Influence of Growth Media on PHA production: A Study on Coconut Rhizosphere soil Bacteria in Minimal salt Media and Tender Coconut Water

Kumari Isha Verma A[1]; Punyashree CM[2]; Suman Kumar Hiremath[3]; Akshatha HC[4]; Prashantha[5]; Tejaswini Theerthapura V[6*]; Bhuvan P[7]; Sharathchandra RG[8]

Department of studies and research in Biotechnology, Jnanasiri Campus, Tumkur University, Tumkur – 572118
*Corresponding Author

Received:- 11 August 2025/ Revised:- 17 August 2025/ Accepted:- 22 August 2025/ Published: 31-08-2025

Abstract— *The study aims to compare the extraction of polyhydroxyalkanoates (PHA), accumulated as intracellular inclusions in rhizosphere bacteria, when cultivated in minimal salt medium (MSM) and tender coconut water (TCW). Soil samples were collected from beneath a coconut tree, and bacterial isolates were obtained. The presence of PHA-producing bacteria was initially screened using Sudan Black B staining and subsequently confirmed with Nile Blue A staining. The confirmed PHA-producing isolates were cultured in MSM (designated as Sample A) and in TCW (designated as Sample B). PHA were successfully extracted from both media. Biofilm formation was observed, and PHA quantification was carried out using the crotonic acid assay. In addition, antioxidant activity was evaluated using the DPPH assay. This investigation provides a comparative assessment of PHA production by coconut rhizosphere bacteria grown in MSM and TCW, highlighting the potential of tender coconut water as a sustainable raw material for large-scale PHA production.*

Keywords— Polyhydroxyalkanoates (PHAs) bio plastics, rhizosphere bacteria, Tender Coconut water media, crotonic acid assay, antioxidant activity and DPPH assay.

I. INTRODUCTION

Plastics have excellent thermal and electrical insulation qualities and are affordable, strong, and resistant to corrosion. A wide variety of goods with multiple benefits are made possible by the diversity of polymers and the adaptability of their qualities. There are serious worries about the negative impacts on both people and wildlife. The production of plastics depends on limited resources, and worries about the compounding effects of various plastic usage patterns are contributing to the world's waste management issues. Acharjee et al. (2023) demonstrate that plastic wastes, such as packaging, electrical equipment, and plastics from end-of-life vehicles, are significant components of both household and industrial wastes. The landfill's capacity is limited, and in certain places, landfills are rapidly approaching, so from a number of angles, it appears that the disposal of plastic is a cause for concern. (Acharjee et al., 2023).

For packaging applications, biodegradable polymers that are similar to conventional petrochemical-based plastics in terms of their functions and processing capabilities have been created; these are usually derived from renewable raw ingredients like cellulose and starch. Biodegradable plastic packaging is popular because it uses renewable raw materials and manages end-of-life trash via anaerobic digestion or composting, which minimises landfilling. Given the increasing emphasis on trash generation and management as crucial environmental components of modern civilisation, the disposal of old waste materials is very vital. Waste management must benefit from biodegradable polymers (Song et al., 2009).

Polyesters called polyhydroxyalkanoates (PHA) are naturally occurring and collect as granules inside bacterial cells. The physiochemical and thermal characteristics of PHA vary depending on the producing organism and the conditions of cultivation. Many bacteria produce poly-3-hydroxybutyrate (PHB), one of the most prevalent types of PHAs. Because of their biodegradability and biocompatibility, these bio-based plastic products are intended to take the place of traditional plastics in

an environmentally responsible and sustainable manner. It is crucial to evaluate the genetic makeup, variety, and end products of bacteria that create PHAs in order to achieve this goal (Vicente et al., 2023).

For the best growth and metabolism, microorganisms need a variety of micronutrients in the right amounts in their ideal growing medium. While trace elements like sulphur, phosphate, vitamins, and so on are micronutrients, carbon and nitrogen are the main sources for microbial development. Additionally, natural medium has long been used as a source for microbial propagation. Because the little salted media is where PHA-producing bacteria are cultivated. 95% water, 4% sugars, 0.1% fat, 0.02% calcium, 0.01% phosphorus, 0.5% iron, significant amounts of amino acids, mineral salts, vitamin B complex, vitamin C, and cytokines make up Tender Coconut Water (TCW), the liquid endosperm found in the coconut fruit's cavity. These nutrients may also provide similar favourable conditions for the growth of bacteria that produce PHAs (Sekar et al., 2013).

It has been established that bacteria are capable of growing in tender coconut water. However, limited information is available regarding the extraction of polyhydroxyalkanoates (PHAs) from coconut rhizosphere soil bacteria cultivated in tender coconut water. Therefore, the present study was undertaken to compare the extraction of PHAs from coconut rhizosphere soil bacteria grown in minimal salt medium (MSM) and tender coconut water (TCW).

II. MATERIALS AND METHODS

2.1 Collection of Soil for bacteria isolation:

Soil samples were collected from agricultural field of coconut *rhizosphere* region, Tumakuru, Karnataka. Sample collected by gently digging them and placed in a sterile polythene bag.(Shraddha et al., n.d.)

2.2 Isolation of bacteria:

The isolation of bacteria was performed by serial dilution method and direct plate technique, using nutrient agar media. Soil sample was serial diluted and placed into nutrient agar plates and incubated at 28℃ for 24 hrs.(Shraddha et al., n.d.)

2.3 Screening for PHA producing bacteria:

Gram's staining was performed to determine whether the bacteria is gram negative or gram positive. The PHAs producing bacteria was confirming by using Sudan black B and Nile blue A staining.(Shraddha et al., n.d.)

2.4 16S rRNA sequencing for Identification of PHA producing Bacteria:

It is a method to identify and characterize bacteria using short, conserved DNA sequences to distinguish different bacterial species within the sample using genetic marker 16S rRNA gene (Mostafa et al., 2020). With the help of MEGA 12 phylogenetic tree was constructed.

2.5 Submerged Cultivation of PHA producing bacteria:

Inoculum of the bacteria was performed by preparing suspension broth using nutrient broth. Further, the suspension broth was inoculated in the minimal salt media which was incubated for a week at 37℃. This media helps to grow PHAs producing bacteria.

Coconut water is used as source of media to grow PHAs producing bacteria. The coconut water is autoclaved under 120°c allowed to cool down and then 5ml of fresh bacterial inoculum is added to the sterilized coconut water and kept for incubation in incubator at 35°c for 7 days.

2.6 Extraction of PHA from cultivated bacteria:

Extraction process was performed by centrifuged the above media like MSM (minimal salt media) and sterilized coconut water media at 7000rpm, 4℃ for 15 mins. Collect the pellet to add 5 ml distilled water. The mixture was sonicated for 30 mins. To that add 5ml of 20% sodium hypochlorite, the mixture was placed on the shaker to get continuous mixing at 150 rpm for 15 mins and incubated for 30mins at 37℃. Again, centrifuge it under 7000rpm at 4℃ for 15mins. The pellet was collected and washed by using acetone: alcohol mixture in the ratio of 1:1 v/v , dissolved the mixture in 10 ml chloroform and placed it at room temperature to get PHAs.(Ibrahim et al., 2025).

2.7 Quantification of PHA by Crotonic acid assay:

The extract (of both sample A which was extracted from MSM and sample B as PHAs extracted from Coconut tender water) was transferred to a clean test tube, and to that add 10 ml of 36N sulfuric acid, capped and heated for 20min at 100℃ in a boiling water bath. Where PHA granules were converted into crotonic acid by dehydration. The resultant, brown-colored solution of crotonic acid was cooled and mixed thoroughly by shaking. The absorbance of the sample was measured at 235nm using a UV-Visible spectrophotometer using sulfuric acid as a control (Ibrahim et al., 2025).

2.8 Biofilm production:

For the preparation of biofilm, two solutions were used, solution A consisted of PHA dissolved in distilled water and solution B consisted of gelatin and agar in 50ml of distilled water, which were separately prepared. Both the solutions were autoclaved at 15lbs of pressure for 15 mins. Later, the solutions were mixed thoroughly with the addition of 2% glycerol and kept it on the magnetic stirrer at 70℃ at 1200 rpm for 20 min. The froth was allowed to settle, and the solution was casted on a glass plate. The obtained biofilm was allowed to dry in a hot air oven at 40℃ for 36h.

2.9 Antioxidant activity:

DPPH assay: The PHAs solution was prepared in condition of 1mg/ml. In test tubes add different Concentration of PHAs solution, dissolve 800 µl of 0.1 M tris-HCL buffer (pH 7.4) and 1 ml of the DPPH solution. In the blank well, add 1.2 ml of ethanol and 800 µl of tris-HCL buffer. Then mix immediately for 10 sec and keep it at room temperature in the dark. After 30min, measure the absorbance of the solution at 517nm.

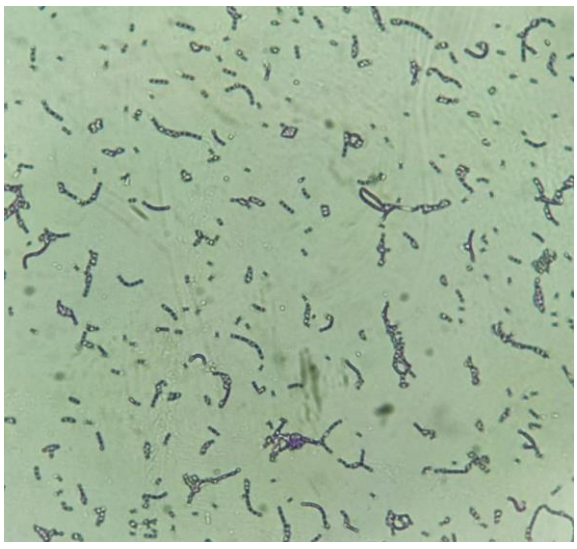
$$Inhibition\ ratio\ (\%) = \{(Ac - As)/Ac\} * 100 \quad (1)$$

Where Ac is the absorbance of blank and as is the absorbance in presence of the test sample.(Mahayothee et al., 2016).

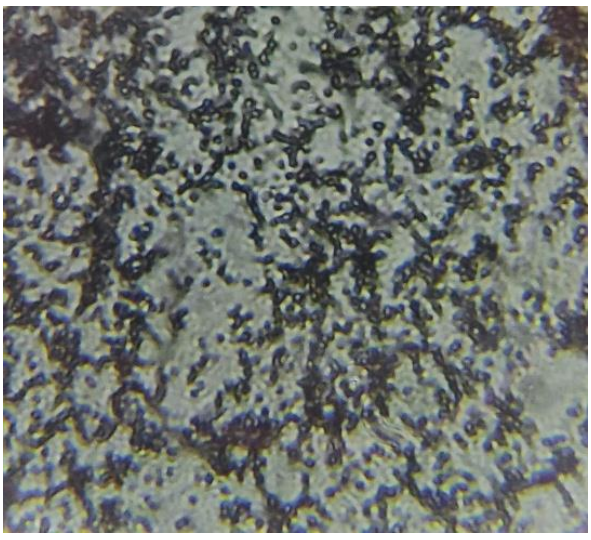
III. RESULT AND DISCUSSION

The presence of PHA-producing bacteria was initially determined using Sudan Black B staining. Sudan Black B is a lysochrome, a fat-soluble dye, commonly employed to detect intracellular lipid inclusions. In PHA-producing bacteria, staining with Sudan Black B results in a distinct black coloration, indicating the presence of PHAs. PHAs are biopolymers composed of hydroxyalkanoate monomers, possessing a lipid-like structure. Being a lipophilic dye, Sudan Black B specifically binds to lipids and other hydrophobic molecules within the cell, making it a useful preliminary screening agent for lipophilic compounds. The PHA granules were identified based on their strong affinity for Sudan Black B, as reported by Ostle and Holt.

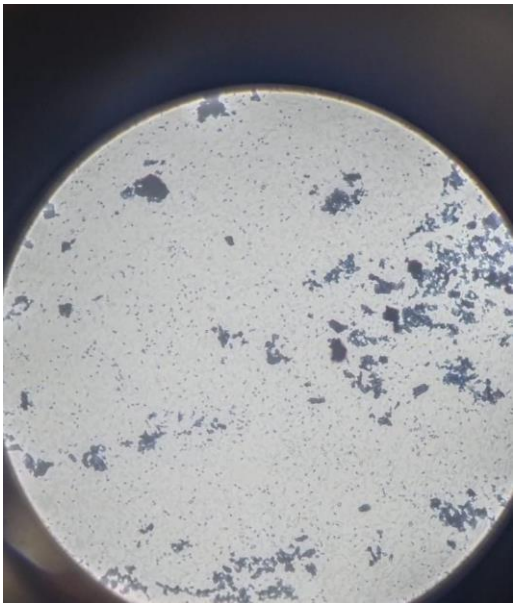
Confirmation of PHA production was performed using Nile Blue A staining. Nile Blue A is a basic oxazine dye, soluble in both water and ethyl alcohol, and is known for its ability to stain PHAs within microbial cells. At the staining temperature of 55 °C, Nile Blue A readily dissolves in neutral lipids, which are in a liquid state, and selectively binds to the hydrophobic PHA granules within bacterial cells. This hydrophobic interaction allows Nile Blue A to serve as a reliable confirmatory stain for PHA-accumulating microorganisms, as described by Ostle and Holt.



(a)



(b)



(c)

(d)

FIGURE 1: (a) Screening for PHAs producing bacteria - gram positive and rod shaped under microscope 40X. Figure 1(b) and (c) indicates PHAs producing Bacteria by Staining with Sudan Black B and Nile Blue A respectively observed under the microscope 40X. Figure 1 (d) pure culture of the PHAs producing bacteria.

3.1 Extraction of PHA from MSM and TCW:

The successful extraction of PHAs was indicated by the presence of a white, powdery residue after solvent evaporation, characteristic of PHA polymers. This visual confirmation strongly supports the presence of intracellular PHA granules within the bacterial cells grown in MSM and TCW.

The PHAs accumulation (%) in the sample A:

= Dry weight of extracted PHAs (mg/ml)/Dry cell weight (mg/ml) X 100

PHAs accumulation (%) = **1325.5**(mg/ml)/**2426**(mg/ml) X100

= **54.637%**

The PHAs accumulation of sample A from the Extracted PHAs by sodium hypochlorite method and using chloroform as solvent was **54.637%.**

The PHAs accumulation (%) in the sample B:

= Dry weight of extracted PHAs (mg/ml)/Dry cell weight (mg/ml) X 100

PHAs accumulation (%) = **138**(mg/ml)/**245**(mg/ml) X100

= **56.326%**

The PHAs accumulation of sample B from the Extracted PHAs by sodium hypochlorite method and using chloroform as solvent was **56.326%.**

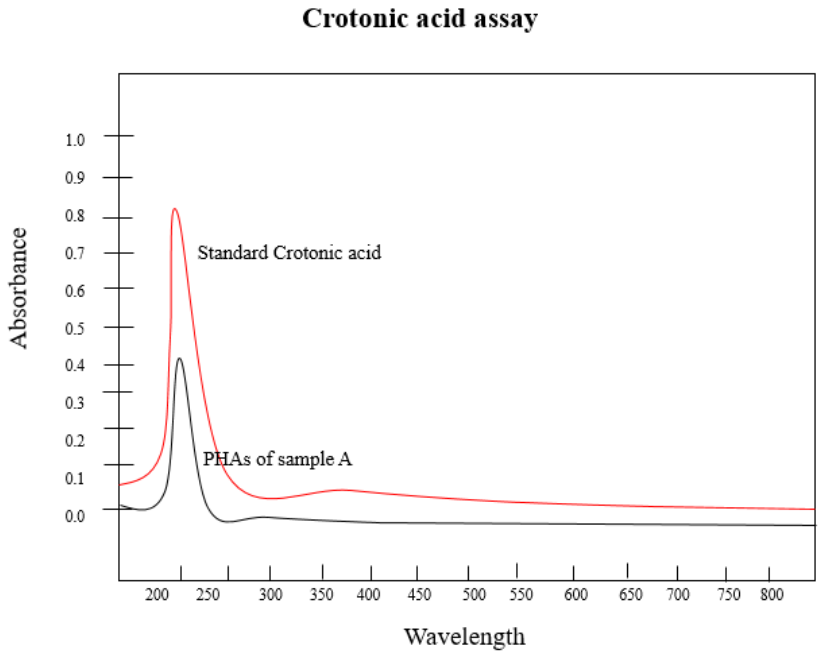
3.2 Quantification of the PHA by crotonic acid assay:

Figure 2 shows the UV-Vis absorption spectra of the extracted PHA sample A (black line) & Sample B (Blue Line) compared with standard crotonic acid (red line), measured across a wavelength range of 200–800 nm. Both the standard and the samples exhibited a prominent absorption peak at approximately 235 nm, which is a characteristic wavelength for crotonic acid, a product formed upon acid digestion of PHA.

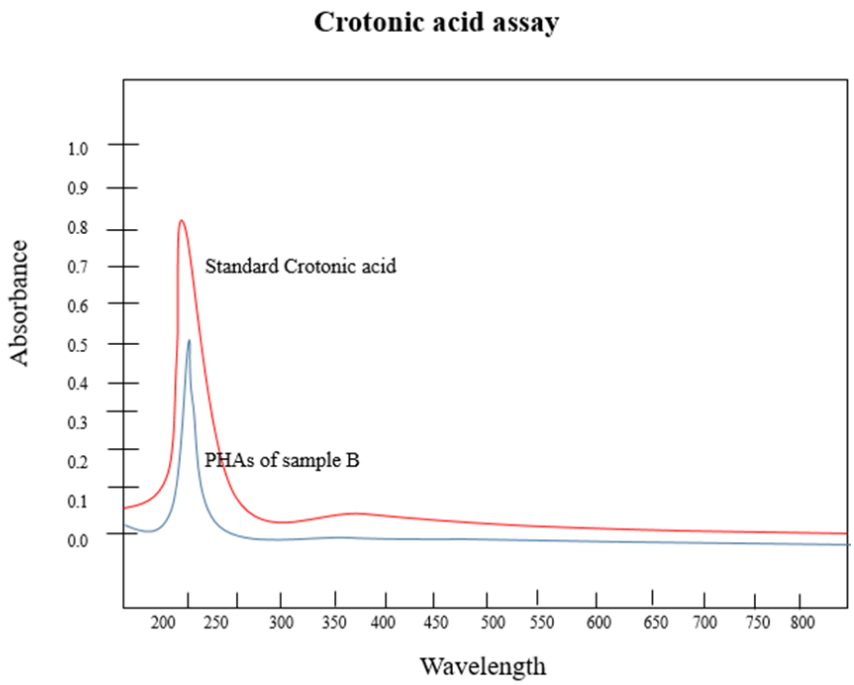
The PHA samples showed a similar absorbance profile to that of the standard crotonic acid, confirming the presence of PHA in the bacterial extract. Although the absorbance intensity of the sample was lower than that of the standard, the peak position

at 235 nm suggests that the extracted compound is structurally consistent with crotonic acid, indicating successful PHA production and extraction.

The difference in peak intensity may be attributed to variations in concentration, partial digestion, or differences in extraction efficiency. Nonetheless, the similarity in spectral behavior between the extracted PHA and the crotonic acid standard validates the presence of PHA in the tested bacterial culture.

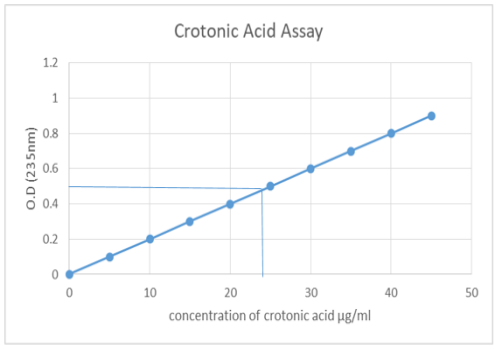


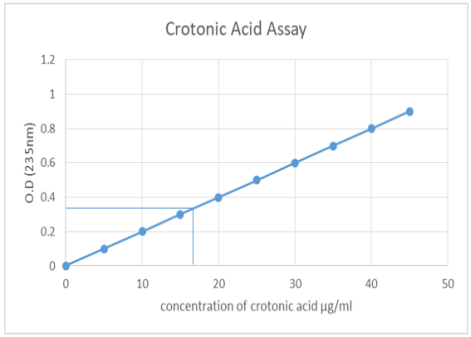
(a)



(b)

FIGURE 2: (a) The UV-Vis spectra illustrate the absorbance peaks of the standard crotonic acid (indicated by red line) and PHAs granules of sample A (indicated by grey line) at a Wavelength of 235nm. (b) The UV-Vis spectra illustrate the maxima absorbance peaks of the standard crotonic acid (indicated by red line) and PHAs granules of sample B (indicated by blue line).





(a) **(b)**

FIGURE 3: (a) The concentration of the PHAs using Standard crotonic acid for the sample A. (b) the concentration of the PHA using standard crotonic acid for the sample B.

Figure 3 illustrates the standard calibration curve generated for crotonic acid, based on absorbance measurements taken at 235 nm. The graph shows a linear relationship between the concentration of crotonic acid (µg/mL) and optical density (OD) at 235 nm, with increasing absorbance corresponding to increasing concentrations of crotonic acid. This linearity confirms the suitability of the assay for quantifying PHA content via acid digestion, wherein PHA were converted to crotonic acid.

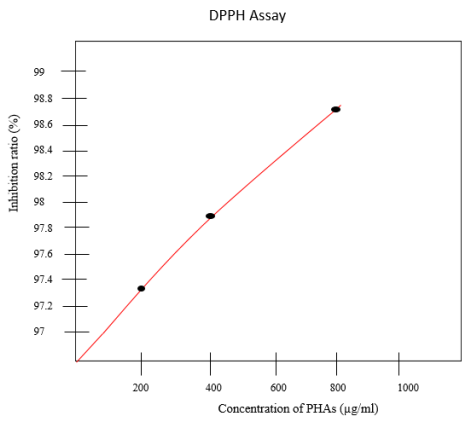
Using this calibration curve, the PHA content in the experimental samples was determined. The absorbance value for the sample A was approximately **0.48**, which corresponds to a crotonic acid concentration of roughly **24 µg/mL** based on the plotted trend line Fig 3 (a) and the absorbance value for the sample B was approximately **0.36**, which corresponds to a crotonic acid concentration of roughly **15 µg/mL** based on the plotted trend line Fig 3 (b). This concentration reflects the amount of crotonic acid derived from the acid digestion of the extracted PHA, and by extension, provides an indirect quantification of PHA content in the bacterial culture.

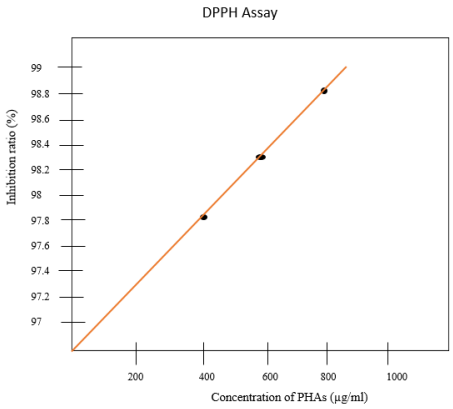
3.3 Antioxidant Activity by DPPH Assay:

Figure 4 illustrates the antioxidant activity of extracted PHA as determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The inhibition ratio (%) was plotted against different concentrations of PHAs (µg/mL), showing a clear dose-dependent antioxidant effect.

As the concentration of PHAs increased from 200 µg/mL to 800 µg/mL, the DPPH inhibition ratio also increased from approximately **97.2% to 98.6% for Sample A and for Sample B 97.8% to 98.8%**, indicating strong free radical scavenging activity. The observed trend suggests that the extracted PHA possesses intrinsic antioxidant potential.

The nearly linear increase in inhibition ratio with concentration demonstrates that the antioxidant activity of PHAs is both consistent and effective, although the changes are relatively small due to the high baseline inhibition. These findings indicates that PHA, beyond their biopolymer applications, may also exhibit bioactive properties, potentially useful in biomedical or packaging industries where oxidative stability was desired.





(a) (b)

FIGURE 4: The UV-Vis spectra readings exhibit the inhibition ratio at the absorbance 517nm for the sample A in (a) and for sample B in (b).

IV. CONCLUSION

The findings of this study demonstrate that PHAs, which are polyesters accumulated in microorganisms, have the potential to serve as raw materials for the production of bioplastics. Enhancing the production of PHA-based bioplastics could promote their use as an environmentally friendly alternative to conventional plastics, which contribute significantly to environmental pollution and harm to wildlife. The replacement of plastics with biodegradable PHAs would reduce environmental contamination and, when disposed of in soil, could even enhance soil fertility. PHA-based bioplastics are eco-friendly, generate zero waste, and are user-friendly, making them a sustainable option for the future.

In conclusion, the rhizosphere bacterium *Bacillus anthracis* was found to produce PHA polyesters possessing desirable characteristics for bioplastic production. These PHAs exhibited antioxidant activity, as determined by the DPPH assay. The PHA accumulation was measured at 54.637% in Sample A (cultivated in minimal salt medium) and 56.326% in Sample B (cultivated in sterilized tender coconut water). The higher yield in Sample B suggests that tender coconut water can serve as a cost-effective and sustainable raw material for large-scale PHA production. Future studies should investigate the characteristics of PHA-based biofilms and their degradability under real-world conditions, which could further enhance their potential as sustainable materials.

REFERENCES

[1] Acharjee, S. A., Bharali, P., Gogoi, B., Sorhie, V., Walling, B., & Alemtoshi. (2023). PHA-Based Bioplastic: A Potential Alternative to Address Microplastic Pollution. Water, Air, and Soil Pollution, 234(1), 21. https://doi.org/10.1007/s11270-022-06029-2

[2] Sekar, N., Veetil, S. K., & Neerathilingam, M. (2013). Tender coconut water an economical growth medium for the production of recombinant proteins in Escherichia coli. BMC Biotechnology, 13, 70. https://doi.org/10.1186/1472-6750-13-70

[3] Song, J. H., Murphy, R. J., Narayan, R., & Davies, G. B. H. (2009). Biodegradable and compostable alternatives to conventional plastics. Philosophical Transactions of the Royal Society B: Biological Sciences, 364(1526), 2127–2139. https://doi.org/10.1098/rstb.2008.0289

[4] Vicente, D., Proença, D. N., & Morais, P. V. (2023). The Role of Bacterial Polyhydroalkanoate (PHA) in a Sustainable Future: A Review on the Biological Diversity. International Journal of Environmental Research and Public Health, 20(4), 2959. https://doi.org/10.3390/ijerph20042959.

[5] Shah, S., & Kumar, A. (2021). Production and characterization of polyhydroxyalkanoates from industrial waste using soil bacterial isolates. Brazilian Journal of Microbiology, 52(2), 715–726. https://doi.org/10.1007/s42770-021-00452-z

[6] Ibrahim, R., Aranjani, J. M., Prasanna, N., Biswas, A., & Gayam, P. K. R. (2025). Production, isolation, optimization, and characterization of microbial PHA from Bacillus australimaris. Scientific Reports, 15, 8395. https://doi.org/10.1038/s41598-025-92146-x

[7] Shraddha, G., Yogita, R., Simanta, S., Aparna, S., & Kamlesh, S. (n.d.). Screening and production of bioplastic (PHAs) from sugarcane rhizospheric bacteria.

[8] Desiante, W. L., Carles, L., Wullschleger, S., Joss, A., Stamm, C., & Fenner, K. (2022). Wastewater microorganisms impact the micropollutant biotransformation potential of natural stream biofilms. Water Research, 217, 118413. https://doi.org/10.1016/j.watres.2022.118413

[9] Mostafa, Y. S., Alrumman, S. A., Otaif, K. A., Alamri, S. A., Mostafa, M. S., & Sahlabji, T. (2020). Production and Characterization of Bioplastic by Polyhydroxybutyrate Accumulating Erythrobacter aquimaris Isolated from Mangrove Rhizosphere. Molecules, 25(1), Article 1. https://doi.org/10.3390/molecules25010179

[10] Mirpoor, S. F., Patanè, G. T., Corrado, I., Giosafatto, C. V. L., Ginestra, G., Nostro, A., Foti, A., Gucciardi, P. G., Mandalari, G., Barreca, D., Gervasi, T., & Pezzella, C. (2023). Functionalization of Polyhydroxyalkanoates (PHA)-Based Bioplastic with Phloretin for Active Food Packaging: Characterization of Its Mechanical, Antioxidant, and Antimicrobial Activities. International Journal of Molecular Sciences, 24(14), Article 14. https://doi.org/10.3390/ijms241411628

[11] Oliveira, G., Petronilho, S., Kapusniak, K., Kapusniak, J., del Castillo, M. D., Coimbra, M. A., Ferreira, P., Passos, C. P., & Gonçalves, I. (2024). Antioxidant and flexible bioplastics based on microwave-assisted extracted coffee fruit cascara pectic polysaccharides. Journal of Cleaner Production, 453, 142264. https://doi.org/10.1016/j.jclepro.2024.142264

[12] Hassan, S., Khudur, L. S., Nahar, K., Dekiwadia, C., & Ball, A. S. (2025). Isolation and Identification of Xylose-Utilising, Polyhydroxyalkanoate-Producing Bacteria from Sugarcane Bagasse: First Report of PHA Production by Mycolicibacterium Smegmatis. Journal of Polymers and the Environment. https://doi.org/10.1007/s10924-025-03600-w