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Comparative study on the extracellular amylase activity of amylolytic bacteria isolated from different soil types and earthworms gut

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Abstract
An analysis for the abundance and diversity of amylolytic bacteria of earthworm gut and nine different soil types *viz*. food waste disposing soil, municipal waste disposing soil, kitchen waste disposing soil, soil from three different wetlands and mangroves, flood affected and unaffected paddy field soil and goat excreta disposing soil was carried out. A total of 52 bacterial isolates (25 from soil and 27 from earthworm gut) were primarily selected for their amylolytic potential. Among them, the percentage of amylolytic bacteria was higher in soil (16) than earthworm gut flora (11). Among soil bacterial flora, the percentage of amylolytic bacteria was higher in waste disposing soil (7) and lower in bacteria from agricultural field (4). Among earthworm gut flora, the percentage of amylolytic bacteria was higher in earthworms isolated from wetlands mangroves (5) and waste disposing area (4) and least in earthworms isolated from agricultural field (4). The highest activity among soil micro biota was shown by Es02s1, 20mm, (flood unaffected soil) followed by Ac01s3, 19.6mm, (kitchen and farming waste dumping soil). The highest activity among earthworm gut microbiota was shown by Cm03m, 18mm (bacteria isolated from the mid gut of earthworm). As the importance of amylase production was increasing in various industrial sectors, it is significant to find more and more cheaper and easily available sources for it. The current work is a small attempt on finding out a better, cheaper and easily available source for the amylolytic bacteria between different soil types and gut flora of earthworms.

Keywords: amylolytic bacteria, earthworm gut flora, flood affected and unaffected soil, waste disposing soil, wetlands and mangroves

Introduction
Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. Amylases are capable of digesting these glycosidic linkages found in starch. It hydrolyses starch and glycogen into simple sugars. There are two types of amylases: α-amylase and β- amylase. α-amylase is an endohydrolase which hydrolyses the α-1, 4 linkage in a random manner within the chain and breaks these polymers into small fragments and it has the ability to by-pass α-1, 6 branch points. Although viscosity reduction of the substrate is faster the production of reducing sugars is slow. In contrast, β-amylase is an exohydrolase and it starts acting from non-reducing end removing two glucose molecules at a time. The products thus formed are a disaccharide, β-maltose and limit dextrin. The viscosity reduction of the substrate due to β- amylase action is slow but the production of reducing sugars is fast [1].

Amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of α-amylase, microbial sources are used for the industrial production due to their advantages such as cost effectiveness, consistency, less time and space required for production as well as ease of process modification and optimization. They are present in all living organisms, but the enzymes vary in activity, specificity and requirements from species to species and even from tissue to tissue in the same organism [2]. Earthworms influence primary soil functions and processes, such as soil structure formation, soil carbon dynamics and biogeochemical cycles [3]. The successful management and exploitation of earthworm bioresources has the potential to deliver significant economic and environmental benefits, especially in light of global concerns regarding sustainable land use, food security and climate change [4]. So, the present study focusses on the isolation of amylase producing microorganisms from various sources such as gut of earthworms and different soil types.

Materials and Methods
Sample collection
The soil samples and the earthworms were collected from three different ecological types; waste disposing sites, wetlands, mangroves and agricultural paddy fields of different localities of Ernakulam, Kerala. A total of nine samples were collected from the various sites. Three samples were collected, from municipality waste disposal area in Kalamassery (latitude: 103'52" N, longitude: 7619'38" E); kitchen and farming waste disposing area in Companippady (latitude: 10 5'31"n, longitude: 7620'23"E) and a food waste dumping area in Thrikkakara (latitude: 101'45"N, longitude: 7620'9"E). Another three samples were collected from wetlands of Thonithode, Mundamveli (latitude: 9.91969^{0}N, longitude: 76.25908^{0}E), Chellanam (latitude: 9.83931936^{0}N, longitude: 76.2591526^{0}E) and Mangalavanam (latitude: 9.988639^{0}N, longitude: 76.273207^{0}E). And the remaining three samples were collected from the flood affected paddy field (latitude: 10^{0}12'59.9" N, longitude: 76^{0}26'36.7" E), goat excreta deposited land area (latitude: 10^{0}13'01.5" N, longitude:

$76^0$26'37.3" E) and a flood unaffected area (latitude: $10^0$13'00.8" N, longitude: $76^0$26'37.1" E) of Manjappra, Angamaly. As the localities varied, there were variation in soil characteristics, distribution of earthworms and the morphology of earthworms.

Waste disposing site

Kitchen and farming waste disposing area- Companippady

The sample was collected from a kitchen and farming waste dumping area of a house in Companippady (Fig: 1a). The biodegradable waste dumped in this area contributed to the abundance of earthworms and the soil was found to black and fertile. The loose texture of the soil made sample collection easier.

Municipality waste disposal area- Kalamassery

The sample was collected from municipality waste disposal area (Fig: 1b). This area was dumped with plastic, food waste, decaying materials from various industries, houses etc. These wastes might have enhanced the acidity of the soil. The soil was found to be black and the abundance of earthworms were least.

Food waste dumping area in BPS convent- Thrikkakkara

The third site was a food waste dumping area of a Convent (Fig: 1c). The soil was black, fertile used for the kitchen garden and was dumped with food waste, vegetable waste, leaves and other decaying matter. The availability of earthworms in this area were abundant.

Wetlands and mangroves

Wetland with a mangrove forest- Mundamveli

This area is a wetland with a mangrove forest (Fig: 1d). The soil of this area was slightly acidic and the earthworms were rarely seen which made the sample collection difficult. The earthworm which was found was present deep inside the soil and it was long, wide, pale brown, and had a prominent clitellum.

Kitchen waste dumping wetland with a mangrove forest- Chellanam

The sample collection from this site (Fig: 1e) was rather easier when compared to the first site. The earthworms were pale brown, long and slender. The earthworms were found on the surface of the soil and were abundant. The soil was black and acidic. The organic waste dumped to this area might have contributed to the acidity and abundance of earthworms.

Mangrove forest- Mangalavanam bird sanctuary

The sample collection from this area was difficult because the earthworms were least abundant in this area (Fig: 1f). The soil was black and acidic. The earthworms were thick, wide, dark brown and had a prominent clitellum.

Agricultural field

The soil samples which were collected from the three sites ie, flood affected paddy field, flood unaffected area and goat excreta deposited area (Fig: 1g, Fig: 1h, Fig: 1I). Soil from these areas had a slightly acidic pH.

The soil samples and earthworms collected from the all the 9 sites were kept in a zip lock cover and labelled with their Site of collection, date of collection, latitude and longitude of the area and was taken to laboratory for the further procedures.

Dissection of earthworms

The earthworms collected from these sites were dissected to separate the gut portions. The dissected samples are labelled separately based on their gut positions.

The earthworms taken from the sampling sites were washed thoroughly. It was then transferred to a petri plate and was kept for freezing for 10 minutes. A chloroform dipped cotton was kept inside the petri plate and made it unconscious. Earthworm was taken from the petri plate and was pinned to a wooden board. The body cavity of the earthworm was cut and opened using dissecting tools. The fore gut, mid gut and the hind gut were taken separately, crushed and were transferred to 3 test tubes containing sterilised peptone water. The test tubes were labelled separately based on their gut positions and was kept for incubation for 24 hours. The procedure was repeated for the remaining earthworm samples.

Isolation of soil and gut bacteria

Isolation of gut bacteria

The fore gut (a), mid gut (m) and the hind gut (h) samples were kept in peptone water (Table 1) and were incubated at 37°c for 24 hours. After about 24 hours the samples were inoculated onto sterile nutrient agar plates (Table 2) and incubated at 37°c for 24 hours. Sampling point and sampling code for earthworm gut microflora were given in Table 3.

Peptone water

It is a microbial growth medium composed of peptic digest of animal tissue and sodium chloride. Peptone water is also a non-selective broth medium which can be used as a primary enrichment medium for the growth of bacteria.

Table 1: Requirements for peptone water.

Sl. No.	Components	Quantity
1.	Peptone	10g
2.	NaCl	5g
3.	Distilled water	1000ml

The media was sterilized by autoclaving at 121°c for 15 lbs pressure.

Nutrient agar

Nutrient agar is a general-purpose growth medium for the cultivation of non-fastidious microorganisms.

Table 2: Requirements for nutrient agar.

Sl. No.	Components	Quantity
1.	Peptone	5g
2.	NaCl	5g
3.	Meat/beef extract	3g
4.	Agar	20g
3.	Distilled water	1000ml

The media was sterilized by autoclaving at 121°c for 15 lbs pressure.

Table 3: Sampling point and sampling code for earthworm gut microflora.

Sl. No.	Sample area type	Sampling point	Sample code
1.	Waste disposing site	Companippady	Ac01a, Ac01m, Ac01h
2.		Kalamassery	Ak02a, Ak02m, Ak02h
3.		Thrikkakara	At03a, At03m, At03h
4.	Wetlands and mangroves	Mundamveli	Cm01a, Cm01m, Cm01h
5.		Chellanam	Cc02a, Cc02m, Cc02
6.		Mangalavanam	Cm03a, Cmo3m, Cmo3h
7.	Agricultural field	Flood affected paddy field	Es01a, Es01m, Es01h
8.		Flood unaffected area	Es02a, Es02m, Es02h
9.		Goat excreta deposited area	Es03a, Es03m, Es03h

Isolation of soil bacteria

1g of soil sample was weighed accurately and mixed well with 10ml of sterilized distilled water contained in a test tube labelled as 10^{-1}.1ml of this solution was taken using micro pipette and was transferred to the second test tube labelled as 10^{-2}, containing 9ml of sterilized distilled water. It was then serially diluted up to 10^{-6}. The remaining soil samples were also similarly serially diluted and inoculated into the agar plates and spread evenly using L rod (spread plate culture). The plates were then incubated at 37°c for 24 hours.

Table 4: Sampling point and sampling code for soil microflora.

Sl.No.	Sampling point	Sample code
1.	Companippady	Ac01
2.	Kalamassery	Ak02
3.	Thrikkakara	At03
4.	Mundamveli	Cm01
5.	Chellanam	Cc02
6.	Mangalavanam	Cm03
7.	Flood affected paddy field	Es01
8.	Flood unaffected area	Es02
9.	Goat excreta deposited area	Es03

Determination of amylase activity

The organisms isolated from soil and gut were inoculated into the starch agar followed by incubation for 24 hours. The amylolytic activity was determined by the formation of halo zone of clearance in the vicinity of the bacterial colony after flooding with iodine solution.

Table 5: Requirements for starch amylase agar

Sl. No.	Components	Quantity
1.	Beef extract	3g
2.	Peptone	5g
3.	Soluble starch	2g
4.	Agar	15g
3.	Distilled water	1000ml

The media was sterilized in an autoclave at 121°c for 15 lbs pressure.

Determination of pH of the soil samples

1g of the soil sample was accurately weighed into a test tube containing 5ml of distilled water and shaken well and the pH was determined.

Results and Discussion

Earthworms are known as the intestine of the soil as they helps in increasing the fertility and aeration of the soil by their turning action [5]. They helps in the decomposition of the materials by enhancing the activity of the microbial population present in their gut and thus the microbes behaves as a bioreactor [6]. Most of the earthworms exhibit a symbiotic relation with their gut microbes for the decomposition of organic matter by producing enzymes such as cellulase, amylase etc. The life of the earthworm is strongly influenced by the texture and p^H of the soil and hence they can live only in slightly acidic and neutral p^H [7]. From the earthworms and the soil samples, a total of 52 bacterial colonies were isolated based on their morphological characters, out of which 27 are from the gut portions of the earthworms and 25 are from the soil samples. The results of the enzymatic activity can be attributed to the pH and the texture of the soil. Earthworms are mostly present and active between the pH range of 5.0 and 7.4 but their growth is substantially limited within the pH range of 3.5–4.5 and ceases under strongly acidic conditions below pH 3.5 [8]. It is clear from the results that the constant enzymatic activity was shown by the soil and gut microbiota of soil with neutral pH. The pH of the 9 soil samples collected from different sites were tested. It was found that the soil samples of had a neutral pH ranging from 6 to 7 except soil of Mangalavanam which had an acidic pH of 4 (Fig 3). The requirement of neutral p^H by the microbes were also reported by [9, 10, 11, 12]. The constant enzymatic activity of this area can also be due to the nature of the waste deposited. 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 [13].

The colour, size and availability of the earthworms isolated from the sampling sites were recorded (Table 6). Earthworm isolated from wetlands and mangroves have a pale brown colour and that from other sites have light to dark red colour. The earthworms were found least abundant in municipal waste disposing area, Mangalavanam, Mundamveli and flood affected paddy field. This may be due to the variation in pH and the soil characteristics. Observing the growth of microflora in agar plates, certain studies were made on the characteristics of these bacteria. Size, shape, opacity, consistency and elevation were the characters which were taken for study.

For determining the amylolytic activity of various microbiota, the samples were inoculated into starch agar. It was observed that some samples formed a halo zone of clearance in the starch agar plates due to their amylolytic activity and their zone were measured.

The highest activity among soil micro biota was shown by Es02s1 (bacteria isolated from the soil of flood unaffected area) produced a zone of diameter 20mm followed by Ac01s3 (soil bacteria isolated from kitchen and farming waste dumping area in Companippady) produced a zone of diameter 19.6mm. Among 27 earthworm gut microbiota 9

bacteria showed produce a zone of clearance above 10 mm. The highest activity among earthworm gut microbiota was shown by Cm03m, 18mm (bacteria isolated from the mid gut of earthworm isolated from Mangalavanam). Midgut microflora showed highest amylolytic activity compared to hind gut and fore gut. The results of the study are given in Table 7. The result of the study can be compared with the study conducted by Albasha *et al.*, they collected earthworms and fed them with dry leaves, plastic waste, kitchen waste and waste paper and the microorganisms were isolated by serial dilution followed by a spread plate culturing in nutrient agar medium. After screening and characterization of the isolates it was observed that high variety of cellulolytic amylolytic and proteolytic microbes were found in the earthworms fed with the kitchen waste and it showed high enzymatic activities [14].

Tables and Figures

Table 6: Characteristics of Earthworm

Sampling site	Sampling code	Characteristics of the Earthworm		
		Colour	Size	Availability
Companippady	AC01	Light Red	Long	Abundant
Kalamassery	Ak02	Red	Short	Least Abundant
Thrikkakara	At03	Light red	Long	Abundant
Mundamveli	Cm01	Pale Brown	Long	Least Abundant
Chellanam	Cc02	Pale Brown	Short	Abundant
Mangalavanam	Cm03	Pale Brown	Long	Least Abundant
Flood affected paddy field	Es01	Light Red	Short	Least abundant
Flood unaffected area	Es02	Dark Red	Long	Abundant
Goat excreta deposited area	Es03	Dark Red	Long	Abundant

Table 7: Amylolytic activity of isolated microflora.

Sampling point	Sampling type	Code	Amylase Production (zone diameter in mm)
Companippady (Ac01)	Soil	Ac01s1	-
		Ac01s2	-
		Ac01s3	+ (19.6mm)
	Gut	Ac01a1	+ (15mm)
		Ac01m	-
		Ac01h	+ (6mm)
Kalamassery (Ak02)	Soil	Ak02s1	-
		Ak02s2	+ (17mm)
		Ak02s3	+ (18mm)
	Gut	Ak02a	-
		Ak02m	-
		Ak02h	-
Thrikkakkara (At03)	Soil	At03s1	+ (14mm)
		At03s2	+ (7mm)
		At03s3	+ (10mm)
		At03s4	+ (18mm)
	Gut	At03a	+ (15mm)
		At03m	+ (17mm)
		At03h	-
Mundamveli (Cm01)	Soil	Cm01s1	+ (15mm)
		Cm01s2	+ (12mm)
	Gut	Cm01a	-
		Cm01m	-
		Cm01h	-
Chellanam (Cc02)	Soil	Cc02s1	+ (18mm)
		Cc02s2	+ (13mm)
	Gut	Cc02a	+ (13mm)
		Cc02m	+ (17mm)
		Cc02h	-
Mangalavanam (Cm03)	Soil	Cm03s1	-
		Cm03s2	+ (10mm)
	Gut	Cm03a	+ (15mm)
		Cm03m	+ (18mm)
		Cm03h	+ (10mm)
Flood affected paddy Field (Es01)	Soil	Eso1 s1	-
		Eso1 s2	+ (12mm)
	Gut	Eso1 a	-
		Eso1 m	+ (15mm)
		Eso1 h	+ (2mm)
		Eso2 s1	+ (20mm)

Flood unaffected area (Es02)	Soil	Eso2 s2	+ (10mm)
	Gut	Eso2 a	-
		Eso2 m	-
		Eso2 h	-
Goat excreta deposited area (Es03)	Soil	Eso3 s1	+ (5mm)
		Eso3 s2	-
	Gut	Eso3 a	-
		Eso3 m	-
		Eso3 h	-

Fig 1: Sampling sites. (A) Companippady, (B) Kalamassery, (C) Thrikkakkara, (D) Mundamveli, (E) Chellanam, (F) Mangalavanam, (G) flood affected paddy, (H) flood unaffected field, (I) goat excreta deposited area.

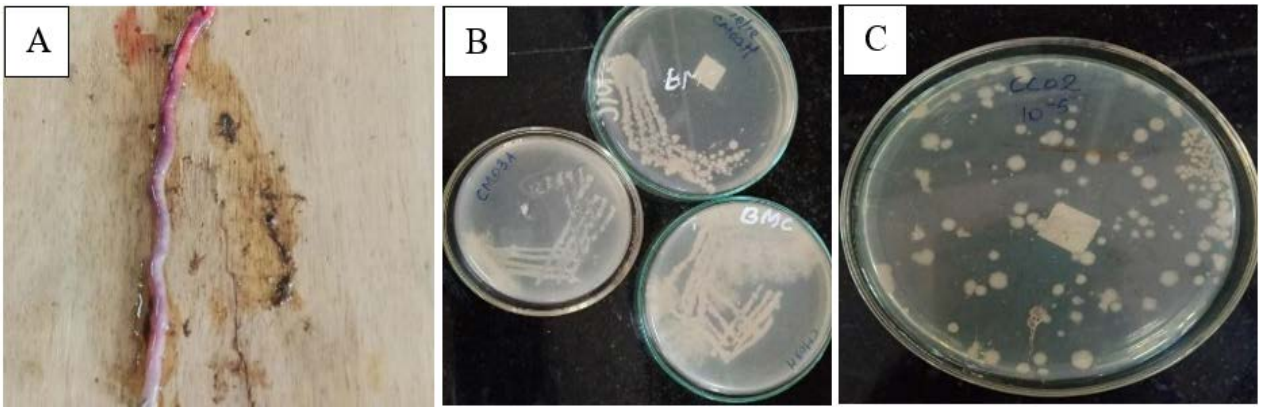

Fig 2: Isolation of bacteria. (A) Earthworm, (B) Earthworm gut flora, (C) Soil bacteria

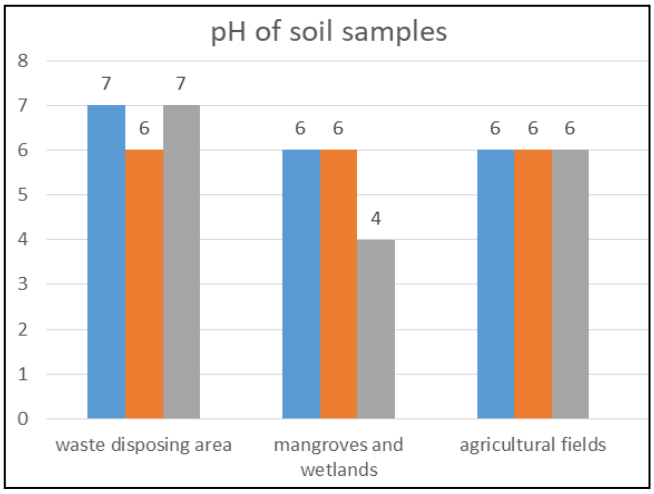

Fig 3: pH of the soil samples

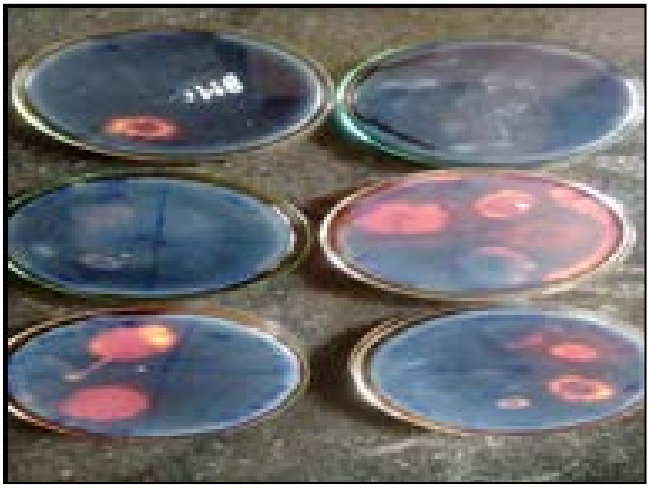

Fig 4: Screening for amylolytic activity

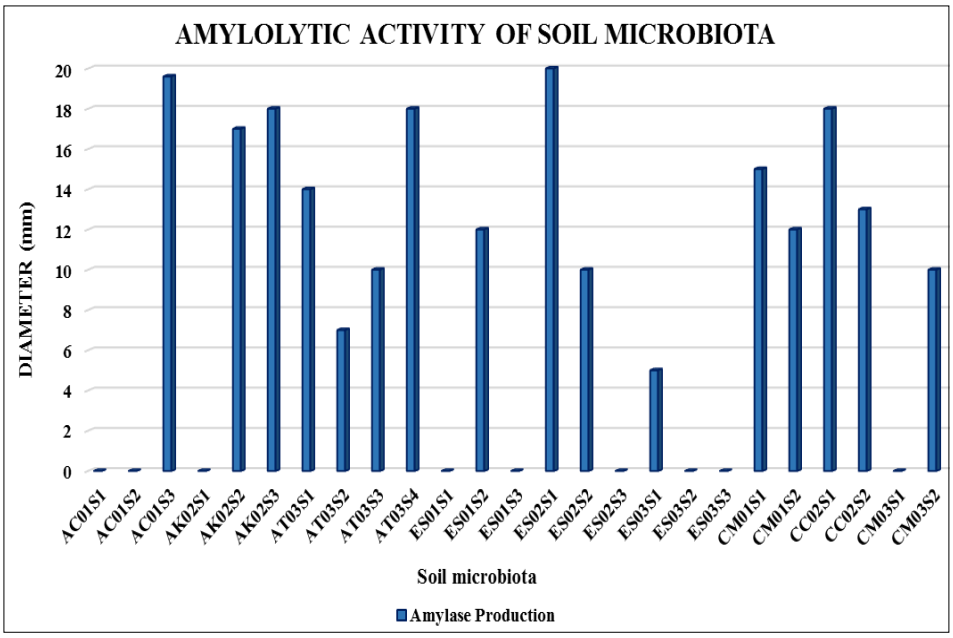

Fig 5: Comparison of amylolytic activity of soil microbiota isolated from different sampling area.

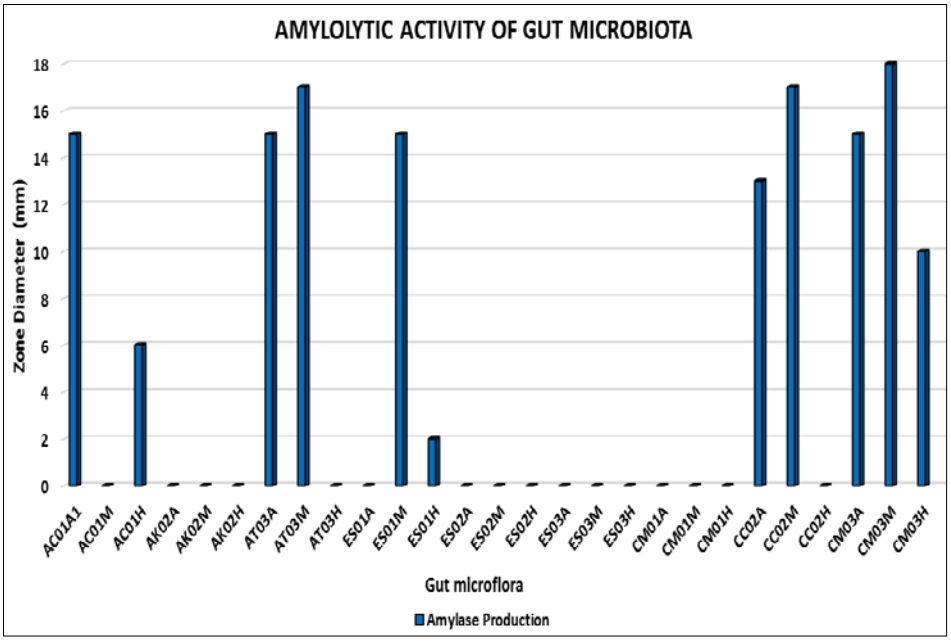

Fig 6: Comparison of amylolytic activity of earthworm gut microflora.

Conclusion

The symbiotic relationship of earthworms and gut microbes plays a pivotal role in the decomposition of organic matter. The work is a comparative study made between the amylolytic activity of bacteria present in the gut of the earthworm and the soil collected from different sampling site. In this work 52 organisms were isolated and screened for amylolytic activity in starch agar. The bacteria which showed higher amylolytic activity were identified and their enzymatic activities were compared. Out of the 52 isolates, 27 isolates (16 soil microbiota and 11 gut microbiota) showed amylolytic activity by forming a zone of clearance in the starch agar. The highest activity among soil micro biota was shown by Es02s1, 20mm, (flood unaffected paddy soil) followed by Ac01s3, 19.6mm, (kitchen and farming waste dumping soil). The highest activity among earthworm gut microbiota was shown by Cm03m, 18mm (bacteria isolated from the mid gut of earthworm).

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