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Isolation, Characterization and Identification of Bacteria from Homemade Fertilizer

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Abstract

The rising demand for natural resources has resulted in the usage of chemical fertilizers. Biological fertilizers must be mandated to safeguard humans and the environment from toxic chemical fertilizers. As a result, this study focuses on isolating, characterizing, and identifying bacteria from homemade biofertilizers, which are environmentally friendly and help boost plant growth. Two distinct media of purple non-sulfur bacteria enrichment medium (PNSBEM) and nutrient agar were used to grow bacteria isolated from the homemade fertilizer. The optimum bacterial growth study was conducted under aerobic, facultative, and obligate anaerobic conditions. Further characterization of isolated bacteria, including Gram staining, catalase assay, pathogenicity assay and partial 16s rRNA sequencing, reflects Gram-positive Clostridium sp. The bacteria isolated from the homemade fertilizer showed its ability to synthesize auxin components such as Indole acetic acid (IAA) and aminolevulinic acid (ALA). Homemade fertilizer has the potential to be an effective biofertilizer since it shows a favourable outcome in the IAA component, which is vital in controlling plant growth and development.

Keywords Homemade fertilizer, purple non-sulfur bacteria, *Clostridium spp.*, indole acetic acid (IAA), aminolevulinic acid (ALA)

1.0 INTRODUCTION

The production of homemade fertilizers is one of the first steps toward improving agriculture and is profitable if done correctly. Organic fertilizers improve soil texture, allowing it to retain water for longer periods and enhancing bacterial and fungal activities, benefiting plants and soil (Kyaw et al., 2018). Continuous and excessive use of pesticides and chemical fertilizers causes damaging effects on our health and environment compared to chemical fertilizers (Santra et al., 2015). Understanding the linked microorganisms is essential for producing an efficient fertilizer. Rhizobium, mycorrhiza, blue-green algae, and purple non-sulfur bacteria (PNSB) are the most prevalent microorganism's involved in biofertilizers.

These microorganisms' operations and interactions are poorly understood, particularly in real-world applications (Mitter et al., 2021). Beneficial microorganisms, such as PNSB, are commonly utilized in biofertilizers but are inconvenient to certain users due to a lack of understanding about their advantages and applications. Furthermore, without additional research, whether phytohormones or plant growth regulators are included in the biofertilizer is unclear. Even though the biofertilizer works well when applied to plants, it is critical to research their functionality thoroughly.

This research aimed to isolate PNSB and other bacteria from previously manufactured homemade fertilizers using PNSB enrichment medium (PNSBEM) and nutrient broth. The isolated PNSB was then identified and described using 16s rRNA and Gram staining. Finally, a spectrophotometer was used to investigate the production of indole acetic acid (ALA) and aminolevulinic acid (IAA), both of which promote plant development (PGPS).

2.0 MATERIALS AND METHODS

2.1 Cultivation and Isolation

Referring to Suhaidah (2019), 5 L unchlorinated rainwater, 3 chicken eggs, 10 g (2 teaspoons) sodium glutamate, 5 g (1 teaspoon) belacan (shrimp paste), and 38.35 g (3 tablespoons) fish sauce were combined to make homemade fertilizer. These components, including the eggshells and water, were well mixed and stored in a light-filled area. The solution was mixed every 2-3 days for 20 days until bloomed.

Samples were taken from the homemade fertilizer stock and performed dilution up to 10^{-5} followed by spreading on agar by spread plate technique on Purple Non-Sulphur Bacteria Enrichment Medium (PNSBEM) agar (1 g/L NH_4Cl, 0.5 g/L Na_2HPO_4, 0.2 g/L $MgCl_2$, 2 g/L NaCl, 2 g/L yeast extract, 6 mL of 80% sodium lactate, 2 % agar powder and distilled water up to 1000 mL). The medium was then adjusted to pH 7 and sterilized via autoclaving) and nutrient agar (NA)(Oxoid). Different colonies detected on each plate were inoculated and streaked on PNSBEM agar and NA. The colony from each plate was transferred and sub-cultured in a new PNSBEM and NA to obtain pure culture. Approximately 10% of the pure culture of each plate was transferred into new media and incubated at room temperature (30°C) with access to light for 4-7 days until purple bloom was formed.

2.2 Bacterial Identification

The DNA of pure cultures was extracted using Wizard Genomic Promega DNA purification kit. The extracted DNA undergoes PCR amplification, which consists of a reaction mixture: 16s rRNA forward primer, reverse primer, DNA template, nuclease-free liquid and GoTaq® Green Master Mix. The PCR amplification reaction was performed as follows: 5 minutes at 94°C (denaturalization), 30 cycles of 1 minute at 94°C (denaturalization), 1 minute at 55°C (annealing), 4 minutes at 72°C (extension), 27 and 10 minutes at 72°C (final extension). The PCR product was observed on 1% agarose gel electrophoresis, and Midori Green was used as a dye. The PCR product was sent for sequencing at Apical Scientific Sdn Bhd. The partial 16S rRNA sequences were compared with GenBank database on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by BLASTN. CLUSTAL W was used for multiple alignments, and a phylogenetic tree was constructed using MEGA X (Ver 10.2.4).

2.3 Characterization of Isolated Bacteria

A loop of pure colonies from each medium was placed on a slide and air-dried before being heated by passing it several times over a flame. Five drops of crystal violet were allowed to rest for 1 minute in the culture. Five drops of iodine solution were then applied, and the area was washed with water after 30 seconds. The slide was inverted and decoloured with an acetone-alcohol solution until the purple hue disappeared. The sample was then cleaned promptly and inspected under a microscope with immersion oil after applying 5 drops of safranin.

The test tube was filled with 1-2 mL of hydrogen peroxide for the catalase test. Several bacteria colonies were selected from each sample and submerged in hydrogen peroxide solution with a sterilized glass rod. Following that, any bubbling formation was recorded.

The bacteria colonies were picked up with an inoculating loop for the pathogenicity test and streaked onto a fresh, sterile blood agar medium. The inoculation plate was then incubated at 35-37°C. After 24 hours, the plate was examined to see if it had darkened or discoloured in the growth location.

2.4 Plant Growth Precursor Assay

All samples were grown in PNSBEM and NA media for 5 days at pH 4.5, with L-tryptophan 3 mM added as a precursor for IAA production. After harvesting, the strain was centrifuged at 8000 rpm for 15 minutes. The mixture was allowed to rest for 30 minutes at room temperature after adding 2 mL of Salkowski's reagent and 1 mL of supernatant to a test tube. The solution was observed using a spectrophotometer with an absorbance wavelength of 535 nm. The testing was carried out at different intervals every 24 hours for 7 days (Gang et al., 2019).

Bacterial samples were isolated and centrifuged at 8,000 rpm for 20 minutes. 2 mL of the bacterial supernatant was mixed with 1 mL sodium acetate buffer (pH 4.7) and 0.5 mL acetylacetone (2,4-pentanedione) in a screw cap tube. The mixture was boiled at 100°C in a water bath for 15 minutes. After 30 minutes, the tube was filled with 3.5 mL of Ehrlich's reagent. The concentration of ALA content was calculated based on the ALA standard curve. ALA content was observed every 24 hours for 7 days.

3.0 RESULTS AND DISCUSSION

3.1 Cultivation and Isolation

Compared to the initial day of preparation, the liquid fertilizer turned to a murky yellowish appearance from clear yellowish after 20 days of incubation (Figure 1). The presence of PNSB from *belacan* or fresh shrimp brain, which can produce bacteriochlorophylls a or b and different carotenoids from the spirilloxanthin, rhodopinal, spheroidene, or okenone series as photosynthetic pigments that undergo anoxygenic photosynthesis, theoretically cause the colour changes to be reddish to purplish (Madigan et al., 2009). However, the fertilizer did not change its colour as expected due to various unexpected circumstances. Rain is a significant element that may contribute to the failure of PNSB growth. PNSB can change their metabolism as long as there is light and oxygen for photosynthesis, according to Chen et al. (2020). Furthermore, biofertilizer formulation accuracy might be regarded as one reason contributing to the failure of blooming. The original measurement for the components is in tablespoons and teaspoons rather than uniform measuring units when referring to the exact formulation (mg, g, kg, mL, L). Aside from that, because PNSB are known to develop the best in anaerobic conditions, there is a chance that the bottle tops are not completely tight, allowing oxygen access.

Table 1 shows the development of bacteria on the streak plate after overnight incubation. The growth finally indicated that diverse bacteria are presented in the homemade fertilizer, but additional testing is required to determine its functioning or usefulness. After the colony had been developed overnight, 5 separate colonies from PNSBEM and NA were chosen for the subsequent investigation. Two of the five colonies were recovered from the PNSBEM plate and designated as A1 and A2, while the other three colonies were acquired from the NA and labelled as B1, B2, and B3.

Figure 1 A: Day 0 of homemade fertilizer; B: Day 20 of homemade fertilizer

3.2 Characterization of Isolated Bacteria

Following the Gram staining processes, the cell shape or unique structure of bacteria strains (B1 and B2) and PNSB strains (A1 and A2) was assessed using LEICA light microscopy. As shown in Table 2, all samples (A1, A2, B1, and B2) tested were Gram-positive by displaying a purple colour with a rod form.

All bacterial strains (A1, A2, B1 and B2) do not show any hydrogen peroxide bubbling and indicated that all strains do not have catalase enzyme. Since the bacteria were cultivated under obligate and facultative anaerobic conditions, they lack the capacity to produce harmful by-products (H_2O_2) and superoxide radicals (O_2^-) through metabolic activity. These products are toxic to organisms and, if it is not broken down, they can induce cell lysis.

Referring to Table 3, all samples (A1, A2, B1 and B2) had a brownish and opaque colony, suggesting γ-hemolysis, which occurs when the organism does not manufacture hemolysin enzyme. As a result, all samples may be considered non-hemolytic (no or low pathogenicity level). The pathogenicity test was also carried out using the biofertilizer, and no alterations in the blood agar were seen, with only brownish colonies forming. As a result, the biofertilizer does not create hemolysin enzyme, suggesting γ-hemolysis with no pathogenic impact, and is deemed to be harmless when applied to plants and soil.

Table 1 The growth of bacteria on PNSBEM and nutrient agar.

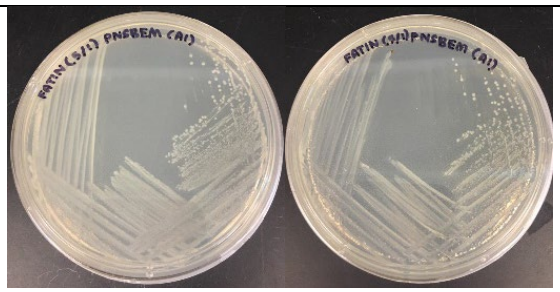
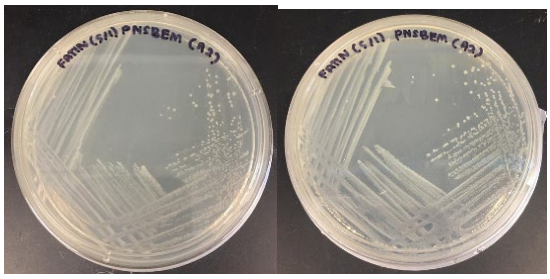
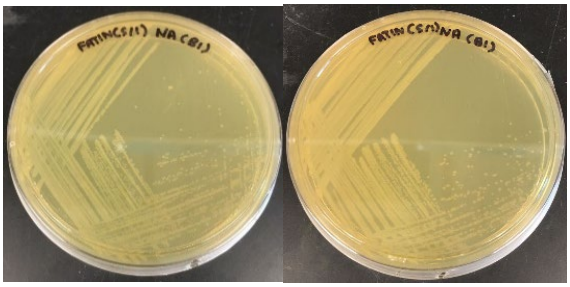
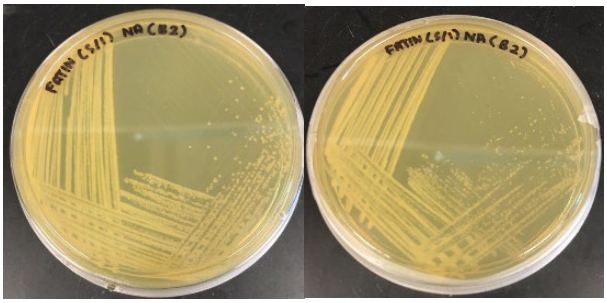
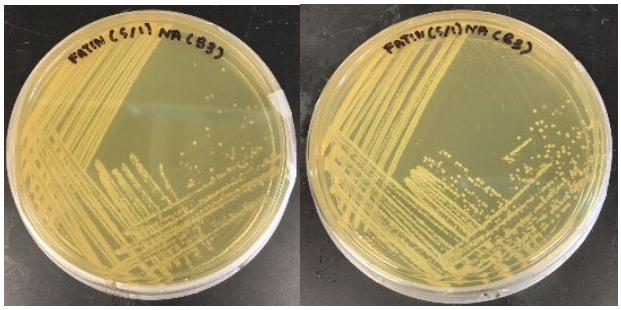
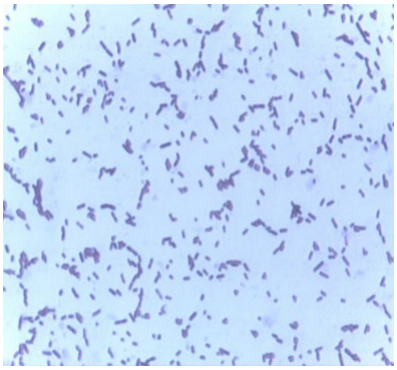
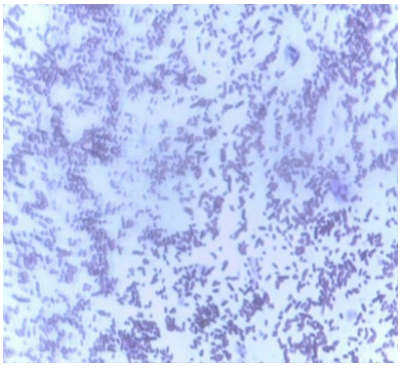
Plates		Results	Characteristics of colony
PNSBEM	A1		• Shape: circular • Elevation: convex • Texture: mucoid • Opacity: translucent • Colour: white
	A2		• Shape: circular • Elevation: convex • Texture: mucoid • Opacity: translucent • Colour: white
Nutrient agar	B1		• Shape: circular • Elevation: convex • Texture: moist • Opacity: opaque • Colour: pale yellow
	B2		• Shape: circular • Elevation: convex • Texture: moist • Opacity: opaque • Colour: pale yellow
	B3		• Shape: circular • Elevation: convex • Texture: moist • Opacity: opaque • Colour: pale yellow

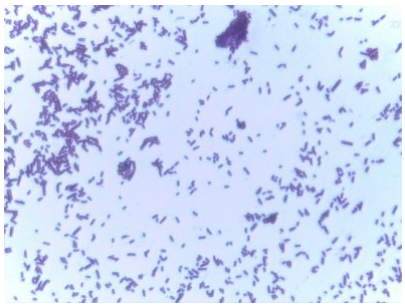
Table 2: Gram staining of PNSB and bacterial colonies.

Plates	Results	Explanation
PNSBEM		

A1		• Total magnification: 1000X
		  ○ Eyepiece magnification: 10X
		  ○ Objective lens magnification: 100X
		• Rod in shape
		• Purplish in colour

A2		• Total magnification: 1000X
		  ○ Eyepiece magnification: 10X
		  ○ Objective lens magnification: 100X
		• Rod in shape
		• Purplish in colour

Nutrient agar		

B1		• Total magnification: 1000X
		  ○ Eyepiece magnification: 10X
		  ○ Objective lens magnification: 100X
		• Rod in shape
		• Purplish in colour

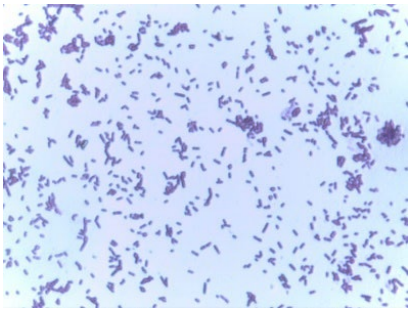
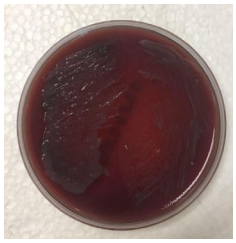
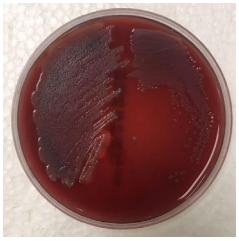
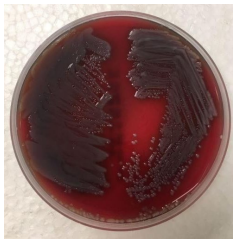
B2		• Total magnification: 1000X
		  ○ Eyepiece magnification: 10X
		  ○ Objective lens magnification: 100X
		• Rod in shape
		• Purplish in colour

Table 3 Pathogenicity test on bacterial samples.

Results	Explanation
A1 	• No change in the blood agar • Brownish colony • β-hemolysis
A2 	• No change in the blood agar • Brownish colony • β-hemolysis
B1 	• No change in the blood agar • Brownish colony • β-hemolysis
B2 	• No change in the blood agar • Brownish colony • β-hemolysis
Biofertilizer 	• No change in the blood agar • Brownish colony • β-hemolysis

3.3 Bacterial Identification

For sample sequencing using NCBI BLAST, all samples could be categorized into the appropriate *Clostridium* species. Sample A1 (Figure 2) had 99% similarity to *Paraclostridium benzoalyticum* along 99% of the query cover. Sample A2 (Figure 3) was 92.14% identical to *Paraclostridium bifermentans* throughout 99% of the query cover. Samples B1 (Figure 4) and B2 (Figure 5) were likewise linked to *Paraclostridium bifermentans*, with 93.63% similarity along 25% query cover and 95.56% similarity along 98% query cover, respectively. MEGA-X was used to construct the phylogenetic tree (ver.10.2.4).

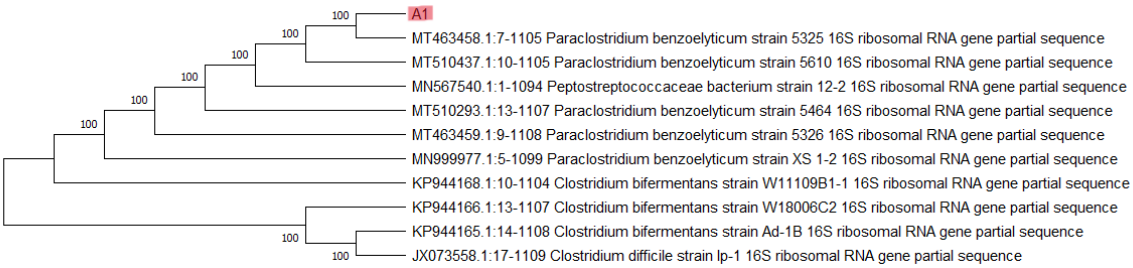


Figure 2 Phylogenetic tree of sample A1

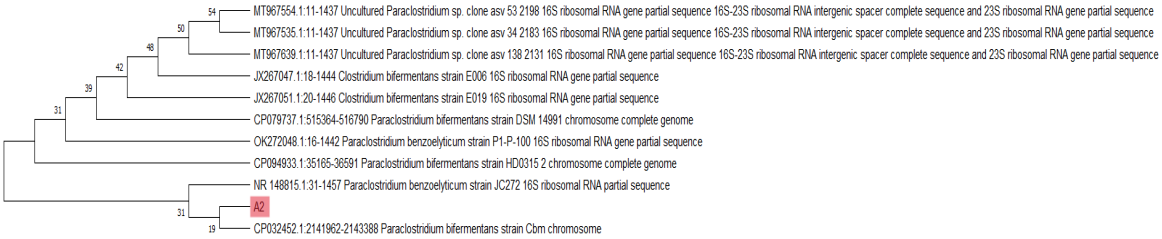


Figure 3 Phylogenetic tree of sample A2

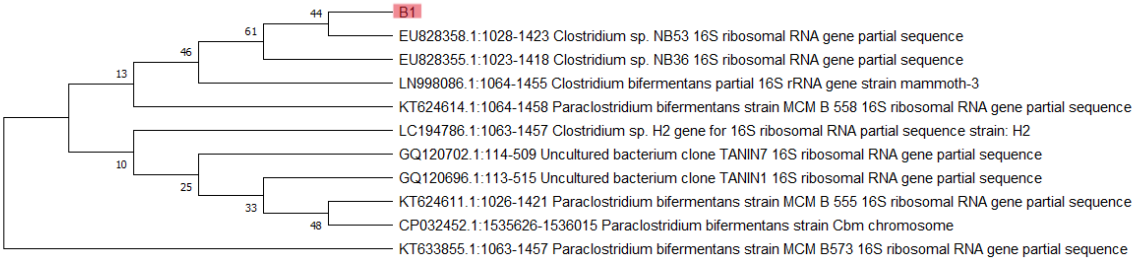


Figure 4 Phylogenetic tree of sample B1

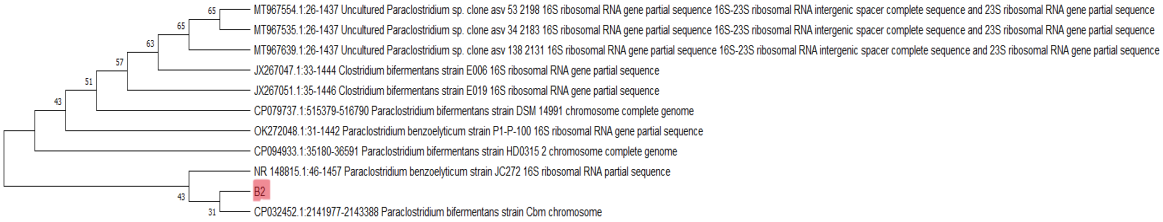


Figure 5 Phylogenetic tree of sample B2

According to Figueiredo et al. (2020), *Clostridium sp*. bacteria functions in a variety of contexts, giving agro-ecological advantages in plant growth promotion and involvement in industrial processes, and substituting chemicals that are damaging to the environment in both situations. This study was also supported by Zeiller et al. (2015) stated that inoculation of *Clostridium botulinum* 2301 spores on clover seedlings resulted in increased plant development and was effective for bacterial and endophytic colonization of the rhizosphere. For that reason, it has been proven that certain *Clostridium* sp. may be beneficial as plant growth-promoting components in biofertilizers to be applied on plants.

3.4 Indole Acetic Acid and Aminolevunilic Acid Assays

Since all samples were categorized as *Paraclostridium* family, the representative bacterial samples isolated from PNSBEM (A1) and nutrient agar (B2) were chosen for IAA detection using Salkowski's technique. As shown in Figure 5, the IAA concentration in A1 sample increased from 2.50 g/mL to 22.17 g/mL, 43.83 g/mL, 49.17 g/mL, 74.50 g/mL, 79.50 g/mL, and 82.17 g/mL after 7 days of observation. For A2 sample, the IAA concentration also displays an improvement from 65.50 µg/mL, 90.50 µg/mL, 123.50 µg/mL, 131.50 µg/mL, 151.17 µg/mL, 150.50 µg/mL and 205.83 µg/mL throughout 7 days of observation as in Figure 6. Figure 7 depicts the escalation of absorbance readings and IAA concentrations over 7 days of observation on biofertilizer. The

IAA concentrations recorded were 334.83 g/mL, 357.17 g/mL, 412.17 g/mL, 472.17 g/mL, 490.5 g/mL, 620.5 g/mL, and 728.5 g/mL.

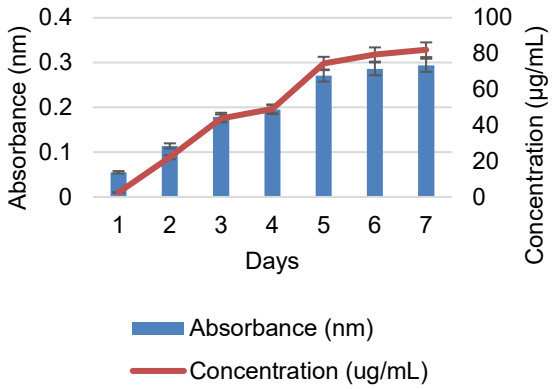


Figure 5 Growth and IAA concentration vs day for sample A1.

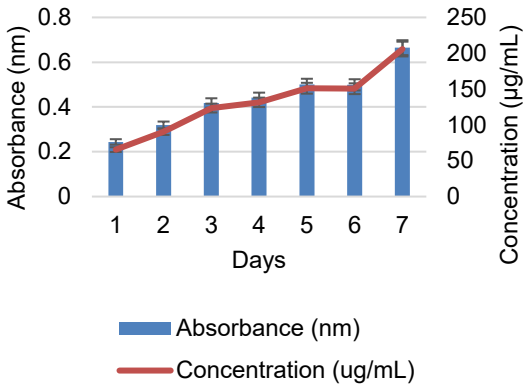


Figure 6 Growth and IAA concentration vs day for sample B2

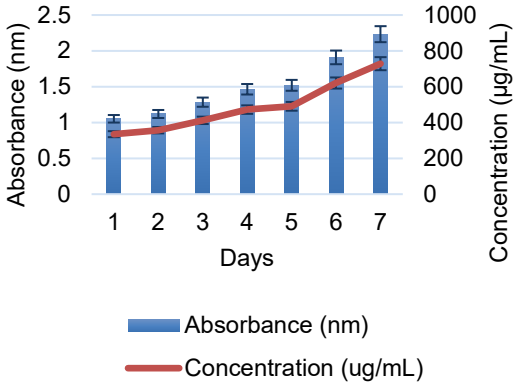


Figure 7 Growth and IAA concentration vs day for biofertilizer.

The augmentation in IAA concentration in fertilizer was much higher than isolated bacteria due to the fact that maybe some more functional bacteria expressed more indolic compounds. To support this, research by Wongkiew et al. (2022) stated that the prominent bacterial groups found in most fertilizers are *Bacteroidia* (0.3–43.8%), *Bacilli* (0.3– 15.8%), *Alphaproteobacteria* (0.0–14.4%) and *Clostridia* (0.2–1.3%).

As stated by Fu et al. (2015), IAA is the primary auxin in plants, controlling cell division and elongation, tissue differentiation, apical dominance, and responses to light, gravity, and pathogens. IAA production by bacteria has been observed to vary between species and strains, and it is also regulated by culture conditions, growth stage, and substrate availability (Sridevi and Mallaiah, 2007). Since IAA is the secondary 49 metabolite, its peaking time is much later compared to ALA. Studies

show that IAA would reach the highest production at day 6-7 (Bunsangiam et al., 2019). Therefore, elucidates the impression that bacteria will metabolize auxin for plants starting from the 7th day after they are added to the plant.

Sample A1, as shown in Figure 8, displays a continuous increment in absorbance and ALA content from day 1 to day 5, followed by a rapid drop on days 6 and 7. The ALA concentrations were recorded as 22.3 g/mL, 30.3 g/mL, 43.9 g/mL, 54.3 g/mL, 83.3 g/mL, 40.3 g/mL, and 48.3 g/mL. These results suggest that *Paraclostridium benzoalyticum* is the best to be used as fertilizer starting from the second day onward. Figure 9 depicts the absorbance reading and ALA concentration for sample B2 from day 1 to day 7 of observation as follows: 107.3 g/mL, 61.3 g/mL, 67.3 g/mL, 42.3 g/mL, 21.3 g/mL, 14.3 g/mL, and 20.3 g/mL.

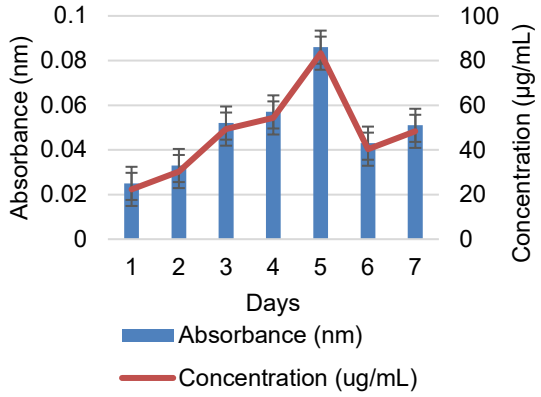


Figure 8 Growth and ALA concentration vs day for sample A1.

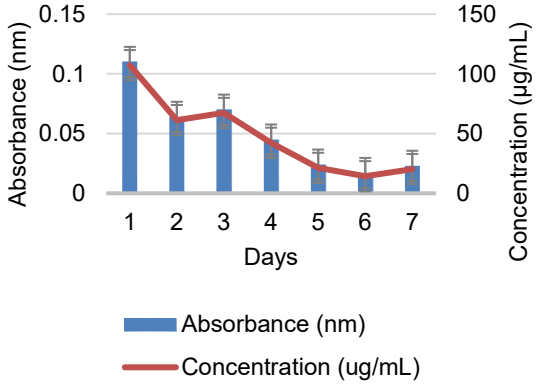


Figure 9 Growth and ALA concentration vs day for sample B2.

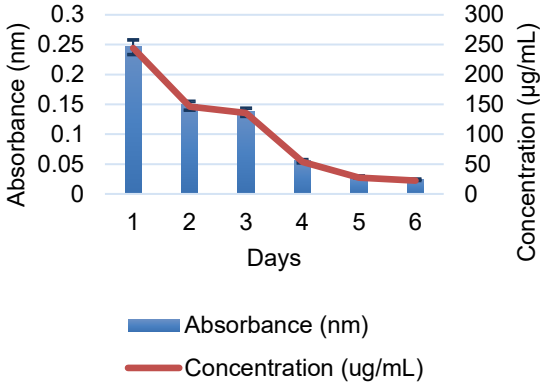


Figure 10 Growth and IAA concentration vs day for biofertilizer.

Using biofertilizer, the absorbance reading, and ALA concentration shows constant reduction throughout 6 days of observation. In particular, the ALA concentration in biofertilizers was recorded as 244.3 µg/mL, 146.3 µg/mL, 135.3 µg/mL, 53.3 µg/mL, 27.3 µg/mL and 22.3 µg/mL as in Figure 10. Even though the ALA concentration recorded a decrease, a research study by Kai et al. (2020) highlighted that at relatively low quantities, ALA increased plant growth. Low ALA concentrations promote chlorophyll synthesis, photosynthetic activity, growth rates, respiration efficiency, and growth control in plants while high levels of ALA cause the formation of numerous chlorophyll intermediates, including protochlorophyllide and protoporphyrin IX (PPIX).

In general, the C4 and C5 pathways regulate ALA production. ALA is produced by the enzyme ALA synthase, which is represented by the hemA or hemT gene and catalyzes the condensation of succinyl-CoA and glycine. According to Zhang et al (2013), the expression of hemA and hemT can be readily repressed, interrupting 5-ALA synthesis, because both genes are extremely sensitive to changes in the mechanism and feedback inhibition. As a result, it may be believed that the bacteria present in the fertilizer may disrupt hemA and hemT expression, resulting in ALA synthesis failure. Furthermore, according to a study by Chen et al (2020), the imbalanced 5-ALA biosynthetic pathway and TCA cycle may have contributed to the decline in 5-ALA production level and biomass. ALA production was first increased and then decreased along with the decrease in intracellular phosphoenolpyruvate carboxylase (PPC) activity, similar to the effect of regulating ALA synthase expression. The excessive consumption of succinyl-CoA by the aminolevulinic acid ALAS enzyme may slow down the TCA cycle and alter energy metabolism, causing a trade-off effect such as growth inhibition (Chen et al. 2020).

4.0 CONCLUSION

Based on the findings, the isolated bacteria from the homemade biofertilizer all showed Gram-positive with negative catalase enzyme and are non-pathogenic. The identified bacteria were *Paraclostridium* spp., especially *Paraclostridium benzoalyticum* and *Paraclostridium bifermentans.* The homemade fertilizer produces IAA in both bacteria and biofertilizers. but implausible ALA production. Since it can express auxin components such as IAA but not ALA, it may be assumed that this biofertilizer has the potential to be an efficient biofertilizer.

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References

Bunsangiam, S., Sakpuntoon, V., Srisuk, N., Ohashi, T., Fujiyama, K., Limtong, S. (2019) Biosynthetic pathway of indole-3-acetic acid in basidiomycetous yeast *Rhodosporidiobolus fluvialis*. *Mycobiology*, 47(3), 292-300.

Chen, J., Wang, Y., Guo, X., Rao, D., Zhou, W., Zheng, P., Sun, J. and Ma, Y. (2020) Efficient bioproduction of 5-aminolevulinic acid, a promising biostimulant and nutrient, from renewable bioresources by engineered *Corynebacterium glutamicum*. *Biotechnology for Biofuels*, 13(1), 1-13.

Chen, J., Wei, J., Ma, C., Yang, Z., Li, Z., Yang, X., Wang, M., Zhang, H., Hu, J., Zhang, C. (2020) Photosynthetic bacteria-based technology is a potential alternative to meet sustainable wastewater treatment requirement. *Environment International*, 137, 105417.

Figueiredo, G.G.O., Lopes, V.R., Romano, T., Camara, M.C. (2020) *Clostridium* in Beneficial Microbes in Agro-Ecology. Academic Press, 477-491.

Fu, S.F., Wei, J.Y., Chen, H.W., Liu, Y.Y., Lu, H.Y., Chou, J.Y. (2015) Indole-3-acetic acid: A widespread physiological code in interactions of fungi with other organisms, *Plant Signaling & Behavior*, 10(8), 1048052.

Gang, S., Sharma, S., Saraf, M., Buck, M., Schumacher, J. (2019) Analysis of indole-3-acetic acid (IAA) production in Klebsiella by LC-MS/MS and the Salkowski method. *Bio-protocol*, 9(9), 3230

Kai, T., Ikeura, H., Ayanokoji, A., Tamaki, M. (2020) Effects of 5-aminolevulinic acid (ala) on zinnia hybrida growth and phytoremediation effects in oil- contaminated soil. *Journal of Agricultural Chemistry and Environment*, 9(03), 97.

Kyaw, E.P., Lynn, T.M., Ko Latt, Z., San Yu, S. (2018) Isolation, characterization and rapid composting of cellulolytic nitrogen-fixing Bacteria for biofertilizer preparation. *International Journal of Plant Biology & Research*, 6(4), 1095

Mitter, E. K., Tosi, M., Obregón, D., Dunfield, K. E., Germida, J. J. (2021) Rethinking crop nutrition in times of modern microbiology: innovative biofertilizer technologies. *Frontiers in Sustainable Food Systems*, 5, 606815.

Santra, S. C., Mallick, A., Samal, A. C. (2015) *Biofertilizer for bioremediation. Recent trends in biofertilizers*. IK International Publishing House Pvt. Ltd, 205-234.

Suhaidah, A. (2019) Cara Buat Bakteria Fotosintetik (PSB) Sendiri Untuk Suburkan Tanah dan Pokok. Mingguan Wanita, https://www.mingguanwanita.my/cara-buat-bakteria-fotosintetik-psb-sendiri-untu k-suburkan-tanah-pokok/

Sridevi, M., Mallaiah, K. V. (2007) Bioproduction of indole acetic acid by Rhizobium strains isolated from root nodules of green manure crop. *Iranian Journal of Biotechnology,* 5(3), 178-182.

Wongkiew, S., Chaikaew, P., Takrattanasaran, N., Khamkajorn, T. (2022) Evaluation of nutrient characteristics and bacterial community in agricultural soil groups for sustainable land management. *Scientific reports*, 12(1), 1-13.

Zeiller, M., Rothballer, M., Iwobi, A.N., Böhnel, H., Gessler, F., Hartmann, A., Schmid, M. (2015) Systemic colonization of clover (*Trifolium repens*) by *Clostridium botulinum* strain 2301. *Frontiers in Microbiology*, 6, 207.

Zhang L., Chen J., Chen N., Sun J., Zheng P., Ma Y. (2013) Cloning of two 5-aminolevulinic acid synthase isozymes HemA and HemO from *Rhodopseudomonas palustris* with favorable characteristics for 5-aminolevulinic acid production. *Biotechnology Letters*, 35(5), 763-8.