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Enhanced Production of Polyhydroxybutyrate (PHB) from *Brevundimonas* Species: Characterization, Optimization, and SEM-EDX Insights

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Abstract: This study focuses on the isolation and characterization of bacterial strains capable of producing polyhydroxybutyrate (PHB) from soil samples collected from garbage dump site. The bacterial isolates, identified as *Brevundimonas vancanneytii* and *Brevundimonas diminuta*, were obtained using the quadrant streak plate technique. PHB production was screened using the Sudan black staining method, revealing that all isolates could accumulate PHB. The PHB extraction was performed using a solvent extraction method, yielding a white powder. Quantification of PHB was conducted through heating the extracted polymer with concentrated sulfuric acid, resulting in crotonic acid. The optimal culture conditions for PHB production were determined, with isolate SW4 demonstrating the highest yield at 76.41%. The effects of temperature, pH, carbon sources, and nitrogen sources on PHB production were systematically evaluated. Additionally, biochemical characterization of the isolates was performed using the HIMEDIA KB003 kit, and molecular identification was confirmed through 16S rRNA gene analysis. This study highlights the potential of utilizing *Brevundimonas* strains for sustainable bioplastic production, offering an eco-friendly alternative to conventional plastics.

Keywords: Polyhydroxybutyrate , Bioplastics, 16S rRNA, *Brevundimonas sp.*, SEM

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Introduction

Several microorganisms produce biodegradable polyesters named as polyhydroxyalkanoates (PHAs). PHAs are produced by a variety of bacteria under nutrient limitation conditions along with excess carbon. These biopolymers get converted into granules inside the bacterial cells and act as energy reserves. Furthermore, while all PHAs are polyesters, their exact qualities differ depending on the biopolymer employed, and not all PHAs have the same characteristics.

The most common example is poly(3-hydroxybutyrate) (from this point on referred to

as simply PHB and thus also as the most common form of a type of polymer called PHAs). It has very good biodegradability and appears to have been used or considered in recent years in instances where that property yields particularly favourable results (Chen, 2009).

Numerous microorganisms synthesize the biodegradable polymer polyhydroxybutyrate (PHB) as a component for energy storage. It belongs to the class of polyhydroxyalkanoates (PHA) and has a number of special qualities. In addition to having a high degree of crystallinity (55–80%) and a high melting temperature of about 175°C, PHB is strong and rigid. It is appropriate for packaging applications due to its strong barrier qualities against gases including oxygen and water vapor. Furthermore, PHB has the potential to be used in medical applications such as tissue engineering and sutures because it is biocompatible and break down naturally in the environment without releasing harmful byproducts (Rai *et al.*, 2011). PHB's brittle nature, however, restricts its use in situations where flexibility is critical (Philip *et al.*, 2007). Further obstacles to wider industrial adoption are the brittleness and comparatively higher production costs (Sudesh *et al.*, 2000).

PHB stands out apart from traditional plastics like polypropylene (PP) and polyethylene (PE) because of its advantages for the environment. Because conventional plastics are not biodegradable and are made from non-renewable petrochemical resources, they pollute the environment over time, especially when they end up in landfills and the oceans as plastic debris. Conventional plastics remain for hundreds of years in the environment while being less expensive to make and having better mechanical qualities, such as flexibility and durability. PHB, on the other hand, is completely biodegradable and decomposes in a few months in the presence of nature, making it a sustainable substitute for transient uses like packaging. But in many places, PHB's brittleness and greater expense continue to be a barrier to replace traditional plastics (Laycock *et al.*, 2013; Reddy *et al.*, 2018).

PHA are basically produced through bacterial fermentation utilizing carbon sources. Some of the most common PHA-producing bacteria include *Cupriavidus necator*, *Pseudomonas species*, and *Bacillus megaterium*. The process involves mainly two stages; first stage is that the bacteria grow during nutrient-sufficient phase and then second phase where their growth is under nutrient limited conditions where they accumulate PHAs as carbon source and as energy reserves. The carbon sources for PHA production may vary starting from simple sugar such as glucose and sucrose to industrial and agricultural waste products such as glycerol from biodiesel production, whey from dairy industries, and agricultural wastes (Koller *et al.*, 2010).

Using these waste carbon sources might be attractive considering their low cost as well as renewability factors (Khanna and Srivastava, 2005a). Moreover, lignocellulosic biomass has gained increasing interest as a major ingredient for PHA production mainly due to its low costs and wide availability. The main drawback of this type of feedstock, however, is the inability to efficiently convert the biomass into fermentable sugars (Choi and Lee, 1997; Dietrich *et al.*, 2019).

Nevertheless, while PHAs can serve as a good and sustainable substitute for traditional petroleum plastics, they still have high production costs mainly attributed to the cost of carbon feedstocks, recovery process, and poorer yields in case of some bacterial strains produced today (Poirier *et al.*, 1995). At present, certain researchers in the field of metabolic engineering, as well as synthetic biology and fermentations, are trying to develop and improve the existing methods of PHA production in order to achieve increased yields and lower the production costs (Chen, 2012; Raza *et al.*, 2018).

Moreover, PHAs can be produced in genetically modified plants, and this application may reduce costs and increase the scalability of

this procedure (Suriyamongkol *et al.*, 2007). Finally, such biodegradable, biocompatible, and renewable polymers could potentially address global environmental issues. These materials will be essential components of sustainable plastics in the future if additional developments in microbial engineering, feedstock selection, and process optimization are made available (Rehm, 2010).

Therefore, the purpose of this study is to isolate and characterize bacterial strains capable of producing polyhydroxybutyrate (PHB) from soil samples, particularly those collected from a garbage dump site. By optimizing the culture conditions, including temperature, pH, carbon sources, and nitrogen sources, this research aims to maximize PHB production. Ultimately, the study seeks to explore the potential of these bacterial strains as a sustainable alternative for bioplastic production, contributing to environmentally friendly solutions for plastic waste.

Materials and Methods

Collection of Samples

Surface soil samples, free of dust and stones, were collected from a garbage dump site using a sterilized spatula wiped with ethanol. The samples were placed in sterile zip-lock bags and immediately transferred to the lab, where they were stored at 4°C for future analysis (Narayanan *et al.*, 2020).

Isolation and Preservation of Bacterial Strains

To isolate bacteria from the soil samples, a standard serial dilution method was applied after the samples had thawed to room temperature. Using the spread plate technique, 50 µl of 10^{-6} dilution of each soil sample was spread onto sterile LB agar plates enriched with 1% glucose as a carbon source. Plates were labelled, and three replicates were prepared for each sample. The plates were incubated for 48 h at 37°C. Afterward, the predominant bacterial colonies were isolated and purified using the quadrant streaking method. The purified bacterial cultures were stored at 4°C for further studies (Narayanan *et al.*, 2020).

Screening of PHA-Producing Bacteria

Each bacterial isolate was streaked onto petri dishes containing Na^+ and 1% glucose media and incubated for 24 h at 37°C. Sudan black stain was used to assess PHA production. To prepare the stain, 200 ml of 70% ethanol was mixed with 60 mg of Sudan black, and the solution was stirred for 24 h. Bacterial colonies were stained by applying the Sudan black solution for 30 min, after which the stain was removed with 96% ethanol.

Production of PHB by Selected Isolates

The chosen bacterial isolates were cultured in a medium containing urea, yeast extract, KH_2PO_4, Na_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2$, glucose, and trace elements like $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$. Before inoculation, glucose and trace element solutions were autoclaved. The bacteria were subcultured in nutrient broth for 24 h, then transferred to the production medium and incubated at 37°C with shaking at 150 rpm for two days.

Extraction and Quantification of Polyhydroxyalkanoates (PHA) from Bacterial Cultures

To extract and quantify polyhydroxyalkanoates (PHA), 10 ml of bacterial culture was centrifuged at 10,000 rpm for 15 min, resulting in a pellet. This pellet was treated with sodium hypochlorite and incubated at 30°C for 2 h. The mixture was then centrifuged, washed with distilled water, acetone, and methanol, and the final pellet was dissolved in boiling chloroform. The chloroform solution was evaporated onto a sterile watch glass and stored at 4°C for further analysis.

For quantification, the extracted PHA was dissolved in concentrated sulfuric acid and heated to 100°C for 10 min, converting poly(3-hydroxybutyrate) (PHB) into brown crotonic acid. The solution was cooled, and its absorbance was measured at 235 nm using a UV spectrophotometer. A standard curve using concentrated sulfuric acid was employed to calculate PHB concentrations based on the absorbance values.

Identification of Soil-Isolated Bacteria Using Biochemical Tests and 16S rRNA Gene Analysis

To explore and identify bacteria from soil, a combination of biochemical and molecular methods was employed. Initially, Gram staining was performed to categorize the bacteria as either Gram-positive or Gram-negative. Bacterial samples were heat-fixed on glass slides, stained with crystal violet, rinsed, and treated with Gram's iodine. After decolorization and another rinse, safranin was applied to reveal the Gram type under a microscope.

Next, the bacteria was cultured in Luria Bertani broth and analysed using biochemical tests. A HIMEDIA KB003 kit was used to conduct 25 different tests, including ONPG, urease, citrate utilization, Voges-Proskauer, and methyl red, helping to partially identify the soil-isolated bacteria.

For a more detailed identification, molecular techniques were employed to analyse the 16S rRNA gene of bacteria that were found to accumulate PHB. Genomic DNA was extracted, its purity checked via spectrophotometry, and verified through agarose gel electrophoresis. The 16S rRNA gene was then amplified using PCR with universal primers (27F and 1492R). After confirming successful amplification by gel electrophoresis, the PCR products were sequenced. The sequences were compared using BLAST and submitted to the NCBI GenBank for reference.

Optimization of Culture Conditions for PHB Production

The optimization of culture conditions for PHB production was explored by examining several factors. Temperature effects were studied by incubating bacterial cultures at 20°C, 35°C, and 50°C, with the aim of identifying the optimal temperature for PHB synthesis. Additionally, the influence of pH on PHB production was assessed by adjusting the medium to pH values of 4, 7, and 10 using 0.1N NaOH and 0.1N HCl. The impact of different carbon sources—mannitol, sucrose, and starch, was also tested to determine their role in enhancing PHB production. Similarly, various nitrogen sources, including ammonium sulfate, peptone, and yeast extract, were evaluated for their effects on PHB yield (Fatimah Alshehrei *et al.*, 2019).

SEM with EDX Analysis of PHB

The PHB granules were studied using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX). The PHB was first dried and placed on a stub, secured with carbon tape, and coated with a thin layer of gold to improve conductivity. SEM was used to capture detailed images of the granules' surface, showing their texture and structure. EDX was then performed to analyze the elements present in the granules. This technique allowed for a better understanding of the granules physical properties and composition, as also seen in previous studies (Hernandez *et al.*, 2019).

Results and Discussion

Isolation and Preservation of Bacterial Strains

In this study, five distinct bacterial isolates, labelled SW1, SW2, SW3, SW4, and SW5, were successfully obtained from soil samples collected at a garbage dump site (Fig. 1). The presence of these isolates highlights the rich microbial diversity in environments with high organic waste concentrations, as previously noted by Chen *et al.* (2015). The isolation process involved using the quadrant streak plate technique, which allowed for the establishment of pure cultures. These isolates were preserved as slant cultures for further experimental analysis, facilitating their use in subsequent assays to assess their potential for polyhydroxybutyrate (PHB) production.

Screening for PHB-Producing Bacteria

To assess the capacity of the isolated strains to synthesize PHB, Sudan Black staining was employed, a widely recognized method for identifying PHB granules in bacteria. All five bacterial isolates exhibited a strong affinity for the Sudan Black stain, which resulted in the absorption of dark blue coloration around the

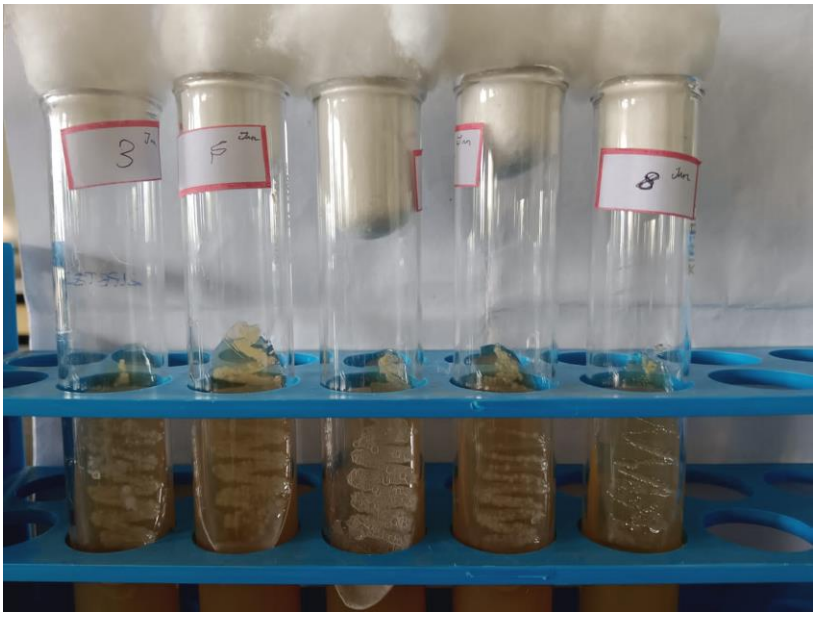

Fig. 1: Slant culture of selected isolates.

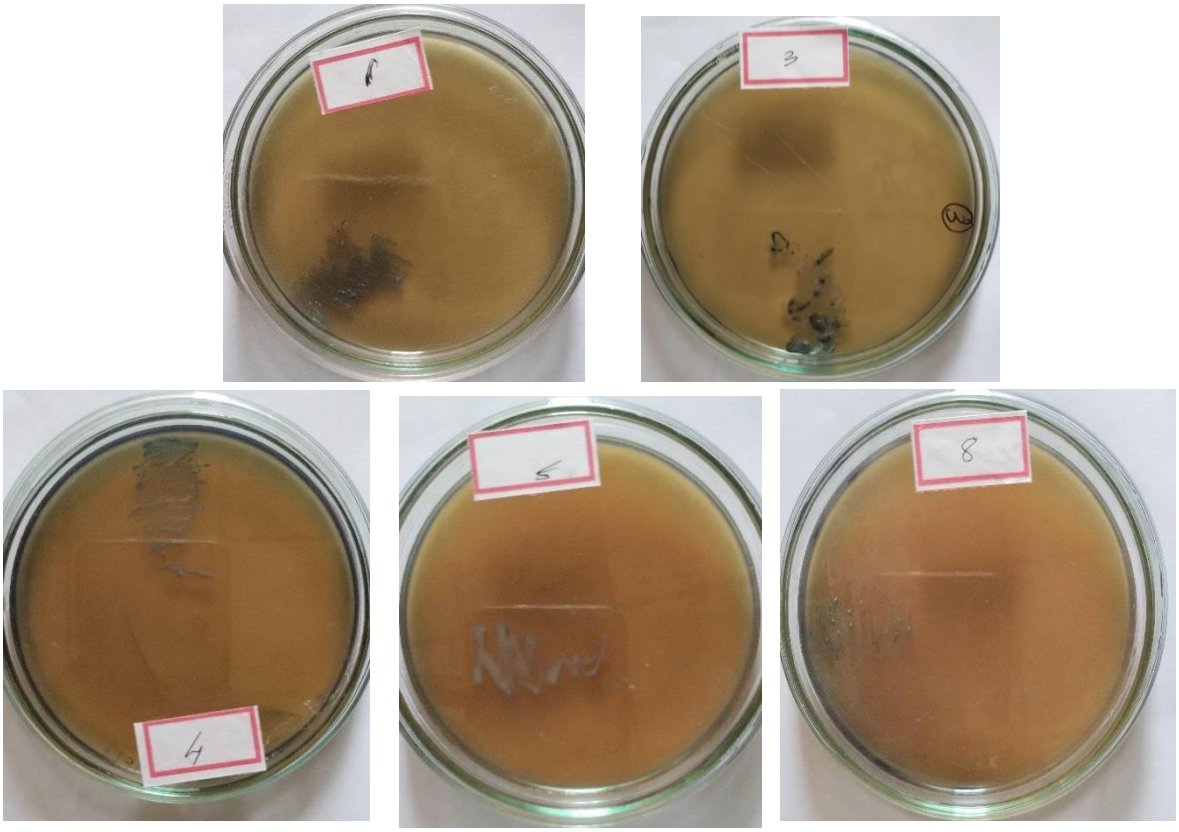

Fig. 2: Screening of strains by Sudan black method.

colonies (Fig. 2). This positive reaction indicated the ability of these isolates to accumulate PHB, a characteristic trait of bacteria thriving in nutrient-limiting conditions, particularly where carbon sources are abundant and nitrogen is limited (Rehm, 2003). The ability to store PHB as an energy reserve has significant implications for biodegradable plastic production, presenting an

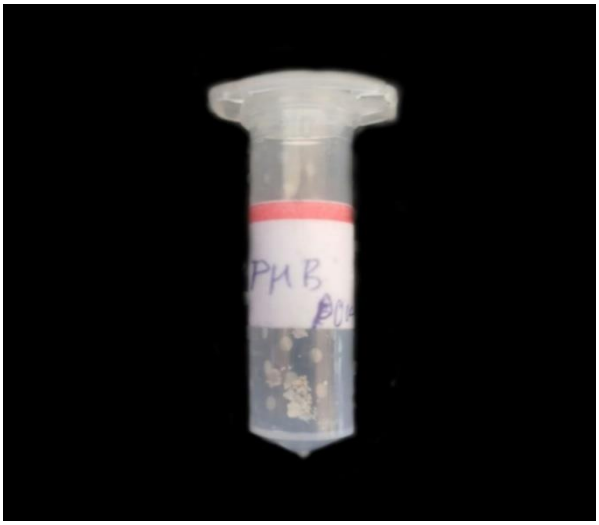

Fig. 3: Extracted PHA powder.

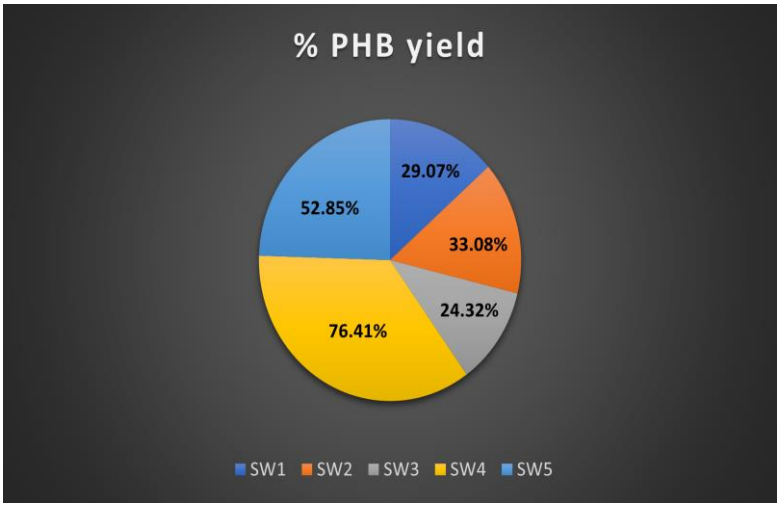

Fig. 4: Percentage of PHB yield by the isolates.

eco-friendly alternative to conventional petrochemical-based plastics (Anderson and Dawes, 1990).

PHB Extraction and Quantification

The extraction of PHB was conducted using a solvent extraction method with chloroform, resulting in a white powder (Fig. 3). The effectiveness of this method has been documented in various studies, emphasizing the utility of chloroform as a solvent for the extraction of intracellular polymers (Poh *et al.*, 2016). Upon treatment with concentrated sulfuric acid, the extracted PHB was converted into crotonic acid, producing a brown coloration. This transformation was quantified using UV-Visible spectrophotometry, where absorbance measurements at 235 nm confirmed the presence of crotonic acid, facilitating the quantification of PHB yields among the isolates. The results demonstrated a range of PHB yields, with isolate SW4 producing the highest yield at 76.41%.

Quantification of PHB yields by the isolate results (Fig. 4) revealed considerable variability in PHB production among the isolates. Isolate SW1

produced 29.07% of PHB, while SW2 exhibited a yield of 52.85%. In contrast, isolate SW3 had a relatively lower yield of 24.32%. Notably, isolate SW4 was identified as the most efficient PHB producer, yielding 76.41% followed by SW5 with 52.8%. These findings suggest that specific strains possess distinct metabolic capabilities for PHB synthesis, which may be attributed to differences in their biochemical pathways and environmental adaptations (Madison and Huisman, 1999). The low yield observed in isolate SW3 indicates a potential limitation in its metabolic capacity for PHB production compared to the other isolates.

Bacterial characterization using biochemical identification and 16S rRNA Gene Analysis

The biochemical characterization of the SW4 and SW5 bacterial isolates was performed as they produced the higher quantity of PHB. The tests were conducted using the HIMEDIA KB003 kit, which provided insights into the metabolic capabilities of these strains. Both the isolates tested positive for ONPG, urease, nitrate reduction, citrate utilization, and esculin hydrolysis, suggesting common enzymatic pathways among them. This uniformity indicates that the isolates may share similar ecological niches or metabolic requirements. This finding aligns with the research conducted by Holt *et al.* (1994), who emphasized the importance of such metabolic traits in bacterial identification. The sugar fermentation patterns revealed additional metabolic diversity among the isolates. While both the isolates tested positive for ONPG, indicating β-galactosidase activity, their capacity to ferment glucose, sucrose, and other carbohydrates varied.

The molecular identification of PHB-accumulating isolates SW4 and SW5 was achieved through 16S rRNA gene amplification, sequencing and submitted to NCBI GenBank (Accession No: PP712915 and PP724751). The sequences revealed that isolates SW4 and SW5 exhibited 99.71% and 99.79% identity with *Brevundimonas vancanneytii* strain PHBJS01 and *Brevundimonas diminuta* strain PHBJS02, respectively. The high degree of similarity to known PHB-producing species reinforces the potential of these isolates for industrial applications in biodegradable plastic production. Previous studies have documented the capability of *Brevundimonas* species to degrade complex organic compounds, further highlighting their significance in biotechnological applications (Sanchez *et al.*, 2011).

Optimization of Culture Conditions for PHB Production

Effect of Temperature on PHB Production

The study examined the influence of temperature on PHB production across the isolates, revealing that 35°C was the optimal temperature for PHB synthesis, yielding a cell dry weight of 5.4 g/L and a PHB concentration of 3.6 g/L, resulting in a yield of 66.7% (Fig. 5). This finding is consistent with the work of Khanna and Srivastava (2005b), who have demonstrated that optimal temperatures significantly enhance bacterial metabolic activity and PHB production. Conversely, lower temperatures (20°C) and higher temperatures (50°C) resulted in reduced bacterial growth and lower PHB yields, emphasizing the importance of maintaining optimal conditions for microbial activity.

Effect of pH on PHB Production

The impact of pH on PHB production was also evaluated, with the highest yield observed at a neutral pH of 7, where cell dry weight reached 5.11 g/L and PHB production was measured at 3.25 g/L, corresponding to a yield of 63.6% (Fig. 6). Lower pH (4) and higher pH (10) resulted in decreased PHB yields of 30.12% and 42.16%, respectively. This trend is supported by previous research indicating that neutral pH conditions favor PHB production due to enhanced enzyme activity and cellular metabolism (Wei *et al.*, 2011). Extreme pH levels can negatively impact cellular processes, leading to reduced PHB synthesis, as also suggested by Palleroni and Palleroni (1978).

Effect of Carbon Sources on PHB Production

The evaluation of different carbon sources revealed that glucose was the most effective,

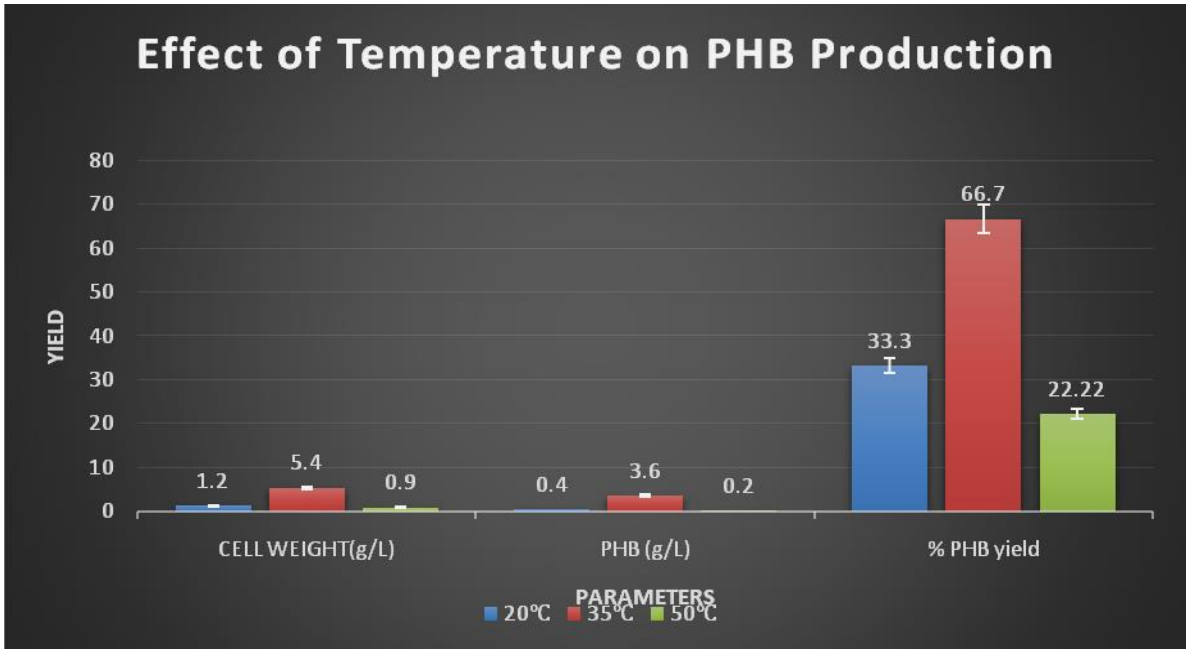

Fig. 5: Effect of temperature on PHB production.

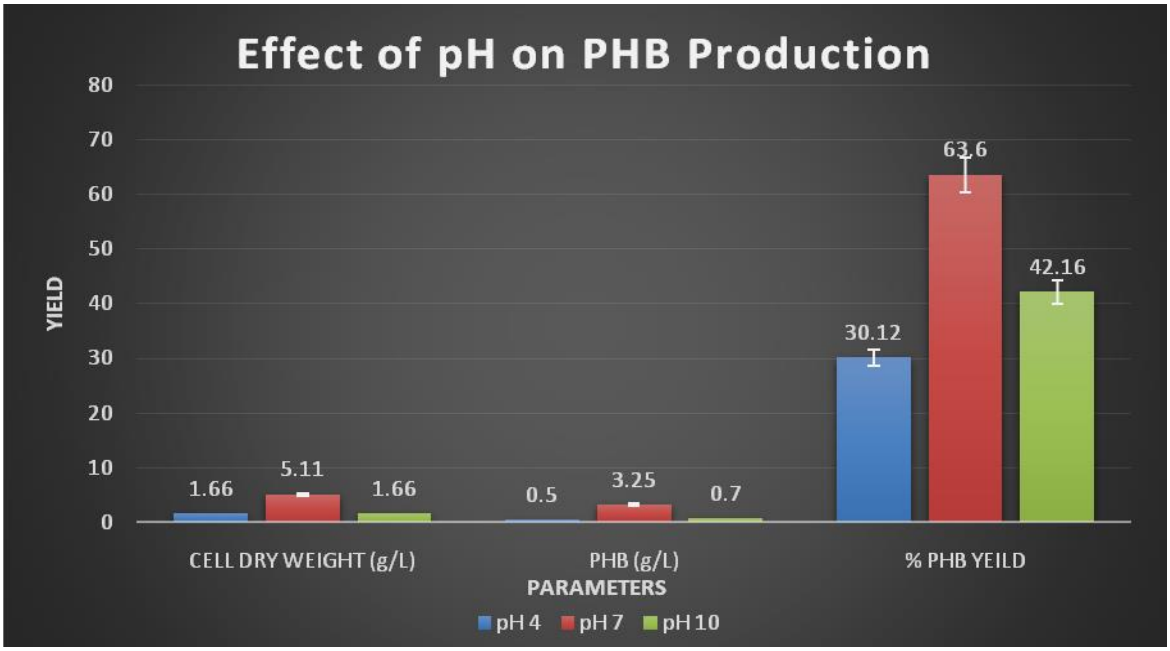

Fig. 6: Effect of pH on PHB production.

yielding a PHB percentage of 66.7% (Fig. 7). In comparison, other carbon sources such as sucrose (48.38%), mannitol (38.5%), and starch (22.2%) produced lower yields. The preferential utilization of glucose can be attributed to its efficient metabolism, which provides a direct substrate for PHB biosynthesis (Madison and Huisman, 1999). The differences in PHB yields among the carbon sources highlight the importance of optimizing carbon source selection for enhanced PHB production in microbial fermentation processes.

Effect of Nitrogen Sources on PHB Production

The influence of nitrogen sources on PHB

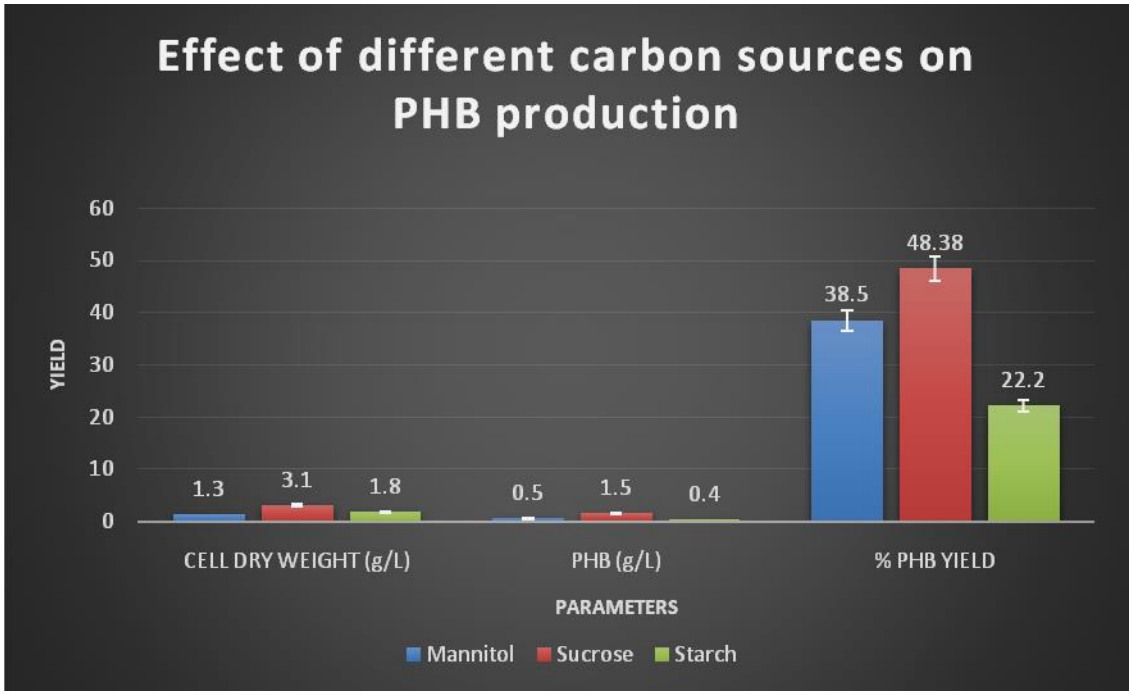

Fig. 7: Effect of different carbon sources on PHB production.

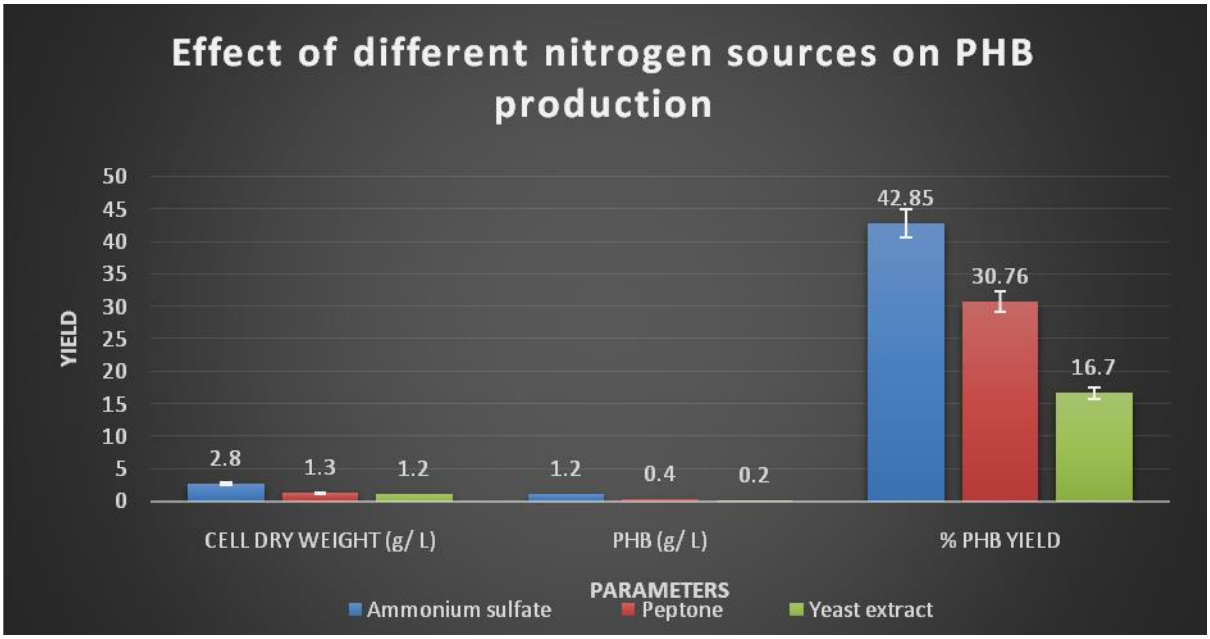

Fig. 8: Effect of different nitrogen sources on PHB production.

production was investigated, revealing that ammonium sulfate yielded the highest PHB production at 42.85% (Fig. 8). In contrast, peptone and yeast extract resulted in lower yields of 30.76% and 16.7%, respectively. The superior performance of ammonium sulfate can be attributed to its consistent nitrogen supply, which is essential for microbial growth and metabolic activities (Rehm, 2003). The lower yields observed with organic nitrogen sources may be due to the presence of complex compounds that could divert metabolic resources away from PHB production.

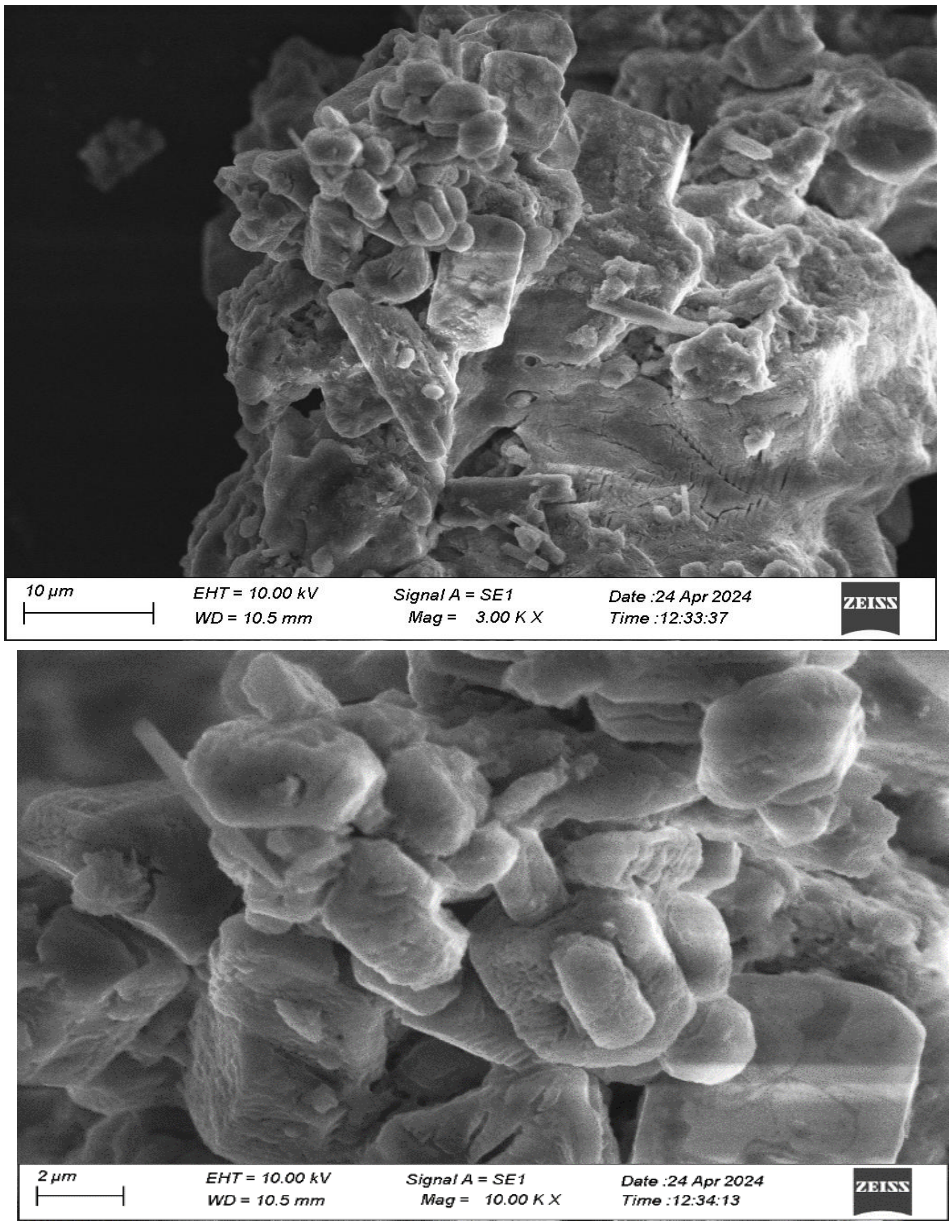

Fig. 9: SEM Analysis of prepared PHB powder.

SEM Analysis of PHB Powder

The surface morphology of the extracted PHB was examined through scanning electron microscopy (SEM), revealing important structural characteristics of the PHB granules. The SEM micrographs depicted an uneven surface texture with scaly and cavity-like formations (Fig. 9). This surface morphology is consistent with previous studies by Hernández *et al.* (2019), where irregularly shaped PHB granules were observed. The variations in granule shape and structure could affect the physical properties and potential applications of the extracted PHB, including its mechanical strength and biodegradability.

Conclusion

The present investigation successfully isolated and characterized five bacterial strains capable of producing polyhydroxybutyrate (PHB) from soil samples at a garbage dump site. The analysis revealed significant variations in PHB production,

with isolate J4 demonstrating the highest yield of 76.41%. The optimization of culture conditions showed that a temperature of 35°C, a neutral pH of 7, glucose as the preferred carbon source, and ammonium sulfate as the nitrogen source markedly enhanced PHB synthesis. These results highlight the potential of utilizing specific bacterial strains for bioplastic production, particularly in waste-rich environments. The findings underscore the importance of optimizing growth conditions to maximize PHB yields, which could contribute to developing sustainable alternatives to conventional plastics.

Ethical Statement

No animal has been used or sacrificed for this study, hence Ethical Approval not required.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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