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Original Research Article

Isolation and characterization of bacteria from solid waste for production and quantification of polyhydroxybutyrate

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Abstract

Background: Polyhydroxyalkanoates (PHA) represent a category of bacterial polyesters synthesized intracellularly under nutrient-stress conditions, particularly with limited essential nutrients and abundant carbon sources, serving as storage compounds. PHBs are currently of significant interest as sustainable bioplastic alternatives. The main objective of the study was to isolate PHB-producing bacteria from the solid waste dumping yard.

Materials and Methods: Five bacterial isolates were screened for PHB granule production by Sudan black B staining method. Estimation and characterization of cellular PHB production was done using different analytical techniques including Fourier transform infrared spectroscopy (FTIR) for molecular analysis and scanning electron microscopy combined with energy-dispersive X-ray spectroscopy (SEM-EDX).

Results: One isolate, a promising PHB producer, was identified by 16s rRNA sequencing and was found to be as *Acinetobacter baumannii*. Quantitative analysis revealed that the isolate was showing capacity to accumulate 48.71% PHB. Characterization by FTIR and SEM-EDX confirmed PHB formation, with elemental composition of 61.65 ± 2.74% carbon and 38.35 ± 4.44% oxygen.

Conclusion: This study helps to understand microbial PHB isolation and purification processes, and its supporting role in advancement of bioplastic production. Further research facilitates the development of sustainable bioplastic alternatives on a larger scale.

Keywords: Polyhydroxyalkanoates, Polyhydroxybutyrate, *Acinetobacter baumannii.*

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1. Introduction

Plastics are essential in today's world, impacting industries like food packaging, technology, and medicine. Plastics are made from more than 98% fossil fuels and contain numerous additives to improve their quality. Numerous of these compounds have harmful effects on both human health and the environment, such as endocrine disruptors and carcinogens.[1] Global aquatic, terrestrial, and atmospheric habitats are contaminated by plastic pollution, and its persistence—especially in oceans—exacerbates environmental harm and disturbs biological processes.[1,2] The situation is made worse by greenhouse gas emissions, non-renewable resource depletion, and insufficient recycling technology.[3] Promising options for a circular lifecycle and less environmental effect include investigating substitutes like biodegradable polyhydroxyalkanoates (PHAs) and polyhydroxybutyrate (PHB).[2,4]

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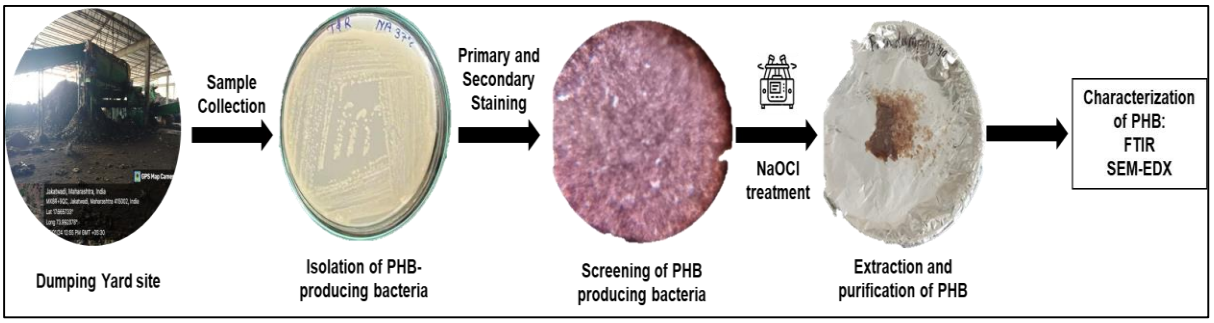

Figure 1:

The broad class of microbial polyesters known as polyhydroxyalkanoates (PHAs) makes up a sizable portion of thermoplastic polymers. Microorganisms produce these microbial polyesters as a reaction to unsuitable nutritional circumstances.[5-7] Bacterial biopolymers have drawn the most attention among these microbes.[6,7] It has been determined that more than 300 bacterial strains are capable of producing biopolymers. A few bacterial genera, such as *Pseudomonas*, *Halomonas*, and *Azotobacter* species, are exhibiting the production of PHA and PHB.[8] However, production expenses related to the use of purified nutritional substrates like glucose, lactose, and sucrose continue to limit the economic viability of biopolymers generated from bacteria.[2,4,5]

The number of carbon atoms in the monomeric components of PHAs mostly determines their classification.[3,4] The first group includes short-chain-length PHA (scl-PHA, C3-C5), which includes PHB and polyhydroxybutyrate-co-hydroxyvalerate (PHBV).[9]

Due to the linear chain, which contains both amorphous and crystalline organization, the Poly (3-hydroxybutyrate) structure is crystalline.[10] PHB is a member of the PHA family and is distinguished by the presence of an ester linkage group (−COOR) and a methyl functional group (CH3). These functional groups are what give the material its high crystallinity, brittleness, hydrophobicity, and thermoplastic properties.[10-12] Because of their nature, these granules are incredibly biocompatible and biodegradable.[13]

The study's main objective was to identify the microorganisms that produce PHB. This study describes the process of isolating bacteria from dumping yards, then thoroughly screening, characterizing, and identifying them. It also discusses the manufacturing, purification, and quantification of PHB. Understanding PHB-producing microbes, PHB yielding techniques, and PHB characterisation aspects would all benefit from the findings of this study.

The novelty of this study lies in its focus on isolating PHB-producing bacteria from an unconventional source—dumping yards—rather than traditional environments. This approach opens up new possibilities for utilizing waste or contaminated environments as sources of microbial strains with high PHB production capabilities. Furthermore, the comprehensive exploration of PHB manufacturing, purification, and quantification techniques provides valuable insights that could advance sustainable production of PHB, a biodegradable plastic, and enhance the overall understanding of its industrial applications.

2. Material and Methods

2.1. Sample collection

Samples of solid waste from the dumping area were collected and stored in a sterile bag. The samples were collected from different dumping yards across various locations.

2.2. Isolation of PHB-producing bacteria

For enrichment, the solid waste sample was inoculated in sterile nutrient broth. The enriched sample was serially diluted up to 10^{-7}. After incubation. Sterile nutrient agar plates were covered with 1 ml of the diluted sample, and they were then incubated at various temperatures. Subculturing on sterile nutrient agar plates produced pure colonies. Stocks of glycerol were made and kept.[6,16] Sterile Mineral salts medium was used for production of PHB granules.

2.3. Screening of PHB-producing bacteria

2.3.1. Primary screening with Sudan black B stain:

On a grease-free slide, a thin bacterial smear was made and air dried. After 10 to 15 minutes of Sudan Black B staining, the smear was rinsed with distilled water. A 20-second counterstain application of safranin was then made. After that, the slide was cleaned with distilled water and allowed to air dry. The oil immersion objective was used for observation.[8]

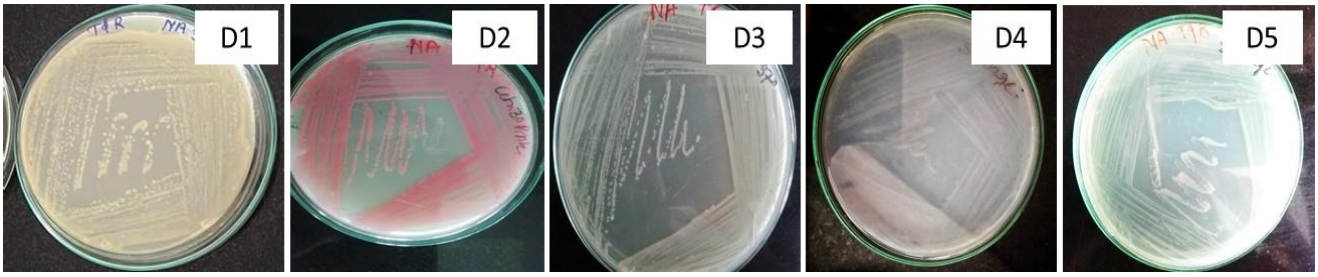

Figure 2: Isolates obtained from solid waste dumping yard

2.4. Extraction of PHB

The isolates selected after primary screening were enriched in sterile Nutrient broth. The medium was harvested and centrifuged at 6000 rpm for 20 minutes. After centrifugation, the cell pellet obtained was washed twice with sterile distilled water. The resuspended pellets were centrifuged at 6000 rpm for 10 minutes at 4°C. The cell pellets were then air-dried for at 100°C for 24 hours to obtain the dry cell weight.[6]

The dried pellets were then treated with sodium hypochlorite for 1 hour. The mixture was centrifuged at 8000 rpm for 10 minutes. The cell pellet was washed with phosphate-buffered saline followed by acetone, ethanol, and water. The purified polymers dried for 2 hours at 105°C, and the extracted polyhydroxybutyrate (PHB) weight was measured.[6,14]

For further purification of PHB, the polymer was subjected to incubation in boiling chloroform for 2 minutes. Subsequently, to determine the amount of poly (3-hydroxybutyrate) [P(3HB)].[6,14,17]

2.5. Quantification of PHB from the isolates

The percentage of intracellular PHB accumulation was estimated as the percentage composition of PHB present in the dry cell weight. The dry weight of extracted PHB was calculated in grams per litre (g/L).[6]

PHB accumulation (%) = Dry weight of extracted PHB (g/L) × 100 / DCW (g/L).[6]

2.6. Identification of PHB-producing isolates by 16s rRNA sequencing

The isolate exhibiting strong polyhydroxybutyrate (PHB) production was identified through 16S rRNA sequencing. This technique involved extracting the bacterial DNA from the isolate and amplifying the 16S rRNA gene. The sequence obtained was then compared to reference databases to accurately identify the bacterial strains at the genus and species level.

2.7. Characterization of microbial PHB

To validate the production of bacterial biopolymer, it was essential to undertake a comprehensive characterization of the extracted biopolymer powder. This characterization procedure entailed the application of analytical methodologies including, Fourier transform infrared spectroscopy (FTIR) for molecular analysis and scanning electron microscopy combined with energy-dispersive X-ray spectroscopy (SEM-EDX). These techniques facilitated the investigation of the chemical, morphological, and structural attributes of the extracted biopolymer powder, thus yielding valuable insights into its formation and composition.[5,10,12,13]

3. Results

3.1. Isolation of PHB-producing bacteria

The diluted samples spread and then streaked on sterile nutrient agar plate showed pure colonies. The cultures were further maintained on slants and glycerol stocks were prepared. (**Figure 2**)

3.2. Screening of PHB producing bacteria

The Sudan Black B staining method is the primary screening technique for PHB-producing bacteria by observing the presence of polyhydroxybutyrate (PHB) under microscope. Among the five isolates, isolate (D1) showed PHB granule accumulation **Figure 3**.

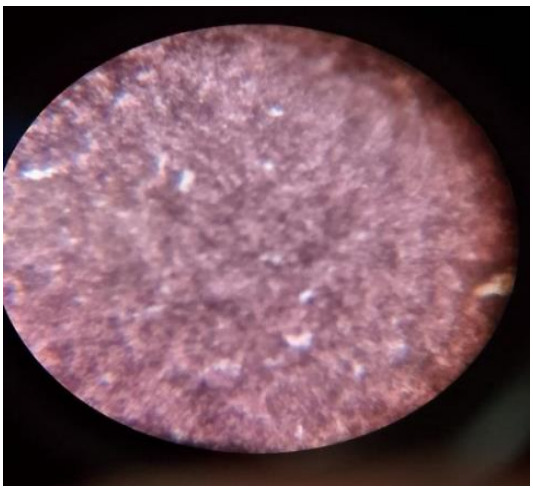

Figure 3: Sudan black B staining of D1 isolate

3.3. Extraction of microbial PHB

The enriched culture was subjected to centrifugation to separate the cell pellets, which harbour PHB granules. After the treatment, the dry cell pellets of each bacterial isolate were obtained. The results showed dry pellets with flaky texture with a brown to green shade (**Figure 4**). The final

PHB extracted from isolate D1 exhibited dry aggregation of PHB granules, and a powdery texture with a brown colour shade as depicted in **Figure 5**.

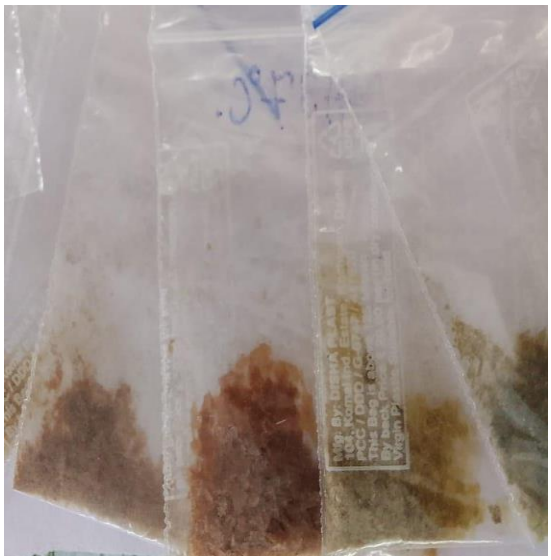

Figure 4: Dry cell pellets

Figure 5: Extracted PHB from isolate D1

3.4. Quantification of PHB

Isolate D1, showed dry weight of extracted polyhydroxybutyrate (PHB) as 0.989 g/L, and dry cell weight (DCW) of 2.03 g/L. Isolate D1 showed PHB accumulation as 48.71%., demonstrating it's potential as a viable source for bioplastic synthesis.

3.5. Identification of PHB producing bacteria and phylogenetic analysis

Sequencing analysis showed that the isolate D1 showed 99.14% homology with *Acinetobacter baumannii*, A phylogenetic tree was constructed to elucidate its relationship with various *Acinetobacter baumannii* strains. (**Figure 6**).

3.6. Characterization of microbial PHB:

3.6.1. Fourier transform infrared spectroscopy analysis

FTIR spectroscopic analysis was employed to elucidate the chemical functional groups present in the biopolymer yield. The FTIR spectra obtained for the isolate, derived from a solid waste dumping yard are depicted in **Figure 7**. The broad absorption peaks observed at 3477.03 cm-1 signify the presence of prominent –NH or –OH group bonds inherent in proteins. The attenuated absorption observed at wavelengths around 2927.41 cm^{-1} arises from the symmetric stretching of C-H bonds within CH_2 groups.

Distinct peaks manifesting at approximately 1643.05 cm^{-1} is indicative of carbonyl (C=O) groups within amides, commonly associated with proteins, or potentially linked to the –CONH– moiety present in amino sugars and proteins. Additionally, the band observed at 1455.99 cm^{-1} in spectra corresponds to CH groups, indicating asymmetric stretching and bending vibrations within CH3 groups. The spectral regions spanning from 1070 to 1243.86 cm^{-1} represent C-O bonding vibrations.

3.6.2. Scanning electron microscopy with energy dispersive x-ray spectroscopy (SEM - EDX) analysis

Scanning Electron Microscopy (SEM) analysis displayed that polyhydroxybutyrate (PHB) possess an amorphous structure. SEM images at different magnifications revealed the combined structure of PHB yield.

The elemental composition of the polyhydroxybutyrate (PHB) sample was determined through Energy Dispersive X-ray (EDX) analysis. **Table 1**, clearly indicate the presence of carbon (C) and oxygen (O) as the primary elements which are present in the PHB structure. The mass percentage of carbon was estimated to be 54.69 ± 2.43%, while oxygen estimated 45.31 ± 5.24% of the total mass. Correspondingly, the atomic percentages were calculated to be 61.65 ± 2.74% for carbon and 38.35 ± 4.44% for oxygen, leading to a total of 100% for both mass and atomic percentages, confirming the sample's composition. (**Figure 9**)

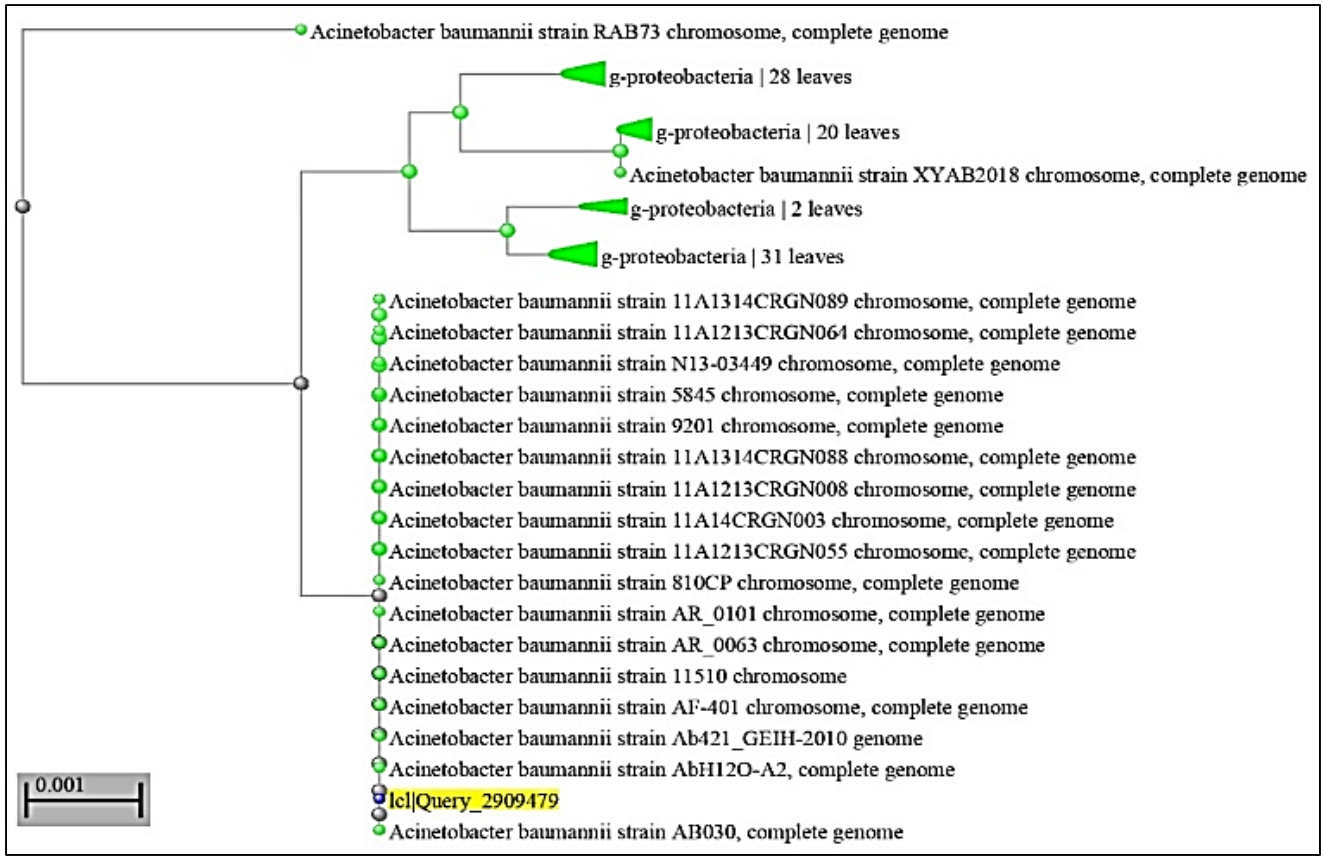

Figure 6: Phylogenetic tree of Acinetobacter baumannii (Isoalte D1)

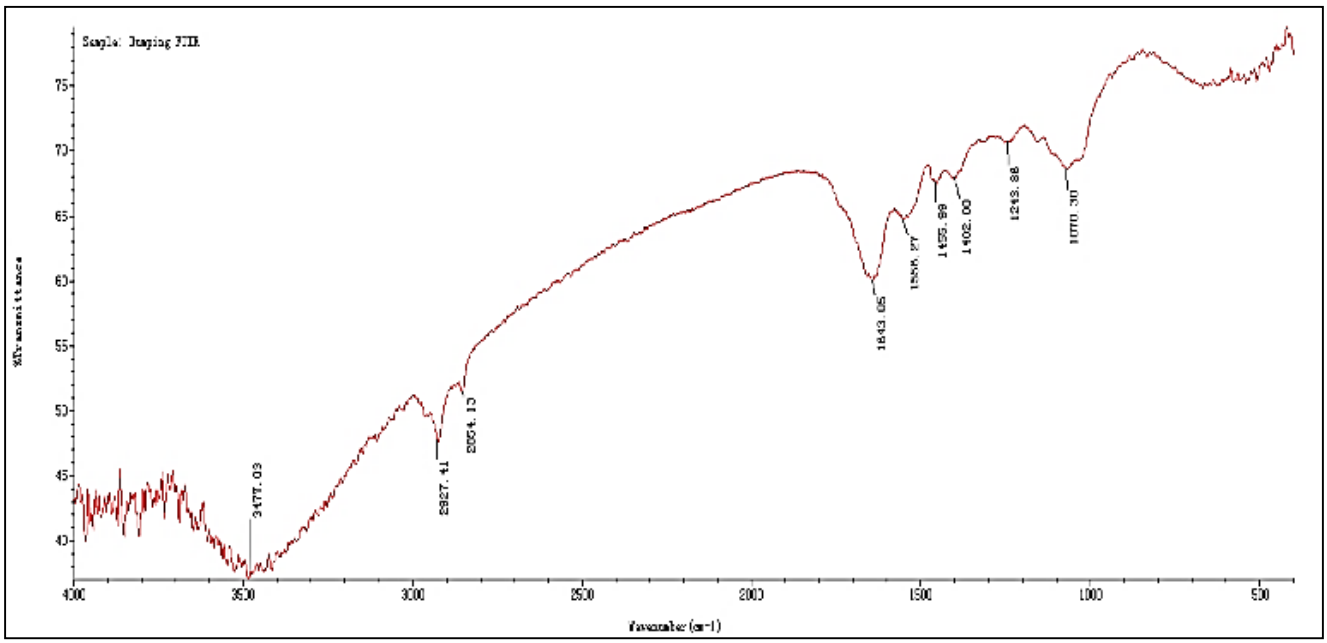

Figure 7: FTIR spectrum for microbial PHB extracted from solid waste dumping yard isolate

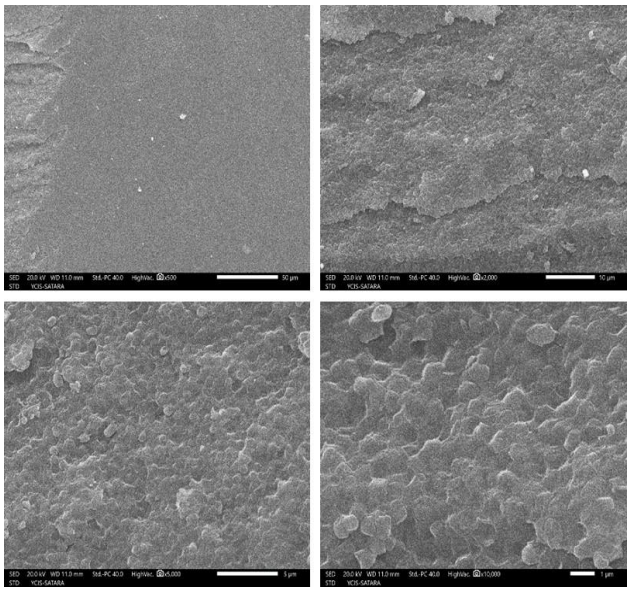

Figure 8: SEM micrograph for PHB at 10kV for different magnifications **a)** at 500X; **b**) at 2000X; **c)** at 5000X and **d**) at 10000X

Table 1: Elemental composition of microbial PHB by EDX analysis

Element	Line	Mass %	Atom %
C	K	54.69 ± 2.43	61.65 ± 2.74
O	K	45.31 ± 5.24	38.35 ± 4.44
Total		100.00	100.00
Fitting ratio 0.6562			

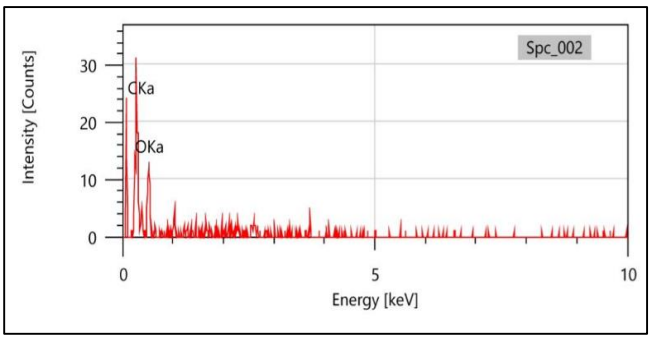

Figure 9: EDX micrograph for PHB at 20kV EHT

4. Discussion

The synthesis and accumulation of PHB in microbes occur as a survival mechanism in response to environmental stress, helping them thrive in challenging conditions. Sudan Black B strongly binds to lipid-associated biopolymers, making it useful for screening PHB producers. Microscopic analysis partially showed that the intracellular lipid and biopolymer content is linked to the cytoplasmic materials and structural components of the isolate.

The broad absorption peak observed at 3477.03 cm-1 indicates the presence of–NH or –OH group bonds, which are typically found in proteins.[5,10,12] In contrast, the absorption peak at around 2927.41 cm-1 corresponds to the symmetric stretching of C-H bonds within CH2 groups.[5,10] Additionally, the distinct peak observed near 1643.05 cm-1 suggests the presence of carbonyl (C=O) groups, commonly found in amides associated with proteins, or potentially linked to the –CONH– group in amino sugars and proteins.[5,10] The band at 1455.99 cm-1 is attributed to CH groups, representing asymmetric stretching and bending vibrations within CH3 groups. Lastly, the spectral range from 1070 to 1243.86 cm-1 reflects C-O bond vibrations.[12] The spectral features align with the composition of PHB, as reported in earlier studies by Hagagy *et al.*, Mostafa *et al.*, and Alarfaj *et al.*[5,10,11]

These findings are consistent with the expected elemental makeup of PHB, as PHB is known to be a biopolymer composed primarily of carbon and oxygen atoms. The fitting ratio of the EDX analysis was 0.6562, which further validates the accuracy of the detected elemental proportions. Previous studies by Vahabi *et al.*, 2019 have similarly reported the dominance of carbon and oxygen in PHB, affirming the reliability and reproducibility of the current EDX results. This alignment with established literature supports the purity and integrity of the synthesized PHB, as no extraneous elements were detected within the sensitivity limits of the EDX analysis.[14]

The EDX spectrum confirms that the synthesized PHB is composed primarily of carbon and oxygen, which is characteristic of its chemical structure. These results corroborate the findings from previous research by Vahabi *et al.*, which similarly identified carbon and oxygen as the predominant elements in PHB biopolymers.[15]

5. Conclusion

In conclusion, this study effectively isolated PHB-producing bacteria from solid waste dumping yards, identifying *Acinetobacter baumannii* as a significant producer with a PHB accumulation of 48.71% as demonstrated by our research. Characterization through FTIR and SEM-EDX confirmed the presence of PHB, with elemental composition showing 61.65% carbon and 38.35% oxygen. These results contribute to the understanding of microbial PHB production and highlight its potential as a sustainable bioplastic alternative. Further research should aim to optimize production conditions and scale-up processes to enhance the commercial viability of PHB for replacing conventional plastics.

6. Source of Funding

NA.

7. Conflict of Interest

No conflict of interest.

References

1. Landrigan PJ, Raps H, Cropper M, Bald C, Brunner M, Canonizado EM, et al. The Minderoo-Monaco Commission on plastics and human health. *Ann Global Health.* 2023;89(1):23
2. Narayanan M, Kandasamy S, Kumarasamy S, Gnanavel K, Ranganathan M, Kandasamy G. Screening of polyhydroxybutyrate producing indigenous bacteria from polluted lake soil. *Heliyon*. 2020;6(10):e05381.
3. McAdam B, Fournet BM, McDonald P, Mojicevic M. Production of polyhydroxybutyrate (PHB) and factors impacting its chemical and mechanical characteristics. *Polymers*. 2020;12(12):2908.
4. Sriyapai T, Chuarung T, Kimbara K, Samosorn S, Sriyapai P. Production and optimization of polyhydroxyalkanoates (PHAs) from *Paraburkholderia sp.* PFN 29 under submerged fermentation. *Electronic J Biotechnol*. 2022;56:1–11.
5. Hagagy N, Saddiq AA, Tag HM, Selim S, AbdElgawad H, Martínez-Espinosa RM. Characterization of Polyhydroxybutyrate, PHB, Synthesized by Newly Isolated *Haloarchaea Halolamina* spp. *Molecules*. 2022;27(21):7366.
6. Adnan M, Siddiqui AJ, Ashraf SA, Snoussi M, Badraoui R, Ibrahim AM, et al. Characterization and process optimization for enhanced production of polyhydroxybutyrate (PHB)-based biodegradable polymer from *Bacillus flexus* isolated from municipal solid waste landfill site. *Polymers*. 2023;15(6):1407.
7. Hamdy SM, Danial AW, Gad El-Rab SM, Shoreit AA, Hesham AE. Production and optimization of bioplastic (Polyhydroxybutyrate) from *Bacillus cereus* strain SH-02 using response surface methodology. *BMC Microbiol*. 2022;22(1):183.
8. Feng L, Yan J, Jiang Z, Chen X, Li Z, Liu J, et al. Characterization of polyhydroxybutyrate (PHB) synthesized by newly isolated rare actinomycetes *Aquabacterium sp.* A7-Y. *Int J Biol Macromolecules*. 2023;232:123366.
9. Trakunjae C, Boondaeng A, Apiwatanapiwat W, Kosugi A, Arai T, Sudesh K, et al. Enhanced polyhydroxybutyrate (PHB) production by newly isolated rare actinomycetes Rhodococcus sp. strain BSRT1-1 using response surface methodology. *Sci Rep*. 2021;11(1):1896.
10. Mostafa YS, Alrumman SA, Otaif KA, Alamri SA, Mostafa MS, Sahlabji T. Production and characterization of bioplastic by polyhydroxybutyrate accumulating Erythrobacter aquimaris isolated from mangrove rhizosphere. *Molecules*. 2020;25(1):179.
11. Alarfaj AA, Arshad M, Sholkamy EN, Munusamy MA. Extraction and characterization of polyhydroxybutyrates (PHB) from Bacillus thuringiensis KSADL127 isolated from mangrove environments of Saudi Arabia. *Braz Arch Biol Technol*. 2015;58(5):781–8.
12. Mahitha G, Madhuri R. Purification and characterization of polyhydroxybutyrate produced from marine bacteria. *Int J Sci Eng Res.* 2015;6(2):71–5.
13. Thapa C, Shakya P, Shrestha R, Pal S, Manandhar P. Isolation of polyhydroxybutyrate (PHB) producing bacteria, optimization of culture conditions for PHB production, extraction and characterization of PHB. *Nepal J Biotechnol*. 2018;6(1):62–8.
14. Vahabi H, Michely L, Moradkhani G, Akbari V, Cochez M, Vagner C, et al. Thermal stability and flammability behavior of poly (3-hydroxybutyrate)(PHB) based composites. *Materials*. 2019;12(14):2239.
15. Raju NN, Sankaranarayanan M, Bharathiraja B. Production of polyhydroxybutyrate (PHB), a biodegradable polymer from seaweed biomass using novel bacterial isolates. *J Mol Struct*. 2024;1303:137511.
16. Reddy AR, Kumar RB, Prabhakar KV. Isolation and Identification of PolyHydroxyButyrate (PHB) producing bacteria from Sewage sample. *Res J Pharma Technol*. 2017;10(4):1065–9.
17. Singh P, Parmar N. Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria. *Afr J Biotechnol*. 2011;10(24):4907–19.

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