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CHARACTERIZATION OF *BACILLUS* SPS FROM GUT FLORA OF EARTHWORM *EUDRILLUS EUGENIAE* FEED ON SUGAR INDUSTRY WASTE

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ABSTRACT: Earthworms are important soil organisms, play important role in modification of physical properties and organic matter decomposition. They carry out these changes due to presence of typical micro flora and enzymes of gut wall. Microorganisms in gut acts as efficient bioreactor. Diversity of bacteria, fungi, actinomycetes play important role in vermicomposting. Bacterial population and more particularly *Bacillus sps* play significant role in vermicomposting. The present study was carried out to find out the types and distribution of microorganisms in gut of *Eudrillus eugeniae* feed on sugar industry waste bagasse and press mud, vermi bed were prepared with cow dung, pre composted bagasse and press mud. The population of bacteria was determined in the fore, mid and hind gut region of earthworm. Distribution of organisms varies in gut region more in fore gut (125 cfu/ml) then in hind gut (94) and lesser in mid gut (72) after 60 days. The predominant bacteria observed were *Bacilus sps*. These organisms along with enzymes plays important role in vermicomposting process. The enzyme production capacity of *Bacillus sps* mainly amylase Nitrate reductase cellulase, xylanase and protease were studied. The Seven bacterial isolates were identified by 16S rDNA sequencing method;four isolates were unique to earthworm gut.

KEYWORDS: Vermicompost, sugar industry wastes, gut micro flora, earthworm.

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1. INTRODUCTION

Vermicompost production by using earthworm is an eco biotechnological process that transforms energy rich and complex organic substrate into a stabilized vermicompost [1]. The Earthworms are important soil invertebrates in terms of their activity [2]. Earthworms have different role in agro ecosystem their borrowing capacity can lead to the humus formation organic matter decomposition

and improving soil structure and enhancing nutrient availability to plants, during the vermicomposting nutrients are released in available forms to plants [3]. Earthworms are considered as ecological engineers [4]. They are key organisms in organic matter decomposition, modifying soil microbial dynamics and nutrient composition. They actively redesign the physical structure of soil environmentby activities like ingesting soil particle and depositing casts on the soil surface and translocation soil particles. The earthworms are acting as bioreactor. The Epigeic earthworms were mainly used for decomposition of organic waste [5].The activities of earthworms in soil have profound effect on the soil ecosystem functioning as well as on the type and number of micro flora and micro fauna [6].The gut of earthworm is the place for the production of the beneficial microorganisms and their products, to understand role of earthworm in the ecosystem the intestinal micro flora must be identified. The [7] found that there is no qualitative difference between the micro flora of the worm gut and surrounding soil, but [8] found that worm gut micro flora differ from the soil. The workers [9, 10] by using an enrichment technique studied specific groups of bacteria in the worm intestine and isolated cellulose decomposers but did not reported nitrifying organisms. The worms feed largely on dead plant and insect material found the selective increase in bacteria and actinomycetes. Researchers [11] found the evidence for presence of cellulose and chitin degrading micro organisms [12] in several earthworms.Earthworm ingests soil microorganisms along with organic residues from the soil and during passage through the earthworm intestinal tract, their population may increase. The ingested microbial population plays a key role in earthworm nutrition by helping in the breakdown of organic matter, particularly the components that the earthworms cannot utilize in their natural state. The composition of micro flora in the earthworm gut varies depending on the species of earthworm studied. Season and feeding habit of the earthworm. The number of microorganisms present in the gut of earthworm depends on the substrate that the earthworm feeds on [13, 14] . The earthworm gut may be enrich with various aerobic and facultative microbes [15,16,17]. There are conflicting reports relative to the proliferation of specific bacterial groups in earthworm gut micro flora, study carried out by [18] shown that presence of variety of species of microorganism in the gut region. The diversity of types and number of fungi, bacteria, actinomycetes, yeasts and protozoa in the gut casts of *Eudriluseugeniae*,*Eisenia fetida, P.excavatus*and*L.mauritii* fed on different substrates were reported.The various industrial wastes which have been vermicomposted and turned into nutrient rich manure include paper waste, sugar industry waste [19], distillery wastes [20].There is growing interest in the bio application of microorganisms from the gut of earthworm on biotransformation of persistent and toxic pollutants [21]. Earthworm ingest waste and acts in symbiosis with their gut microbes to detoxify waste or effluent [22] Some key microorganisms that have been isolated in gut of *Eisenia fetida* includes genera of *Klebsiella, Bacillus, Streptomyces, Microbacterium, Agromyces, Rhodococcus and Pseudomonas*[23]. The present study was carried out to find out the types and distribution of

Bacillus sps in gut of *Eudrilus eugeniae* feed on sugar industry waste bagasse and press mud.

2. MATERIALS AND METHODS

Materials

i) Culture pots, Blender machine, scissor,

ii) Sterile Petridishes,pipettes

iii) Cowdung, urine, Bagasse, press mud sample

iv) Ethanol, distilled water

v) Culture media - Nutrient agar

vi)Biochemical media for detection of enzymes amylase, nitrate reductase ,cellulase ,xylanase and protease and biochemical for identification

Methods

1) Collection of earthworms: The earthworm *Eudrilus eugeniae* was collected from the vermicomposting unit of Ajinkyakrishisevapadali, an agricultural research centre.

2)Earthworm culture: *Eudrilus eugeniae* from centre brought to laboratory and mass cultured in the culture pot containing urine free cow dung, which was collected from the nearby cattle shed, sun dried and powdered, worms acclimatized in cow dung were used for various experimental studies.

3) **Collection of raw material**: The bagasse and press mud samples were collected from Kisanveersatara sugar factory, Bhuinj. They were chopped into small pieces 3 to 5 cms and kept in shed for 15 days.

4) **Pre composting of the sample**: The shed dried sample was blend, mixed with cow dung, urine of cow to increase C:N ratio, moisture content was adjusted to 80% by sprinkling water. This sample was kept for three weeks.

5) **Vermi bed preparation**: The pre composted waste material were taken from vermin bed. The vermin beds were prepared with cow dung, pre composted bagasse, and pre composted press mud in the ratio 1:1:1. Water was sprinkled over the vermi bed to hold the moisture content 60%to 70% and kept for 24 hrs. Ten healthy matured *Eudrilus eugeniae* of 10-12 cm length and 0.4 to0.8gm weight were taken from culture tank and were introduced into the pots.

6) **Collection of specimen:** Earthworms collected from vermin bed was washed with sterile distilled water and placed in sterile Petri dish with moistened filter paper for 24 hrs. They were then cleaned externally with 75% ethanol, again washed with sterile distilled water 2-3 times and dissected.

7)**Dissection of earth worm**: The specimen to be dissected was washed in sterile distilled water placed across the second ,third and fourth fingers of the left hand with the anterior end pointing forward. The fine edge of the flamed pair of the dissecting scissor was inserted into ventral surface at the region of the clitella and with the body walls slightly raised up with the scissor; an incision was made longitudinally along the earth worm. Sterile dissecting pins were used to hold the earthworm down on the board. Stretching out the body wall to expose the internal structures. The

gut was then freed from surrounding blood vessels and nephridia with flamed forceps and separated into three sections fore gut, mid gut and hind gut. The gut sections were washed in sterile distilled water to free their contents before being suspended in the other tube containing sterile distilled water.

8) **Isolation of microorganisms from worm gut :**The different gut contents were suspended in three sterile 10 ml sterile saline solutions. They were serially diluted up to 10^{-5}. The 0.1 from 10^{-5}.Thedilution was taken using sterile pipette and placed on nutrient agar media for bacterial isolation. The plates were then incubated at room temperature for 24 hrs. Enumeration process was carried out at regular intervals up to 60 days

9)Identification of bacteria

The bacteria isolated by the standard procedure were primarily by studding their morphological and biochemical characteristics. Burgey's Manual of Determinative Bacteriology. The other bacteria were indentified up to genus level, while *Bacillus sps* were identified by 16S r DNA sequencing method.

16S r DNA sequencing method

For identification all bacterial strains were grown in nutrient broth at37^{0c} and cells were harvested at exponential phase by centrifugation at 8000 x g for 10 min. The DNA was extracted using method (Wilson, 2001)and used as template for 16S r DNA gene amplification by polymerase chain reaction (PCR) using primers forward 27F-5-GAGAGTTTGATCCTGGCTCAG-3 and reverse 1495r5-CTACGGCTACCTTG-3(Bachate et al ,2012).the PCR reactions were performed in a final volume of 50 uland contained approximatly10 ng of DNA, 1x PCR buffer,1.5U OF Taq polymerase,0.2mm of each primer,200 mm of each dNTPs and1.75Mm ofMg Cl 2.The DNA amplification conditions were, initial denaturation at 95^{oC} for 5 min,$55^{0C}$for 40sec,72^{Oc} for 1 min40sec, and final extension step at72^{0C} for 10 min. The amplified PCR products were checked by electrophoresis and purified using a PEG- NaCl method.(Hi media) The PCR products were sequenced using a Big Dye terminator kit (Microgen) The16S r DNA the gene sequence was searched for homology by using the NCBI-Blast 2- Nucleotide Database Query program.

3. RESULTS AND DISCUSSION

The gut flora of *Eudriluseugeniae* was studied and the number of bacteria present in different parts of gut was shown in [Table No-1)

Table No. 1: Number of bacteria present in different parts of gut

Days	Fore gut	Mid gut	Hind gut
0	24	25	32
15	45	38	49
30	70	45	62
45	85	48	77
60	125	72	94

The gut of earth worm having unique micro environment. The selective activity of gut fluid could be significant factor. The survival of microorganisms in the gut depends on their capacity to resist digestive enzymes of microbial or earthworm origin. In present study the gradual increase in CFU/ml was found from initial day to final day (60 days). The maximum CFU was found in the fore gut and minimum in mid gut region. The result indicate that the organic substrate used in the study(bagasse, press mud, cow dung) could initiate proliferation of bacteria. Earthworm gut also acted as organic nutrient for rapid bacterial colonization. Other bacterial genera also isolated includes *Pseudomonas, Enterobactor, Vibrio, Staphylococcus* and *Proteus*sps but number and types of organisms are less in number so it was decided to study *Bacillus* species(TableNo2,3), the dominant Bacterial micro flora of the earthworm gut..

Table No 2: Colony characters of *Bacillu*s sps on Nutrient agar incubated at RT for 24 hrs

Isolate	Size	Shape	Color	Margin	Elevation	Consistency	Opacity
EMG 3a	1-2 mm	circular	Pale yellow	regular	flat	moist	opaque
EMG 4b	1mm	circular	Pale yellow	regular	flat	moist	opaque
EMG 5	pinpoint	circular	Pale yellow	entire	convex	moist	opaque
EMG 7a	1mm	circular	Pale yellow	entire	convex	moist	opaque
EMG 7c	1mm	circular	Pale yellow	entire	flat	moist	opaque
EMG 11	pinpoint	irregular	Pale yellow	entire	flat	dry	opaque
EMG 12	1-2mm	circular	Lemon yellow	regular	convex	moist	opaque

Table No 3: Enzyme activity of different isolates

Sr.No	Isolate	Amylase	Nitrate reductase	Cellulase	Xylanase	Protease
1	EMG 3a	+	+	+	+	+
2	EMG 4b	+	+	+	+	+
3	EMG 5	-	+	-	-	+
4	EMG 7a	-	-	-	-	-
5	EMG 7c	-	+	-	-	+
6	EMG 11	-	+	-	-	+
7	EMG 12	+	-	+	+	+

Table No 4: Identification of bacteria

The16S r DNA the gene sequence was searched for homology by using the NCBI-Blast 2-Nucleotide Database Query program.

Sr. No	Isolate	Organism	Query length bp	Percentage Identity
1	EMG 3a	*Bacillus tropicus* strain MCCC1A014016	968	96%
2	EMG 4b	*Bacillus aerius* strain 24K	1392	99%
3	EMG 5	*Bacillus safensis* strain NBRC100820	892	99%
4	EMG 7a	*Bacillus xiamenensis* strain MCCC1A00008	1115	99%
5	EMG 7c	*Bacillus safensis* FO-36b	1400	99%
6	EMG 11	*Bacillus subtilis* strain SBMP4	1015	99%
7	EMG 12	*Bacillus licheniformis* strain DSM13	1192	98%

Out of seven isolates (TableNo4) identified *Bacillus subtilis* strain SBMP4, *Bacillus licheniformis* strain DSM13, *Bacillus safensis* FO-36b.these species were previously reportd but strains are different while, other species *Bacillus xiamenensis* strain MCCC1A00008, *Bacillus safensis* strain NBRC100820 , *Bacillus aerius* strain 24K, *Bacillus tropicus* strain MCCC1A014016 reported first time from gut of earthworm *Eudrilus eugeniae*, The number of bacteria isolated by [24]from earthworm gut found that isolates obtained also present in surrounding soil therefore, it was assumed that earthworm might not have their own normal flora in the gut[25].The certain species of microorganisms after their ingestion survive, grow and become dominant type in the gut content of earthworm.Many investigations show that certain types of microorganisms might develop mutualistic relationship with earthworm. There are some evidences of resident micro flora. The microorganisms passing un harmed and undigested through the gastrointestinal tract of earthworm. The researchers [26] studied intestinal micro flora of earthworm *Pheretinasp* and isolated *Psudomonas,Nocardia, Sreptomyces and Bacillus* species from earthworm gut. Earthworm make suitable condition for microorganisms adding considerable amount of water (80-150% dry wt of soil) and intestinal mucus (5-43%) play role in mutualystic digestive process, stimulate to digest complex material [27].The cellulolytic organisms increase from mouth and attain maximum level

in anterior and posterior intestine [28]. The nitrogen fixing microorganisms were reported in gut. The workers.[29] isolated Nitrogen fixing anaerobic *Clostridium sps.* The earthworm in its gizzard portion of gut might digest some of the bacteria and fungi present in feed material, survived bacteria might get conducive environment for their growth in posterior region. Maximum percentage of bacteria isolated having capacity of secretion of various enzymes reflects their potential role in biochemical transformation that occur in gut ecosystem The bacterial isolates- 42'85 % secrete amylase, cellulase and xyllanase, 71.4% % secrets nitrate reductase while 85.71% secrets protease. The (30) studied origin of digestive enzymes in gut and reported cellulase produced by microorganisms present in gut and not by earthworm. Another researcher (31) reported increase in nitrogenase activity in cast as compare with soil indicate presence of nitrogen fixing microorganisms in gut Further studies are necessary to clearly establish the role of microorganisms in the vermicompost and also their mutualystic relation with earthworm *Eudrilus eugeniae*.

4. CONCLUSION

Bacterial population was found more in foregut region; seven isolates were studied up to species level by 16S r DNA gene sequence method. The four strains were reported first time in *Eudrilus eugeniae* earthworm. The *Bacillus* being spore former resist digestive enzyme of earthworm and enzyme synthesizing ability of organisms helps in degradation of complex organic matter it helps in formation of vermicompost

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CONFLICT OF INTEREST

Authors have no any conflict of interest.

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