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***Research paper***



# Isolation and Characterization of Bacteria from Earthworms' Intestines

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**Abstract**

Vermicomposting; breaking down of organic material by earthworms that feed on wastes and converts them to soil-like mass and liquid, is an alternative to reduce waste into the environment. Nitrogen fixing and Phosphorus solubilizing bacteria in the earthworms' intestines are important in promoting plant growth by increasing the Nitrogen (N) and Phosphorus (P) uptake when used as biofertilizers. The objectives of this study are to isolate, identify and characterize N-fixing and P-solubilizing bacteria from the earthworms' intestines. The preparation of vermibeds included the introduction of leachate taken from Sungai Ikan Landfill, Kuala Terengganu. Standard serial dilution procedure was performed to isolate the microorganisms. Next, identification and characterization of bacteria were conducted via gram staining and bacteria morphological characteristic studies. Qualitative screening of N-fixing bacteria was performed by Hach Method 8039 while screening of P-solubilizing bacteria used Pikovskaya's Agar containing insoluble tri-calcium phosphate (TCP). Six isolates, (A1, A2, B1, B2, C1 and D1) were successfully isolated. However, only single colony of A1, A2, B1 and B2 were obtained. All were gram negative and bacilli except for A1. A2 showed the highest nitrogen fixing and phosphate solubilizing activity though with the nitrate content (148.6 mg/L) and high phosphate solubilization efficiency (10.6).

***Keywords***: *Bacteria; Intestines; Nitrogen Fixing; Phosphorus Solubilizing; Vermicomposting*

## 1. Introduction

In Malaysia, the generation of municipal solid waste has amplified for more than 91% for the past decade [1]. As a result, these solid wastes are being disposed to the landfill site as it is the most preferred method due to technical feasibility, ease of operation, minimum supervisions and low operation expenditure [2]. However, the practice of landfilling has a major consequence, which is the excessive generation of leachate. Leachate is made up of rain that passes through a landfill site and liquids that are generated by the breakdown of the waste within the landfill [3]. The toxic materials and heavy metals in leachate may cause prolonged harmful health problems to human body. If not properly treated and safely disposed, landfill leachate could bring significant threat to surface water and groundwater as it may percolate throughout soils and subsoils, causing adverse impacts to receiving waters and subsequently living organisms [2] .

Therefore, a viable option is proposed, which is vermicomposting. This alternative waste treatment method consists of utilizing earthworms to break down organic wastes in the leachate and convert them to soil-like masses and liquid, which can be used as a soil conditioner [4]. The biochemical decomposition of waste materials is primarily attributed to the microbial activity including Nitrogen (N) fixing and Phosphorus (P) solubilizing bacteria activity during vermicomposting; where the earthworms contribute to fragmentation and conditioning of the substrate which stimulate the microbial population thereby increasing the surface area available for microbial activity [5]. The participation of microorganisms which are N-fixing and P-solubilizing bacteria, within the digestive tract of the earthworms is of great importance as they play the key role in degrading wastes and stabilizing nutrient availability in the processed materials.

Under these perspectives, the present study was designed to isolate and characterize productive N-fixing bacteria (NFB) and P-solubilizing bacteria (PSB) from the intestinal microflora of earthworms, *Eisenia fetida* reared in vermibeds containing landfill leachate. These bacteria could be further exploited not only in the degradation of wastes but also as beneficial biofertilizers to enhance plant growth.

## 2. Experimental

### 2.1. Preparation of vermibeds

Two vermibeds were prepared for the breeding of earthworms within a plastic vermibin for 21 days, both containing 1 kg of soil and 90 earthworms. Each vermibin was added with 150 mL of leachate at the initial of the vermicomposting process and followed by another 150 mL leachate in the middle of the process. The leachate was procured from Sungai Ikan Landfill, Kuala Terengganu, Malaysia. The Organic Agriculture Centre of Canada highlighted that worm will die if its skin dries out [6]. Thus, in order for the worms to have a livable environment, the moisture content for both vermibeds was maintained at 65 to 75 % by sprinkling water occasionally. Each vermibin was covered with a black plastic to shield the vermicompost from the sun and left at room temperature. Vermicompost was manually turned every week to aerate the soil and maintain good porosity in the vermibin.

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Nitrogen and Phosphorus content in the vermibeds were continuously monitored and the earthworms living in the one showing higher concentration of N and P at the end of vermicomposting period were used for further experiments.

### 2.2. Isolation of N-fixing and P-solubilizing microbe from earthworms' intestines

Five well grown matured specimens of earthworms, with the average length of 10 cm, were collected after 21 days of vermicomposting for microbial analysis. The earthworms were washed with sterile water and placed on moist filter papers and allowed to starve overnight for intestine / gut evacuation. After starvation, the earthworms were disinfected with 70% ethanol for 30 s and blended. The gut content was suspended in 10 mL of sterile 0.85% NaCl solution and vigorously stirred for 15 min [7]. The gut content mixture was then diluted by using serial dilution technique. Dilution factors of $10^1$ to $10^{10}$ were tested to observe the development of microbes on Nutrient Agar plates. The microbial population in the earthworms' intestines was enumerated using pour plate technique on the Nutrient Agar. The microbes underwent 24 hr of incubation at 37°C. Subsequently, every present microbe with different form was transferred onto new nutrient agar plate and purified by streak-plate technique until single colony was obtained.

### 2.3. Identification and characterization of microbes

The isolated pure microbe colonies were identified and characterized by observing their morphological characteristics and bacterial shape via gram staining technique[8]. A thin smear of bacterial isolates was separately made on clean glass slides. The smear was fixed by rapidly passing through the flame of a Bunsen burner for three times. Then, the smear was stained by crystal violet for one minute and rapidly washed with water followed by flooding with gram's iodine. After one minute, the slide was washed again with water and decolorized with alcohol. The smear was washed immediately with water and covered with safranin for one minute. The slide was then washed and air dried, and finally observed under microscope. The gram-negative organisms would show pink stain while the gram-positive organisms would be dark violet in colour. Mix isolate (mixture of gram negative and gram positive) obtained were repeatedly sub-cultured until pure isolate was obtained**.**

### 2.4. Qualitative screening of N-fixing and P-solubilizing bacteria

The pure isolates were tested for nitrate by applying a modified method by Sivanskari and Anandharaj [11], whereby the nitrogen fixing activities were observed on the basis of the formation of turbidity in the flasks containing initially nitrogen-free medium. In this study, the isolates were priorly inoculated in nutrient broth separately at 37°C for 24 hr and were then checked for the nitrate concentration by using Hach Spectrophotometer DR/2400 (Method 2039). Nitraver 5 Nitrate Reagent Powder Pillows were used as standard in this method to detect the presence of nitrate concentration (mg/L) in the bacteria inoculum. Meanwhile, Phosphate solubilization test was conducted by inoculating the bacteria (10 µL) at the center of Pikovskaya's agar (PKV) via hole punch method and incubated at 37°C. The diameter of the halo zone formed around the colony was measured after 7 days. The Phosphate Solubilization Efficiency (PSE) was determined by measuring the total halo zone of the colony and the colony diameter [9].

$$\mathbf{PSE} = \frac{\mathbf{Colony\ diameter + Halo\ zone\ diameter}}{\mathbf{Colony\ diameter}}$$

## 3. Results and Discussions

### 3.1. Isolation and identification of isolates

In total, four pure isolates were obtained from the repeated streaking process (A1, A2, B1 and B2). These isolates exhibited various morphologies in terms of form, elevation and margin (Table 1). All four isolates were found to be gram negative as the isolates were stained in pink. Three of them (A2, B1 and B2) exhibited rod shape, which strongly suggested that they are bacilli (Table 2). These findings indicated that the isolates might be NFB or PSB, as several researchers claimed that both the N-fixing and P-solubilizing bacteria display the gram negative colour and are rod-shaped [7,8]. The finding of Sharma[10] in his study of P-solubilizing microorganism from soil also concurred with the present results, thus supporting that the bacteria isolates here might be PSB.

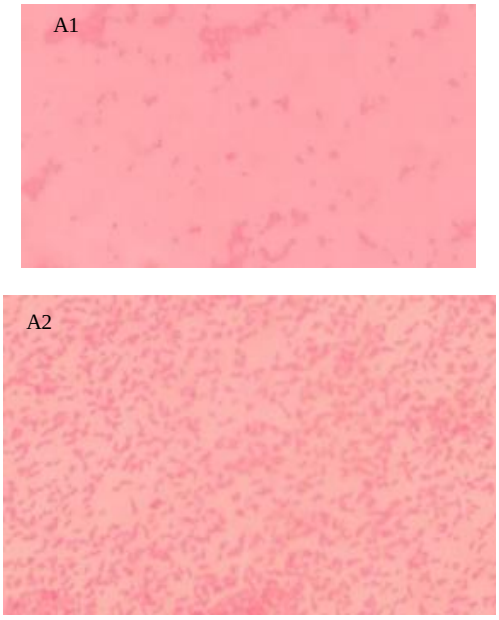
**Table 1:** Morphological characteristics of the isolates

| Isolate | Form | Elevation | Margin |
|---|---|---|---|
| A1 | Circular | Convex | Entire |
| A2 | Spindle | Flat | Undulate |
| B1 | Irregular | Flat | Undulate |
| B2 | Circular | Convex | Entire |

**Table 2:** Gram staining results of the isolates

| Isolate | Gram reaction | Bacterial shape | Pure/ Mix |
|---|---|---|---|
| A1 | Negative | Cocci | Pure |
| A2 | Negative | Bacilli | Pure |
| B1 | Negative | Bacilli | Pure |
| B2 | Negative | Bacilli | Pure |

Figure 1 depicts the bacterial shape of the isolates observed under the microscope. The shape of A1 was more circular and physically diverse from the other isolates which appeared to be long and rod-shaped (A2, B1 and B2). Similar observations were also reported by Khan and Uma [9,10]. Gram negative bacteria are referred to as having two membranes, having inner cell membrane and thinner peptidoglycan layer compared to gram positive bacteria [13]. Due to this thin peptidoglycan layer, it was easily broken when being decolorized during gram staining tests, therefore causing the gram negative microbes to take up the pink colour of the safranin dye.



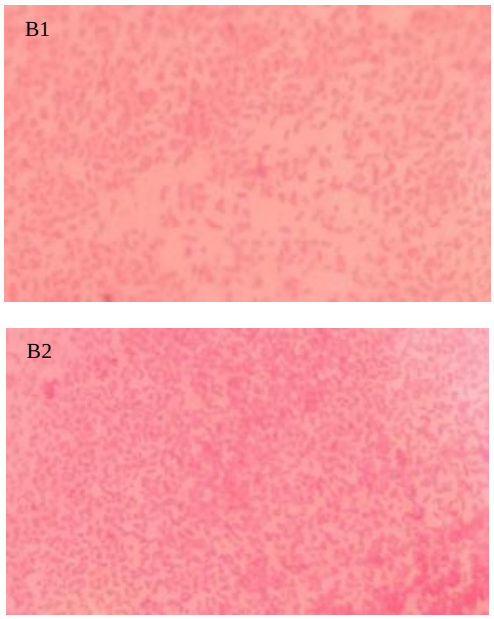



**Fig. 1:** Bacterial shape of the isolates obtained under microscope: A1) Gram negative, cocci A2), B1) and B2) Gram negative, bacilli

### 3.2. Qualitative analysis of N-fixing bacteria

Table 3 shows the different nitrate concentration of each isolate. A2 has the highest concentration of nitrate (148.6 mg/L), followed by A1 (135.3 mg/L), B1 (23.7 mg/L) and B2 (13.9 mg/L). High concentration of nitrate indicated that these bacteria strains possessnitrogen fixation ability. Nitrogen fixation is a process by which the nitrogen is converted to a plant available form. It is one of the basic requirements for the growth, productivity, and yield of plants. The ultimate source of the nitrogen used by plants is Nitrogen ($N_2$) gas, which constitutes 78% of the Earth's atmosphere. Unfortunately, majority of the plants could not metabolize $N_2$ directly into protein[14]. Therefore, the N-fixing bacteria is vital in promoting plant growth by converting the $N_2$ into usable forms, such as nitrate. Figure 2 further demonstrated that an increment in nitrate concentration resulted in increased turbidity. As seen in the figure, A2 was the most turbid while B2 was the least turbid. This signified that the high nitrate content in the NFB was successfully detected by the Hach method.

**Table 3:** Nitrate concentration of each isolate

| | A1 | A2 | B1 | B2 |
|---|---|---|---|---|
| $NO_3^-N$ (mg/L) | 135.3 | 148.6 | 23.7 | 13.9 |

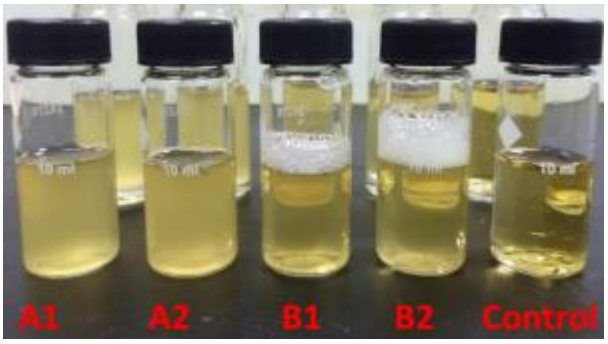



**Fig. 2:** Comparison of nutrient broth containing isolates against blank as control.

### 3.3. Qualitative analysis of P-solubilizing bacteria

Phosphorus is a plant macronutrient that plays a significant role in plant metabolism, second only to nitrogen in requirement for plants. However, most soil phosphorus, around 95 to 99 percent, is present in the form of insoluble phosphates and hence cannot be utilized by plants [15]. Several studies have been carried out to investigate the ability of bacterial species from the earthworms' gut to solubilize the insoluble phosphate compounds, such as tricalcium phosphate of Pikovskaya's medium [12][7]. The phosphorus solubilizing activity is determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, with the latter being converted to soluble forms [9]. This was signified by the formation of halo zones using specified PKV plate as portrayed in Figure 3.

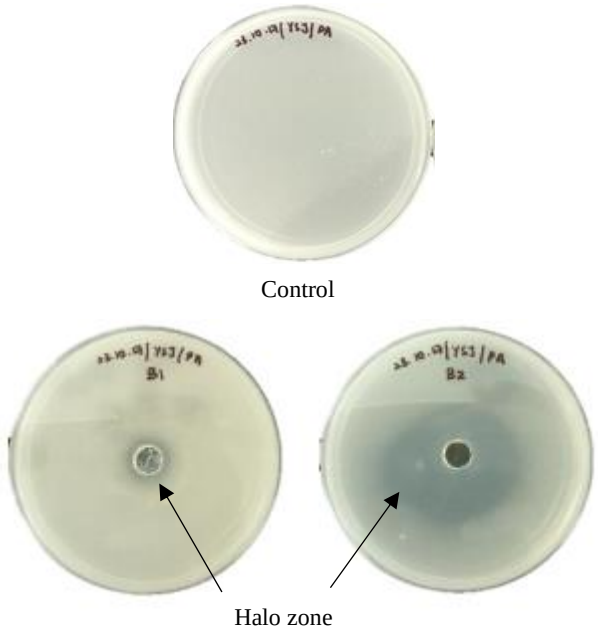



**Fig. 3:** Halo zone developed by B1 and B2 compared against a blank PKV plate as control

Upon screening, all isolates showed varying levels of phosphate solubilizing activity in agar plate using PKV medium. Thus, all were found to be potent phosphate solubilizers. The PSE measurement was presented in Table 4. Among these four potent isolates, A2 showed maximum PSE (10.6) while B1 showed the least efficiency (2.6). Strains developing clear zones around their colonies could easily be identified as PSB [9], and large zone of clearance indicated high efficiency of Phosphorus solubilization [16]. Figure 3 also showed different diameters of clear halo zones developed, indicating the extent of phosphate solubilization efficiency. According to Sivasankari, the halo zone was formed due to solubilization of insoluble phosphates by organic acid secretion [14].

**Table 4:** Phosphate solubilization efficiency of each isolate

| | A1 | A2 | B1 | B2 |
|---|---|---|---|---|
| Clear Zone | + | + | + | + |
| Colony Diameter (cm) | 0.8 | 0.8 | 0.8 | 0.8 |
| Halo Zone Diameter (cm) | 2.6 | 7.7 | 1.3 | 4.3 |
| Phosphate Solubilization Efficiency | 4.3 | 10.6 | 2.6 | 6.4 |

## 4. Conclusion

Four bacterial strains (A1, A2, B1 and B2) were successfully isolated from the intestines of earthworms, *Eisenia fetida* grown in vermibeds containing landfill leachate. These isolates were identified as Nitrogen fixing and Phosphorus solubilizing bacteria based

on specified characterization works. All isolates were found to be gram negative and bacilli except for A1 which was cocci. A2 was discovered having the highest Nitrogen content (148.6 mg/L) as well as demonstrating the highest phosphate solubilization efficiency. These information is essential for further exploration of the NFB and PSB in waste degradation or as potential biofertilizers for future agriculture.

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## References

[1] Periathamby A, Hamid FS, K Khidzir (2009), Evolution of solid waste management in Malaysia: Impacts and implications of the solid waste bill, J. Mater. Cycles Waste Manag. 11, 96–103, available online: doi:10.1007/s10163-008-0231-3.

[2] Kamaruddin MA, Yusoff MS, Aziz HA, Hung YT (2015), Sustainable treatment of landfill leachate. Appl. Water Sci. 5,113–126, available online: doi:10.1007/s13201-014-0177-7.

[3] Wiszniowski J, Robert D, Surmacz-Gorska J, Miksch K, Weber JV (2006), Landfill leachate treatment methods: A review. Environ. Chem. Lett. 4, 51–61, available online: doi:10.1007/s10311-005-0016-z.

[4] Marangon KC, Henrique E, Silva DF, Lucas K, De I, Rodrigues S (2014), Impact of Vermicomposting products in soil fertility of degraded Brazilian Cerrado and the perspective to minimize water pollutants release.

[5] Revolution G, Earthworm T, District E, Prabha L (2006), 4. results and discussion,.

[6] Pfiffner L (2014), Earthworms – Architects of fertile soils. Their significance and recommendations for their promotion in agriculture. Tech. Guid. Earthworms 9.

[7] Hussain N, Singh A, Saha S, Kumar MVS, Bhattacharyya P, Bhattacharya SS (2016), Excellent N-fixing and P-solubilizing traits in earthworm gut-isolated bacteria: A vermicompost based assessment with vegetable market waste and rice straw feed mixtures. Bioresour. Technol 222, 165–174, available online: doi:10.1016/j.biortech.2016.09.115.

[8] Gislin D, Sudarsanam D, Raj GA, Baskar K (2018), Antibacterial activity of soil bacteria isolated from Kochi, India and their molecular identification. J. Genet. Eng. Biotechnol, available online: doi:10.1016/j.jgeb.2018.05.010.

[9] Karpagam T, Nagalakshmi PK (2014), Original esearch Article Isolation and characterization of Phosphate Solubilizing Microbes from Agricultural soil. Int. J. Curr. Microbiol. Appl. Sci 3, 601–614.

[10] Sharma S, Kumar V, Tripathi RB (2011), Isolation of Phosphate Solubilizing Microorganism (PSMs) From Soil. J. Microbiol. Biotechnol. Res. Sch. Res. Libr. 1, 90–95.

[11] Khan HR, Rahman M (2008), Enumeration , Isolation and Identification of Nitrogen-Fixing Bacterial Strains at Seedling Stage in Rhizosphere of Rice Grown in Non-Calcareous Grey Flood Plain Soil of Bangladesh, 13, 97–101.

[12] Maheswari NU, Sudha S (2013), Enumeration and detection of phosphate solubilizing bacteria from the gut of earthworm varieties. J. Chem. Pharm. Res. 5, 264–267.

[13] Vollmer W, Höltje J, Ho J (2004), The Architecture of the Murein ( Peptidoglycan ) in Gram-Negative Bacteria : Vertical Scaffold or Horizontal Layer ( s )? MINIREVIEW The Architecture of the Murein ( Peptidoglycan ) in Gram-Negative Bacteria : Vertical Scaffold or Horizontal Layer ( s )?†, 186, 5978–5987. available online: doi:10.1128/JB.186.18.5978.

[14] Sivasankari B, Anandharaj M (2014), Isolation and Molecular Characterization of Potential Plant Growth Promoting Bacillus cereus GGBSTD1 and Pseudomonas spp. GGBSTD3 from Vermisources, available online: doi:10.1155/2014/248591.

[15] Kadiri DD, Gorle N, Varada K, Peetala R, Peela S (2013), Isolation , Screening and Identification of Phosphate Solubilizing Bacteria From Different Regions of, 4, 518–526.

[16] Shanti RI, Rekha S, Anitha AA (2012), Analysis of Nitrogen and Phosphate in Enriched and Non Enriched, J. Environ. Res. Dev. 7, 899–904.