Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Impact of organic rich diet on gut enzymes, microbes and biomass of earthworm, *Eudrilus eugienea*

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Publication Info

Paper received:
07 April 2011

Revised received:
29 November 2011

Re-revised received:
03 March 2012

Accepted:
27 July 2012

Abstract

Vermitechnology provides scope and opportunities in the field of Biotechnology. The sudden decline in earthworm biomass may mainly be due to the over use of chemicals. Steps had been taken to enhance the production of biomass of earthworms by providing organic wastes rich in major organic constituents such as cereals, pulses and skin of chick. Earthworms (*Eudrilus eugienea*) were fed with organic constituents individually, and in combination of organic rich diet. The biomass of the earthworm was steadily increasing in the individual treatment was found when fed with organic rich diet at 10, 20 and 30 days respectively 09.987, 13.569 and 18.212. The bacterial counts in the gut of earthworms were 543 x 10^5 CFU ml^{-1}. The bacteria identified were *Bacillus* spp., *Lactobacillus* spp and *Flavobacterium* spp. Enzymes screened in the gut were amylase, endoglucanase, cellulase, sucrase and protease. From the present investigation, it was found that the organic rich diet is the ideal medium in which the biomass of earthworms are high, their enzymatic activity was also high with variety of microbes which will enhance the efficiency of the soil.

Key words

Eudrilus eugienea, Organic rich diet, Enzyme, Microbes

Introduction

Earthworms are the members of the class Oligochaeta of phylum Annelida. These are one of the major macrofauna of soil and are considered as natural bioreactors since they redesign the physical structure of soil environment by ingesting litter and soil particles by depositing casts on the soil surface (Ansari, 2011; Pederson and Hendrikson, 1993; Verma and Shweta, 2011). Earthworms ingest soil microorganisms along with organic residues from the soil and during passage through the worm's intestinal tract, their population may increase. Therefore earthworm casts have been reported to be much more microbiologically active and richer in microflora than their surrounding undigested soils (Parthasarathi *et al.*, 2007). Gut microbial population plays an important role in earthworm nutrition by helping in the breakdown of organic matter. It also determines the microbial density in the cast and microbial dynamics in soil (Lattaud *et al.*, 1999; Prabha *et al.*, 2007).

An increasing appreciation of the synergistic interactions between earthworms and microorganisms is observed. The main interest is focused on microorganisms that are ingested from soil and transit the gut by employing culture-based and molecular methods (Parthasarathi *et al.*, 2007). Despite those recent studies, the real existence of symbionts in the earthworm gut is still controversial (Curry and Schmidt, 2007). However, other studies show some evidence of earthworm gut symbionts (Sampedro and Whalen, 2007). They found some microorganisms in the earthworm intestine that are absent in the surrounding soil and important changes in the fatty acid concentration and composition in the gut of the earthworm, *L. terrestris* (Sampedro and Whalen, 2007). They along with microorganisms play a major role in degrading organic waste and thus, maintain the nutritional flux in the system. It has been demonstrated that microorganisms in the gut of some tropical earthworm species, using mucus secretion from the gut epithelium as an energy source, may fix atmospheric

nitrogen in quantities that are significant for the earthworm metabolism and as well as a source of nitrogen for plant growth (Lee, 1992). They excrete a large part of these consumed waste materials in a half-digested form (Edwards, 1995). Under such circumstances, the use of selected species of earthworms for vermicompost production in a most economical way as well as evolving methods to substantially improve the quality of manure is essential (Pizl and Novakova, 2003).

The activity of the earthworm gut is like a miniature composting tube that mixes, conditions and inoculates the residues. Moisture, pH, enzymes and microbial populations in the gut are favorably maintained for a synergistic relationship, and then a terrific by-product (Bill Becker, 1991). The present work was undertaken to study the enhanced biomass production of earthworm fed with organic rich diet (pulse, cereals and combination of pulse cereals and skin of chicks).

Materials and Methods

Preparation of experimental troughs : Earthworm, *Eudrilus eugienea* was chosen as experimental animal, while waste form of cereals, pulses and skin of chick were selected as diet. Five troughs were prepared with cow dung and sawdust of 1:1 ratio as common base medium. Trough I was treated as control, where as trough II, III, IV and V were added waste cereals, waste pulses, waste skin of chick and combination of waste cereals, pulses and skin of chick in the ratio of 1:1:1. All the experiments were conducted in triplicates. The content of the troughs were allowed for semi decomposition with the sprinkling of water and mixing of the content daily. Earthworms were inoculated in the semi decomposed medium on 20th day in a manner of 6 worms (approximately 6 g) per kg of substrate. The day of inoculation was treated as day 1.

The biomass of the earthworms was calculated on day 10, 20, and 30 by taking the weight of the worms on the respective days of exposure, and the variation in weight from day one was calculated percent.

The number of bacteria per ml of the original suspension was calculated as Organism ml^{-1} per Number of colonies. Each gram of sample was calculated by the average of three replicates divided by the amount plated and it was multiplied with dilution.

In the IMViC tests, the indole test was performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction was detected by adding "Kovac's reagent" (dimethyl amino benzaldehyde) which produces a "Cherry-red reagent layer". The methyl-red and Voges-Proskauer test**s** were used to differentiate 2 major types of facultative anaerobic "enteric bacteria". The citrate Utilization test was done to differentiate the enteric organisms on the basis of their ability to ferment citrate as sole carbon sources. In Simmon's citrate agar slants the isolated organism was streaked and incubated at 37ºC for 2 days. Growth was visible on the surface of the medium; the blue colour indicated citrate positive and no change in colour indicated citrate negative

Hydrolysis tests : This test determines the ability of the microorganisms to degrade the polysaccharide starch by using hydrolytic extra cellular enzyme. A loop ful of test culture was streaked on starch agar medium and incubated at room temperature for 24 hrs. After incubation the plates were flooded with Gram's iodine solution. Plate was observed for the presence of clear zones surrounding the bacterial colonies. The casein hydrolysis test was done to determine the ability of the organism to produce proteolytic enzyme capable of degrading casein. The organism was streaked in milk plates and incubated at 37ºC for 24 hrs and results were observed.

Tributrin agar was originally formulated by Anderson for the detection and enumeration of lipolytic microorganisms such as *Staphylococci* and *Clostridia*. A 10ml of tributrin (Glycerol trybutrate) was mixed with distilled water and heated to boiling to dissolve the medium completely. After sterilization, the content was poured in the individual plates and the culture was streaked. After 24-48 hrs of incubation, the result can be noted as positive or negative based on the formation of zone in the streaked culture Urease test was performed to confirm the presence of urease enzyme. Urea agar slant was inoculated with the isolated culture and slant was kept for incubation at 37ºC for 24hrs. Urea hydrolysis was confirmed by the development of pink colour.

The test culture was inoculated into fermentation medium with inverted Durham's tubes and incubated for 24 hrs. The medium colour was changed from red to yellow which was taken as a positive test for carbohydrate fermentation. This catalase test was performed to determine the ability of the organism to produce catalase degrading hydrogen per oxide. The enzyme catalase present in some microorganisms breaks down hydrogen per oxide to water and oxygen.

The organism was inoculated into the tube containing sterile nitrate broth and incubated at 37ºC for 20 hrs. After incubation alpha-naphtha amine and sulfanilic acid reagents were added to the tube. Development of red colour was the indication of positive result.

Some microorganisms contain deaminase enzymes capable of removing the amino group from amino acids.

During the course of experiment, the amino acid phenylalanine will be deaminated by phenylalanine deaminase and converted to the keto acid phenyl pyruvic acid and ammonia. The organisms are cultured on phenylalanine agar slant medium and after 24-48 hrs incubation ferric chloride solution were added. Development of green colour at the slant showed positive result while no colour change indicated negative result.

Identification of bacteria : After subculturing, these plates of microbes were tested for Gram positive or Gram negative with the use of Gram's staining method. The bacteria were isolated by the method of Basha and Ulaganathan (2002) and identified physically and biochemically following the Bergey's manual (1986).

Results and Discussion

The earthworm biomass percentage after 10, 20 and 30 days of exposure in pulse medium was noted as 53.4, 123.8 and 203.3; cereals 31.3, 66.3 and 107.2 ; Organic rich diet 66.5, 126.15 and 203.7; and in control diet 18.7, 37.5 and 57.03 respectively (Table1). These worms were also exposed to skin of chick and there was no vermicast production in this medium. CFU ranges of organic rich diet was high (543 x 10^5 CFU ml^{-1}) compared to pulse (350 x 10^5 CFU ml^{-1}).Cereal (131 x 10^5 CFU ml^{-1}) and control diet (31 x 10^5 CFU ml^{-1}) (Fig. 1).

Control and cereal diets have only gram positive bacteria for their total number of plates. Pulse has a total of 6 plates (Gram positive 3; gram negative 3); Organic rich diet has 9 plates, (gram positive -5; Gram negative- 4). The total numbers of plates were 18(Gram positive -11; gram negative-7). The Gram positive bacteria are high in all the diets, particularly in Organic rich diet (Fig. 2).The Gram negative bacteria are low in number than the Gram positive bacteria.

In IMViC tests all the identified bacteria gave negative result for the indole and VP test. But positive result was obtained for methyl red and citrate tests. In catalase test all bacteria gave positive results. In hydrolysis test, control and pulse diet gave a positive result and some of them gave 2 tests positive and 2 tests negative but in phenylalanine test, all of them gave a negative result (Table 2). Carbohydrate fermentation test was also carried out for glucose test, many of them have a Gas positive and all are acid positives in some was gas negative. Same result for sucrose test. In Galactose all microbes were acid negative and gas negative. Most of the tests of lactose, fructose and mannitol were gram negative, acid positive and some of them are gram positive and acid negative. All bacteria gave negative results in nitrate reduction tests. *Lactobacillus viridescense, L.minor* and *Bacillus pumilus, B.licheniformis*, and *Flavobacterium* were also identified. Species differentiation has also seen on these identified bacteria (Table 3). Microbial identifications of Gram Positive Bacteria are Bacillus pumilus, *Bacillus licheniformis*, *Lacto bacillus minor*, *Lacto bacillus viridescense* and also the identification of Gram Negative Bacteria as Flavobacterium. Enzymes were also identified in different feeding diets of cereals, pulse and in combination. The identified enzymes were endoglucanase, amylase, cellulase, sucrase and protease. The status of microbes in the gut of Eudrilus engienea fed with different diets is shown in Fig. 1.

Several workers have compared the mineral composition of the earthworm's food substrate with those of its excreted casts and reported that the earthworm's activities significantly increase the nitrogen mineralization in the soil (Sharon and Paul, 2002). Few soil ecological studies have focused on the prospects of linking microbes and fauna (Brown and Doube, 2004; Coleman *et al.*, 2004). These is not doubt that earthworms are the most important soil invertebrates in the soil ecosystem in terms of biomass and activity (Römbke *et al.*, 2005). Moreover, soil contains a large diversity of microorganisms (Torsvik and Ovreas, 2002.). Microorganisms are an unavoidable constituent of earthworms' natural diet. Three earthworm ecological groups are generally defined and earthworms feeding behavior is clearly associated to their ecological group (Edwards and Bohlen, 1996; Brown *et al.*, 2000). This is in concordance with the present investigation. The different diets have been provided to earthworm *(Eudrilus* sp.*)*. Hong *et al* (2011)

Table 1 : Increase in biomass percent of *Eudrilus eugienea* on different days of exposure to various diets medium

Day	Diets				
	Control	**Cereals**	**Pulses**	**Skin of chick**	**Organic rich diet (pulses, cereals and skin of chick)**
1	06.000	06.000	06.000	06.000	06.000
10	07.121 (18.7)	07.877 (31.3)	09.204 (53.4)	07.352 (22.5)	09.987 (66.5)
20	08.247 (37.5)	09.978 (66.3)	13.427 (123.8)	07.924 (32.1)	13.569 (126.15)
30	09.422 (57.03)	12.432 (107.2)	18.197 (203.3)	06.481 (8.01)	18.212 (203.7)

Values in parentheses indicate percent

Table 2 : The results of IMViC, Catalase, Hydrolysis and Phenyl- alanine test in subculture plates

No. of Subculture plates	Different diets in subculture plates	IMVIC tests				Catalase test	Hydrolysis tests				Phenyl-alanine test
		I	M	V	iC		S.A	S.C	UR	T.A	
1	C1	-	+	-	+	+	+	+	+	+	-
1	CE1	-	+	-	+	+	-	-	+	+	+
2	CE2	-	+	-	+	+	-	-	-	+	+
1	P1	-	+	-	+	+	-	-	+	-	+
2	P2	-	+	-	+	+	-	-	+	-	+
3	P3	-	+	-	+	+	-	-	-	+	+
4	P4	-	+	-	+	+	-	-	+	-	+
5	P5	-	+	-	+	+	-	+	+	+	+
6	P6	-	+	-	+	+	+	+	+	+	+
1	O1	-	+	-	+	+	-	-	-	+	+
2	O2	-	+	-	+	+	-	+	+	+	+
3	O3	-	+	-	+	+	-	+	-	+	+
4	O4	-	+	-	+	+	+	-	-	-	+
5	O5	-	+	-	+	+	-	-	-	-	+
6	O6	-	+	-	+	+	-	-	+	-	+
7	O7	-	+	-	+	+	-	-	+	-	+
8	O8	-	+	-	+	+	-	-	+	-	+
9	O9	-	+	-	+	+	-	-	+	-	+

C = Control; CE = Cereal; P = Pulses; O = Organic rich diet; I = Indole test; M = Methyl Red test V = Voges Proskauer test; iC = Citrate Utilization test; SA = Starch Agar Test; SC = Starch Casein Test UR = Urease Test; TA = Tributrin Agar Test

Table 3 : The results of the tests on Carbohydrate fermentation, Nitrate Reduction, Gram's staining tests and Bacterial identification in the gut of earthworms fed with organic rich diets..

No. of subcu-lture plates	Different diets in subculture plates	Carbohydrate fermentation tests						Nitrate Reduction	Identification of bacteria plates
		Glucose	Trypticase Broth test	Lactose	Fructose	Galactose	Mannitol	Trypticase Broth test	
1	C1	A+ G+	A+ G+	A- G-	A+ G+	A- G-	A+ G-	-	*Lacto bacillus minor*
1	CE1	A+ G+	A+ G-	A- G-	A+ G+	A- G-	A- G-	-	*Lacto bacillus viridescense*
2	CE2	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Bacillus pumilus*
1	P1	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Flavobacterium*
2	P2	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Flavobacterium*
3	P3	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Bacillus pumilus*
4	P4	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Flavobacterium*
5	P5	A+ G+	A+ G-	A- G-	A+ G+	A- G-	A- G-	-	*Lacto bacillus viridescense*
6	P6	A+ G+	A+ G+	A- G-	A+ G+	A- G-	A+ G-	-	*Lacto bacillus minor*
1	O1	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Bacillus pumilus*
2	O2	A+ G+	A+ G-	A- G-	A+ G+	A- G-	A- G-	-	*Lacto bacillus viridescense*
3	O3	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Bacillus pumilus*
4	O4	A+ G+	A+ G+	A- G-	A- G-	A- G-	A- G-	-	*Bacillus licheniformis*
5	O5	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A- G-	-	*Bacillus pumilus*
6	O6	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Flavobacterium*
7	O7	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Flavobacterium*
8	O8	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Flavobacterium*
9	O9	A+ G+	A+ G-	A+ G-	A- G-	A- G-	A+ G-	-	*Flavobacterium*

C = Control; CE = Cereal; P = Pulses; O = Organic rich diet; A = Acid; A (+) = Acid Positive; A (-) = Acid Negative; G = Gas; G (+) = Gas Positive, G (-) = Gas Negative

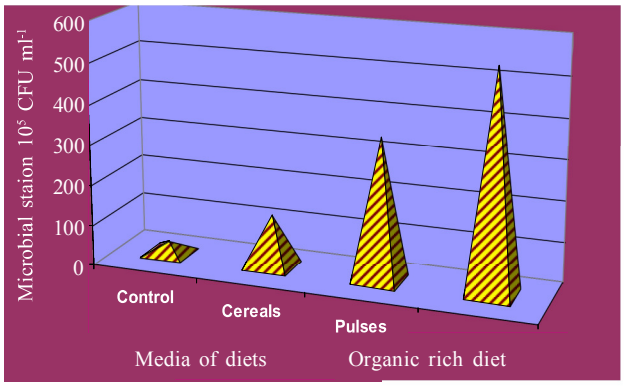

Fig. 1 : Status of microbes in the gut of *Eudrilus eugienea* exposed to different media of diets

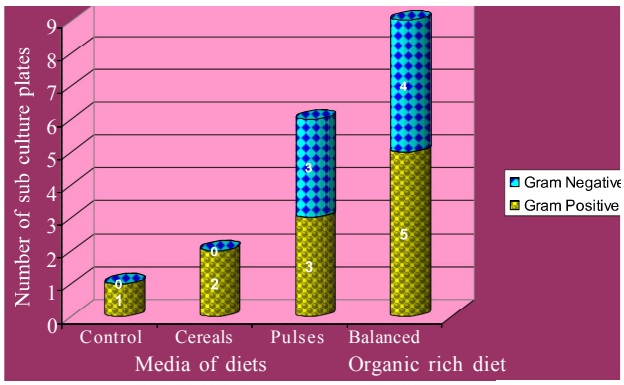

Fig. 2 : Identification of Gram positive or Gram negative bacteria from the subculture plates

suggested that when organic materials passes through the earthworm gut, the resulting vermicast is rich in microbial activity, plant growth regulators and pest repellants. In the present investigation CFU ranges in different diets of control, cereal, pulse and organic rich diet were also made. The Organic rich diet has a high range of 543 x 10^5 CFU ml^{-1} than other diets. So, Organic rich diet had been differentiated and more colony variations were seen than other diets. A large number of microbes were seen on the Organic rich diet, than the other diets of pulses & cereals. But, control diet had very low microbial colonies.

Fraser *et al.* (1993) investigated the quantity and distribution of organic carbon, microbial biomass carbon, protease, arylsulphatase and arylphosphatase activity, and earthworm numbers and biomass in the soil. The survival of microorganisms in the earthworm gut depends on their capacity to resist to digestive enzymes of microbial or earthworm origins, intestinal mucus, $CaCO_3$ or to bacteriostatic and microbial substances (Brown, 1995). The increase in the counts of bacteria and yeasts along the gut of all earthworms analyzed, especially in those of the refuse dump area and arboretum, suggests the growth of microbial population in the gut probably due to increase in availability of nutrients in the gut. This could have been as a result of digestion of ingested materials either by microbial enzymes produced within the gut or by the gut wall of these earthworms. In the present investigation, activity of endoglucanase, amylase, cellulase, protease and sucrase enzymes increased in all diets compared with standard values in organic rich diet of earthworms. The enzymes activities cellulase, endoglucanase, sucrase and protease were high except for amylase. In *Eudrilus eugienea* it was proved that the carbohydrate consumption was lesser as compared to other feed (pulse diet+organic rich diet). It was also tested and found that the amylase activity was lesser and the earthworms fed with this diet were found to be docile. Atiyeh *et al.* (2000) reported that during ingestion of organic substrates, earthworms not only fragment them, but also stimulate microbial activity and increase humic acid content by enhancing rates of mineralization. Earthworms produce an enormous amount of intestinal mucus composed of gluco- proteins and small glucosidic and proteic molecules (Morris, 2005). The microbes entering the worm guts consume these nitrogenous compounds of mucus (Zhang *et al.*, 2000), which mainly increase their activity, which in turn enables them to contribute enzymes to the digestive processes of the earthworms. These enzymes come with the ejected materials of earthworms. In *E.fetida*, a variety of intestinal microorganisms that produce enzymes, such as amylase, protease, lipase and cellulose, enhance the biodegradation of organic matter (Aira *et al.*, 2006).

Rice based cropping system showed superior microbial biomass and enzyme activities after organic manuring (Araujo *et al.*, 2009). Similarly soil fertility potential has also been found to be restored by organic manuring mainly due to greater biological activities and nutrient mobilization (Chukwuka and Omotayo, 2009). In another study higher bacterial, fungal and actinomycetes count were also found in organic agriculture field soil than in conventional farming system (Bettiol *et al.*, 2002). However, suitable application rate of organic matter in relation to crop growth and soil moisture level is essential to minimize anaerobic activity (Affendy *et al.*, 2011).

From the present investigation it was found that the organic rich diet was an ideal medium in which the biomass of earthworms and their enzymatic activity were high due to variety of microbes which enhanced the efficiency of the soil.

Acknowledgment

We are grateful to G. Manivannan, H.O.D. of Microbiology, N.M.S.S.V.N. College, Madurai for technical guidance in the field of microbiology.

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