

Research Article

Microbial Production of PHB from Rhizospheric Bacteria

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Abstract

The use of non-degradable polymers is generally inexpensive but has been creating havoc and has led to very serious environmental problems, as these non-degradable polymers take many years to degrade due to the hardness of this polymer. In the last few decades, there has been much research in the generation and advancement of new materials to address the problem presented by fossil-based polymers. This study focuses on The isolation, screening, and evaluation of PHB-producing bacteria from a soil sample collected from the rhizosphere. A total of heterotrophic bacterial isolates was obtained and subjected to screening using a fluorescence-based staining method involving Nile red dye. Approximately most of the isolates demonstrated significant fluorescence, indicating the presence of intracellular PHB granules. Selected strains were further cultivated under a glucose-rich medium to promote polymer development, and their PHB production was quantitatively monitored using spectrofluorometry. The findings indicated that PHB accumulation in all examined strains escalated over time and reached a plateau after approximately 48 hours of incubation. Comparative analysis using different carbon sources, such as glucose, showed similar levels of polymer production after 72 hours in most isolates. However, certain strains exhibited enhanced accumulation, suggesting variability in metabolic efficiency. Among the isolates studied, one strain demonstrated notably higher PHB production compared to others. The results show that using Nile red staining with spectrofluorometry is a quick and accurate way to find and count microorganisms that make PHB. Furthermore, rhizosphere soils are identified as promising natural sources for isolating efficient PHB-producing bacteria with potential industrial applications. The study also emphasizes the importance of environmental isolates in improving the accuracy of predictive models related to microbial activity and polymer production.

Keywords

Polyhydroxybutyrate (PHB), Bioplastic, Rhizosphere Soil, Nile Red, Sudan Black B, FTIR Characterization, Bacterial Isolates

1. Introduction

Soil microorganisms harbour an enormous diversity of microbial species, particularly within the rhizosphere, which is a

highly active and nutrient-rich habitat influenced by plant roots [1-3]. This unique ecological niche fosters a dynamic microbial

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Received: 31 December 2025; Accepted: 19 January 2026; Published: 17 October 2026



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community well-adapted to fluctuating environmental conditions and nutrient availability, making it a promising reservoir for novel biotechnological applications [4]. Consequently, rhizosphere bacteria represent an invaluable and largely untapped resource for the discovery of bio compounds that are important for industry, such as polyhydroxyalkanoates, which are biopolymers that store carbon and energy inside cells when growth conditions aren't ideal [5]. Polyhydroxyalkanoates (PHAs) are a well-known group of biopolymers that some types of microbes make as intercellular carbon and energy storage inclusions when they are not growing well [6]. They have received considerable attention as an alternative thermoplastic due to their inherent biodegradability and biocompatibility [7, 8]. Given the high production costs associated with PHAs, exploring novel microbial sources, particularly those from the rhizosphere, could significantly reduce expenses and facilitate large-scale applications [1]. Specifically, the rhizosphere of certain plants, such as groundnut plants, has demonstrated a notable prevalence of PHB-accumulating bacterial isolates, indicating its potential as a prolific source for such microbes [9]. Detailed investigation into these rhizospheric communities is crucial for identifying efficient PHB producers, thereby addressing the escalating demand for sustainable bioplastics and mitigating environmental pollution caused by conventional petrochemical-derived plastics [5]. PHB is a versatile polymer and has extensive use in medicine, food industry and flourishing in the field of drug delivery based on nanoparticles [10]. The mechanical characteristics of PHB closely resemble those of prevalent non-biodegradable synthetic thermoplastics, such as polypropylene. The growing appeal of PHB, particularly over synthetic or other bio-based plastics, is largely attributable to a convergence of factors, including complete biodegradability, biocompatibility, intrinsic hydrophobicity, adaptable hydrophilicity, and the availability of surface functional groups [11-13]. Adjusting the properties of PHB molecules to fit certain needs makes them even more useful as smart materials in the future [14]. PHB-based nanoparticles for targeted drug delivery are becoming more popular because they are very suitable for drugs and are easy to find in nature. It exhibits a significantly longer degradation time compared to polymers of the poly(α -hydroxyacid) group (e.g., PLA or PLGA) and demonstrates robust compatibility with diverse cell lines, supporting the proliferation and adhesion of mesenchymal stem cells, epithelial cells, chondrocytes, and fibroblasts ([15] Shaikh *et al.*, 2024).

2. Materials and Methods

2.1. Collection of Samples

Rhizosphere soil samples were collected from the agricultural fields of GIET University, Gunupur, Odisha (Latitude: 21°63'20.11" N, Longitude: 87°54'11.37" E). Sample was collected from the farm lands, mostly from the rhizosphere of plants like sunflower, coconut and sugarcane which have di-

verse microbial populations. The surface layer of soil is removed and the sample is collected from a depth of 5-10cm with sterile tools. The samples were transferred to the laboratory in a sterile container and kept in refrigerated conditions. The samples were processed within a short time frame for physicochemical and bacteriological studies to retain their original characteristics.

2.2. Bacteriological Analysis

Tenfold serial dilutions followed by the spread plate culture technique were used to isolate various bacteria from the representative samples. Required amount of Nutrient agar (NA) medium is prepared and poured into petri plates by maintaining aseptic conditions. Then 0.1ml of serial diluted samples from each tube were spread on each petri plate and the plates were incubated at 37 °C for 24 hours. From these, colonies with different morphological characters were picked for sub-culture on NA and incubated at 37 °C for 24 hours to obtain pure culture.

2.3. Screening of Bacteria by Sudan Black B

Sudan black B staining is used for screening of the intracellular PHB present inside the PHB producing cells. Prior to screening the isolates were incubated 72 hours for accumulation of PHB. Smear was prepared on a clean glass slide. After heat fixation the slide is stained with 0.3% of Sudan black prepared in 70% ethanol. Excess staining was removed by gentle washing and immersing it in xylene. Counterstain is done with safranin (0.5% w/v in deionized water) and the slide was then rinsed, dried and observed under microscope at 100x magnification to detect dark intracellular PHB granules ([16] Bhawal *et al.*, 2014).

2.4. Screening of Bacteria by Nile Red Staining Method

The Nile red viable colony staining was employed for the detection of PHB granules in the bacterial cytosol. Briefly, selected bacterial isolates were streaked onto a nitrogen-limiting mineral salt medium (MSM) supplemented with 0.2% Nile red solution (prepared in DMSO) and incubated at 37 °C for 48 hours. Following incubation, the plates were examined under UV transillumination at an excitation wavelength of 312 nm to identify fluorescent colonies indicative of PHB accumulation.

2.5. PHA Production and Extraction Under Submerged Fermentation

PHA production was carried out using a one-stage batch cultivation method in shake flasks. The PHB positive bacteria is grown under optimized conditions for PHA biosynthesis and accumulation. Briefly, 1 L of modified growth medium (pH 8.0) with a C: N ratio of 6:1 was inoculated with 10% (v/v)

culture and incubated at 37 °C for 72 hours in a shaker incubator at 180 rpm. 4 different carbon sources are used for the culture that are sodium acetate, glucose, glycerol and waste cooking oil. The Media are named as MSM-Glucose (MGL), MSM-Glycerol (MG), MSM-Oil (MO), MSM-Sodium acetate (MS) After incubation, the biomass was harvested by centrifugation. The resulting pellet was dried at 50 °C overnight to determine cell dry mass (CDM). The dried pellet was further treated with a sodium hypochlorite-distilled water mixture (2:3), vortexed for 1 minute, and incubated at 37 °C for 30 minutes to achieve cell lysis, followed by centrifugation at 10,000 × g for 15 minutes. The pellet was then washed sequentially with distilled water and a solvent mixture of diethyl ether, acetone, and methanol (1:1:1) to remove residual non-PHA materials, with centrifugation at 10,000 rpm for 10 minutes ([17] Maity *et al.*, 2020).

2.6. Fourier Transform Infrared (FTIR) Spectroscopic Analysis of Extracted PHA

The extracted PHA sample was analyzed using Fourier Transform Infrared (FTIR) spectroscopy. Briefly, the sample was mixed with potassium bromide (KBr) (2%) and compressed into translucent pellets. The pellets were then subjected to FTIR analysis using a spectrometer (PerkinElmer RX-I). The infrared spectra were recorded in transmission mode over the range 4000–400 cm⁻¹ to identify the distinctive functional groups in the polymer ([18] Dash *et al.*, 2013; [19] Lau *et al.*, 2011).

2.7. Molecular Identification and Characterization of PHAs

Genomic DNA was extracted from overnight bacterial culture using the phenol-chloroform method, followed by enzymatic lysis and purification ([20] Ahmed *et al.*, 2014). The DNA was precipitated, washed, resuspended in the TE buffer, and its purity was checked using a NanoDrop spectrophotometer. The extracted DNA was verified on 1.2% agarose gel, and the 16s rDNA gene was amplified using universal primers (27F and 1429R). The PCR product was purified, sequenced, and analyzed using BioEdit software before submission to NCBI (<http://blast.ncbi.nlm.nih.gov/blast.cgi>). Phylogenetic analysis was performed using ClustalW and MEGA software with the neighbor-joining method and bootstrap analysis. Secondary structure of rRNA was predicted using the mfold web server based on minimum free energy (ΔG). The phaC gene was amplified under optimized PCR conditions and confirmed

by agarose gel electrophoresis.

3. Results and Discussion

3.1. Detection of PHB Accumulation Using Sudan Black B, Nile Red Staining

The positive Sudan Black B staining, characterized by bluish-black coloration, provided a preliminary indication of lipid accumulation in bacterial cells, thereby distinguishing potential PHB producers from non-producers ([21] Narayanan *et al.*, 2020). identified as PHB producers by both rapid plate screening and Sudan Black B staining, with microscopic analysis confirming the presence of lipophilic black granules in the stained bacterial cells ([22] Madhumathi & Velan, 2016). Nile Red produces characteristic orange fluorescence under UV light. Subsequent analysis revealed that these granules ranged from 0.2 to 0.5 μm in diameter, consistent with typical PHA granule dimensions ([23] Ranjan *et al.*, 2025). This finding was corroborated by prior research demonstrating that Nile Red staining, under specific excitation conditions, effectively visualizes PHB granules as bright orange fluorescence, enabling quantitative assessment of intracellular accumulation ([24] Baria *et al.*, 2023; [25] Trakunjae *et al.*, 2021).

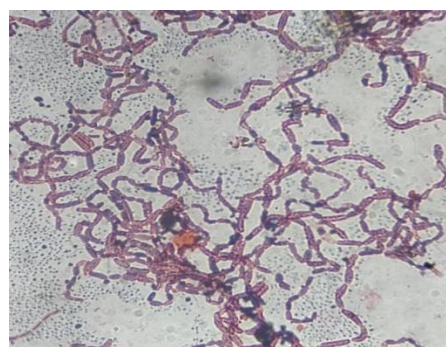


Figure 1. Sudan black B staining under microscope.

3.2. Screening for PHB Production

The dark intracellular granules showed PHB positive. The bacteria was further evaluated for PHA production in mineral salt medium (MSM) with sodium acetate, glucose, waste cooking oil and glycerol in submerged fermentation (SMF) conditions. Biomass yield and PHA production were measured and are presented in Table 1.

Table 1. Comparative evolution of biomass, PHB yield, and PHB accumulation under submerged Fermentation.

SL No.	Media code	Biomass (SMF) (g/L)	PHB yield (SMF) (g/L)	PHB % (SMF)
1	MG	3.2	0.882	26.77

SL No.	Media code	Biomass (SMF) (g/L)	PHB yield (SMF) (g/L)	PHB % (SMF)
2	MO	4.6	1.323	28.29
3	MS	2.8	0.907	31.69
4	MGL	5.1	2.522	49.37

There is more production of PHA in the glucose medium compared to other two mediums. There is 49% of PHA production in Submerged fermentation in glucose medium followed by sodium acetate, waste cooking oil and glycerol media respectively. Moreover, exploring the impact of varying carbon sources and their concentrations on PHB yield, particularly agricultural wastes, presents a viable avenue for cost-effective and sustainable production ([26] Sirohi, r., Pandey,2024; [27] Rizvi *et al.*, 2023).

3.3. Fourier Transform Infrared (FTIR) Spectroscopic Analysis

FTIR characteristics peak at 1719.45 cm⁻¹ further corroborated the presence of the (C=O) ester carbonyl group, consistently identifying the synthesized biopolymer as polyhydroxybutyrate across all tested carbon sources, aligning with prior structural confirmations of PHB homopolymers ([28] Quintero-Silva *et al.*, 2023). This consistent observation of a strong absorbance band at approximately 1719 cm⁻¹ is a definitive indicator of the C=O ester carbonyl stretches, which is characteristic of PHB ([17] Maity *et al.*, 2017).

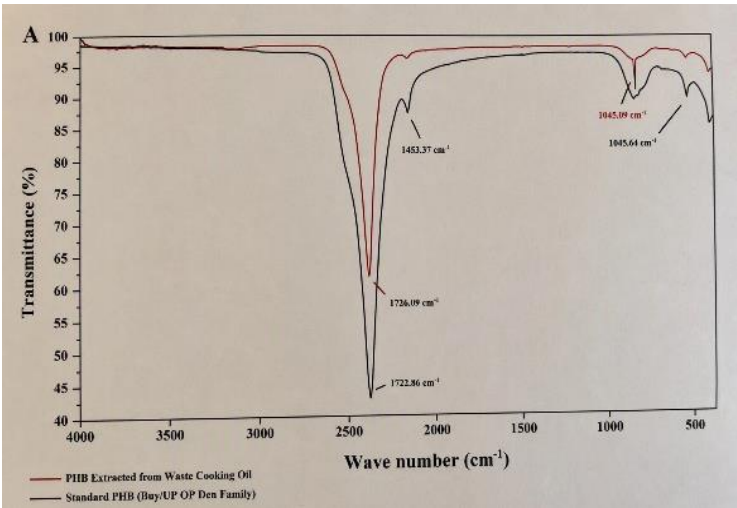


Figure 2. FTIR analysis of PHB.

3.4. Molecular Identification and Phylogenetic Analysis

The 16s rDNA analysis identified the isolate as *Bacillus altitudinis* (accession number: MN794433.1), showing high similarity with related *Bacillus altitudinis* strains, confirming

its taxonomic position with strong bootstrap support. The tree also indicated a clear evolutionary relationship with other bacillus members, while *Escherichia coli* served as an outgroup. The stable clustering pattern suggests genetic conservation within the species. These results validate the molecular identification and evolutionary placement of the isolate ([29] hal-iru *et al.*,2024).

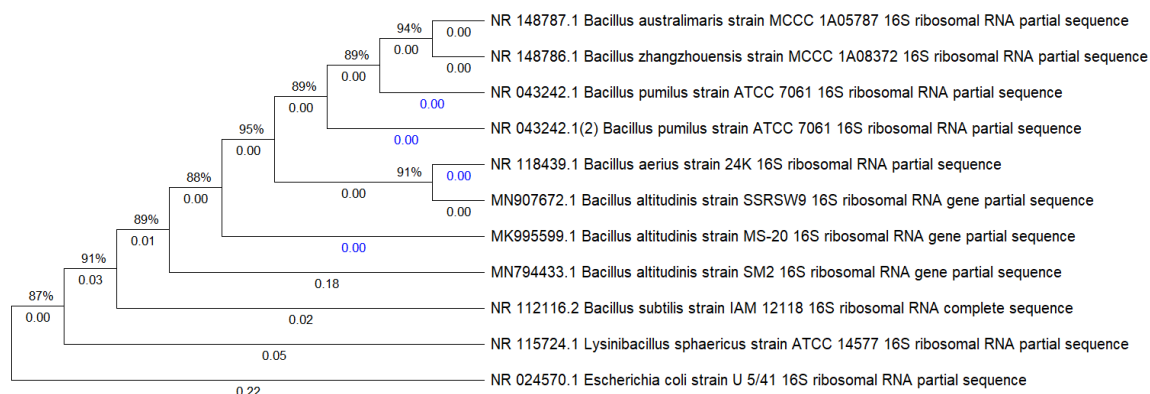


Figure 3. Phylogenetic Tree.

4. Conclusion

This study successfully demonstrated the isolation and characterization of polyhydroxybutyrate (PHB) producing bacteria from rhizospheric soil, highlighting the rhizosphere as a rich source of biopolymer producing microorganisms. Screening methods such as Sudan black B and Nile red staining proved to be rapid and reliable for the detection of intracellular PHB granules. Among the isolates, one potent strain showed significantly higher PHB accumulation, particularly under glucose supplemented conditions, indicating the importance of carbon source optimization for enhanced production. FTIR analysis confirmed the synthesized polymer as PHB through the presence of characteristic functional groups. molecular identification using 16s rRNA sequencing revealed the isolate as *Bacillus altitudinis* (MN794433.1), further supported by phylogenetic analysis. The detection of the *phaC* gene also validated its genetic potential for PHB biosynthesis. Overall, the findings emphasize the potential of rhizospheric bacteria for sustainable and eco-friendly bioplastic production with promising industrial application.

Abbreviations

PHB	Polyhydroxy Butyrate
PHA	Polyhydroxy Alkanoates
FTIR	Fourier Transform Infrared Spectroscopy
MSM	Mineral Salt Media
SMF	Submerged Fermentation

Acknowledgments

The authors acknowledge BIRAC E-Yuva Center and Department of Biotechnology GIET University for providing laboratory facilities and encouragement for the research work.

Author Contributions

Subhrojeet Singha Roy: Conceptualization, Resource
Anjali Panigrahi: Data curation, Methodology, Writing – original draft, Visualization
Rinki Maharana: Formal Analysis, Investigation, Software
Bodroth Rabi Prasad: Supervision, Formal Analysis
Sudipta Maity: Project administration, Software, Validation, Writing – review & editing

Funding

This research was financially supported by Ministry of Micro, Small and Medium Enterprises, India under the grant ‘Idea Hackathon 2.0’ and Biomat Innovations Private Limited.

Data Availability Statement

The data supporting the outcome of this research work has been reported in this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Biography



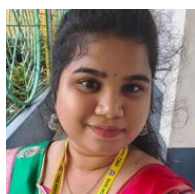
Subhrojeet Singha Roy is an innovator and researcher in biotechnology, nanotechnology, and materials science, holding professional roles in research and technology development. Educational background includes biotechnology with ongoing research focused on electrochemical sensing

systems and the sustainable fabrication of screen-printed electrodes. Current work involves designing, fabricating, and optimizing electrochemical and printed electrochemical biosensors and printed electronic platforms for applications in point-of-care diagnostics, environmental monitoring, and using scalable manufacturing techniques to achieve high sensitivity, selectivity, and reproducibility. Contributions include interdisciplinary research and development aimed at translating laboratory-scale sensor technologies into robust, application-oriented analytical systems for next-generation diagnostic and monitoring applications.



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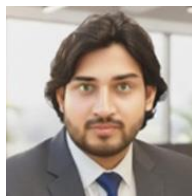


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Sudipta Maity is driving as the EYC Coordinator at the DBT-BIRAC E-Yuva Center. He is an accomplished researcher in biotechnology and environmental microbiology with expertise in bioenergy, biocomposites, biomaterials, drug delivery, and environmental remediation. His research primarily focuses on polyhydroxyalkanoates (PHAs), sustainable biopolymers, microbial biotechnology, and waste valorization. He has authored numerous high impact research publications with significant citations in reputed international journals. He has actively contributed to innovative research on biofertilizers, microbial fermentation, and sustainable agricultural applications, promoting eco-friendly technologies and interdisciplinary scientific advancement.

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Research Field

Subhrojeet Singha Roy: Microbiology, Environmental Biotechnology

Anjali Panigrahi: Microbiology, Environmental Biotechnology

Rinki Maharana: Microbiology, Nanotechnology

Bodroth Rabi Prasad: Immuno-biotechnology, Microbiology

Sudipta Maity: Microbiology, Material Science

Pankaj Kumar Parhi: Aqueous Chemistry, Environmental Biotechnology