Isolation, Characterization of Fungal and Bacterial Isolates from Gut of *Eudrilus Eugeniae*

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Abstract

In the present study the earthworms (Eudriluseugeniae) were collected from vermiculture farm Kanyakumari. The healthy worms were identified and gut was dissected for enumeration of fungus and bacteria. The bacterial population in the gut was identified first, followed by fungus isolated from Eudrilus eugeniae. The Heterotrophic bacteria in the gut was identified by morphological, biochemical characterization. The predominant bacteria was identified and found to be Bacillus.sp, Staphylococcus sp in Eudriluseugeniae. The fungal population in the gut was identified and found that they were Penicillium.sp in Eudriluseugeniae.

Keywords: *Eudrilus eugeniae, earthworms, Bacillus.sp, and Staphylococcus.sp.*

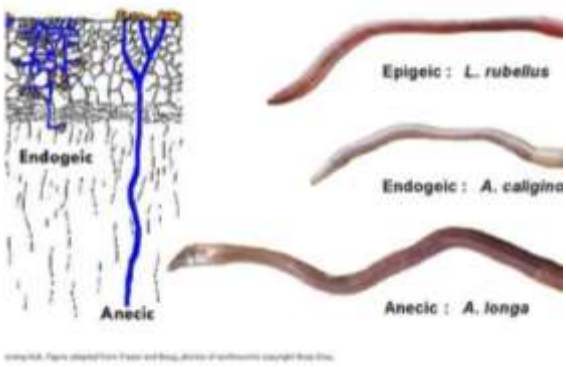
Introduction

Vermicomposting is the process by which earthworms are used to convert organic materials, usually wastes, into a humus-like material known as vermicompost. The term vermitechnology is used in a general sense to refer to the utilization of surface and subsurface varieties of earthworms in composting and management of soil. Vermicast is the faecal matter released by the earthworms. The term vermicast is also used in a general sense to mean the product of vermicomposting, the vermicompost. Vermicomposting is a non thermophilic biological oxidation process in which organic materials are converted into vermicompost which is a peat like material, exhibiting high porosity, aeration, drainage, water holding capacity and Vermiculture is a cost-effective tool for environmentally sound waste management (Asha*et al*., 2008).

Earthworms are capable of transforming garbage into ‘gold’. Charles Darwin described earthworms as the ‘unheralded soldiers of mankind’, and Aristotle called them as the ‘intestine of earth’, as they could digest a wide variety of organic materials. Earthworms play an important role in carbon turnover, soil formation, participates in cellulose degradation and humus accumulation. Earthworm actively profoundly affects the physical, chemical and biological properties of soil. Earthworm’s intestine contains a wide range of microorganisms, enzymes and hormones which aid in rapid decomposition of half digested material transforming them into vermicompost in a short time (nearly 4-8 weeks) (Nagavallema*et al*., 2004).

They can efficiently utilize energy from poor soils, hence can be used for soil improvements. Endogeics include *Aporrectodeacaliginosa*, *Octolasioncyaneum*, *Dontoscolexcorethrurus*, etc. Aneceic earthworms are larger dorsally pigmented worms with low reproductive rate, sensitive to disturbance, nocturnal, phytogeophagous, bury the surface litter, forms middens and extensive deep, permanent vertical burrows and live in them. *Lumbricusterrestris*, *Lumbricuspolyphemus* and *Aporrectodea longa* are examples of aneceic earthworms (Kooch and Jalilvand, 2008). Earthworms thus act as natural bioreactors, altering the nature of the organic waste by fragmenting them.

Figure 1 Types of Earthworm



In the present study it was further extended to evaluate biological parameters, such as total count of gut micro flora of *Eudriluseugeniae*. In gut tissues of earthworm, micro flora was found to be high.

Materials and Methods

Collection of Earthworms

The specimens selected for the present investigation were the adult earthworms namely *Eudriluseugeniae*. The worms were collected from Koonpura-The house of Earthworms, Trivandrum and from Vivekananda centre, Kanyakumari. Species identification was confirmed using the general characters of the worms (Table 1).

Figure 1 *Eudrilus eugeniae*

Dissection of the Selected Organisms – *Eudrilus eugeniae*

Healthy adults from each type was collected and allowed to starve for 24 hours. They were then disinfected with 50% ethanol. A sterile surgical blade was used for dissection. Bell pins was inserted into the ventral surface of the clittelar region and with the body slightly raised up. With the sterile surgical blade an incision was made longitudinally along the worm. The gut was then freed from the surrounding blood vessels. With a flamed foreceps the gut section was removed. This was then transferred to saline solution (0.85% NaCl solution). Then it was homogenized for 5 minutes in a vortex. This serves as the samples for further analysis.

Figure 2 Dissecting earthworm

Microbial Analysis of the Gut of *Eudrilus eugeniae*

The gut isolated from the selected earthworms *Eiseniafoetida and Eudriluseugeniae*were used for microbial analysis.

Enumeration of Total Heterotropic Bacteria

The samples were serially diluted by serial dilution agar pour plating techniques. 1.0ml of each dilutions of 10^{-4}, 10^{-5} and 10^{-6} were plated in sterile nutrient agar media and one plate served as control. For each dilution original and duplicate plates were maintained. The plates were incubated at 37ºc for 24 hours.

Enumeration of Fungi

The samples were serially diluted by serial dilution agar pour plating technique. 1.0ml of each dilutions of 10^{-4}, 10^{-5} and 10^{-6} were plated in the sterile Rose Bengal agar media and one plate served as control. For each dilution, original and duplicate plates were maintained. The plates were incubated at 28ºc for 3 days.

Identification and Characterization of the Efficient Earthworm Bacteria from Gut Tissue

Morphological Characterization

Morphological characteristics such as abundance of growth, pigmentation, optical characteristics, size and shape were studied on nutrient agar plates.

Spore Staining (Schaeffer-Fulton Method)

The isolate was smeared in the slide and heat fixed. Then the slide was flooded with malachite green and stream heat the slide for 2-3 minutes. Cool the slide and washed in running tap water. It was then added with counter stain safranin and kept for 30 seconds. Wash the slide with running tap water. Air dried the slide and examined under oil immersion, in microscope. The spores appeared green in colour while the vegetative cells appeared red in colour.

Biochemical Characterization

Biochemical characterizations were performed in accordance with Bergey's manual of determinative bacteriology.

Indole Production Test

Indole production test is used to test whether the organism can have the ability to produce indole. Peptone broth was prepared, sterilized and cooled. Inoculate the test organism to the sterile peptone broth and incubate the tubes at 37°C for 24 hrs. The culture producing the cherry red colour ring following the addition of kovac's reagent indicated as positive. The absence of red coloration indicated a negative result.

Methyl Red Test

Methyl red test is employed to detect the ability of microorganisms to oxidize glucose with the production of high concentration of acid end products. The isolated organisms were inoculated into test tubes containing sterile MR-VP broth and incubate the tubes for 24 to 48 hrs 37°C. After incubation, add 7-8 drops of methyl red indicator and appearance of red colour indicated the positive result.

Voges-Proskauer Test

This test is also known as the acetoin production test. This test is used to differentiate the capacity of organisms to produce some nonacidic (or) neutral end product such as acetyl methyl carbinol (or) 2,3,-butanediol. The isolated organisms were inoculated into sterile MR-VP broth tubes and incubate for 24 hrs at 37°C. Development of deep rose colour following the addition of Barritt's reagent A and B indicated the positive result. The absence of deep rose colour is a negative result.

Citrate Utilization Test

Some of the organisms were capable of utilizing citrate as the sole carbon source and mono ammonium phosphate at the sole source of nitrogen. As a result, the pH of the medium change, this was indicated by changes in the indicator present in the medium. Simmon's citrate medium was prepared, sterilized and kept in a slanting position and allowed the tubes to solidify. The test organisms was streaked on the slant and incubated at 37°C for 24 hrs. The change of color green to Prussian blue coloured slant incubated the positive result.

Triple Sugar Iron Test

TSI test is used to differentiate the isolate according to the ability to ferment lactose, sucrose and glucose and production of hydrogen sulfide. Triple sugar iron agar medium was prepared and sterilized. Kept the tubes as slant and butt and allow it to solidify. Streak a loop full of test organisms on the surface of the slant and incubate at 37°C for 24 hrs. Acidification of the medium caused by the isolates attacking one of the sugars causes the phenol red indicator to change to yellow colour. Gas production in indicated by bubble formation in the butt. Hydrogen sulphide production causes the formation of a black precipitate at the junction between the slope and the butt.

Nitrate Reduction Test

This test is used to detect whether the organisms reduced the nitrates to nitrites or not. Nitrate broth was prepared and sterilized. Inoculate one loop full of test culture and incubate at 37°C for 96 hrs. Following incubation, add 0.1 ml of test reagent (Sulphanilic acid and α-napthalamine) to the test culture. A red colour developing within a few minutes the presence of nitrites and hence the ability of the organisms to reduce nitrates.

Identification of Fungi from the Gut of *Eudrilus eugeniae*

From the Rose Bengal agar plates the fungal colonies were identified by colony morphology, pigmentation, lactophenol cotton blue mounting technique.

Result

Microbial Analysis of the Gut of *Eudrilus eugeniae*

Enumeration of Heterotrophic Bacteria Isolated from the Gut of *Eudrilus eugineae*

Innutrientagar plates colony count was determined both manually and also with a colony counter and the colony count (CFU/ml) was found to be tabulated (Table 1) (Plate 1A, 1B, 1C). The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{200.5 \times 10^4}{1} = 200.5 \times 10^4 \text{ CFU/ml.}$$

Enumeration of Fungi Isolated from the Gut of *Eudrilus eugeniae*

The fungal count was determined by serial dilution spread plating technique in Rose bengalagar plates. The colony count was determined by macroscopic observation and tabulated (Table 2) (Plate 1D, 1E, 1F).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{179.5 \times 10^4}{1} = 179.5 \times 10^4 \text{CFU/ml.}$$

Enumeration of Heterotrophic Bacteria Isolated from the Gut Tissue of *Eudrilus eugeniae*

In nutrient agar plates colony count was determined both manually and also with a colony counter and the colony count (CFU/ml) was found to be tabulated (Table 3) (Plate 2A, 2B, 2C).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{180 \times 10^4}{1}$$

$$= 180 \times 10^4 \text{CFU/ml.}$$

Enumeration of Fungi Isolated the Gut of *Eudrilus eugeniae*

The fungal count was determined by serial dilution spread plating technique in Rose bengalagar plates. The colony count was determined by macroscopic observation and tabulated (Table 4) (Plate 2D, 2E, 2F). The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{189 \times 10^4}{1}$$

$$= 189 \times 10^4 \text{CFU/ml.}$$

Isolation of Microorganisms from Gut Tissue of*Eudrilus eugeniae*

Staphylococcus sp

The isolate EE1 was identified by morphological and biochemical characterization methods. The bacterial isolate EE1 was gram positive, nonspore forming, round shaped (cocci) organism. Indole was not produced. Glucose was not oxidized in methyl red test. Acetoin was not produced in VP test. Citrate was utilized as its sole carbon and energy source. Nitrates were reduced to nitrites. Starch and gelatin were hydrolyzed. It ferments sugars like Dextrose, fructose, Mannitol and Sucrose. From the observation, the bacterial isolate EE1 was identified as *Staphylococcus sp*. The results(Table 6) were compared with Bergey's manual of determinative bacteriology.

Bacillus sp

The strain EE2 was a gram positive, short rod shaped spore forming organism. Further biochemical tests were conducted to ascertain the genus of the bacteria. The results were depicted in Table 6. Indole was not produced. It oxidized glucose with the production of acid end products in methyl red test. It did not produce acetoin and butane diol in vogesproskauer test. Citrate was utilized as its sole carbon source. Nitrates were reduced to nitrites. Gelatin and starch were hydrolyzed by the organism. Strains fermented sugars like dextrose. From the obtained results, the strain EE1 was confirmed as *Bacillus sp.*

Identification of Fungi Isolated from the Gut Tissueof *Eudrilus eugeniae*

From the RBA plates fungal isolate were obtained. They were identified by microscopic examination by LCB mounting technique and by colony morphology (Table 15) (Plate 10). They showed grey colour colony on RBA plates.

Table 1: Enumeration of Heterotrophic Bacteria Isolated from the Gut of *Eudrilus eugeniae*

S.NO	Dilution	CFU/ml		
		Original	Duplicate	Average
1.	10^{-4}	191	169	180
2.	10^{-5}	55	69	65
3.	10^{-6}	33	21	27

Table2: Enumeration of Fungi Isolated from the Gut of *Eudrilus eugeniae*

S.No.	Dilution	CFU/ml		
		Original	Duplicate	Average
1.	10^{-4}	199	179	189
2.	10^{-5}	98	61	79.5
3.	10^{-6}	23	11	17

Table 3: Biochemical Characterization of Bacteria Isolated from Gut Tissues of Earthworm *Eudrilus eugeniae*

Biochemical test	EE1	EE2
Gram Staining	G^+ rod	G^+ rod
Spore Staining	Present	Present
Peptone	Negative	Negative
Methyl Red Test	Negative	Positive
VogesProskauer	Negative	Negative
Simmon Citrate Agar	Negative	Positive
Triple Sugar Iron Agar	Alkaline slant & acid butt	Alkaline slant & acid butt
Nitrate reduction	Positive	Positive
Gelatin	Positive	Positive
Starch	Positive	Positive
Carbohydrate fermentation		
1. Dextrose	Positive	Positive
2. Fructose	Positive	Negative
3. Mannitol	Positive	Negative
4. Sucrose	Positive	Negative

Table 4: The Microorganism Isolated from Gut Tissues of Earthworm *Eudrilus eugeniae*

Different areas of gut tissue	Bacteria
EE1	*Staphylococcus sp*
EE2	*Bacillus sp*

Table 5: Identification of Fungi Isolated from the Gut of *Eudrilus eugeniae*

S. No.	Isolate	Colony Morphology	Microscopic Examination by LCB Mounting Technique
1.	EE_1	Blue green colony	

Discussion

In the present study Microbial analysis of the gut tissue of two different Earthworms *Eudriluseugeniae* were studied. The two different earthworms and its gut tissue was dissected and screened for Microbial analysis. The vermicomposting ability of the worm is enhanced by the gut microorganisms namely Hetrotrophic bacteria, fungi and actinomycetes. The earthworm intestine contains wide range of microorganisms, enzymes and hormones which aid in rapid decomposition of half digested material

transforming them into vermicompost in the short time (Ghosh*et al.,* 1999). Compared to traditional composting its state the advantage of micro alone and thereby required a prolonged period for compost production (Sanches/Moneder*et al., 2001*).

As the organic matter passes through gizzard of earthworm it was grounded into a fine powder after which the digestive enzymes, microorganisms and other fermenting substances act on them aiding their breakdown within the gut and finally passes in the form of casts, which the later acted upon by earthworm gut associated microbes converting them into manure product, the vermicompost (Edward *etal*.,2004).

The other important isolate EE_1 was a gram positive, short rod shaped spore forming organism. Biochemical characterization revealed negative, for indole, glucose, voges proskauer. The isolate EE_1 was found to be positive for citrate utilization test, nitrate utilization, strach hydrolysis and gelatin hydrolysis test. Besides, it also ferment sugars like dextrose, fructose and mannitol. It was confirmed as *staphylococcus.*

The second important microbe isolated from the gut tissue of *Eudriluseugeniae*fromgram positive, spore forming, fermentation aerobic and rod shapedbacteria (Perez *et al.,* 2000) isolated from the gut of both*and Eudrilus eugineae(EE2). Bacillus sp*found mostly in soil substratum, play an important role in the decomposition of organic materials and this strain was considered metabolically diverse. (Ara*et al.,* 2007). The second important microbe isolated from the gut tissue of *Eudriluseugeniae is Staphylococcus sp.* In tune with above discussion the enteric microbes play an important role in the activities of earthworm. These microbes are not just passes, but permanent twellers in definte regions of earthworms gut (singleton, 2003).

The gut microbes were found to be responsible for the cellulose and mannose activities (Munnoli*et al.,* 2010). Earthworm commiuties the substrate thereby increases the surface area for microbial degradation constituting to the active phase of vermicomposting. As this crushed organic meterials passes through the gut, it get mixed with gut associated microbesand the digestive enzymes and finally leaves the gut in partially digested form as casts after which the microbes takes up the process of decomposition contributing to the maturation phase (Lazcano*et al.,* 2008).

Earthworm possesses an immense bacterial diversity within their digestive/e tracts and is very little explored mainly because of the non-cultivatable character of a large quantity of microorganisms which mainly come from soil. Earthworm activity engineers the soil by forming extensive burrows which loosen the soil and makes it porous).Earthworm feeding reduces the survival of plant pathogens such as *Fusarium* sp. *and Verticillium sp*and *increases* the densities of antagonistic fluorescent pseudomonads and filamentous actinomycetes while population density of *Bacilli* and *Trichoderma*sp. Remains unaltered (Elmer 2009).The gut microbes of earthworm were found to be responsible for the cellulase and mannose activities (Munnoli*et al*., 2010).

The fungal isolate obtained from the gut of *Eudriluseugeniae*was identified by morphological (Colony morphology, LCB) examination. It was identified as *Penicillium* sp. Thus the microorganisms present in the gut of earthworms are the major cause for casting. These organisms produce enzymes which are responsible for the breakdown of the waste products into valuable casting or vermicompost.

Conclusion

Earthworm's composting ability is enhanced by the microbial activities in the gut. The earthworms can be used in the management of number of organic waste generated in agriculture, horticulture, rural industries including household section creating environmental population and problem. Vermicomposting seems to be a natural tool for waste management since it convert the waste into wealth in form of compost and the environmental population will be mitigated. Thus the way of management of organic waste seems to be vermicomposting technique. Vermicomposting is an efficient eco-friendly method of waste management.

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