Toxicology International
DOI: 10.18311/ti/2026/v33i3/55044
INFORMATICS JOURNALS
eISSN: 0976-5131 | Vol 33(3), 2026 | pp. 635-648

Isolation and Biochemical Screening of PHB-Producing Bacteria with Quantitative Assessment of PHB Yield

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Abstract

Environmental pollution from synthetic plastics has accelerated the search for sustainable, eco-friendly alternatives, such as Polyhydroxybutyrate (PHB). PHB is a biodegradable and biocompatible biopolymer that accumulates intracellularly in several bacterial species as a carbon and energy storage compound under nutrient-limited conditions. This research aimed to screen, isolate, and quantify efficient PHB-producing bacterial strains obtained from various environmental sources. Serial dilutions followed by spread plating were used to isolate bacterial colonies from soil samples collected at industrial and agricultural sites. PHB accumulation was screened using Sudan Black B stain. For the quantitative measurement of PHB accumulation in potential PHB producers, the crotonic acid test was conducted after acid digestion. A small number of isolates exhibited exceptional PHB accumulation capabilities. The present research highlights the diversity of PHB-producing bacteria in nature and their ability to synthesise bioplastics from inexpensive substrates. Thus, this study paves the way toward sustainable plastic alternatives by providing new insights into microbial biotechnology.

Major Findings:

- J7 and J9 showed the highest PHB yield (0.240 and 0.210 g/100ml).
- Optimal PHB at glucose, ammonium sulphate (1.0 g/L), C: N 20:1, pH 7.0.

Keywords: Bioplastic, Isolation, Polyhydroxybutyrate (PHB), Quantification, Screening, Sustainable Production

1. Introduction

Over the last few decades, industrialisation and the extensive use of petroleum-based plastics have caused serious environmental and ecological problems. Commonly used synthetic plastics such as polyethylene, polypropylene, polystyrene, and polyvinyl chloride are non-biodegradable and persist in the environment for several hundred years[1]. Their accumulation in terrestrial and aquatic ecosystems contributes to severe pollution and ecological imbalance[2]. Furthermore, degradation of these plastics produces microplastics, nanoplastics, toxic additives, carcinogens, and endocrine disruptors that threaten both wildlife and human health[3,4]. Consequently, there has been growing interest in sustainable and biodegradable alternatives to conventional plastics[5].

Biodegradable polymers derived from biological sources, particularly Polyhydroxyalkanoates (PHAs), have emerged as promising substitutes for synthetic plastics[6]. PHAs are intracellular polyesters synthesised by numerous bacterial species under nutrient-limited conditions in the presence of excess carbon sources[7]. Among the different types of PHAs, polyhydroxybutyrate (PHB) is the most extensively studied due to its biodegradability, thermoplasticity, biocompatibility, and renewability[8]. PHB is a linear polyester composed mainly of 3-hydroxybutyrate units and exhibits properties comparable to conventional thermoplastics such as polypropylene. It is highly crystalline, Ultra Violet (UV)-stable, hydrophobic, and biodegradable, making it suitable for applications in biomedical, agricultural, packaging, and industrial sectors[9,10].

Article Received on: 23.02.2026 **Revised on:** 25.05.2026 **Accepted on:** 03.06.2026

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The polymer is a linear polyester consisting mainly of 3-hydroxybutyrate units and possessing physical properties comparable to those of some traditional thermoplastics, including polypropylene[11]. In contrast to many artificial polymers, PHB is completely degraded by microbial enzymes, producing environmentally friendly substances, including carbon dioxide and water in the presence of oxygen or methane in the absence of oxygen. A wide range of bacteria, such as *Cupriavidus, Bacillus, Pseudomonas, Azotobacter, Ralstonia, Alcaligenes,* and *Burkholderia,* have been known to produce PHB within their cells as intracellular granules. Of all the bacteria mentioned, *Cupriavidus necator* is among the best at synthesising PHB, as it can accumulate large amounts of PHB under nutrient-limited conditions[12,13]. In the synthesis of PHB, various enzymatic reactions occur through the use of enzymes like β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase[14].

The production and accumulation of PHB are strongly influenced by various environmental and nutritional conditions, including the sources of carbon and nitrogen, the Carbon/Nitrogen (C/N) ratio, pH, temperature, dissolved oxygen, incubation period, and salinity. The sources of carbon strongly affect PHB formation, as an abundance of carbon promotes polymer formation under nutrient stress. Various low-cost carbon sources such as glucose, sucrose, molasses, whey, starch hydrolysates, agro-industrial wastes, wastewater, and lignocellulosic waste have been used to reduce the high cost of PHB[15,16] production. Microbial species thriving in stressed soils are likely to exhibit greater metabolic adaptability and resistance mechanisms, including PHB production. Thus, soils polluted by industrial chemicals, hydrocarbons, pesticides, or heavy metals become highly favourable habitats for the isolation of robust PHB producers with efficient metabolic activity[17].

Modern biotechnological, metabolic, and molecular approaches to studying PHB biosynthesis pathways have advanced the field, providing powerful tools for screening and optimising PHB production in microorganisms. Qualitative screening techniques, such as Sudan Black B staining and Nile Blue A fluorescence staining, are routinely used for detecting PHB inclusion bodies in microbial cells, whereas quantification of PHB content can be achieved via crotonic acid assay, gas chromatography, high-performance liquid chromatography, and spectroscopic analysis[18]. The application of molecular biology approaches, specifically 16S rRNA gene sequencing, has greatly contributed to the identification and classification of effective PHB producers among bacterial species[19].

Despite the immense possibilities PHB offers as an environmentally friendly bioplastic, its commercial use remains constrained by high production costs, low production efficiency, and difficulties in downstream processing[20]. It is therefore necessary to continually look for efficient strains and optimise production conditions to enhance PHB production and reduce its manufacturing cost. Isolation of indigenous strains from various habitats might be a significant factor in the discovery of robust strains with excellent PHB accumulation capacity under economically viable conditions[21].

In this study, bacterial strains were isolated from distinct soil habitats, including agricultural wastelands, contaminated soil samples from Jodhpuriya Dham, and soil samples from the Brahma Temple in Banasthali, Rajasthan, India. They were examined for their ability to produce PHB using the Sudan Black B and Nile Blue A methods, with subsequent quantification through the crotonic acid method. Additionally, the effects of various carbon and nitrogen sources, nitrogen concentration, pH, and C: N ratio were evaluated to optimise production conditions. Selected PHB-producing strains were identified morphologically, biochemically, and genetically using 16S rRNA sequencing and phylogenetic analysis[22].

The present study aimed to isolate and screen efficient PHB-producing bacteria from diverse soil habitats and to evaluate their PHB production potential using qualitative and quantitative assays. The study further investigated the effects of different nutritional and environmental parameters on PHB production and characterised selected high-yielding isolates through morphological, biochemical, and 16S rRNA-based molecular identification for their potential application in sustainable bioplastic production[23].

2. Materials and Methods

2.1 Isolation of PHB- Producing Bacteria

Soil samples were collected from three different locations: Newai agricultural wasteland (N), Jodhpuriya Dham contaminated site (J), and Brahma Temple, Banasthali (B). These sites were selected to explore microbial diversity under varying environmental conditions, including contaminated and non-contaminated ecosystems[24].

Each soil sample (1g) was serially diluted in 9mL of sterile distilled water to a final concentration of 10^{-5}. 100µL of the appropriate dilutions was plated evenly on nutrient agar plates. The nutrient agar plates consisted of peptone (5 g/L), beef extract (3 g/L), sodium chloride (5 g/L), and agar (15 g/L). These media were sterilised at 121°C for 20 min at 15 psi. The inoculated nutrient plates were incubated at 30°C for 24 hours under aerobic conditions. Distinct colonies were observed after incubation, with the highest number in the contaminated soil sample (J), indicating a high level of microbial diversity in this environment[25,26].

Morphologically distinct colonies from each nutrient agar plate were selected and further purified by streak plating. The isolated pure cultures were sub-cultured in nutrient broth medium and incubated at 30°C for 24 hours with shaking (120rpm). Short-term preservation of these cultures was done at 4°C. All the experiments were carried out in triplicate (Figures 1a, b, and c).

2.2 Screening for PHB- Producing Bacteria

Preliminary screening of bacterial isolates for PHB-producing ability was performed using the Sudan Black B staining method, as described by Jeran *et al.* (1998). Nutrient agar medium (ingredients: peptone 5 g/L, beef extract 3 g/L, NaCl 5 g/L, agar 15 g/L) with an additional 1% (w/v) glucose was prepared and sterilised by autoclaving at 121°C for 20 minutes at 15 psi. Sterilised nutrient agar medium was poured into sterile Petri dishes and left to solidify. The Petri dish was divided into five parts, and purified bacterial isolates were streaked onto medium[27]. *Cupriavidus necator* and *Escherichia coli* were used as positive and negative controls, respectively, to confirm the effectiveness of the staining process[28].

The Petri dishes inoculated with the bacterial strains were cultured for 24 hours at 30°C in aerobic conditions. Petri dishes cultured with bacterial isolates were flooded with 0.02% (w/v) Sudan Black B solution in ethanol and stained for 30 minutes. The excess stain was removed by flushing the plates with 96% ethanol. Blue-black colonies indicated PHB accumulation within the bacterial cells, whereas colonies lacking PHB did not show any colour change after the staining process[29]. The experiment was performed three times to confirm the result.

A second screening using Nile Blue A staining was performed to assess the ability of the isolated microorganisms to accumulate PHB. Preparation of mineral salts medium agar for Nile blue screening was done. Mineral Salt Medium (MSM) agar was mixed with 2% (w/v) glucose and 0.5 µM Nile Blue A dissolved in dimethyl sulfoxide to a final concentration. The Sudan black B dye-positive isolates were inoculated onto MSM agar plates. The Petri dishes were then incubated for 3 days at 30 °C. Following Nile Blue A staining, the agar plates were irradiated using UV light to identify PHB-accumulating isolates. Isolates that fluoresce orange were selected as PHB accumulators[30].

2.3 PHB Extraction and Quantitative Analysis

PHB content quantification was performed through crotonic acid analysis as described by John and Ralph (1961), with some modification. Cultures of the bacteria were grown in nutrient broth containing 1% glucose for

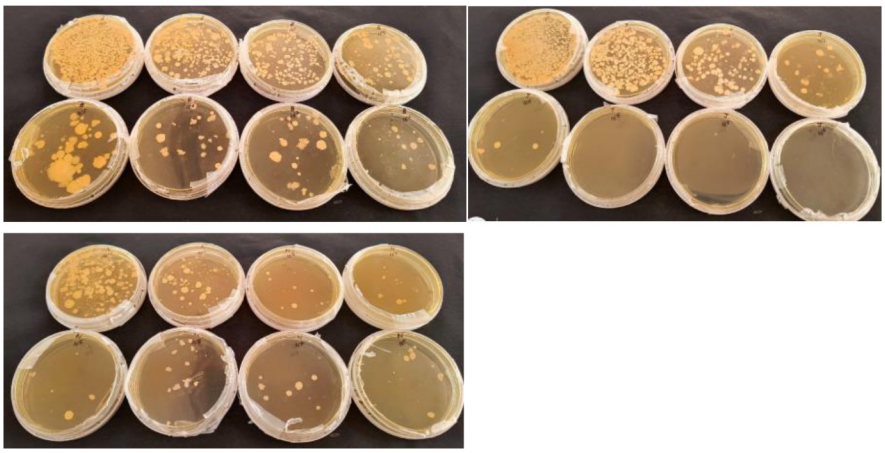


Figure 1. Isolation of PHB-producing bacterial isolates obtained from different environmental samples collected from **(a)** Brahma Temple (B), **(b)** Jodhpuriya contaminated site (J), and **(c)** agricultural wasteland, Newai (N).

48 hours at 30°C while shaking at 120 rpm. Cells were collected via centrifugation at 10,000 rpm for 10 minutes at 4°C. This bacterial cell mass was subsequently washed twice using distilled water before treatment with an equal volume of a solution comprising acetone and ethanol (1:1, v/v), followed by another round of centrifugation at 10,000 rpm for 10 minutes at 4°C. This process helps remove lipids from cells. Then, 10mL of 4% (w/v) sodium hypochlorite was added to the cell mass for cell lysis.

The mixture after incubation was then centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was discarded. The PHB pellets obtained were then washed sequentially with distilled water, acetone, and ethanol to remove impurities. The pure pellet was then dissolved in 5mL of hot chloroform (60°C). The filtrate was further filtered to eliminate cell remnants[31]. Next, 1mL of the chloroform extract was mixed with 10mL of concentrated sulfuric acid (H_2SO_4) and heated at 100°C for 10 min, yielding crotonic acid, which formed a characteristic brown colour. The absorbance of the reaction mixture was measured at 235nm using a UV–Visible spectrophotometer, with concentrated sulfuric acid as the blank solution[32].

A calibration graph was plotted using commercial PHB (0–100 µg/mL) processed under identical conditions to the samples. The amount of PHB in the samples was determined using the calibration curve and reported in mg/L of culture medium and as a percentage of dry cell weight (% DCW)[33].

$$\text{PHB (\%)} = \frac{\text{Dry weight of extracted PHB (g L)}}{\text{DCW (g L)}} \times 100\%$$

All experiments were performed in triplicate, and results were expressed as mean ± Standard Deviation (SD)

2.4 Screening Techniques

The 24-hour-old culture was stained with Gram stain and examined microscopically for Gram stain reaction and morphology. The biochemical characterisation of the isolate was performed using Indole, Methyl Red, Voges-Proskauer, and Citrate utilisation (IMViC) tests. Indole production was determined using Kovac's reagent; Methyl Red (MR) and Voges-Proskauer (VP) were tested by the change in colour following the addition of the appropriate reagents; citrate utilisation was demonstrated by the change in colour from green to blue in the Simmons citrate medium[34].

The oxidase test was performed by adding tetramethyl-p-phenylenediamine solution; The appearance of a dark purple colour indicated a positive reaction. The peroxidase (catalase-like) test was conducted by adding hydrogen peroxide to the organism; the immediate bubbling indicated a positive result. Although this test is not usually used for identification, it was performed here to assess oxidative stress resistance among isolates[35].

2.4.1 Optimisation of Culture Parameters

The microbial isolates were streaked onto gelatine agar slants and incubated at 37°C for 2 days.

Important factors affecting PHB biosynthesis were optimised. Selected bacteria were grown in 250mL Erlenmeyer flasks with 100mL of mineral salt medium fortified with different carbon sources (glucose, fructose, sucrose, maltose, and cellulose; each at 10 g/L)[7]. The flasks were incubated for 48 hours at 30°C in a shaking incubator at 150rpm. The amount of PHB synthesised was estimated using the spectroscopic method[36].

2.4.2 Effects of Various Nitrogen Source Concentrations

To examine the effect of nitrogen sources, the isolated cultures were inoculated into MSM containing an optimal carbon source and varying nitrogen sources, including (NH4)2SO4, NH4Cl, NH4NO3, and yeast extract (each at 1 g/L). The cultures were then incubated in the same manner as mentioned above, and PHB production was determined after 48 h of incubation

2.4.3 Effect of Different pH Sources

The influence of pH on PHB production was assessed by varying the initial pH of MSM (pH 5-9) through suitable buffer systems. The cultures were grown under optimal conditions, and PHB production was estimated after 48 hours[37].

2.4.4 Impact of Various C: N Ratios

Carbon-nitrogen (C: N) ratios were optimised by varying the amounts of the best sources for both elements. C: N ratio was calculated in grams per gram. The cultures were grown under standard conditions, and the PHB yield was determined at 48 hours. All experiments were run in triplicate.

2.4.5 *Genomic DNA Isolation and Phylogenetic Studies*

DNA extraction was performed, and PHB-producing strains were characterised by their 16S rRNA gene sequences. The extracted DNA was then used as the template for amplifying the 16S rRNA gene[38]. For this process, 16S rRNA genes were specifically amplified using oligonucleotide primers that anneal to conserved regions of bacterial 16S rRNA genes. In this case, the primer sequences were as follows: UNI16S-L (5'ATTCTAGAGTTTGATCATGGCTTCA) and UNI16S-R (5'ATGGTACCGTGTGACGGGCGGTGTTGTA). Sequencing of the 16S rRNA gene was performed using Applied Biosystems[38].

3. Results

3.1 Screening for PHB-Producing Bacteria

A total of 29 bacterial isolates were isolated from three sampling locations. Screening with Sudan Black B staining identified 13 bacterial isolates capable of producing PHB. The most positive bacterial isolates (5/8; 62.5%) were obtained from the Jodhpuriya polluted location, followed by the agricultural wasteland (5/12; 41.7%) and the Brahma temple location (3/9; 33.3%) (Table 1 and Figures 2a and b; Figures 3a and b). This finding suggests that polluted sites may harbour a higher proportion of PHB-producing bacteria.

3.2 Quantification of PHB Production and Selection of Isolates

Among the isolated strains, J7 and J9, which produced the highest PHB yields, were subsequently studied. Strain J7 gave the highest yield of 0.240 g/100mL, while strain J9 gave a yield of 0.210g/100mL (Table 2). The strains selected from the above results were J7 and J9.

3.3 Screening Test

Both the organisms (J7 and J9) have been characterised as Gram-negative rods. These organisms were positive for citrate, indole, methyl red, oxidase, and peroxidase reactions, whereas they were negative for gelatin liquefaction and the Voges–Proskauer reaction (Table 3).

3.4 Optimisation of Culture Parameters for Maximum PHB Production

All experiments were conducted in triplicate, and results are presented as mean values (± SD).

3.4.1 *Impact of Various Carbon Sources on PHB Yield*

Of all the carbon sources investigated, glucose yielded the highest production for both bacterial isolates. In the case of isolate J7, glucose gave a maximum value of 0.540g/100mL, followed by fructose (0.390g/100mL), with no production from cellulose. Isolate J9 gave its

Table 1. Sudan black positive isolates

S.no.	Sample Description	Isolates	Sudan Black Positive Isolates
1.	Agriculture waste land, Newai (N),	12	5
2.	Jodhpuriya contaminated site (J)	8	5
3.	Brahma Temple Banasthali (B),	9	3

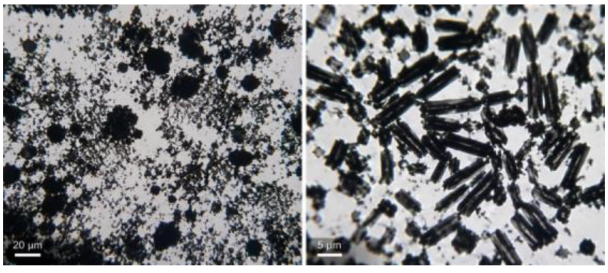


Figure 2. Sudan black B-stained microscopic images showing intracellular PHB granules in bacterial isolates: **(a)** isolate J7 and **(b)** isolate J9.

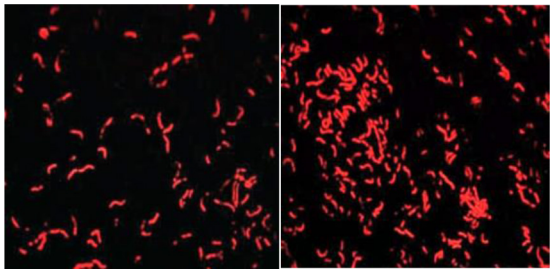


Figure 3. Nile blue fluorescence microscopic images showing intracellular PHB granules in bacterial isolates: **(a)** isolate J7 and **(b)** isolate J9.

maximum yield with glucose (0.330g/100mL) (Table 4 and Figure 4). These observations make it clear that simple sugars are better sources for PHB formation.

3.4.2 *Impact of Various N Sources on PHB Yield*

Among the tested nitrogen sources, ammonium sulphate supported the highest PHB production in both isolates, yielding 0.630g/100mL in J7 and 0.530g/100mL in J9 (Table 5 and Figure 5). Lower PHB production was observed with ammonium chloride and ammonium nitrate, while yeast extract showed minimal yield. These results indicate that inorganic nitrogen sources favour PHB biosynthesis more effectively than organic nitrogen sources.

Table 2. Isolates with maximum yield

S.no.	Source	Isolates	Yield (g/100ml)
1.	Jodhpuriya contaminated site (J)	J7	0.240
2.	Jodhpuriya contaminated site (J)	J9	0.210

3.4.3 *Impact of Various Concentrations of Ammonium Sulphate*

Ammonium sulfate was found to be the most suitable nitrogen source for PHB production. Isolate J7 exhibited the highest yield (0.630g/100mL), followed by ammonium chloride and ammonium nitrate. Yeast extract resulted in minimal PHB production. A similar trend was observed for isolate J9, with a maximum yield of 0.530g/100mL using ammonium sulfate (Table 6 and Figure 6). This suggests that inorganic nitrogen sources favour PHB accumulation.

3.4.4 *Impact of Various C: N Ratios on PHB Yield by the Selected Isolates*

The C: N ratio significantly influenced PHB production. The highest yield was observed at a 20:1 ratio, with isolate J7 producing 1.120g/100mL and isolate J9 producing 0.840g/100mL (Table 7). Lower and higher ratios resulted in reduced PHB accumulation, indicating that an optimal balance of carbon and nitrogen is critical for maximising yield (Figure 7).

Table 3. Biochemical characterisation of the bacterial strain

S.no.	Tests	J7	J9
1.	Gram Staining	Gram-negative rods	Gram-negative rods
2.	Gelatin Liquification	Negative	Negative
3.	Citrate test	Positive	Positive
4.	Indole test	Positive	Positive
5.	Methyl Red	Positive	Positive
6.	Voges-Proskauer	Negative	Negative
7.	Oxidase	Positive	Positive
8.	Peroxidase	Positive	Positive

Table 4. The highest yield was observed in the presence of glucose as a carbon source in both J7 and J9

S.no.	Isolates	Glucose	Fructose	Sucrose	Maltose	Cellulose	Mean Yield (g/100ml)
1.	J7	0.540 ± 0.020	0.390 ± 0.020	0.230 ± 0.020	0.180 ± 0.020	0.000 ± 0.000	0.268 ± 0.016
2.	J9	0.330 ± 0.020	0.350 ± 0.020	0.140 ± 0.020	0.110 ± 0.020	0.000 ± 0.000	0.186 ± 0.014

Table 5. Effect of different N sources on PHB yield by the selected isolates

S.no.	Isolates	Ammonium Sulphate	Ammonium Chloride	Ammonium Nitrate	Yeast Extract	Mean Yield (g/100ml)
1.	J7	0.630 ± 0.020	0.350 ± 0.020	0.230 ± 0.020	0.002 ± 0.002	0.303 ± 0.016
2.	J9	0.530 ± 0.020	0.270 ± 0.020	0.080 ± 0.020	0.008 ± 0.002	0.222 ± 0.014

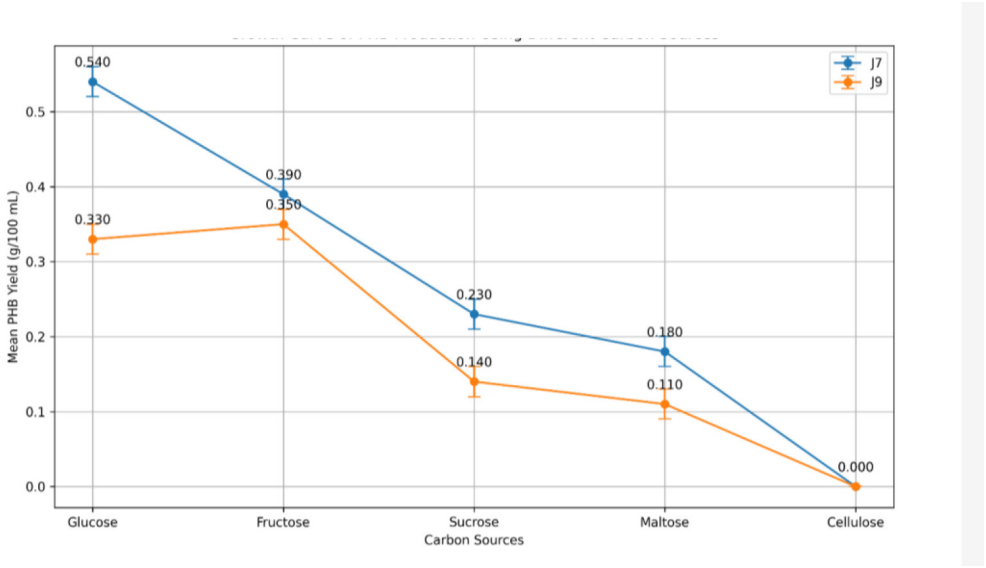


Figure 4. Growth curve showing the effect of different carbon sources on PHB production by isolates J7 and J9.

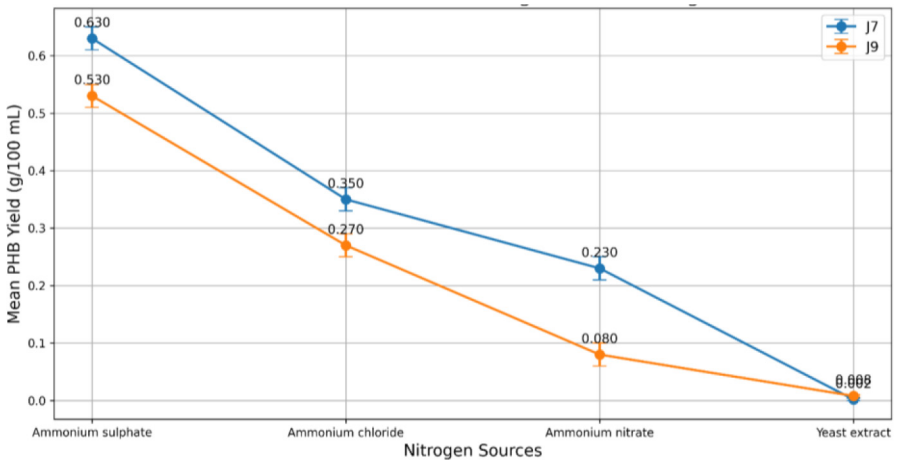


Figure 5. Growth curve of PHB production by isolates J7 and J9 using different nitrogen sources.

Table 6. The maximum yield of PHB-synthesising bacteria was obtained at pH with an ammonium sulphate concentration of 1.0

Ammonium Sulphate Concentration (%)	J7 (Mean ± SD) g/100mL	J9 (Mean ± SD) g/100mL
0.5	0.530 ± 0.008	0.460 ± 0.019
1.0	0.590 ± 0.016	0.520 ± 0.011
1.5	0.380 ± 0.017	0.250 ± 0.025
2.0	0.300 ± 0.009	0.220 ± 0.012

3.4.5 Impact of Various pH Levels

The effect of pH on PHB production showed that neutral pH (7.0) was optimal. Isolate J9 exhibited the highest yield (1.20g/100mL) at pH 7, followed by J7 (0.78g/100mL) (Table 8). Both acidic (pH 6) and alkaline (pH 8) conditions reduced PHB production, suggesting that the enzyme activity involved in PHB biosynthesis is pH-dependent (Figure 8).

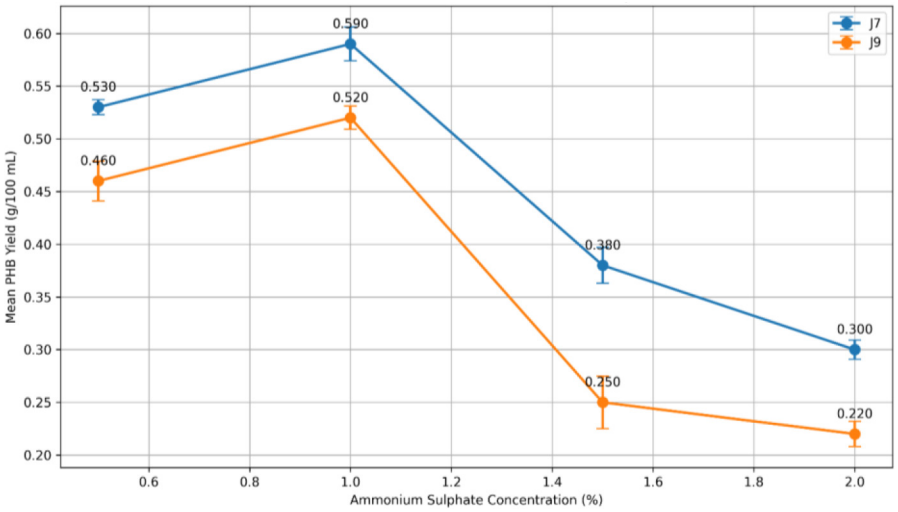


Figure 6. Growth curve of PHB production by isolates J7 and J9 at different ammonium sulphate concentrations. Maximum PHB yield was observed at 1.0% ammonium sulphate.

Table 7. The highest bacteria production was observed at a C: N ratio of 20:1

C: N Ratio	J7 (Mean ± SD) g/100mL	J9 (Mean ± SD) g/100mL
5:1	0.410 ± 0.009	0.460 ± 0.014
10:1	0.580 ± 0.013	0.520 ± 0.014
15:1	0.740 ± 0.014	0.640 ± 0.015
20:1	1.120 ± 0.017	0.840 ± 0.017
25:1	1.050 ± 0.018	0.740 ± 0.014

Table 8. Effect of different pH on the production of PHB

pH	J7 (Mean ± SD) g/100mL	J9 (Mean ± SD) g/100mL
6	0.000 ± 0.000	0.240 ± 0.015
7	0.780 ± 0.014	1.200 ± 0.020
8	0.650 ± 0.016	0.800 ± 0.020

3.4.6 PHB Extraction and Quantitative Analysis

The amount of PHB produced by isolates was measured with the crotonic acid spectrophotometric method and

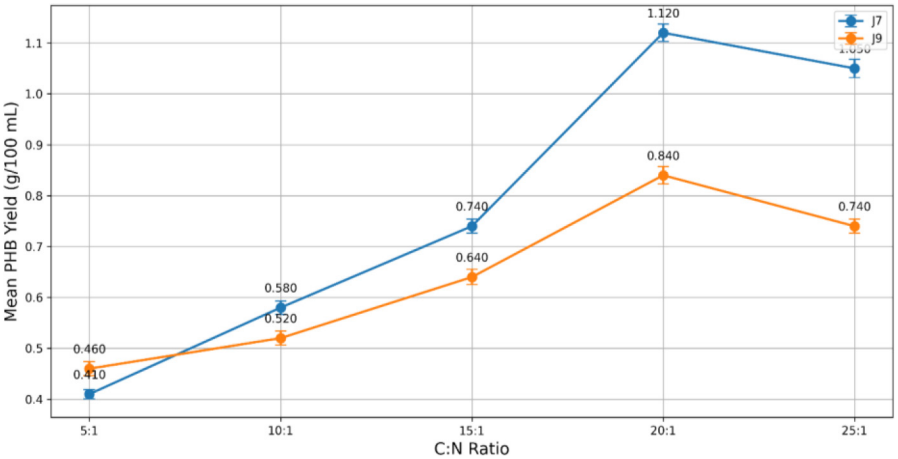


Figure 7. Growth curve of PHB production by isolates J7 and J9 at different C: N ratios. Maximum PHB production was observed at a 20:1 C: N ratio.

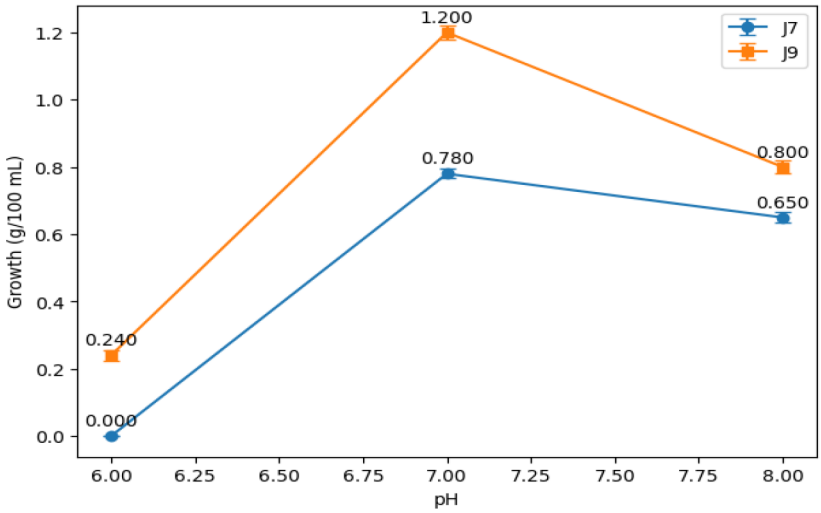


Figure 8. Effect of pH on the growth of J7 and J9 isolates expressed as g/100mL (mean ± SD). Maximum growth for both isolates was observed at pH 7, with J9 showing higher growth compared to J7 across all tested pH levels.

Table 9. Concentration-dependent response values represented as mean ± standard deviation (SD), showing a progressive increase with increasing concentration and saturation at higher concentrations

Concentration	Mean ± SD
0	(0.000 ± 0.001)
5	(0.539 ± 0.019)
10	(0.677 ± 0.016)
15	(0.781 ± 0.021)
20	(0.872 ± 0.022)
25	(0.879 ± 0.019)
30	(0.938 ± 0.018)
40	(0.948 ± 0.018)
50	(0.977 ± 0.017)

was expressed as average ± SD for three independent trials. The PHB production by isolates was higher in J7 and J9 than in the others. The concentration of PHB was calculated based on the absorbance at 235 nm according to the standard curve (Table 9 and Figure 9).

3.4.7 Genomic DNA Isolation and Phylogenetic Studies

The genomic DNA of bacteria that produce PHB was extracted successfully and used to amplify the 16s rRNA genes using universal primers. Amplification by Polymerase Chain Reaction (PCR) generated an amplicon of about 1500 base pairs. Analysis and sequencing of the amplified fragments were carried out using NCBI BLAST for the identification of bacteria. Phylogenetic analysis using MEGA software confirmed their relatedness to other bacterial organisms (Figures 10 and 11).

4. Discussion

In this study, it is evident that contaminated environments can be an excellent source for selecting metabolically flexible PHB-producing bacteria. Of the three selected sites for this experiment, the highest percentage of PHB-positive bacterial isolates was observed in the contaminated soil sample from Jodhpuriya, suggesting that contaminated environments favour the selection of microorganisms that are metabolically flexible and able to cope with stress by storing intracellular carbon. Such results are not new; in other studies, PHB-producing bacteria were found in polluted soil samples, industrial wastelands, and other hydrocarbon-rich environments, and these bacteria showed high PHB accumulation due to stress adaptation and metabolic modulation[40,41].

From the thirteen PHB-producing isolates, isolates J7 and J9 produced the highest amounts of PHB at the beginning of the experiment, with values of 0.240g/100mL and 0.210g/100mL, respectively. Their high efficiency in PHB synthesis may be linked to efficient carbon assimilation pathways, enzyme activity in PHB biosynthesis, and metabolic regulation of carbon partitioning. It was previously established that microorganisms capable of efficiently metabolising

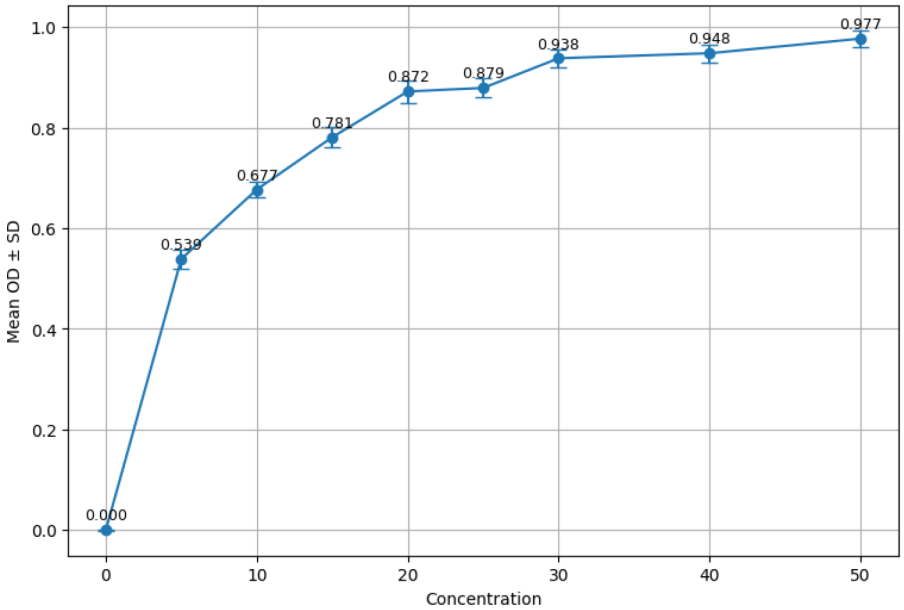


Figure 9. Quantitative analysis of PHB production by bacterial isolates represented as mean ± SD.

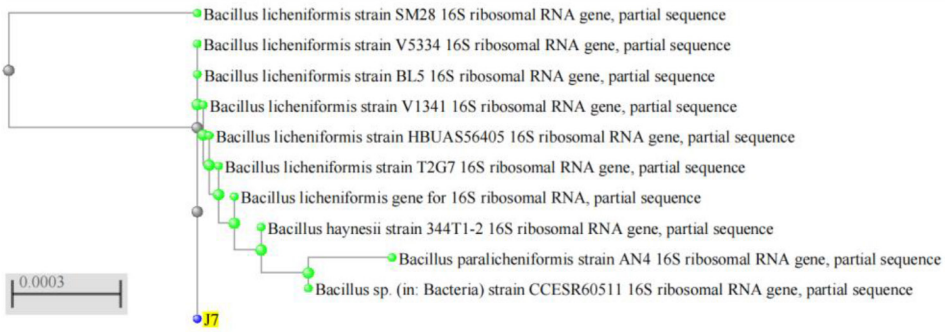


Figure 10. Phylogenetic tree based on 16S rRNA gene sequence showing the evolutionary relationship of bacterial isolate J7 with closely related bacterial species.

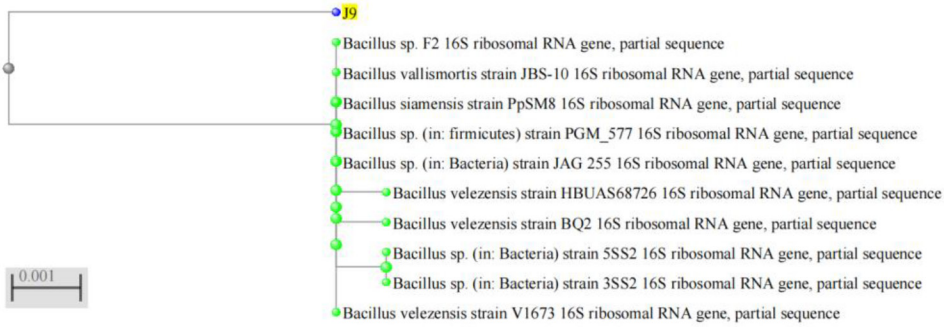


Figure 11. Phylogenetic tree based on 16S rRNA gene sequence showing the evolutionary relationship of bacterial isolate J9 with closely related bacterial species.

carbon sources into intermediates such as acetyl-CoA exhibit improved ability to accumulate PHB during growth in nutrient-limited environments[39,40].

From the optimisation experiments, glucose was found to be the best carbon source for PHB synthesis in both isolates. The highest quantity of PHB produced

by isolate J7 using glucose in the culture medium was 0.540g/100mL, while isolate J9 produced 0.330g/100mL. Glucose can be easily metabolised using glycolysis to produce reducing equivalents and other precursors needed for PHB biosynthesis. PHB synthesis is more efficient in bacteria using simple carbohydrates as the carbon source than complex polysaccharides. Cellulose could not sustain significant PHB synthesis in this experiment because the isolates lack cellulases[15,21].

Optimisation of the nitrogen source showed that ammonium sulfate was more effective for the synthesis of PHB than ammonium chloride, ammonium nitrate, or yeast extract. The maximum PHB production was achieved using ammonium sulfate at a concentration of 1.0 g/L, yielding 0.590g/100mL for strain J7 and 0.520g/100mL for strain J9. It is widely known that inorganic nitrogen sources result in greater nitrogen limitation, causing extra carbon to accumulate as PHB rather than in cell growth[41]. It can be assumed that the reduced levels of PHB produced using yeast extract were due to the abundant supply of amino acids, which supported cell growth. Similar results have been obtained in other experiments on *Cupriavidus, Bacillus*, and *Pseudomonas.*

The C: N ratio was important in determining the amount of PHB produced. Both isolates reached maximum polymer production at a ratio of 20:1; the maximum amounts produced were 1.120g/100ml and 0.840g/100ml for isolates J7 and J9, respectively. Nitrogen deficiency in the presence of excess carbon has been found to stimulate PHB production by altering cell growth and metabolism, thereby promoting intracellular storage. The trend in PHB production observed in this study is common to others reported earlier; maximum PHB production is achieved when the C: N ratio exceeds 15:1, reaching up to 30:1 [4,8].

The effect of pH on PHB production indicated that a nearly neutral pH was ideal for PHB accumulation. The strain J9 was the best producer of PHB at neutral pH 7.0, with a PHB yield of 1.20g/100mL, whereas isolate J7 produced 0.78g/100mL of PHB at pH 7.0. Decreased PHB production at acidic and basic pH values can be attributed to reduced enzyme activity, impaired transport mechanisms, and metabolic stress. Previous studies have shown an increased yield of PHB under neutral conditions because enzymes are stable under these conditions[42].

Biochemical characterisation studies of isolates J7 and J9 showed that the isolates had similar characteristics to each other and were identified as gram-negative, rod-shaped organisms showing positive results in the citrate utilisation test, indole test, methyl red test, oxidase, and peroxidase tests. All of these biochemical characteristics reveal that the isolates have diverse metabolic abilities and can tolerate varying environmental conditions. Such physiological characteristics are usually displayed by efficient PHB-producing bacteria that utilise different substrates during stress conditions[43]. Molecular identification of these strains is needed using 16S rRNA sequencing.

In terms of industrial and environmental implications, this work is extremely important because PHB can serve as a sustainable alternative to conventional oil-based plastics. In contrast to artificial polymers, PHB has numerous advantages, including biodegradability, biocompatibility, and non-toxicity during degradation[3,8], which make its application feasible in fields such as biomedical engineering, agriculture, food packaging, and environmental protection. The capacity of isolates J7 and J9 to form PHB from simple, inexpensive carbon sources suggests their great potential for the economic production of bioplastics. Besides, the ability of these strains to utilise cheap carbon sources indicates the potential for using agricultural and industrial wastes in PHB biosynthesis.

However, despite these encouraging results, there are some limitations to this study. For example, the research was carried out on a laboratory scale using batch cultures only, and factors such as aeration rate, dissolved oxygen control, fed-batch fermentation, and the recovery process were not considered. Furthermore, molecular identification and genomic analysis of the isolated bacteria were not performed, thereby preventing elucidation of the genetic aspects associated with the biopolymer production process. It is also noteworthy that methods for polymer characterisation, including Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA), and Scanning Electron Microscopy (SEM), have not been employed.

It is therefore recommended that future investigations aim to isolate and perform genome-wide analyses of potent strains to identify the genes associated with PHB synthesis and stress tolerance. Further improvement in PHB production can be achieved through metabolic engineering and synthetic biology approaches. Moreover, optimising the process using bioreactors, utilising cheap, renewable carbon sources, and employing advanced

downstream processing techniques are vital to ensuring economic feasibility. Finally, detailed characterisation of the synthesised polymer and determination of its biodegradability, toxicity, and environmental safety are important steps towards establishing the suitability of PHB for use in industries and biomedical applications. This study, therefore, makes an important contribution to the growing global trend in sustainable bioplastic production.

5. Conclusion

In terms of quantitative results, sample (J) performed best among the three soil samples. A total of eight optimal colonies were collected; five tested positive for PHB-producing bacteria, with J7 and J9 yielding the best results. The physical and chemical conditions of the two samples were varied to determine the optimal conditions for maximum yield. Results from this research study have shown that polluted areas are excellent sources of PHB-producing bacteria, as the highest number of positives was found at the polluted site in Jodhpuriya (J). J7 and J9 produced the highest yields among the samples, with initial yields of 0.240g/100mL and 0.210g/100mL, respectively. Significant improvements have been made through the optimisation process, as evidenced by the high yields achieved with the optimal parameters. Optimal growth was observed when the medium contained 10g/L of glucose (as carbon source), 1g/L of ammonium sulfate (as nitrogen source), at pH 7.0, and at a C: N ratio of 20:1. Under these conditions, the yield reached a maximum value of 1.120g/100mL (J7) and 0.840g/100mL (J9).

The high efficiency of PHB accumulation by isolates J7 and J9 under optimal culture conditions indicates that these organisms can be used to produce industrial biopolymers. Notably, the utilisation of simple carbon compounds and inorganic nitrogen sources suggests the possibility of economically scaling up PHB production using agro-industrial wastes. This research lays the groundwork for devising sustainable systems for bioplastic production and waste valorisation for PHB synthesis. In future studies, it would be prudent to concentrate on identifying strains at the molecular level, manipulating metabolic pathways, and scaling up processes from benchtop to industrial scales.

6. Acknowledgements

The authors are highly obliged for the kind and needful support of the DBT- funded

The Bioinformatics Centre and the "Workshop in Bioinformatics" project, along with the Department of Science and Technology- Fund for Improvement of Science and Technology (DST-FIST) program at Banasthali Vidyapith, are providing the necessary facilities to conduct this study. The authors also express their deepest gratitude to Prof. Ina Shastri, Vice Chancellor, Banasthali Vidyapith, for her support.

7. Data Availability Statement

Data supporting the findings are available from the corresponding author upon reasonable request.

8. References

1. Abid S, Azeem M, Hussain S, Shahid M, Riaz M, Alotaibi SS, *et al.* Identification, biochemical characterization, and safety profiling of locally isolated probiotic candidates. BioMed Res Int. 2022; 2022:1-12.
2. Galego N, Rozsa C, Sánchez R, Fung J, Vázquez A, Santo Tomás J. Characterization and application of poly(β-hydroxyalkanoates) family as composite biomaterials. Polym Test. 2000; 19(5):485-492. https://doi.org/10.1016/S0142-9418(99)00011-2
3. Getino L, Martín JL, Chamizo-Ampudia A. A review of polyhydroxyalkanoates: Characterization, production, and application from waste. Microorganisms. 2024; 12(10):2028. https://doi.org/10.3390/microorganisms12102028 PMid:39458337 PMCid:PMC11510099.
4. Hadri SH, Tareen N, Hassan A, Naseer M, Ali K, Javed H. Alternatives to conventional plastics: Polyhydroxyalkanoates (PHA) from microbial sources and recent approaches - A review. Process Saf Environ Prot. 2025. https://doi.org/10.1016/j.psep.2025.106809
5. Cheng KC, Lee WH. Comparative evaluation of biochemical tests and molecular methods for bacterial identification. J Microbiol Methods. 2019; 158:30-38. https://doi.org/10.1016/j.mimet.2019.04.007 PMid:30978364.
6. Alagunachiyar M, Jeevitha RA, Handique M, Kesavan SS. Isolation and screening of polyhydroxybutyrate (PHB) producing bacteria from salt pan soil. Res J Pharm Technol. 2015; 8(9):1276-1280. https://doi.org/10.5958/0974-360X.2015.00231.0
7. Ladhari S, Vu NN, Boisvert C, Saidi A, Nguyen-Tri P. Recent development of polyhydroxyalkanoates (PHA)-based materials for antibacterial applications: A review. ACS Appl Bio Mater. 2023;6(4):1398-1430.https://doi.org/10.1021/acsabm.3c00078 PMid:36912908.
8. Li D, Wang F, Zheng X, Zheng Y, Pan X, Li J, *et al.* Lignocellulosic biomass as promising substrate

for polyhydroxyalkanoate production: Advances and perspectives. Biotechnol Adv. 2024; 108512. https://doi.org/10.1016/j.biotechadv.2024.108512 PMid:39742901.

9. Liu Y, Li GR, Guo FF, Jiang W, Li Y, Li LJ. Large-scale production of magnetosomes by *Magnetospirillum gryphiswaldense*. Microb Cell Fact. 2010; 9:99. https://doi.org/10.1186/1475-2859-9-99 PMid:21144001 PMCid:PMC3019156.
10. López-Cuellar MR, Alba-Flores J, Rodríguez JG, Pérez-Guevara F. Production of PHAs with canola oil as carbon source. Int J Biol Macromol. 2011; 48(1):74-80. https://doi.org/10.1016/j.ijbiomac.2010.09.013 PMid:20920524.
11. Malashin I, Martysyuk D, Tynchenko V, Gantimurov A, Semikolenov A, Nelyub V, *et al*. Machine learning-based process optimization in biopolymer manufacturing: A review. Polymers. 2024; 16(23):3368. https://doi.org/10.3390/polym16233368 PMid:39684112 PMCid:PMC11644666.
12. Muneer F, Rasul I, Azeem F, Siddique MH, Zubair M, Nadeem H. Microbial polyhydroxyalkanoates (PHAs): Replacement of synthetic polymers. J Polym Environ. 2020; 28(9):2301-2323. https://doi.org/10.1007/s10924-020-01772-1
13. Palmeiro-Sánchez T, O'Flaherty V, Lens PN. PHA bioproduction and its rise as biomaterial of the future. J Biotechnol. 2022; 348:10-25. https://doi.org/10.1016/j.jbiotec.2022.06.004 PMid:35716887.
14. Qie Z, Sivashankari RM, Miyahara Y, Tsuge T. Biosynthesis and property evaluation of PHB-co-2HA polymer. Polym Degrad Stab. 2025; 236:111277. https://doi.org/10.1016/j.polymdegradstab.2025.111277
15. Sachan RS, Devgon I, Karnwal A, Mahmoud AE. Valorization of wheat straw sugar for eco-friendly PHA production. Bioresour Technol Rep. 2024; 25:101770. https://doi.org/10.1016/j.biteb.2024.101770
16. Shah AT, Favaro L, Alibardi L, Cagnin L, Sandon A, Cossu R, *et al*. Bacillus sp. for biohydrogen from municipal waste. Appl Energy. 2016; 176:116-124. https://doi.org/10.1016/j.apenergy.2016.05.035
17. Shah TA, Li Z, Li Z, Zhang A. Energy status, anaerobic digestion and metabolic engineering for hydrogen/methane. J Water Process Eng. 2025; 69:106725. https://doi.org/10.1016/j.jwpe.2024.106725
18. Li MN, Han Q, Wang N, *et al*. 16S rRNA gene sequencing for bacterial identification and infectious disease diagnosis. Biochem Biophys Res Commun. 2024; 739:150974. https://doi.org/10.1016/j.bbrc.2024.150974 PMid:39550863.
19. Shah TA, Zhihe L, Zhiyu L, Zhang A, Lu D, Fang W, *et al*. *Clostridium butyricum* CBT-1 for agro-food waste hydrolysis to biohydrogen. Environ Sci Pollut Res. 2023; 30:4853-4865. https://doi.org/10.1007/s11356-022-22443-9
20. Aneja KR. Experiments in microbiology, plant pathology and biotechnology. 4th ed. New Delhi: New Age International Publishers; 2003.
21. Surendran A, Lakshmanan M, Chee JY, Sulaiman AM, Thuoc DV, Sudesh K. PHA production from waste plant and animal oils. Front Bioeng Biotechnol. 2020; 8:169. https://doi.org/10.3389/fbioe.2020.00169 PMid:32258007 PMCid:PMC7090169.
22. Thamarai P, Vickram AS, Saravanan A, Deivayanai VC, Evangeline S. Recent advancements in PHAs: Biosynthesis, production and applications. Bioresour Technol Rep. 2024; 27:101957. https://doi.org/10.1016/j.biteb.2024.101957
23. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. J Clin Microbiol. 2007; 45(9):2761-2764. https://doi.org/10.1128/JCM.01228-07 PMid:17626177 PMCid: PMC2045242
24. Getachew A, Woldesenbet F. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low-cost agricultural waste material. BMC Res Notes. 2016; 9:509. https://doi.org/10.1186/s13104-016-2321-y PMid:27955705 PMCid: PMC5154074.
25. Thomas P, Sekhar AC, Upreti R, Mujawar MM, Pasha SS. Optimisation of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured and easy approach for bacterial and yeast CFU enumeration and single-colony isolation from diverse samples. Biotechnol Rep. 2016; 8:45-55. https://doi.org/10.1016/j.btre.2015.08.003 PMid:28352572 PMCid:PMC4980700.
26. Madigan MT, Bender KS, Buckley DH, Sattley WM, Stahl DA. Brock biology of microorganisms. 15th ed. Boston: Pearson Education. 2018. https://doi.org/10.1093/icb/icy062 PMid:29897574
27. Madkour MH, Heinrich D, Alghamdi MA, Shabbaj II, Steinbüchel A. PHA recovery from biomass. Biomacromolecules. 2013; 14(9):2963-2972. https://doi.org/10.1021/bm4010244 PMid:23875914.
28. Kumar P, Singh M, Sharma R. Screening and characterization of PHB-producing bacteria isolated from contaminated soil samples. Int J Biol Macromol. 2022; 194:103-112. https://doi.org/10.1016/j.ijbiomac.2021.11.089 PMid: 34800522.
29. Balasubramanian VK, Prakash M, Kumar PS, *et al*. Biosynthesis of bioplastic polyhydroxybutyrate (PHB) from soil bacterial isolates and optimization of culture conditions. Process Biochem. 2024; 137:110-121.
30. Malashin I, Martysyuk D, Tynchenko V, Gantimurov A, Semikolenov A, Nelyub V, *et al*. Machine learning-based process optimization in biopolymer manufacturing: A review. Polymers. 2024; 16(23):3368. https://doi.org/10.3390/polym16233368 PMid:39684112 PMCid:PMC11644666
31. Tan GYA, Chen CL, Li L, Ge L, Wang L, Razaad I, *et al*. Start a research on biopolymer polyhydroxyalkanoate (PHA): A review. Polymers. 2014; 6(3):706-754. https://doi.org/10.3390/polym6030706.

32. Patel JB, *et al*. Manual of clinical microbiology. 12th ed. Washington (DC): ASM Press; 2021. https://doi.org/10.1128/9781555817381
33. Kaur G, Roy I. Strategies for large-scale production of polyhydroxyalkanoates. Chem Biochem Eng Q. 2015; 29(2):157-172. https://doi.org/10.15255/CABEQ.2014.2255
34. Kshirsagar PR, *et al*. Optimization of culture conditions for polyhydroxybutyrate production by *Bacillus* species. J Environ Biol. 2015; 36:789-794.
35. Shrimali G, Sharma R, Sharma S, *et al*. Optimized polyhydroxybutyrate production by *Neobacillus niacini* GS1 using agricultural residues as low-cost substrates. BioTech. 2024; 4(4):64. https://doi.org/10.3390/biomass4040064
36. Hoşafcı E, Ateş C, Volkan AE, Koku H, Erguder TH. Effect of pH and salinity on photofermentative hydrogen and poly-hydroxybutyric acid production via Rhodobacter capsulatus YO3. Int J Hydrogen Energy. 2025; 118:331-342. https://doi.org/10.1016/j.ijhydene.2025.03.198
37. Clarridge JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev. 2004; 17(4):840-862. https://doi.org/10.1128/CMR.17.4.840-862.2004 PMid:15489351 PMCid:PMC523561.
38. Liu Y, Li GR, Guo FF, Jiang W, Li Y, Li LJ. Large-scale production of magnetosomes by *Magnetospirillum gryphiswaldense*. Microb Cell Fact. 2010; 9:99. https://doi.org/10.1186/1475-2859-9-99 PMid:21144001 PMCid:PMC3019156.
39. Kourmentza C, *et al*. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering. 2017; 4(2):55. https://doi.org/10.3390/bioengineering4020055 PMid:28952534 PMCid:PMC5590474.
40. Mohanrasu K, Rao RGR, Dinesh GH, Zhang K, Pugazhendhi A. Optimization and production of polyhydroxybutyrate biopolymer from novel bacterial strains: Current advances and future perspectives. Bioresour Technol. 2025; 10.1201/9781003304142-5
41. Raza ZA, Abid S, Banat IM. Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. Int Biodeterior Biodegradation. 2018; 126:45-56. https://doi.org/10.1016/j.ibiod.2017.10.001
42. Yan Y, Kuramae EE, Klinkhamer PGL, van Veen JA. Revisiting the dilution procedure used to manipulate microbial biodiversity in terrestrial systems. Appl Environ Microbiol. 2015; 81(13):4246-4252. https://doi.org/10.1128/AEM.00958-15 PMid:25888175 PMCid:PMC4475865.
43. Franco-Duarte R, Černáková L, Kadam S, Kaushik KS, Salehi B, Bevilacqua A, *et al*. Advances in chemical and biological methods to identify microorganisms-From past to present. Microorganisms. 2019; 7(5):130. https://doi.org/10.3390/microorganisms7050130 PMid:31086084 PMCid: PMC6560418.

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Conceptualisation: K.S. and A.P.; Methodology: K.S.; Investigation: K.S., C.G.P. and B.S.; Data Curation: K.S. Writing – Original Draft: K.S., C.G.P., and B.S.; Writing – Review and Editing: R.B. and A. P.; Supervision: A.P. All authors have read and approved the manuscript.

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