganisms:

- MacConkey Agar
- Luria Broth Agar (Luria-Bertani Agar)
- Nutrient Agar

Spread plate method

The medium is transferred into the petri plates under aseptic condition in the laminar air flow chamber. Then the petri plate upper lid is removed and about 0.1 ml of diluted sample from respective dilutions is placed at the centre of the plate. The L rod is sterilized by dipping it in ethanol and heating it in flame. The glass rod is cooled and inoculum placed in the petri plate is spread uniformly by swirling the rod over the petri plate. The petri plates are inverted and incubated at 37º C for 24 hours for bacteria and 23-26ºC for fungi.

Pour plate method

The upper lid is removed and about 0.1ml of diluted free sample from the respective dilutions is placed at the center of the place. The sample is uniformly distrubuted among the plate, the medium was transferred into petriplate understand aseptic condition over petriplate. The plate were inverted and incubated at37ºC for 24hours for bacteria and 23-26ºC for fungi.

Microbial Identification

Gram staining

Wipe the clean glass slide with 95% ethanol. Dry the slide and label it regarding which bacteria is stained. From the bacterial suspension broth take sterile loop full of broth culture on the slide. Spread by means of circular motion of the inoculating loop to about 1 cm in diameter. Then place sterile water or saline solution on the slide using a dropper and gently stir on the slide. Allow the smear to air dry or heat fix it by passing the entire slide through the flame two to three times. Place the slide with heat fixed smear on the staining tray. Then gently flood smear with crystal violet reagent and let stand for 1 minute. Here crystal violet acts as the primary stain. Rinse the slide with sterilized water. After this gently flood the slide with Grams Iodine solution and let stand for 1 minute. Grams Iodine acts as the mordant. Rinse the slide with sterile water. Then decolourize the slide with 95% ethanol. Immediately rinse it with sterile distilled water. After this gently flood the smear with safranin reagent and let stand for 45 sec to 1 minute. Here safranin acts as the secondary stain. Rinse with sterile water. Dry the slide. Then view the slide under Oil Immersion Microscope. If the bacteria stained blue or violet, then they are gram positive and if they are stained pink, they gram negative [9].

Fungal staining

Place one to two drops of 70% ethanol on a clean, dry glass slide. Use a sterile inoculation loop to transfer a small piece of the fungal colony or sample into the ethanol drop. Gently use a needle or mounter to tease the sample and spread it evenly in the drop. One to two drops of lactophenol cotton blue solution to the sample while the ethanol is still wet. Carefully place a coverslip over the mixture, making sure to avoid air bubbles. Allow the slide to sit for a minute or two before observing it under a microscope to see the blue-stained fungal structures [9].

Biochemical Tests

Biochemical tests were performed on the obtained colonies to further identify the microorganisms.

Catalase test

Nutrient agar medium was prepared. The medium was poured into culture tubes. It was sterilized by autoclaving at 15 lbs. pressure for 15 minutes. The nutrient agar slants were inoculated with bacterial suspension culture. The cultures were incubated at 37º C for 24 hours and 3 – 4 drops of hydrogen peroxide was added on the growth of each slant culture. The culture was observed for the appearance or absence of gas bubbles [6].

Indole production test

1% Peptone broth was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. The peptone broth was inoculated with bacterial suspension culture. The tubes were incubated at 37ºC for 24 hours. After this 1 ml of kovac's reagent was added. The tubes were shaken gently after intervals of 10 to 15 minutes. The tubes were allowed to stand for few minutes to permit the reagent to come to the top. The tubes were observed for cherry red layers in the top layer. The result is positive is the cherry layers are there and negative if not [6, 7].

Methyl Red test

Methyl red broth was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. 5 ml of broth was poured in each tube. The tubes were inoculated with the bacterial suspension culture. All the tubes were incubated at 37ºC for 24 hours. 5 drops of methyl red indicator was added to the tubes of each set. The change in colour of methyl red was observed. The result is positive if red colour appears and negative if yellow colour appears [6, 8].

Voges-Proskauer test

Voges-proskauer broth was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. 5 ml of broth was poured into each tube. The tubes were inoculated with the bacterial suspension culture. All the tubes were incubated at 37ºC for 24 hours. 12 drops of VP reagent-I and 2-3 drops of VP reagent-II was added to the tubes. The tubes were gently shaken for 30 seconds with the caps off to expose the media to oxygen. The tubes were kept aside for 15-30 minutes and observed for change in colour for the VP test. The result is positive if red colour appears and negative if yellow colour appear [6, 8].

Citrate Utilization Test

Simmons citrate agar medium was prepared and sterilized using an autoclave at 15 lbs pressure for 15 minutes. The media was poured into sterile test tubes in a slanted position and allowed to solidify to form agar slants. The bacterial suspension was inoculated onto the surface of the slant using a sterile inoculating loop, taking care not to stab the butt of the medium. The tubes were then incubated at 37°C for 24–48 hours. After incubation, the slants were observed for change in colour and growth. The result is positive if there is growth on the slant along with a colour change of the medium from green to Prussian blue, and negative if there is no growth and no colour change, with the medium remaining green [6, 9].

Starch hydrolysis test

Starch agar medium was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. The media was poured into petri plates and allowed to solidify. The bacterial suspension culture was inoculated on to the plate with a sterile transfer loop. The plate was incubated at 37°C for 24 hours. After incubation the plate was flooded with Gram's Iodine. Plate was observed for clear zone around the inoculated organism. The result is positive if there is a clear zone around the line of growth and negative if there is dark blue colouration of the medium [6, 10].

Observation and Results

The earthworm gut was observed to contain a highly diverse bacterial and fungal community, with diversity influenced by soil type and the earthworm species [3, 5, 7].

Isolated Bacteria

A total of twelve bacterial types were isolated and identified, including fermenters and common soil and gut inhabitants:

- *Bacillus subtilis*
- *Bacillus cereus*
- *Clostridium spp. (fermenters)*
- *Bacillus pumilus*
- *Pseudomonas spp.*
- *E. Coli*
- *Proteus vulgaris, P. mirabilis*
- *Staphylococcus*
- *Micrococcus*
- *Actinobacter*
- *Acinetobacter*
- *Nocardia spp., Aeromonas*

Isolated Fungi

A variety of fungal species were isolated, with some noted as being rare:

- *Aspergillus*
- *Rhizopus stolonifer*
- *Penicillium chrysogenum*
- *Trichoderma*
- *Mucor*
- *Fusarium*
- *Candida (Rare)*
- *Cladosporium (Rare)*
- *Saccharomyces (Rare)*

Observation of biochemical test results

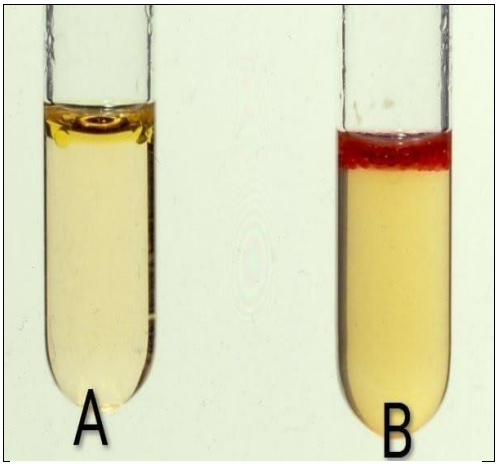

Fig 1: Indole test: A) Control B) Positive for *P. vulgaris*

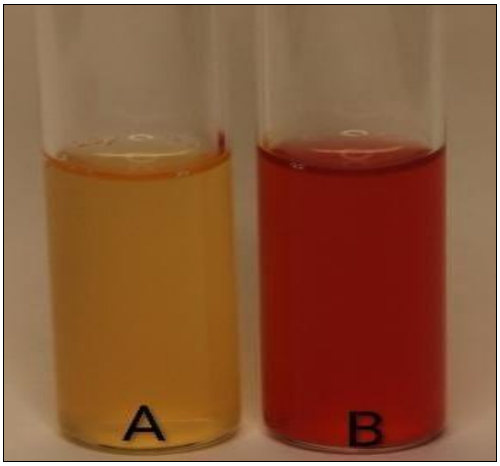

Fig 2: Methyl red test: A) Control B) Positive for *E coli*

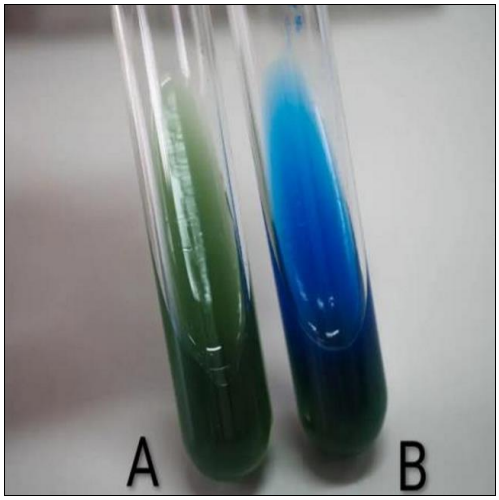

Fig 3: Citrate Utilization test: A) Control B) Positive for *P. mirabilis*

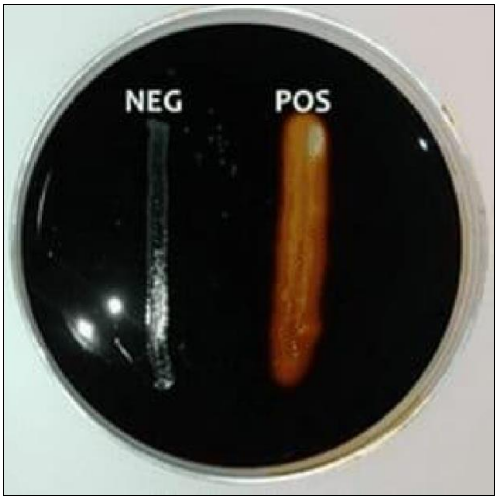

Fig 4: Starch hydrolysis test

Biochemical Test Results

The results of the biochemical tests (IMVIC and others) for key bacterial isolates are presented in the tables below, which help to confirm their identities and metabolic pathways.

Table 1

Isolate	Indole	Methyl Red	Voges-Proskauer (VP)	Citrate
1. E. coli	+	+	-	-
2. Proteus sp.				
2.1 *P. vulgaris*	+	+	+	+
2.2 *P. mirabilis*	-	+	-	+
3. Aeromonas sp.	+	-	-	+
4. Enterobacter sp.	-	+	+	Rare

Table 2

Isolate	Catalase	Oxidase	Starch Hydrolysis (Starch)
1. B. subtilis	+	-	+
2. Pseudomonas sp.	+	+	-
3. Micrococcus spp.	+	-	-
4. Acinetobacter spp.	+	-	-
5. B. cereus	+	-	+
6. B. pumilus	+	-	-

Conclusion

The isolation procedure successfully identified a highly diverse and metabolically active microbial community within the earthworm gut, reinforcing its role as a crucial bioreactor in soil ecology. The presence of bacteria known for polysaccharide breakdown (e.g., *Bacillus* and *Clostridium spp*.) and potential detoxification (e.g., *Pseudomonas* and *Aeromonas*) directly supports the established understanding of the earthworm microbiome's contribution to digestion, nutrient cycling, and xenobiotic degradation. The diverse fungal population further suggests a significant role in organic matter decomposition. The data derived from this study, particularly the identification of specific, beneficial microbes, provides a foundation for future research aimed at developing bio strategies to enhance soil health, improve nutrient availability, and ultimately advance the field of soil bioremediation. Further studies using advanced molecular techniques would be beneficial to fully characterize the functional genes and complex symbiotic relationships within this vital micro-compartment [1, 2, 7].

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