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Research Paper

Isolation, identification, and characterization of gut microflora of *Perionyx excavatus* collected from Midnapore, West Bengal

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Agriculture is an important part of the economy of the undivided Midnapore district. Agricultural land is its asset and most importantly its means of sustenance as well as survival. Earthworms are invertebrates that play a key role in recycling organic matters in soils. Since the intestines of earthworms harbor wide ranges of microorganisms, enzymes, hormones etc., these half digested materials decompose rapidly and are transformed into a stabilized material called vermicompost which is very useful for increasing the soil fertility. One has to look for these characters before recommending any species for vermiculture. In the present study, *Perionyx excavatus* specimens were collected from the undivided Midnapore district and from the Earthworms gut, bacteria, fungus, actinobacteria, and yeast were isolated and identified using various morphological and biochemical tests. All the bacterial isolates were identified using morphological study, staining techniques, and different biochemical tests such as catalase test, KOH test, H_2SO_4 test, Starch hydrolysis test, oxidase test, and sucrose hydrolysis test. All the fungal, actinobacteria, and yeast isolates were subjected to staining and morphological characterization (color and texture of fungal colony). Bacterial isolates of genus *Bacillus* sp., *Staphylococcus* sp., *Enterococci*, *Micrococcus* sp., *Enterobacter* sp., and *Citrobacter* sp. were identified. Among the fungal isolates *Aspergilus* sp., and *P. boydii* were identified. *Streptomyces* sp., *Nocardia* sp. among the actinobacteria and *Candida* sp. among yeast were also found to be present in earthworm gut and these might play an important role along with the earthworm to increase the quality and fertility of soil.

Keywords: Perionyx excavatus / Gut microflora / Bacillus / Aspergilus / Yeast

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Introduction

Earthworms have been studied scientifically by man from the time of Darwin [1] and a lot of attention has been paid to the understanding of the relationship between earthworm and microbes. Earthworms (class Oligochaeta) comprise approximately 800 genera and 80,000 species that account for almost 90% of invertebrate biomass present in soil [2]. Earthworms while ingest organic waste and soil, consume heavy metals through their intestine and their skin. As the intestines of earthworms harbor wide range of microorganisms, hormones, enzymes etc., these half digested materials decompose rapidly and are transformed into a stabilized material called vermicompost [3]. Vermicomposting involves bio-oxidation and stabilization of organic matter through the interactions between earthworms and microorganisms [4]. There is no doubt that in India, where on one side pollution is increasing day by day due to accumulation of organic waste and on the other side there is a great shortage of organic manure, which could increase the fertility and productivity of the land and produce nutritive and safe food, the scope for vermicomposting is enormous [5]. Currently soil appears to degrade more rapidly than it is replenished [6]. Higher microbial population and activity in the casts of earthworms, compared to surrounding soil, have been demonstrated by Parle [7], Scheu [8], Ghosh [9], Edwards and Bohlen [3], and Parthasarathi and Ranganathan [10]. From a numerical perspective, the occupation of 1 g of

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soil is comparable to the occupation of the Earth's globe by humans [11]. Microorganisms are the main cause of the biochemical degradation of organic matters but earthworms also play a critical role in this process through fragmenting the substrates, increasing the surface area for growth of microorganisms, aeration, and changing their activities [12].

Studies show that during the first stage of organic matter decomposition, the interactions between the earthworms and microorganisms are somehow mutualistic [13]. These earthworms are considered as bioreactors which in their gut, microorganisms proliferate and therefore provide required conditions for the biodegradation of wastes [14]. The gut contents usually comprise mucus, organic, and mineral matter. An analysis of gut contents in earthworms revealed the occurrence of different kinds of symbiont-like microfungi, bacteria, protozoa etc; gradually decreasing in number in the mid and hindgut with fewest in freshly laid casts [15]. It is well established that the earthworm gut provides suitable conditions for the development of different bacterial colonies since earthworm casts contain significantly higher counts of bacteria than in the surrounding soil [14, 16–19]. The impact of earthworms gut enzyme in biodegradation and biodecomposition of wastes have been mentioned in several literatures [20–22]. These enzymes are either originated from their intestinal juice or are the result of microbial activity in their gut. Microorganisms having high enzymatic activities form symbiotic and synergistic relationships with the earthworms [23].

West Bengal is the state of India which is mainly dependent on the agriculture fields. Midnapore districts (*Purba* and *Paschim*) are basically the districts of the farmers. Here, people are highly dependent on agriculture land which is of only monocropped and less diversified. Land differs markedly in their values and is influenced by its different properties like type of soil, type of microbes, and microflora residing in the soil and on the flora and fauna of the particular region. With the introduction of high-yielding seeds and chemical fertilizers the agricultural land is losing its natural flora and fauna which indirectly affects the crop productivity. Earthworms affect the ecosystem structure and function directly by ingesting, altering, and mixing organic residues and mineral soil and they change the structure, chemistry, and biology of soil [24]. It is desirable to know about species of earthworms that may be as efficient or better in their performances over the mentioned species in a country having rich diversity of fauna for *in situ* and *ex situ* vermiculture. Most of the species that are included under genus *Perionyx* show great potential to work on organic matter [9]. In order to gain an insight of the abovementioned threat and microbe-earthworm relationship, a comprehensive study was made on the gut of *Perionyx excavatus* from both the districts of Midnapore to isolate and identify the different microbial populations present in normal or natural environmental conditions of these two regions.

Materials and methods

Organism for the study

Perionyx excavatus specimens were collected from the locality in and around our college campus (Paschim Medinipur) and Mecheda (Purba Medinipur).

Collection of the earthworm species

Earthworms are commonly found in the upper layer of the earth down to 30–50 cm in depth. The appropriate time for their collection was found to be early in the morning in summer, and noon time during the winter. Freshly collected worms were stored in plastic containers with pores, filled with suitable quantity of wet compost soil.

Test conditions

A total of 150 worms were collected and maintained in separate cement containers ($50 \times 35 \times 35\,cm^3$). The worms were allowed to acclimatize for the next 20 days and then they were separated in different containers in a group of 5 earthworms per container. For both Purba and Paschim Medinipur, 10 replicates were maintained for the experimental purpose. All these containers contained 4 Kg of feed material, which is mainly prepared from cow dung, at 28 ± 2°C, 65% moisture, 12L/12D photoperiod and the containers were kept at normal room tempertare. The feed was not changed during the experiment period. Only adult (Clitellate) worms with an individual body weight of 300–600 mg were used in the study. Microbial analysis was done after 20 days of commencement of the experiment.

Preparation of the earthworm extract

After the exposure, the earthworms were collected, washed with sterile tap water and then placed on a sterile Petriplate moistened with filter paper and subjected to starvation for 24 h. Further, they were then disinfected with 70% ethanol. Complete gut of each earthworm was dissected out, weighed and homogenized in sterile 0.85% NaCl solution. We maintained the replicates in microbial analysis process also by analysing the gut content of the each specimen, present in each of the container,

separately. The resultant homogenates were then used as the extract for the microfloral isolation.

Isolation of gut microflora

The resulting suspension of each earthworm from each test condition was serially diluted with sterile water and used as inoculums. About 0.1 ml of the inoculums of each earthworm was separately inoculated into Nutrient agar (NA) and Mackonkey agar (MA) plates and spread over each media for bacterial growth. For fungal and yeast growth, Sabouraud's dextrose agar (SDA) plates were used and actinobacteria agar (AA) and SDA plates were used for actinobacteria growth.

The plates were then incubated at 30–37°C for 18–24h for bacteria, 25–28°C for 4–7 days for fungi, 30–37°C for 10–12 days for actinobacteria, and 25–37°C for 12–14 days for yeast.

Enumeration of the isolated bacteria

The CFU was done for each isolated strain of bacteria by dilution pour plate method [25].

Identification of the microflora

To identify the bacteria, actinobacteria and yeast at genus level, Gram's staining, spore staining, and various biochemical tests like catalase test, KOH test, starch hydrolysis test, Voges–Proskauer test, oxidase test, and sucrose hydrolysis test were performed as described in Mahon and Manuselis [26].

All the fungal isolates were identified using Lactophenol blue staining and morphological characterization (color and texture of fungal colony). The morphological and cultural features of each fungus were compared with descriptions given by Bryce [27] and Kwon-Chung and Bennett [28] for identification.

Results

The results were observed after the respective days of incubation. Different types of bacteria, fungus, and actinobacteria were observed (Fig. 1). Firstly, they were characterized in order to identify them as a part of earthworm's gut microbial population. A total of 7 bacteria, 14 fungi, 2 actinobacteria, and 1 yeast were obtained, out of which only 5 bacteria (B1, B2, B3, B4, B5), 3 fungi (F1, F2, F3), 1 actinobacteria (F4), and 1 yeast (F5) were identified depending on their morphological and biochemical characteristics.

Characterization of bacteria

Out of the five identified bacteria, four were found Gram positive and only one was found to be Gram negative (Fig. 2). All the isolates gave negative result for oxidase test and VP test. All the isolates were positive for catalase activity except bacterium B2. All the isolates were found to ferment sucrose except isolate B3. Only isolates B1 and B4 were found to exhibit starch hydrolysis ability.

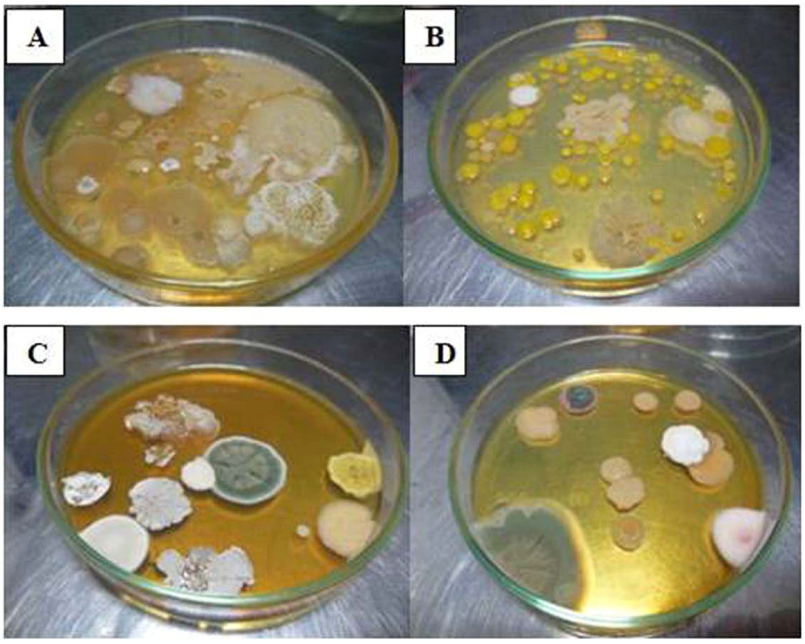

Figure 1. Bacterial and fungal isolates on Nutrient agar and SDA plates before identification. A, B: bacterial isolates; C, D: fungal isolates.

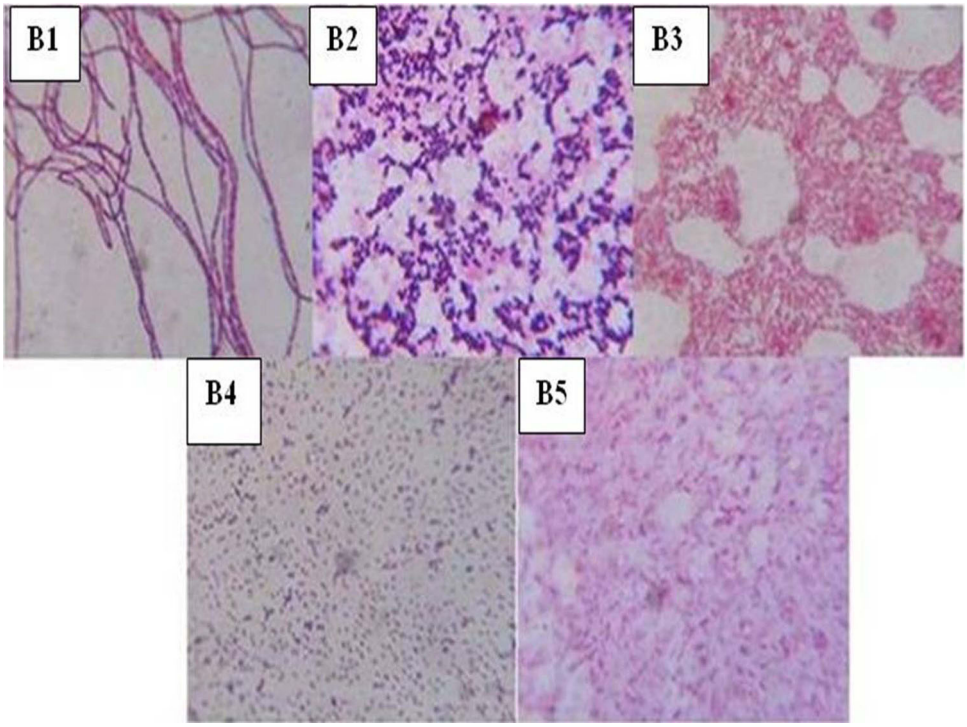

Figure 2. Gram staining of the isolated bacterial strains (magnification 100×).

CFU calculation

CFU has been calculated for the isolated bacteria by dilution plating method. The CFU of the bacterial isolates B1, B2, B3, B4, and B5 were observed as 5×10^6, 366×10^8, 421×10^8, 70×10^7, and 202×10^7, respectively.

The results for bacterial isolates have been summarized in Table 1.

Characterization of fungi, actinobacteria, and yeast

All the fungal isolates were cultured on Sabouraud's Dextrose agar and their morphology was quite different from each other (Fig. 3).

Out of the five isolated fungi, only two were subjected to Gram's staining which were suspected to be actinobacteria, for others, Lactophenol cotton blue staining was done (Fig. 4).

Depending on the above characteristics of fungi, the results have been summarized in Table 2.

Probable nomenclature based on the above studies

Based on the observations made in the biochemical tests and on their comparisons with Mahon and Manuselis [26] and Bryce [27], the probable nomenclature of the isolates have been tabulated in Table 3.

Table 1. Summarized results of biochemical tests performed on the bacterial isolates.

	Bacterial isolates				
Biochemical tests	**B1**	**B2**	**B3**	**B4**	**B5**
Gram's staining	+	+	+	+	−
Shape	Bacillus	Coccus	Coccus	Bacillus	Bacillus
Cultural characteristics on agar plate	Abundant, opaque, white waxy growth	Clear, smooth, small, round	Soft, smooth, yellow growth	Abundant, opaque, white waxy	White, fluffy, irregular
Catalase test	+	−	+	+	+
Oxidase test	−	−	−	−	−
Starch hydrolysis test	+	−	−	+	−
Sucrose fermentation test	+	+	−	+	+
Voges–Proskauer test	−	−	−	−	−

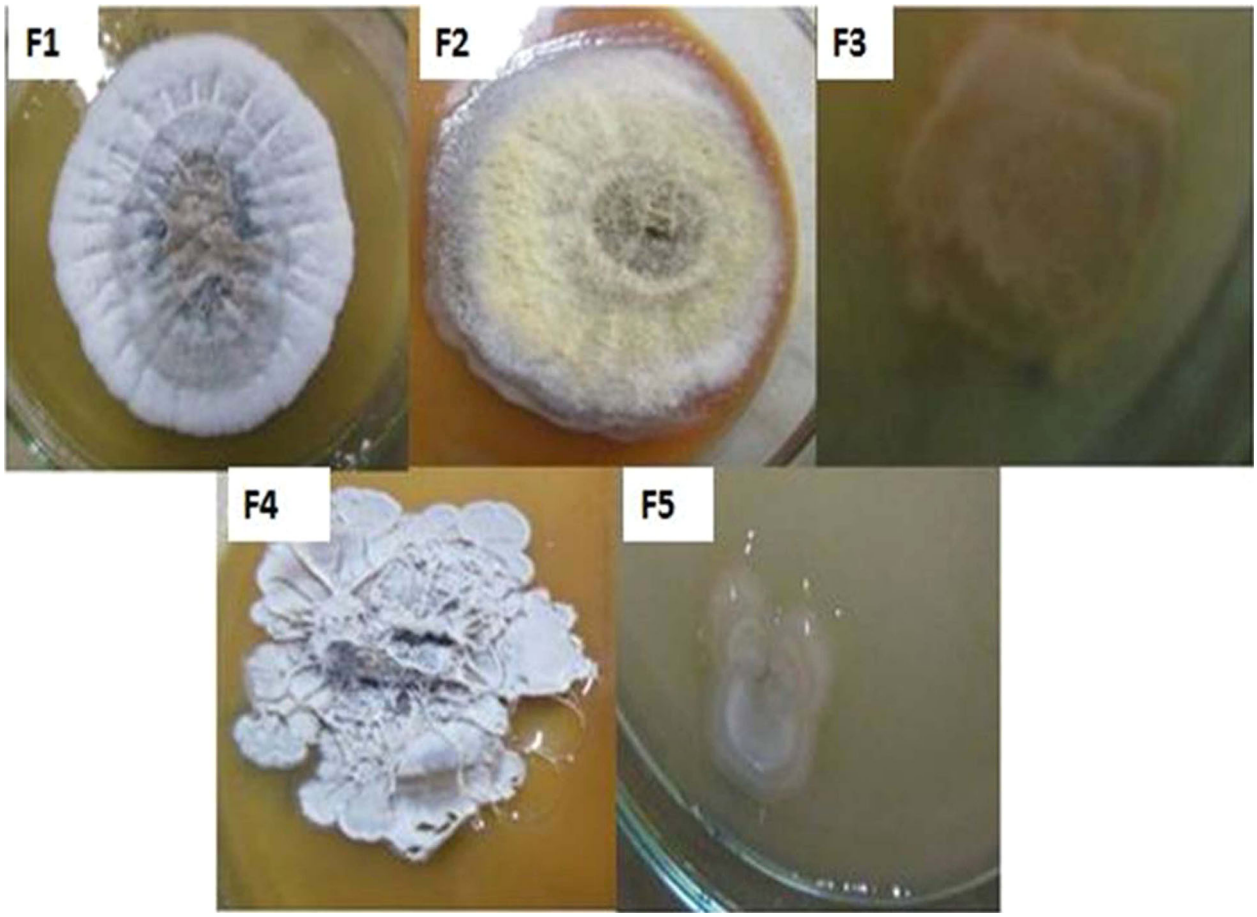

Figure 3. Pure cultures of the fungal isolates on SDA plates for morphological characterization.

Discussion

Role of microbes and earthworms in decomposition of organic matter and particularly in humification is well known [3, 29] and this humification has been shown to be predominantly a microbial process [30–33]. Recently, many earthworms including *Perionyx excavatus* have been shown to aid in humification [34]. The variation in the microbial populations in the earthworm gut may be due to their nutritional needs and digesting ability of the earthworms. Pederesen and Hendriksen [35] reported qualitative and quantitative changes in the bacterial flora of ingested food materials during gut transit. Many authors have studied the microbial community in the gut of earthworms [36, 37]. It is well known that Gram-negative bacteria are common inhabitants of the intestinal canal of earthworms [38]. Edwards [39] reported that vermicompost is rich in microbial populations and diversity, particularly fungi, bacteria, and actinobacteria. The presence of fungal isolates in the earthworm gut and in cast material has been known for some time [7] and earthworm have been implicated in both the reduction and dispersal of soil-borne animal and plant fungal diseases and the spread of beneficial group such as mycorrhizal fungi [40]. Parle [7] reported that population of yeast and fungi did not proliferate during passage through the gut, although actinobacteria and bacteria did. Also, the gut extracts of earthworms have antibacterial and antifungal activity [41]. Edward and Fletcher [2] showed that the number of bacteria and actinobacteria contained in the ingested material by earthworms increased up to 1000-fold by transit through the gut. Whether it is the earthworm, microorganisms stimulated in their gut, or a collective action of both organisms that are responsible for the mineralization of soil is still controversial [21].

In the present study, it was found that the gut of *Perionyx excavatus* collected from both the regions

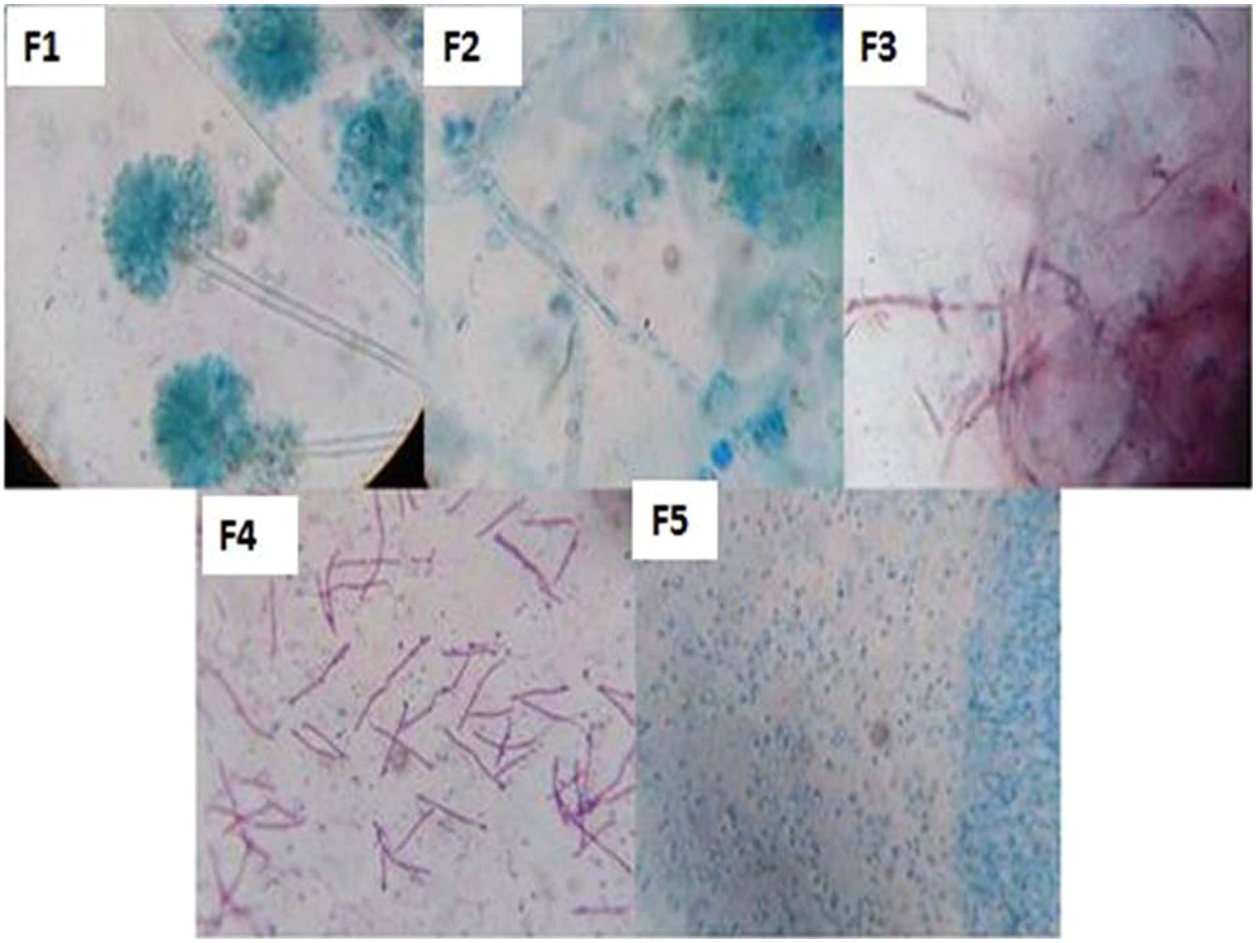

Figure 4. Gram's and lactophenol cotton blue staining of the fungal isolates (magnification 100×).

contain many different species of bacteria, fungi, actinobacteria, and yeast. Five different bacterial and five different fungal species which includes two actinobacteria and one yeast were isolated. This microflora contains many different enzymes like amylase and catalase. The bacterial isolates of genus *Bacillus* sp., *Staphylococcus* sp., *Enterococci*, *Micrococcus* sp., *Enterobacter* sp., and *Citrobacter* sp. were identified and the fungal isolates such as *Aspergilus* sp., *P. boydii* and *Streptomyces* sp., *Nocardia* sp. among the actinobacteria and *Candida* sp. among Yeast were identified and these results are similar to the findings by Parthasarathi [42].

The present scenario in India shows that there is good response from the farmers to adopt the technology for producing vermicompost to use as soil amendment. Present study showed that the gut of *Perionyx excavatus* collected from the undivided Midnapore district contains similar kinds of bacterial and fungal isolates which plays an important role in increasing the soil fertility. Having determined that the gut of *P. excavatus* house distinct gut wall-associated bacterial and fungal communities, the challenge is to further determine the functional significance of these isolates. Understanding the composition and function of the earthworm gut microflora will help designing appropriate management practices for sustainable agriculture and other land uses. The studies are at preliminary stage and it will require some more time to draw any specific conclusions based on the available data. Interdisciplinary applications of earthworm research will further help to understand the importance of these gut-microbes in agriculture as the species that is promising under protected laboratory conditions in a small scale may fail to perform under field conditions when it is expected to work on large amount of organic matter.

Table 2. Summarized results of biochemical and morphological studies on the fungal isolates.

Biochemical and morphological study	Fungal isolates				
	F1	F2	F3	F4	F5
Gram's staining	NA	NA	+	+	NA
Lactophenol cotton blue staining	Blue conidiophores observed	Branched conidiophore	NA	NA	Globose, elliptical and oval cells
Color	Grayish white	creamy	Off white to cream colored	Chalky white	Shiny white
Fungal texture	Circular, rough	Circular, furry	Irregular edges, creamy	Irregular to highly wrinkled colony	Shiny, slimy, irregular

Table 3. Probable nomenclature of the isolated species.

Isolates	Probable nomenclature
B1	*Bacillus cereus*
B2	*Enterococcus fecalis*
B3	*Micrococcus luteus*
B4	*Bacillus megaterium*
B5	*Enterobacter cloacae*
F1	*Aspergilus fumigates*
F2	*Pseudollescheria boydii*
F3	*Streptomyces* sp.
F4	*Nocardia* sp.
F5	*Candida* sp.

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Conflict of interest

The authors have declared no conflict of interest.

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