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Isolation and Characterisation of Polyhydroxybutyrate (PHB) Producing Bacteria for Sustainable Bioplastic Development

Darshit Rameshbhai Goyani, Tankariwala Aneesha Aiyub[1], Dr. A.A. Raval[2] (Guide)
[1, 2]*Undergraduate Student, Department of Microbiology, Arts, Science and Commerce College, Kholwad, Kamrej, Surat, Gujarat, India*
[3]*Assistant Professor, Department of Microbiology, Arts, Science and Commerce College, Kholwad, Kamrej, Surat, Gujarat, India*

Abstract: ***Polyhydroxybutyrate (PHB), a biodegradable biopolymer produced by certain bacteria, is one of the sustainable alternatives being sought after due to the growing environmental burden posed by traditional plastics. In this study, environmental samples were taken from Dumas Beach and Kholwad College Garden in South Gujarat in order to isolate, identify, and optimize PHB-producing bacterial strains. Using the Carbol Fuchsin, Sudan Black B, and Nile Blue A staining methods, a total of 25 bacterial isolates were acquired and tested. Based on morphological, biochemical, and staining investigations, two strains—Bacillus spp. and Pseudomonas spp.—were determined to be effective PHB producers.***
Molasses, leftover cooking oil, orange and potato peels, and other low-cost carbon sources were assessed for PHB production. The largest PHB concentration was found in potato peels, where Pseudomonas spp. could reach 89%. PHB yield was considerably increased by a pH of 7, a temperature of 37°C, and shaking conditions, according to the results of production parameter optimization. Fourier Transform Infrared Spectroscopy (FTIR), which showed distinctive peaks of ester carbonyl and C–H functional groups, verified that the isolated polymer was PHB.
This research highlights the potential of using regionally isolated bacteria and agro-waste materials for low-cost, eco-friendly PHB production. These findings support sustainable bioplastic development and offer promising avenues for industrial-scale biopolymer synthesis using locally sourced resources.
Keywords: PHB production, bioplastic, Bacillus spp., Pseudomonas spp., agro-waste substrate, biodegradable polymer

I. INTRODUCTION

A. Background

Global plastic production is expected to nearly triple to 590 million tonnes by 2050, having increased dramatically to 413.8 million tonnes in 2023 [1]. Recycling rates are still shockingly low—less than 10% of all plastic generated is currently recycled—despite this sharp rise [3].

The great majority wind up in natural areas, streams, or landfills. The growing problem of microplastic contamination is fuelled by the estimated 19 to 23 million tonnes of plastic that leak into aquatic environments each [2]. This poses serious threats to human health and the stability of entire ecosystems in addition to endangering marine species.

Polyhydroxybutyrate (PHB), a biodegradable polymer synthesized by a variety of bacteria has the potential to substitute the petroleum-based plastic because it is environmentally benign, biocompatible and is a thermoplastic. M. Adnan et al. (2022) compared Agromyces indicus as a distinct and effective producer of PHB. The study aimed at maximizing the process of fermentation and choice of substrate in an attempt to increase the production PHB. PHB was seen to be comparable with other polymers such as polypropylene and techniques of characterising them justified the statement. Despite problems of expenses, these microbial solutions provide environmentally viable means of mass production of PHB that assists in the preservation of the environment and the reliance on the use of non-degradable synthetic polymers [5].

In order to ensure that PHB becomes commercially viable, its researchers are interested in identifying cost effective bacterial strains together with low-cost substrates (e.g. agro-industrial waste) to lower the cost barriers significantly. The need to identify PHB producers of local sources that can have high and sustainable yields is thus of great essence in realizing sustainable bioplastic production [4].

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B. Research Gap

Despite notable progress in microbial biotechnology and bio-based polymer research, significant challenges persist in the identification and optimisation of potent PHB-producing bacterial strains. While genera such as *Cupriavidus*, *Ralstonia*, *Bacillus*, and *Pseudomonas* are well-established producers, these represent only a fraction of the microbial biodiversity capable of PHB synthesis. Numerous indigenous and underexplored bacterial strains, especially from organic-rich environments like compost, sugar industry sludge, and agricultural soils, have not been thoroughly investigated for their PHB-producing potential [6].

Additionally, most existing studies focus on model organisms or genetically modified strains under standardised laboratory conditions, which may not reflect the true performance of native bacteria under varied environmental circumstances. The lack of research on region-specific microbial sources and their capacity to produce PHB from locally available low-cost substrates limits the scalability of eco-friendly bioplastic production.

Furthermore, detailed studies on media optimisation—especially the use of glucose-supplemented nutrient agar or agro-waste-based media—are sparse or inconsistent. As a result, current laboratory yields are suboptimal, impeding industrial application. Addressing these gaps is essential to advancing sustainable and economically viable PHB production technologies.

C. Aim of the Study

This research aims to screen, isolate, and characterise PHB-producing bacteria from various environmental samples to contribute to sustainable solutions for plastic waste. The study involves:

- Isolation of bacterial strains from natural sources such as soil and organic waste.
- Screening for PHB production using staining techniques.
- Characterisation of selected isolates based on morphological, biochemical, and physiological traits.
- Optimisation of growth medium (including glucose-enriched nutrient agar) to enhance PHB yield.

By identifying novel or highly efficient PHB-producing bacteria, this study seeks to provide a foundation for low-cost bioplastic production technologies, ultimately contributing to environmental sustainability and waste reduction. This work will help bridge the existing research gap in discovering diverse PHB producers from underexplored ecological niches and improve bioplastic feasibility on a commercial scale.

II. METHODOLOGY

A. Collecting and isolating PHB-producing bacteria

To identify possible PHB-producing bacteria, soil and water samples were gathered from a variety of ecological niches in the South Gujarat region. Dummas Beach (soil and water), Kholwad Garden (soil), and Tapi River (soil and water) were among the chosen test locations. 9 mL of sterile distilled water was used to dissolve about 1g of each soil sample, which was then serially diluted and spread out onto nutrient agar plates that had been fortified with 2% glucose. Likewise, prior to plating, 1 millilitre of river and marine water samples was combined with 9 millilitres of sterile water. To encourage the growth of bacterial colonies, the plates were incubated for 24 hours at 37°C [6] .

B. Screening of PHB-Producing Bacteria

To identify potential PHB-producing bacterial isolates, a series of staining techniques were employed for effective visualisation of intracellular PHB granules. Initially, Carbol Fuchsin staining was performed by heat-fixing bacterial smears and staining with Carbol Fuchsin for 45 seconds. The presence of dark intracellular granules indicated PHB accumulation. To confirm this finding, Sudan Black B staining was used—a lipid-specific stain prepared as a 0.3% solution in 60% ethanol. The permanent smears were also stained and counter-stained to ensure the presence of lipids using safranin stains taking 10 and 5 minutes consecutively, respectively (Sneha B. et al., 2017). To make it more specific, Nile Blue A stain was done. Carbon enriched agar was supplemented with this fluorescent dye (0.5 ug/mL in DMSO). Under UV exposure, colonies that had a bright orange fluorescence signal were said to be PHB-positive and the correctly-produced PHB was accumulated intracellularly in large amounts [8].

C. Morphological and Biochemical Characterisation

The PHB producing isolates were characterised through biochemical and morphological studies. Metabolic characteristics and enzyme profiles were determined by a battery of biochemical tests in series; TSI, citrate utilisation, nitrate reduction, gelatin hydrolysis, urease, indole production, methyl red, Voges-Proskauer, catalase and sugar utilisation. Such tests helped in differentially identifying the bacterial species according to their biochemical make-ups.

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Furthermore, other characteristics of culture were noted including colony size, shape, elevation, surface texture, margin, consistency, opacity and pigmentations on nutrient agar.. Gram stain coloration was also done and the isolates classified as Gram-positive or Gram-negative depending on their cell wall characteristics in oil immersion microscope [9].

D. Production of PHB Using Organic Wastes

The possibility of utilizing organic wastes as carbon sources of PHB production was evaluated following two steps. The preparation of an inoculum was done by using a carbon-rich media composed of peptic digest (5g), sodium chloride (5g), beef extract (1g), yeast extract (1g), 2 % of glucose, and 1000mL of distilled water. The bacterial isolates producing PHB were inoculated into the medium and incubated at the temperature of 37o C over 24 hours to guarantee that the bacteria had grown actively. A mineral salt medium (MSM) was prepared and each was supplemented with various organic wastes such us molasses, crude oil, orange peel, potato peel and mixed fruit waste (5g each in 100 mL MSM) to obtain the PHB. Each flask was inoculated with 5 mL volume of inoculum and incubated in a shaking (200 RPM) incubator set at 37o C temperature until 3 days, to allow the maximum activity of the bacteria and PHB synthesis [10].

E. Extraction of PHB

The solvent dispersion method was used to extract PHB. After incubation, cultures were centrifuged for 10 minutes at 3000 rpm in order to extract the biomass. To lyse the cells, the resultant pellet was treated with 2.5 mL of 4% sodium hypochlorite and 2.5 mL of chloroform, and it was then incubated for an hour at 37°C. The mixture was divided into three layers following a second centrifugation step: the top aqueous phase, the middle debris layer, and the bottom chloroform layer, which contained PHB. After carefully collecting the chloroform phase, PHB was precipitated using a 7:3 methanol:water combination. PHB was obtained in powder form by drying the final polymer at 30°C [11].

F. Quantification of PHB

PHB traces were measured to assess manufacturing efficiency. Dry cell weight (DCW) and PHB were determined grammatically. The following formula was used to calculate the residual biomass value: DCW (g/L) minus PHB (g/L) equals residual biomass (g/L). Then, using the formula PHB Accumulation (%) = (PHB / DCW) × 100, the percentage composition of PHB was determined. This made it possible to precisely estimate the PHB intracellularity based on total biomass.

G. Optimization of PHB Production

Depending on the difference in environmental and nutritional conditions, optimisation studies were carried out to increase PHB yield. The parameters were changed systematically by the incubation temperature, pH value by nitrogen independently or in combination, aeration (static or shaking), and varied concentrations of carbon source. Regarding this, cultures were incubated at each condition and 48 hours after incubation, PHB extraction and quantification were carried out.. The findings gave an understanding of the best conditions that can be used in order to maximize the synthesis of PHB in the bacterial strains chosen.

H. Characterisation via FTIR

The chemical structure of the PHB extracted was characterised using Fourier Transform Infrared (FTIR) Spectroscopy. The pure PHB was dissolved in chloroform followed by casting it on KBr plate to produce thin films of the polymer. Spectral analysis was performed in the range of 400 to 4000 cm^{1} to identify functional groups associated with PHB. Peaks corresponding to ester carbonyl (C=O), methyl (CH_3), and methylene (CH_2) stretching confirmed the polymer's identity and purity.

III. RESULTS AND DISCUSSION

A. Survey and Soil Sample Collection

Extensive sampling was conducted at two selected sites in Surat, Gujarat—Dumas Beach and Kholwad College Garden in Kamrej. These locations were chosen to capture a contrast between a saline coastal environment and a terrestrial inland site. Sterile zip-lock plastic bags were used to collect soil samples, which were then aseptically transferred to the lab. The temperature at Kholwad College Garden was comparatively lower at 25°C during collection than the 35°C temperature at Dumas Beach.

In the laboratory, standard serial dilution techniques were used, followed by plating on nutrient agar supplemented with 2% glucose. After a 24-hour incubation at 37°C, pure cultures were obtained by repeated streaking.All these cultures were maintained through periodic sub culture [6].

B. Isolation of Soil Bacteria

Out of the processed soil samples obtained in Dummas beach and Kholwad College garden, a total of 25 different bacterial colonies were isolated. Primary cultures were taken by serial dilution and nutrient agar. It undertook sub-culturing after defined intervals of one or two weeks in order to secure the sustenance as well as the genuineness of the isolates. The variation in colony morphology was significant such as the differences in shape, appearance, size, pigmentation, elevation, and consistency that indicated the existence of varied bacterial species in the samples. These isolates were again screened to know the possible polyhydroxybutyrate (PHB) producing strains to be analyzed further.

C. Screening of PHB Producing Bacteria

Primary screening for microorganisms that produce PHB was done with Carbol Fuchsin staining. PHB accumulation was indicated by dark-stained intracellular granules in 10 of the 25 isolates. A shortlist of these isolates was created for additional verification.

Sudan: The 10 initial PHB-positive isolates were subjected to Black B staining, which is renowned for its selectivity for lipid inclusions. The existence of PHB was confirmed by the strong black to purple-stained granules seen in eight of them.

Blue Nile: The presence of PHB was further confirmed by a staining that showed orange fluorescence in eight bacterial colonies when exposed to UV light. Strains 3 and 4 were chosen for in-depth analysis and PHB production trials based on the fluorescence intensity.

D. Identification of PHB Producing Bacteria

1) Identification of Isolate 3 (Bacillus spp.)

Morphologically, Isolate 3 had small, circular colonies with flat elevation, translucent appearance, and no pigmentation. Gram staining showed violet-colored bacilli arranged singly or in pairs, identifying the strain as Gram-positive.

Biochemical profiling (Table 1) revealed positive results for glucose, sucrose, citrate, catalase, urease, and nitrate reduction tests. VP test was also positive, while tests for mannitol, maltose, xylose, and indole were negative.

Table 1: Biochemical Test Results of Isolate 3 (Bacillus spp.)

Test	Result
Glucose	Positive
Sucrose	Positive
Mannitol	Negative
Xylose	Negative
Maltose	Negative
Urease	Positive
Methyl Red	Negative
VP Test	Positive
Citrate	Positive
Indole	Negative
Catalase	Positive
Nitrate Reduction	Positive

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2) Identification of Isolate 4 (Pseudomonas spp.)

Isolate 4 formed large, raised, bluish-green opaque colonies. Gram staining revealed red-colored rods arranged singly or in pairs, confirming it as Gram-negative. Biochemical results (Table 2) matched the profile of Pseudomonas spp.

Table 2: Biochemical Test Results of Isolate 4 (Pseudomonas spp.)

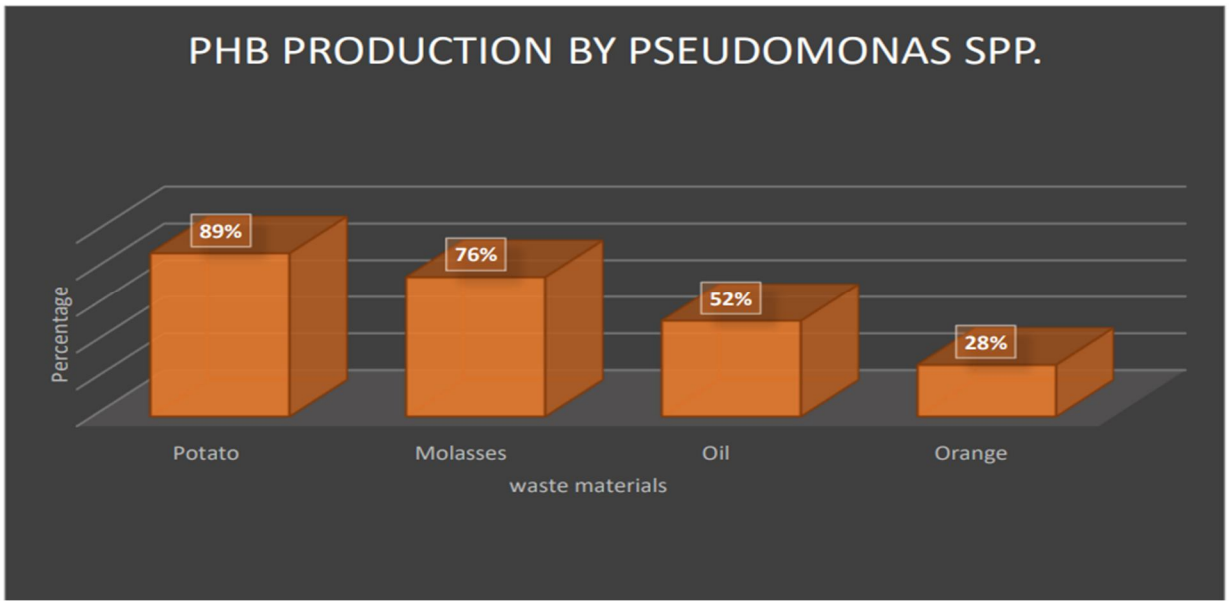
Test	Result	Test	Result
Glucose	Positive	TSI	K/K
Sucrose	Negative	H_2S	Negative
Mannitol	Negative	Urease	Negative
Xylose	Negative	Catalase	Positive
Maltose	Negative	Indole	Negative
Lactose	Negative	Gelatinase	Positive
MR	Negative	VP	Negative
Citrate	Positive		

E. Production and Extraction of PHB

1) Carbon Source Screening

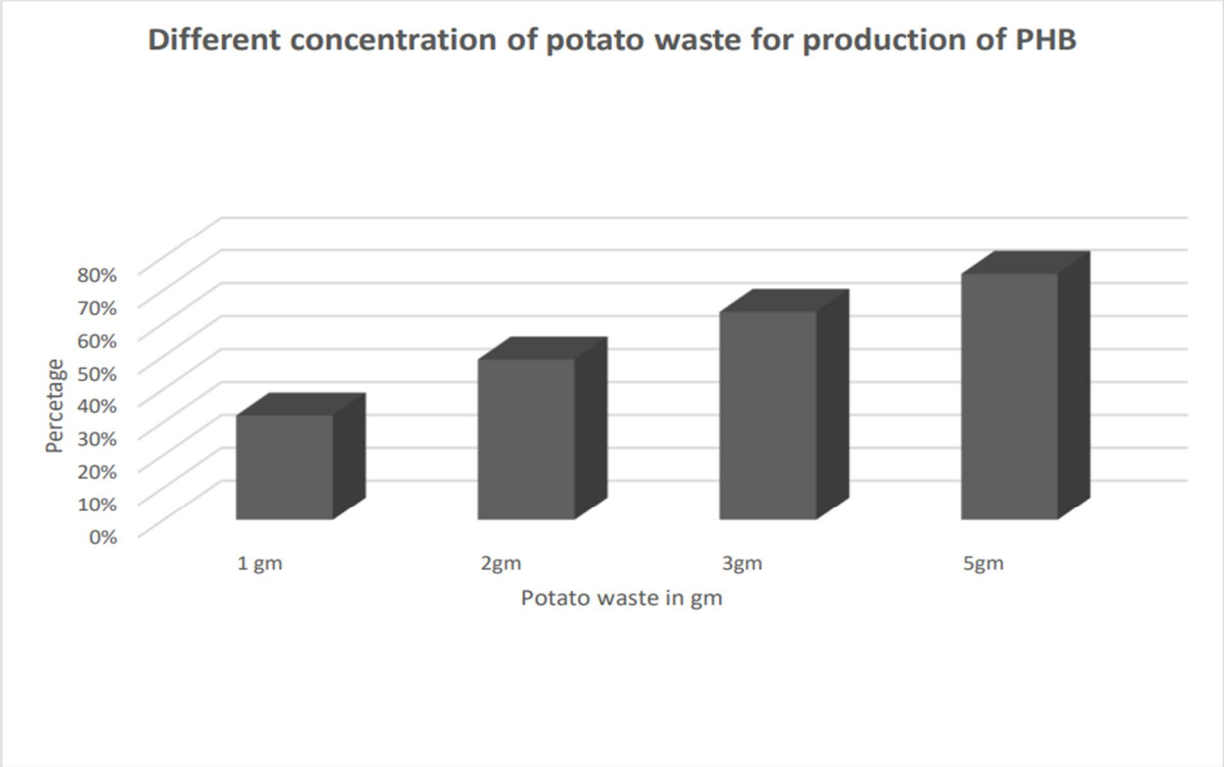
Four low-cost carbon sources—potato peels, orange peels, molasses, and waste cooking oil—were evaluated. For Bacillus spp., potato peels yielded the highest PHB production (76.86%), followed by molasses (53%), waste oil (30.42%), and orange peels (12.65%).

Figure 1: PHB Yield from Bacillus spp. Using Different Carbon Sources



For Pseudomonas spp., the trend was similar with potato peels producing the highest yield at 89%, followed by molasses (76%), oil (52%), and orange peels (28%).

Figure 2: PHB Yield from Pseudomonas spp. Using Different Carbon Sources

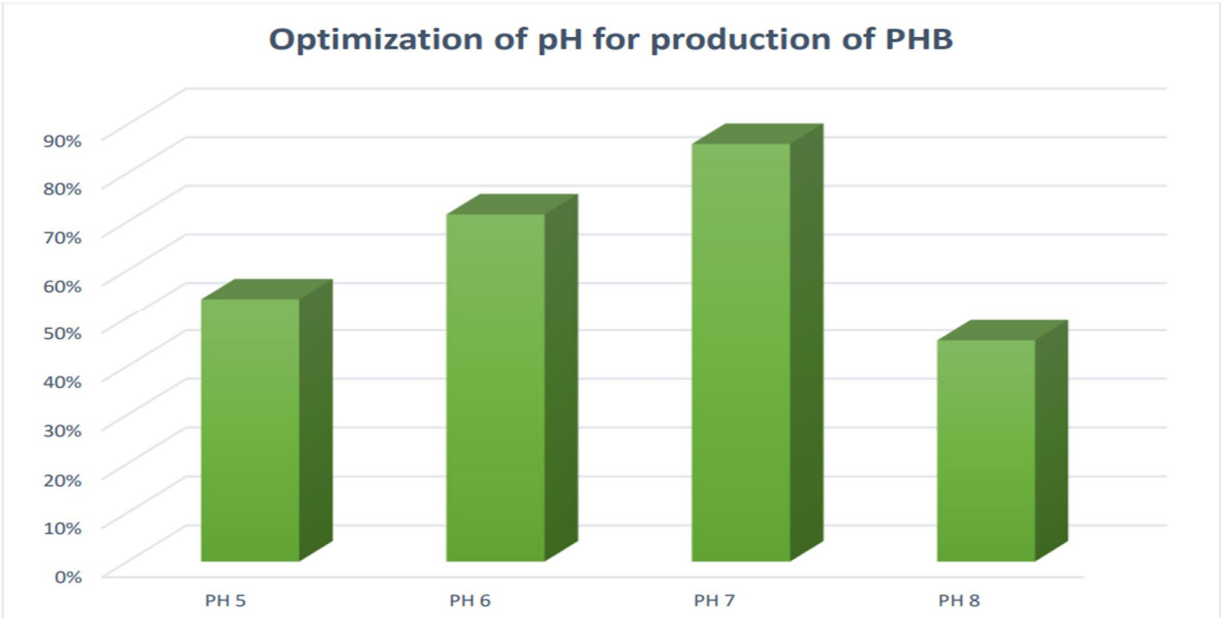


2) Optimisation Studies

Effect of Carbon Source Concentration:

Increasing concentrations of potato peel (1g to 5g) enhanced PHB yield from 32% to 70%, with optimal production at 5g.

Figure 3: Effect of Potato Peel Concentration



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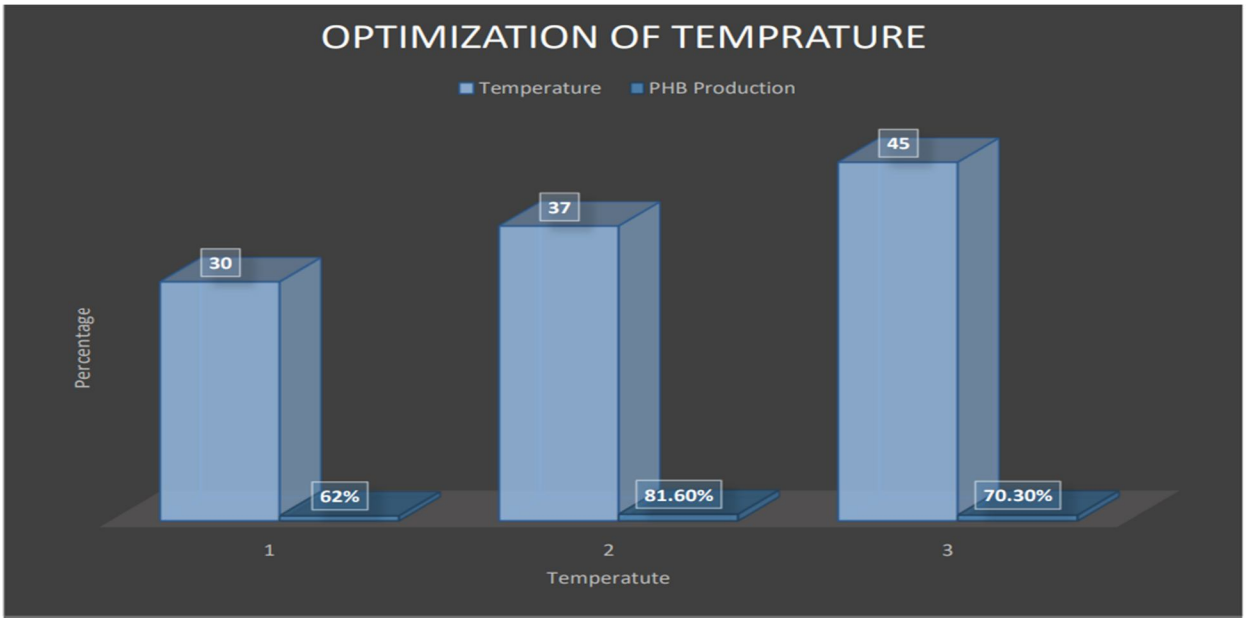
3) Effect of pH

Optimal PHB production occurred at pH 7 (86%). Production decreased under both acidic (pH 5: 54%, pH 6: 71.5%) and basic conditions (pH 8: 45.3%).

Figure 4: Effect of pH on PHB Production



4) Effect of Temperature

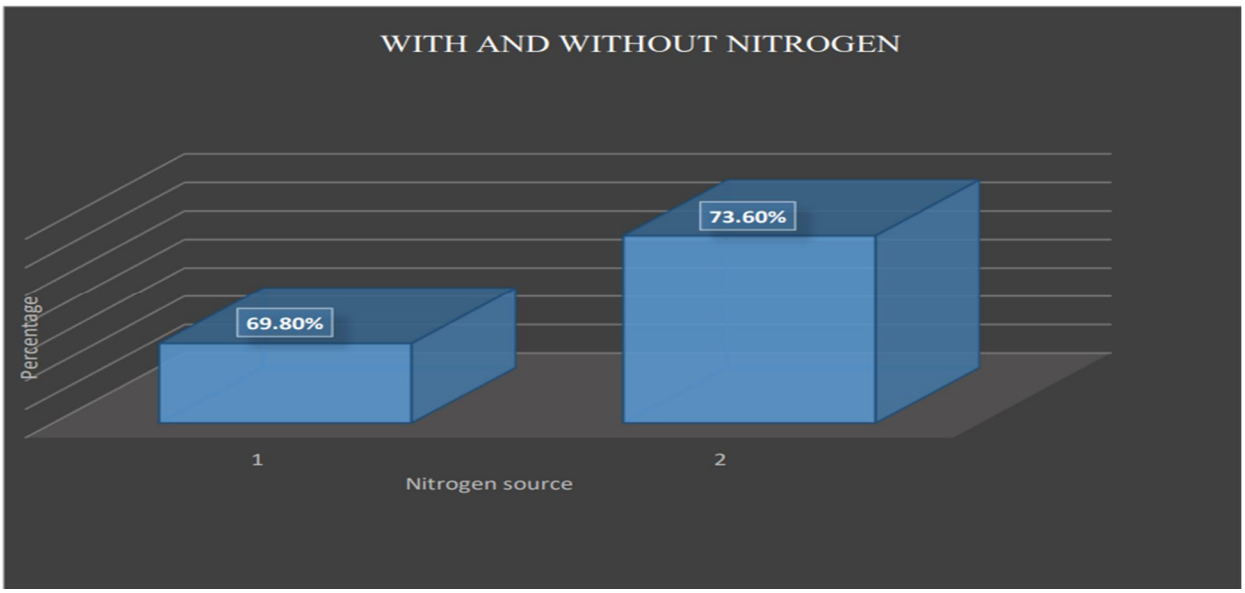
Maximum PHB was obtained at 37°C (81.6%), while reduced production was observed at 30°C (62%) and 45°C (70.3%).

Figure 5: Effect of Temperature

5) Nitrogen Source Effect

Presence of nitrogen yielded slightly more PHB (73.6%) compared to nitrogen-deprived media (69.8%), suggesting that nitrogen enhances biomass growth, indirectly supporting PHB accumulation.

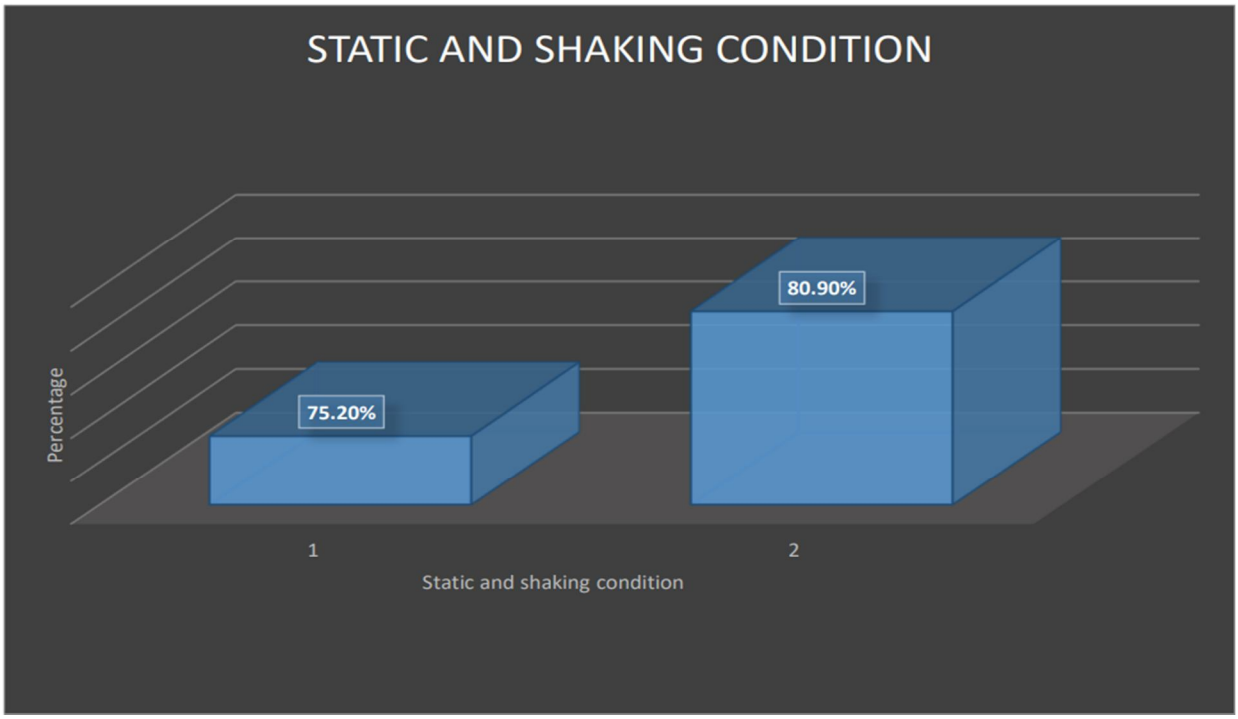
Figure 6: PHB Production With and Without Nitrogen



6) Static vs. Shaking Conditions

Shaking culture conditions (200 rpm) resulted in 80.9% PHB, while static conditions yielded 75.2%, showing that aeration enhances metabolic activity and PHB synthesis.

Figure 7: Aeration Condition Comparison



7) FTIR Characterisation of PHB

FTIR analysis confirmed the identity of extracted PHB with characteristic peaks at **1730.21 cm^{-1}** (C=O stretching) and around **1280 cm^{-1}** (C-O stretching), consistent with reported spectra for PHB.

Figure 8: FTIR Spectra from Molasses Medium

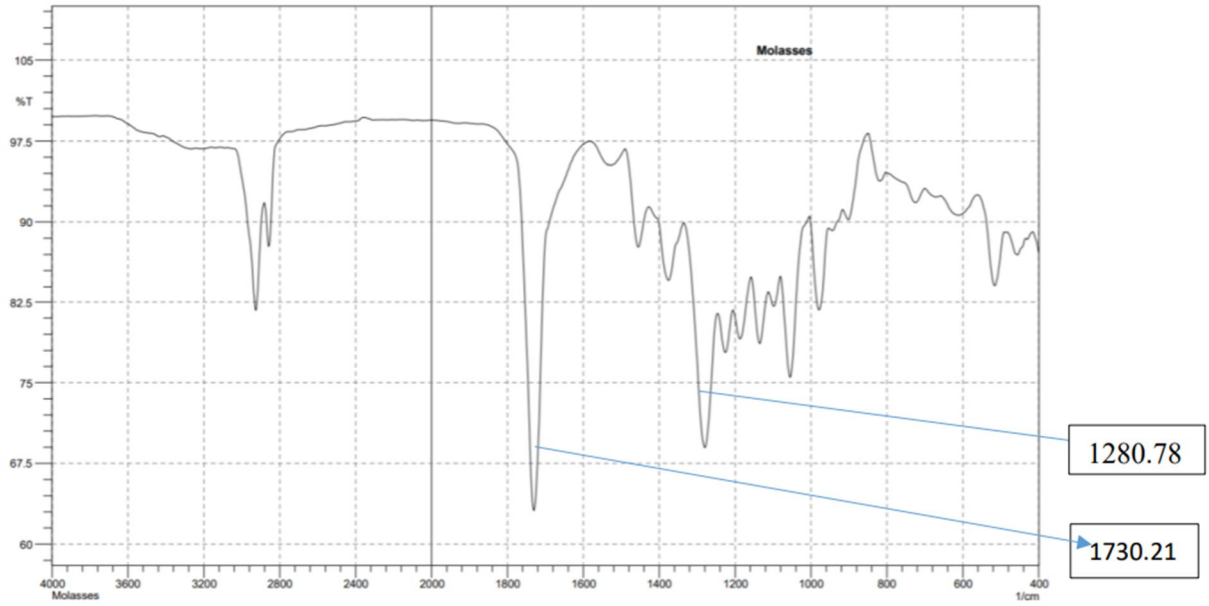
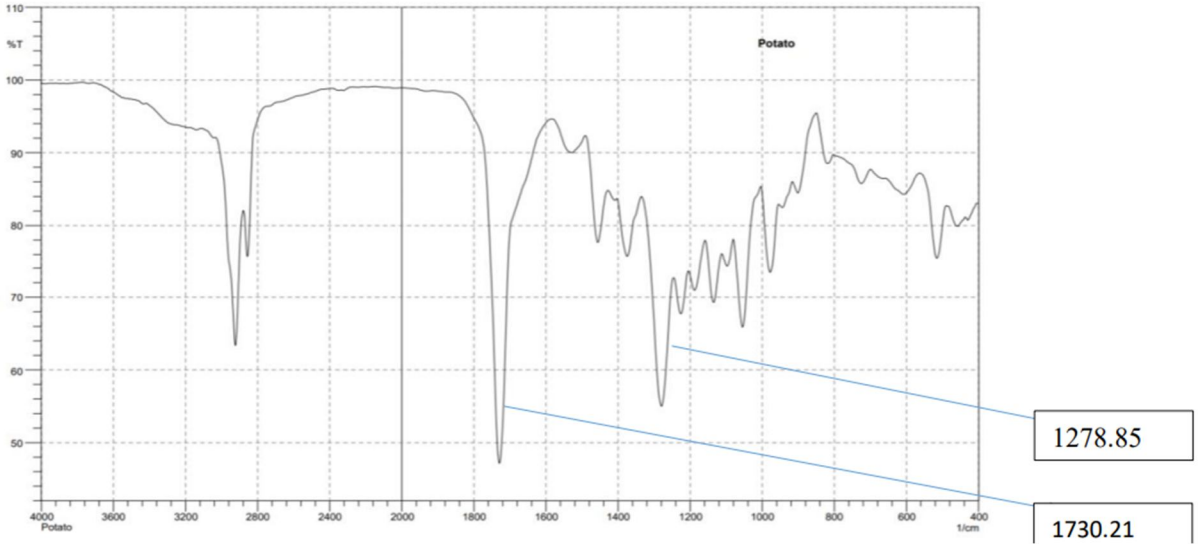


Figure 9: FTIR Spectra from Potato Peels Medium



The results demonstrate that both Bacillus spp. and Pseudomonas spp. isolated from Dumas Beach and Kholwad Garden are efficient PHB producers. Among various tested carbon sources, potato peels showed the highest potential as an economical substrate. Optimised production conditions for Pseudomonas spp. were pH 7, temperature 37°C, and 5g carbon source under shaking conditions, leading to maximal yield. These findings offer a promising foundation for sustainable biopolymer production using low-cost agro-waste [12] .

IV. CONCLUSION

The goal of the current study was to identify and isolate native bacterial strains that might produce polyhydroxybutyrate (PHB), a biodegradable biopolymer that could eventually take the place of traditional plastics. Twenty-five bacterial isolates were found in the various environmental samples collected in South Gujarat's Dumas Beach and Kholwad College Garden. Two promising organisms, Bacillus spp. (Isolate 3) and Pseudomonas spp. (Isolate 4), were found by primary screening utilising Carbol Fuchsin, Sudan Black B, and Nile Blue A staining procedures. These strains demonstrated a notable intracellular buildup of PHB.

The characterisation of these isolates was concomitantly done by biochemicals, and more on their PHB production was done using specific low cost organic waste sources. The tested four carbon sources that include potato peels, orange peels, molasses, and waste cooking oil showed that potato peels produced greater PHB accumulation in both isolates; 89% PHB accumulation in Pseudomonas spp. and 77% in Bacillus spp. Optimisation trial experiments showed that optimal PHB production was obtained at pH7, a temperature of 37o C and shaking (aerobic) conditions. Also, the second factor contributed to a small-scale biomass and PHB improvement due to the presence of nitrogen. These results present the idea that the nutritional and environmental conditions have a strong effect on the PHB synthesis.

FTIR analysis of the extracted polymer confirmed the structural integrity of PHB through characteristic peaks corresponding to ester carbonyl and alkyl groups. This validates the biopolymer identity and supports the effectiveness of the extraction and purification method.

Overall, this study demonstrates that native bacterial strains can be harnessed for cost-effective PHB production using agro-industrial waste. The use of regionally available organic materials not only reduces production costs but also supports circular waste management. Future work should explore scale-up strategies, bioreactor development, and molecular-level strain optimization to advance commercial applications. This research contributes meaningfully to sustainable plastic alternatives and localised bioeconomy development.

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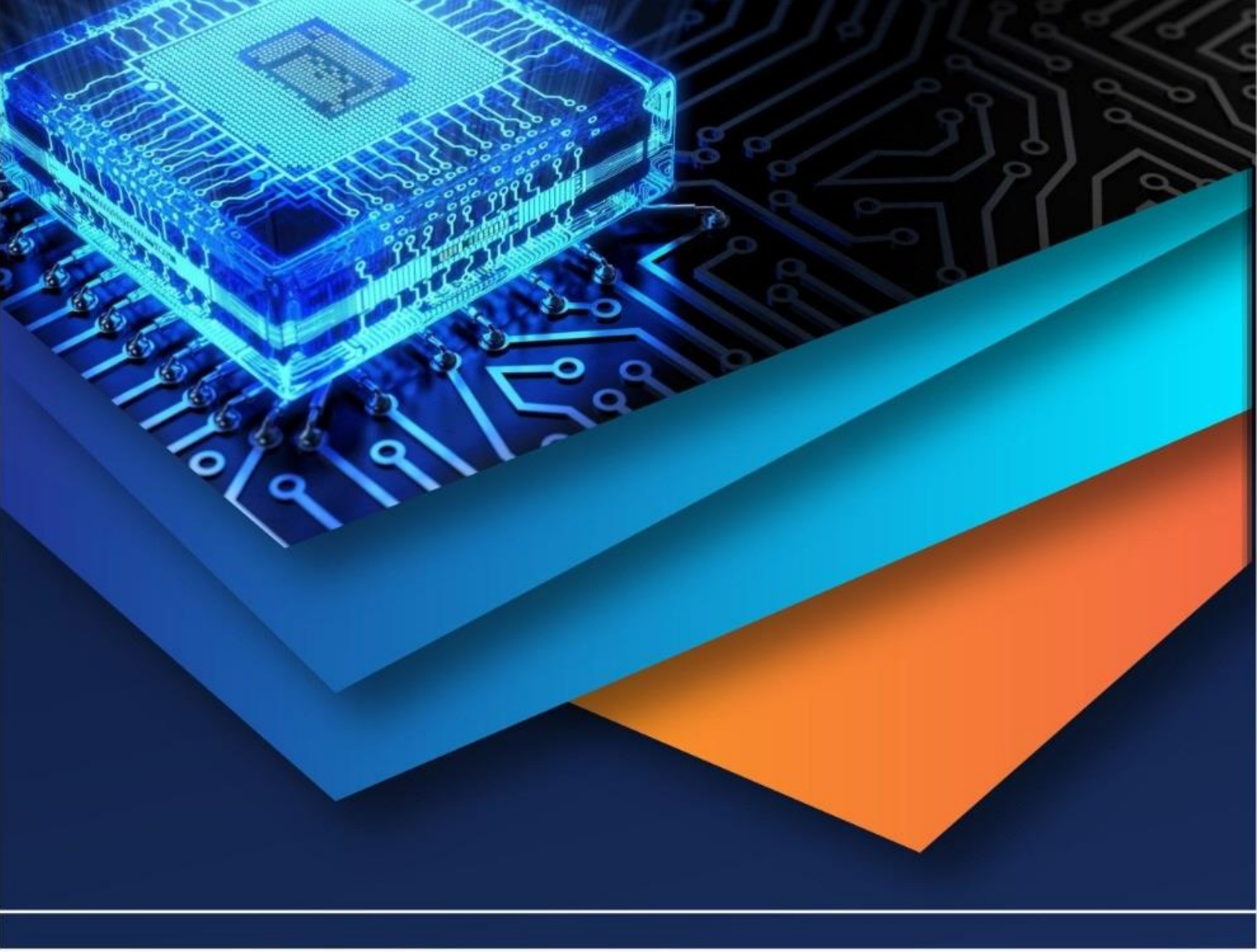

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