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# "VALORIZING LIGNOCELLULOSIC BIOMASS FOR SUSTAINABLE PHA PRODUCTION: A PROCESS OPTIMIZATION STUDY WITH *PRIESTIA MEGATERIUM*"

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## ABSTRACT
The accumulation of synthetic plastic waste has accelerated the demand for eco-friendly alternatives like Polyhydroxyalkanoates (PHAs). However, the high production cost of PHAs remains a major bottleneck for commercialization. This study investigates the bioconversion of affordable and abundant lignocellulosic waste into high-value PHAs using the bacterial strain *Priestia megaterium*. To maximize biopolymer yield, a systematic process optimization was conducted during controlled batch fermentation. Various lignocellulosic materials were screened as primary carbon sources, alongside the optimization of critical process parameters, including carbon source concentration, nitrogen sources, pH, and culture temperature. Following optimization, the accumulated PHA was harvested from the final batch culture and subjected to detailed molecular-level characterization. The chemical structure, functional groups, and thermal stability of the extracted biopolymer were comprehensively verified using Fourier Transform Infrared (FTIR) Spectroscopy and Nuclear Magnetic Resonance (NMR) analysis. The results demonstrate that *Priestia megaterium* effectively metabolizes optimized lignocellulosic residues, yielding high-quality PHAs with favorable physicochemical and thermal properties. This research highlights a sustainable, green biorefinery approach to valorizing agro-industrial residues, offering a viable pathway for the cost-effective production of biodegradable biopolymers.

**KEYWORDS**: Polyhydroxyalkanoates (PHA); *Priestia megaterium*; Lignocellulosic waste; Carbon and nitrogen source optimization; Fermentation optimization; Molecular characterization; Bioplastic production

## 1. INTRODUCTION
Many bacteria produce and store polyhydroxyalkanoates (PHAs), which are polyesters made of 3-, 4-, 5-, and 6-hydroxyalkanoic acids, as carbon and energy reserves. This is especially true when carbon is abundant but nutrients like nitrogen, phosphorus, or sulfur are scarce. These polymers can have piezoelectric, thermoplastic, or elastomeric qualities in addition to being biodegradable, water-insoluble, non-toxic, and biocompatible. These qualities make PHAs a sustainable substitute for traditional petroleum-based plastics and promising materials for packaging applications.[1] Many bacteria and archaea accumulate polyhydroxyalkanoates (PHAs), a large family of polyesters, as intracellular carbon and energy storage materials. This is usually the result of unbalanced growth conditions with excess carbon and a lack of other nutrients like nitrogen, phosphorus, or oxygen. In light of the global push to lessen reliance on petrochemical plastics and reduce plastic pollution, these granules—which are produced from renewable substrates through microbial metabolism—have garnered a lot of attention as sustainable biopolymers.[2, 3] PHAs solve end-of-life issues related to traditional plastics because they are completely biodegradable in a variety of settings, such as soil, compost, and marine ecosystems, and their breakdown does not produce hazardous or persistent residues. Furthermore, a number of PHA homopolymers and copolymers have tunable mechanical and thermal properties as well as outstanding biocompatibility, making them suitable for a variety of applications, including drug delivery systems, biomedical devices, tissue engineering scaffolds, packaging, and agricultural films. Their thermoplastic nature, which is frequently similar to that of polypropylene and other commodity polymers, facilitates processing using conventional melt-based techniques like injection molding and extrusion.[4,5,6] However, despite their favorable properties, PHAs currently occupy only a niche share of the plastics market because their production costs remain significantly higher than those of fossil-based polymers. The major cost drivers include the price of carbon substrates, which can contribute up to approximately half of the total production cost, along with relatively low productivities, energy-intensive downstream processing, and scale-up limitations. To improve the economic feasibility of PHA bioplastics, recent research has increasingly focused on utilizing low-cost and renewable feed stock, such as lignocellulosic crop residues, food waste, agro-industrial by-products, and glycerol—as well as engineering robust microbial strains and optimizing fermentation and recovery strategies. [36,8] Against this background, the present study investigates PHA production using alternative feedstocks and improved bioprocess conditions, aiming to reduce overall production costs while maintaining or enhancing polymer yield and quality. Such advancements are essential to enable broader industrial adoption of PHAs and to support the transition toward a circular bioeconomy based on renewable, biodegradable materials.[8.9] According to the 2019 PHAs Market

Research Report, the market for PHAs is expected to grow to nearly USD 98 million by 2024. [10] Microorganisms under stressful or nutrient-limited conditions typically synthesize polyhydroxyalkanoate (PHA). The availability of other vital nutrients, such as nitrogen, phosphorus, dissolved oxygen, sulfur, or trace metals necessary for cellular metabolism, is limited in these circumstances even though the environment has an excess of carbon sources. Microorganisms build up PHAs in their cells as carbon and energy storage molecules as a result of this nutrient imbalance.[11] When nutrients are scarce but carbon sources are plentiful, both Gram-positive and Gram-negative bacteria can produce polyhydroxyalkanoates (PHAs) as intracellular carbon and energy reserves. PHAs are known to be highly productively accumulated in the cytoplasm of a number of genera, including Alcaligenes, Bacillus, Pseudomonas, recombinant Escherichia coli, and Methylotrophs. Polyhydroxybutyrate (PHB) is the most widely produced type of polymer. Additionally, copolymers like P (HB-co-HV) and polyhydroxyvalerate (PHV) can be produced by microorganisms. These biodegradable polymers are used in a wide range of products, such as packaging materials, tissue and skin implants, 3D printing, photographic components, pharmaceuticals, drug coatings, and nutritional supplements. PHAs are also frequently utilized in tissue engineering and drug delivery systems because of their superior biocompatibility.[7,8,11] Although the great potential of the PHA-producing bacteria for the industrial-scale production of bioplastics exists, the process as a whole is currently impaired by the high price of raw materials. In order to increase the economy of the PHA process, the possibility of using low-priced and renewable agro-industrial by-products as alternative carbon sources has been considered. Organic wastes from the food industry, agriculture, municipal solid waste, as well as wastewater, among other sources, have been studied as prospective carbon-containing materials for PHA.[12] Furthermore, the current limitations in the industrial production of bioplastics could be removed by using high PHA-producing bacterial strains and ideal culture conditions. The use of these kinds of bacteria is currently restricted by the low productivity of bacterial cells and the high cost of refined sugars used, even though the industrial production of these plastics has been going on for several decades. [7,12] Thus, the objective of this study was to isolate bacteria capable of accumulating PHAs and to optimize the nutritional and physical culture conditions to enhance polymer production using a one-factor-at-a-time (OFAT) approach. Different carbon and nitrogen sources, along with variations in pH and temperature, were tested individually to determine their effects on PHA yield. The isolate that showed the greatest potential for PHA production was further evaluated for its ability to utilize low-cost agro-industrial residues—such as kitchen waste, sugarcane molasses, bakery waste flour, and fruit pulp from juice shops—as economical carbon sources for fermentation-based PHA production. [11] Use of such renewable waste materials has dual benefits: it helps in the efficient management of the large amount of agro-industrial wastes, and it also helps in the conversion of the same wastes to valuable biodegradable bio-plastics. The harvested biomass was authenticated as PHA through the crotonic test, followed by FTIR, NMR spectroscopic techniques, as well as chromatography and thermoanalytical techniques such as GC, TGA, in order to understand the properties.[11,12,14]

## 2. MATERIALS & METHODS

The bacterial strain *Priestia megaterium* (PS2) used in this study was isolated from petroleum-contaminated soil collected near the Coco Vasna Indian Oil Petrol Pump, located on Akota–Vasna Road, Vadodara, Gujarat, India (22.294780° N, 73.157444° E).

### 2.1 Microbial Inoculation and Bacterial Growth Process.

Basal mineral salt medium (MSM) was used in the fermentation process to produce polyhydroxyalkanoates (PHAs). The medium was autoclaved at 121 °C under 15 lb of pressure for 15 minutes after its pH was brought to 7.0. Following sterilization, a sterile 2% inoculum was added to the fermentation medium. For 120 hours, the inoculated flasks were kept at 37 °C with agitation at 120 rpm in an incubator shaker. Sterile nutrient broth with 1% glucose was utilized as the seed medium to prepare the inoculum. The seed medium composition included glucose (10 g/L), peptone (3 g/L), beef extract (5 g/L), and sodium chloride (5 g/L), with the pH adjusted to 7.0. In this study, the bacterial isolate PS2 was utilized.
Urea (1.0 g/L), yeast extract (0.16 g/L), $KH_2PO_4$ (1.52 g/L), $Na_2HPO_4$ (4.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.52 g/L), $CaCl_2$ (0.02 g/L), glucose (10 g/L), and 0.1 mL of trace element solution were all included in the production medium. $ZnSO_4 \cdot 7H_2O$ (0.13 g/L), $FeSO_4 \cdot 7H_2O$ (0.02 g/L), $(NH_4) \cdot Mo_2O_{24} \cdot 4H_2O$ (0.06 g/L), and $H_3BO_3$ (0.06 g/L). The production medium was supplemented with a trace element solution. For efficient polyhydroxyalkanoate (PHA) production, fermentation was carried out at 37 °C for 120 h under shaking conditions at 120 rpm, while maintaining the pH of the medium at 7.0 .

### 2.2 Extraction and Quantitative Analysis of Polyhydroxyalkanoates (PHAs).

The production broth, with a volume of forty five milliliters, was centrifuged at 8,500 × g for fifteen minutes.After discarding the supernatant, the pellet was washed with pH 7.4 phosphate buffered saline. The dry cell weight was determined by precisely weighing the cell pellet after it had been air-dried for two hours. The cell pellet was then suspended in a solvent that contained 12.5 µL of 4% sodium hypochlorite solution and 12.5 µL of chloroform per milligram of pellet weight. For 90 minutes, the suspension was shaken at 37°C to ensure that all cell components—aside from PHAs—were completely digested. Different phases were formed when the dispersion was centrifuged at 6500 × g for 10 minutes at room temperature after 90 minutes. The volume of the bottom chloroform phase containing PHAs was measured after it was moved to a new tube.
A precipitate of PHAs was formed by centrifuging the mixture at 8500 rpm for 15 minutes after adding five volumes of methanol and water (7:3 v/v). The precipitates were then allowed to air dry for an entire night after the solution was transferred into a petri plate. The dry weight of extracted PHAs was determined by weighing the precipitate. The following formulas were used to estimate Cell Dry Weight (g/l), PHA Concentration (g/l), and PHA Yield (%) for quantitative analysis of PHAs:

**Cell Dry Weight (g/l)** = Total Cell Dry Weight Extracted (g) / Total Volume of sample taken (l)

- **PHA Concentration (g/l) =** Total PHA extracted (g) / Total Volume of sample taken (l)
- **PHA Yield (%)** = [PHA Concentration (g/l) / Cell Dry Weight (g/l)] * 100

### 2.3 Optimization Studies Using OFAT Method

The physical and nutritional culture conditions for PHAs production were optimized using the one-factor-at-a-time (OFAT) approach. This experimental design method evaluates the effect of individual factors separately by altering one variable while keeping all other conditions constant, rather than testing multiple factors simultaneously.

#### 2.3.1 PHA Production Optimization and Assessment with Various Carbon, Nitrogen, and Lignocellulosic Substrates.

Using a variety of carbon substrates, nitrogen sources, and lignocellulosic wastes at different temperatures, the isolate PS2 was chosen to assess its potential for improved PHA production. The culture was first cultivated in nutrient broth at 37 °C with shaking at 120 rpm for a full day in order to conduct the carbon substrate experiment. Following incubation, the actively proliferating cells were moved to PHA production medium (MSM medium) that contained 2% (w/v) of the chosen carbon substrate.

During the optimization of carbon and nitrogen sources, the temperature (37°C), pH (7), and agitation speed (120 rpm) were maintained constant to ensure consistent experimental conditions. Common lignocellulosic wastes such as coconut fibers, corn cob, wheat bran, rice straw, sugarcane bagasse, vegetable peels, and wood bark were among the carbon substrates examined. Furthermore, despite not being categorized as lignocellulosic materials, sucrose and mannitol were included as reference carbon sources.

Each experiment used 500 ml flasks filled with 120 ml of medium. For each of the seven carbon substrates, measurements were made of total biomass and PHA production.

To boost PHA yield, fermentation experiments using different nitrogen sources were conducted using the PS2 strain. Yeast extract, beef extract, ammonium chloride, ammonium sulphate, potassium nitrate, sodium nitrate, peptone, urea, and casein were the nine nitrogen sources that were tested separately. Each nitrogen source was added separately to flasks filled with Mineral Salt Medium (MSM). The flasks were filled with the PS2 strain and shaken at 120 rpm for 120 hours at 37 °C. The effects of different nitrogen sources on PHA production were then evaluated.

#### 2.3.2 Pretreatment Methods for Different Carbon Substrates

**Acid Treatment:**Seven lignocellulosic wastes were treated with 1.5% (w/v) $H_2SO_4$ at a 10% (w/v) liquid-to-solid ratio and autoclaved at 121°C for 20 minutes. After cooling, 10% activated charcoal was added to remove furfural, and the solution was filtered through Whatman No.1 paper. Higher acid concentrations reduced sugar yield, while 10% charcoal removed ~98% of by-products.

**Alkali Treatment:** Seven lignocellulosic wastes were autoclaved for 20 minutes at 121°C after being treated with 2% (w/v) NaOH at a 10% (w/v) liquid-to-solid ratio. Following autoclaving, the solution was filtered and 10% activated charcoal was added. Sugar yield was reduced by increasing alkali above 2%, and the majority of furfural was eliminated by 10% charcoal.

**Enzymatic Treatment:** After applying 10% cellulase at a 10% (w/v) liquid-to-solid ratio to seven lignocellulosic wastes, the mixture was shaken at 120 rpm for two hours at 37°C. The ideal enzyme concentration was 10%, as higher concentrations did not enhance hydrolysis.

#### 2.3.3 Analysis of PHAs Production under Varying Initial pH and Temperature

Various initial pH and temperature conditions were used to assess PHA production. PHA production was investigated in relation to physical factors, such as initial pH values between 5 and 9 and incubation temperatures between 25 and 40 °C.

### 2.4 Sequencing the 16S rRNA gene and using phylogenetic analysis to identify bacteria

Bacterial Identification using 16S rRNA Sequencing The bacterial strain was taxonomically identified at the molecular level by amplifying the 16S rRNA gene using universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) was performed in a thermal cycler under the following conditions: initial denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s; with a final extension at 72°C for 10 min. Amplicons were verified via 1% agarose gel electrophoresis, purified, and sequenced using the Sanger method. The resulting raw sequences were aligned and quality-trimmed using ClustalW , and subsequently identified through BLASTn analysis against the NCBI GenBank database

### 2.5 "Characterization of Polyhydroxyalkanoates Synthesized by bacterial isolate"

#### 2.5.1 Fourier Transform Infrared Spectroscopy Analysis of Polyhydroxyalkanoates for Structural and Functional Characterization.

Attenuated total reflection–Fourier transform infrared (ATR-FTIR) spectroscopy was used to analyze the functional groups and chemical makeup of the extracted PHAs. A Bruker ATR-FTIR spectrophotometer with a Zn-Se crystal was used to record infrared spectra in the 600–4000 $cm^{-1}$ range.

#### 2.5.2 $^1H$ and $^{13}C$ NMR Analysis of Polyhydroxyalkanoates Synthesized by bacterial isolate.

The extracted PHAs were structurally characterized using $^1H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy. Following the dissolution of the purified polymer samples in deuterated chloroform ($CDCl_3$), the spectra were recorded at room temperature using a Bruker NMR spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Proton ($^1H$) and carbon ($^{13}C$) NMR spectra were analyzed to identify the repeating units, functional groups, and overall polymer structure of the generated PHAs.

## 3. RESULT & DISCUSSION

### 3.1 Growth and PHA Production Profile of PS 2 *(Priestia megaterium)* Bacterial Strain.

The effect of cultivation time on PHA production by *Priestia megaterium* was investigated over a period of 120 h. The results demonstrated that both biomass formation and intracellular PHA accumulation were significantly influenced by the duration of cultivation. At 24 h, the culture exhibited a cell dry weight (CDW) of 2.80 g/L and a PHA concentration of 1.11 g/L, corresponding to a PHA yield of 39.20%. The gradual increase in these parameters during the early stages of cultivation indicates active cell growth and efficient conversion of the carbon source into biomass and storage polymer.[8,39] Biomass production increased progressively and reached a maximum CDW of 4.88 g/L at 72 h. Likewise, the highest PHA concentration (2.66 g/L) was also recorded at 72 h, suggesting that this period represented the most active phase of polymer biosynthesis. The concurrent increase in CDW and PHA concentration indicates that *P. megaterium* efficiently accumulated intracellular PHA during the exponential growth phase. Similar observations have been reported in several PHA-producing bacteria, where excess carbon availability promotes rapid biomass formation accompanied by enhanced polymer accumulation. Interestingly, although both CDW and PHA concentration decreased after 72 h, the PHA yield continued to increase and reached its maximum value of 59.40% at 96 h. This result suggests that polymer accumulation remained active despite a reduction in overall biomass production. Such behavior is commonly observed in PHA-producing microorganisms under nutrient-limited conditions, where excess carbon is redirected toward intracellular PHA storage rather than cellular proliferation. The higher PHA yield observed at 96 h therefore indicates a greater proportion of polymer accumulated within the cells during the transition from exponential to stationary growth phase.[35]

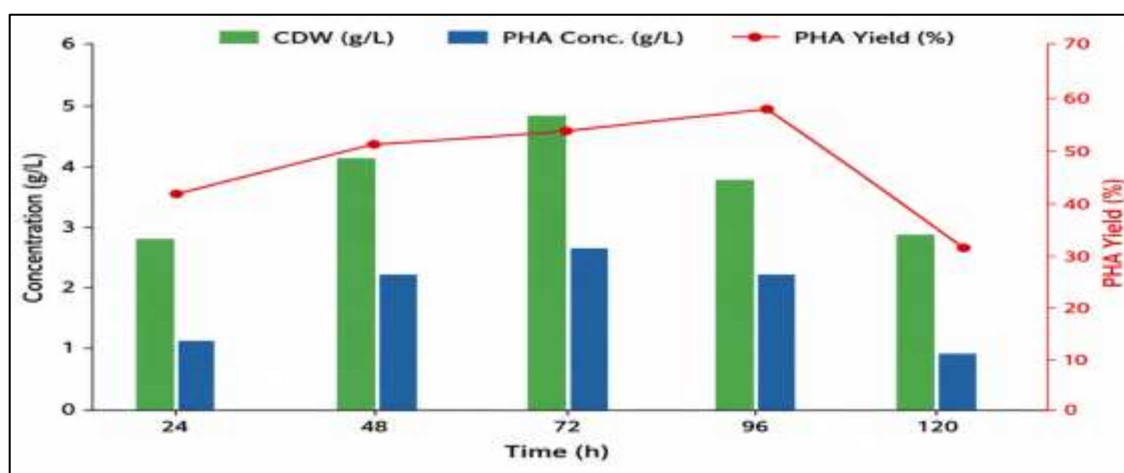



**Figure. 1- Growth and PHA Production Profile of PS 2 *(Priestia megaterium)* Bacterial Strain.**

A substantial decline in CDW (2.88 g/L), PHA concentration (0.88 g/L), and PHA yield (28.50%) was observed at 120 h of cultivation. This decrease may be attributed to nutrient depletion and the subsequent mobilization of intracellular PHA reserves to support cellular maintenance. Previous studies have reported that under prolonged stationary-phase conditions, stored PHA is utilized as an endogenous carbon and energy source, resulting in a reduction in polymer content and overall biomass. Overall, cultivation time had a pronounced effect on PHA production by *Priestia megaterium*. Maximum biomass production and PHA concentration were achieved at 72 h, whereas the highest PHA yield was obtained at 96 h. These findings suggest that 96 h is the optimal harvesting time for maximizing intracellular PHA accumulation under the conditions employed in the present study.[35,40]

### 3.2 Effect of Nitrogen Source Variation on PHA Production by *priestia megaterium*

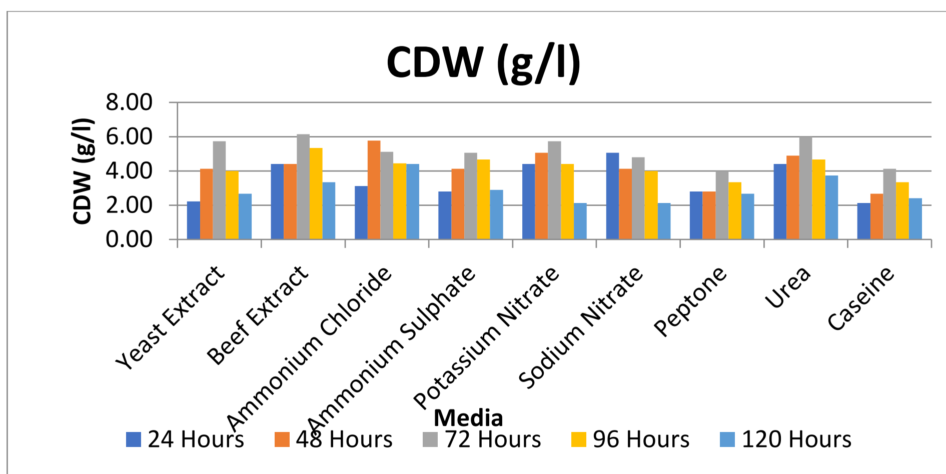



**Figure 2. Effect of Different Nitrogen Sources on Cell Dry Weight of PS2 Strain**

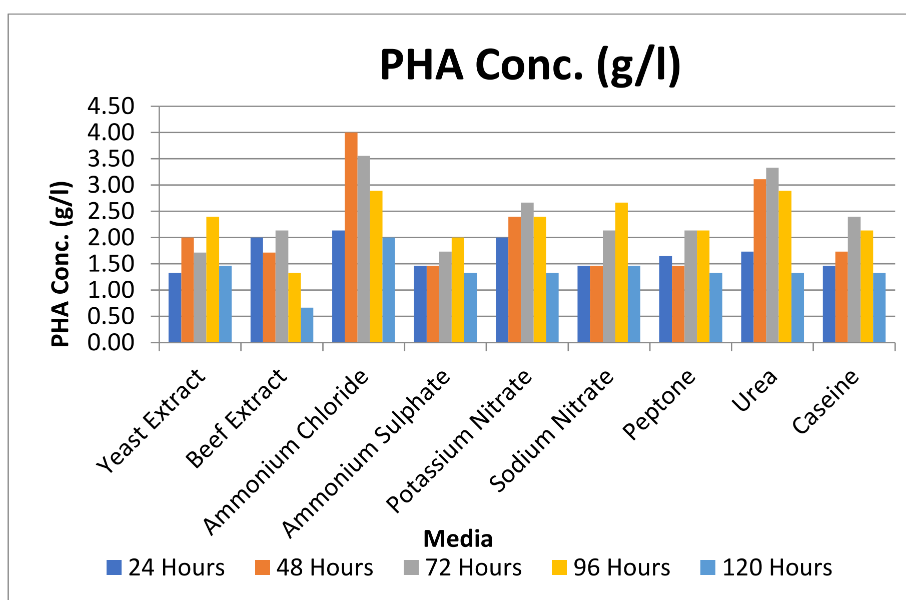



**Figure 3. Effect of Different Nitrogen Sources on PHA Concentration of PS2 Strain**

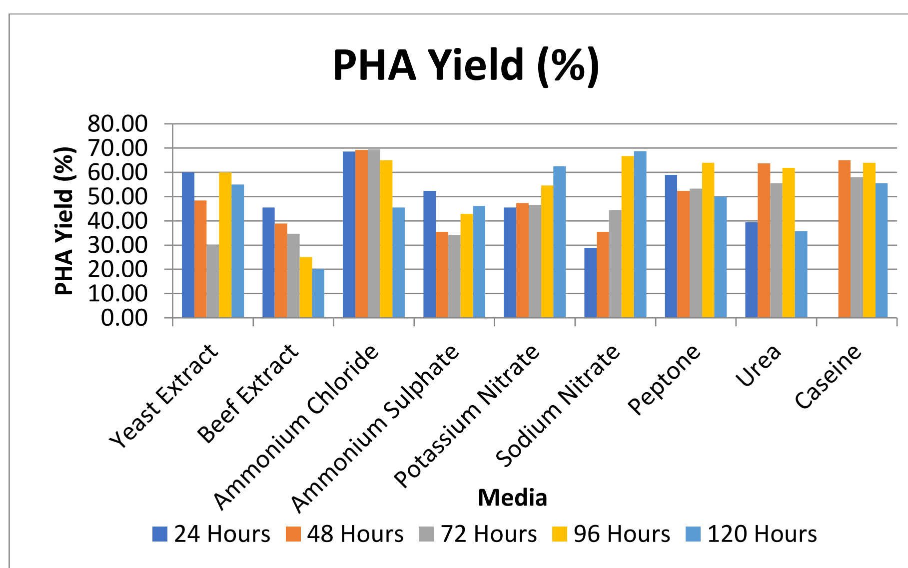



**Figure 4. Effect of Different Nitrogen Sources on PHA Yield of PS2 Strain**

The present study demonstrated that the nature of the nitrogen source significantly affected cell growth and PHA accumulation. Among the tested nitrogen sources, ammonium chloride yielded the highest PHA concentration (4.00 g $L^{-1}$) and PHA yield (69.57%), indicating its suitability for PHA biosynthesis. Although beef extract and urea supported higher biomass production, their PHA yields were comparatively lower, suggesting that excess nitrogen availability favored cell proliferation over polymer accumulation. Similar observations have been reported by Koller and co-workers,

who noted that readily assimilable inorganic nitrogen sources enhance PHA accumulation by promoting nitrogen-limited conditions while maintaining sufficient metabolic activity for polymer synthesis.[15]

The highest PHA production was observed between 48 and 72 h of cultivation, corresponding to the late exponential and early stationary growth phases. This trend agrees with previous reports that PHA accumulation is stimulated when cells experience nutrient imbalance, particularly nitrogen limitation in the presence of excess carbon. Studies by Madison and Lenz showed that microorganisms redirect excess carbon toward intracellular PHA granules under nutrient-stress conditions as a survival mechanism. The decline in PHA concentration after 96 h may be attributed to intracellular polymer degradation and utilization as an alternative carbon and energy source during prolonged cultivation and nutrient exhaustion.[20]

Comparison of organic and inorganic nitrogen sources revealed that inorganic nitrogen compounds, particularly ammonium chloride and urea, were more favorable for PHA production than complex organic nitrogen sources such as beef extract, peptone, and casein. Similar findings have been reported for several PHA-producing bacteria, including Cupriavidus necator and Bacillus megaterium, where ammonium-based nitrogen sources enhanced PHA accumulation due to their rapid uptake and controlled nitrogen availability. Therefore, the superior performance of ammonium chloride observed in the present study suggests that it provides an optimal balance between cellular growth and nitrogen limitation, making it a promising nitrogen source for efficient and cost-effective PHA production.[20,11]

### 3.3 Effect of Pretreatment Methods on Different Carbon Substrates for PHA Production.

| **Treatment** | **Source** | **Absorbance (540nm)** | **Concentration (mg/ml)** |
|---|---|---|---|
| **Acid** | Coconut Fibres | 1.11 | 1.44 |
| | Corn Cob | 0.19 | 0.29 |
| | Wheat Bran | 0.73 | 0.96 |
| | Rice Straw | 0.12 | 0.20 |
| | Sugarcane Bagasse | 0.39 | 0.54 |
| | Vegetable Peels | 0.43 | 0.58 |
| | Wood Barks | 1.16 | 1.50 |
| **Alkali** | Coconut Fibres | 1.09 | 4.16 |
| | Corn Cob | 1.26 | 1.62 |
| | Wheat Bran | 1.19 | 1.53 |
| | Rice Straw | 0.37 | 0.51 |
| | Sugarcane Bagasse | 1.14 | 1.47 |
| | Vegetable Peels | 1.08 | 1.40 |
| | Wood Barks | 1.29 | 16.17 |
| **Enzymatic** | Coconut Fibres | 1.06 | 1.37 |
| | Corn Cob | 0.24 | 0.34 |
| | Wheat Bran | 0.65 | 0.86 |
| | Rice Straw | 0.23 | 0.33 |
| | Sugarcane Bagasse | 0.31 | 0.42 |
| | Vegetable Peels | 0.39 | 0.43 |
| | Wood Barks | 0.84 | 1.10 |

**Table 1. Effect of Pretreatment Methods (Acidic, Alkali, and Enzymatic) on Substrate Conversion**

Alkali treatment produced the greatest conversion of polysaccharides into simple sugars, according to the pretreatment data. As a result, the samples that underwent alkali pretreatment were chosen as the substrate for the polyhydroxyalkanoates (PHAs) that were subsequently produced. The pretreatment technique used has a significant impact on how well lignocellulosic biomass is converted into fermentable sugars. The efficiency of three pretreatment methods—acidic, alkali, and enzymatic—in turning agricultural residues into simple sugars was assessed in this study. As evidenced by the higher absorbance values at 540 nm and corresponding sugar concentrations, the results showed that alkali pretreatment produced the greatest sugar release. It is well known that alkaline pretreatment breaks up lignin structures, solubilizes hemicellulose, and increases the porosity of lignocellulosic biomass, making cellulose more accessible for hydrolysis. [21] Among the tested substrates, wood bark and coconut fibres subjected to alkali pretreatment showed the highest sugar concentrations, indicating that alkaline treatment effectively breaks down the complex lignocellulosic structure. Previous studies have reported that alkaline pretreatment removes lignin and breaks ester linkages between lignin and hemicellulose, which significantly improves the enzymatic digestibility of biomass.[22] On the other hand, for a number of substrates, including corn cob and rice straw, acid pretreatment produced relatively lower sugar concentrations. While acid treatment works well to hydrolyze hemicellulose, it is typically less effective at removing lignin and, under extreme treatment conditions, may also result in the formation of inhibitory by-products like furfural and hydroxymethylfurfural [23]. Similarly, enzymatic pretreatment resulted in moderate sugar release, which may be attributed to the limited accessibility of cellulolytic enzymes to cellulose in the absence of effective delignification. Overall, the findings show that among the tested strategies, alkali pretreatment is the most successful way to improve the conversion of polysaccharides into fermentable sugars. Alkali-treated substrates yield more sugar, which makes them appropriate for further microbial fermentation procedures. In order to produce polyhydroxyalkanoates (PHAs) in the next phase of this study, alkali-pretreated samples were chosen as substrates.

### 3.4 Effect of Different Carbon Substrates for PHA Production by *Priestia megaterium*

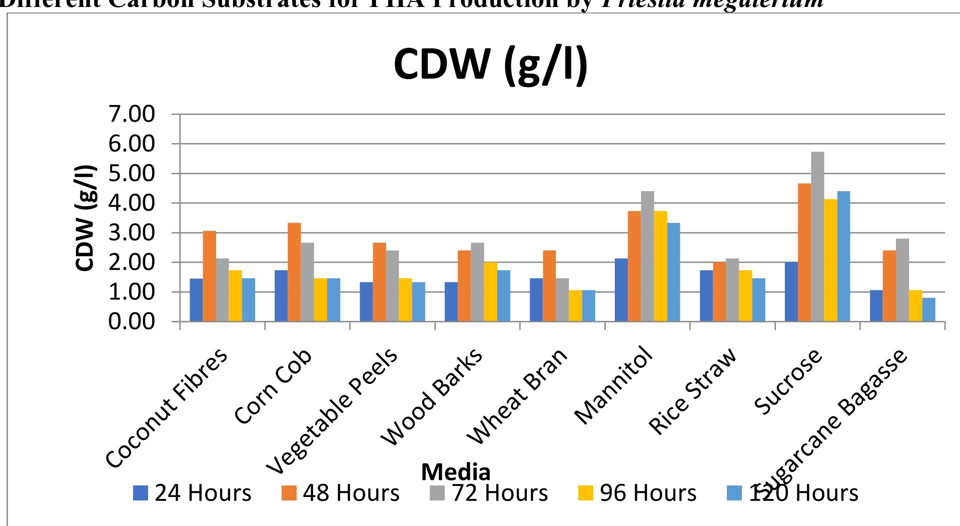



**Figure 5. Effect of Different Carbon Sources on Cell Dry Weight of PS2 Strain**

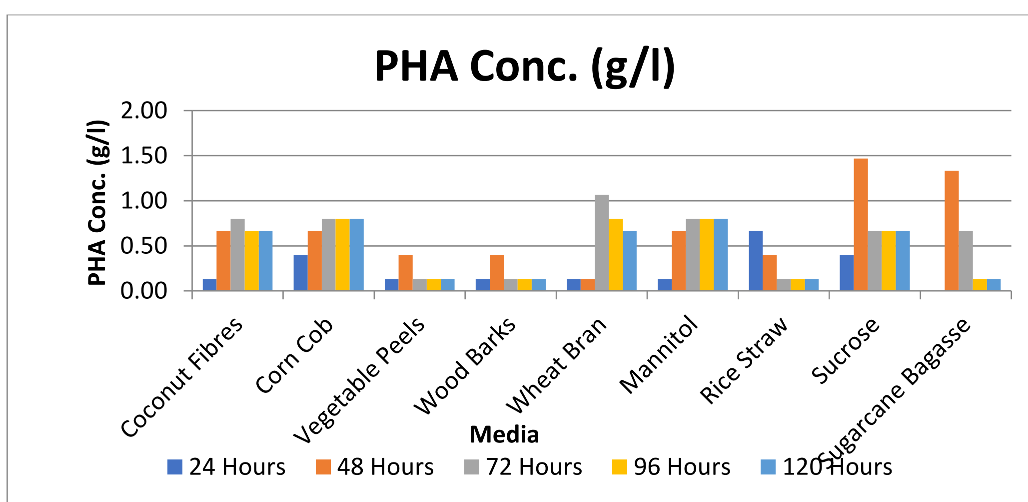



**Figure 6. Effect of Different Carbon Sources on PHA Concentration of PS2 Strain**

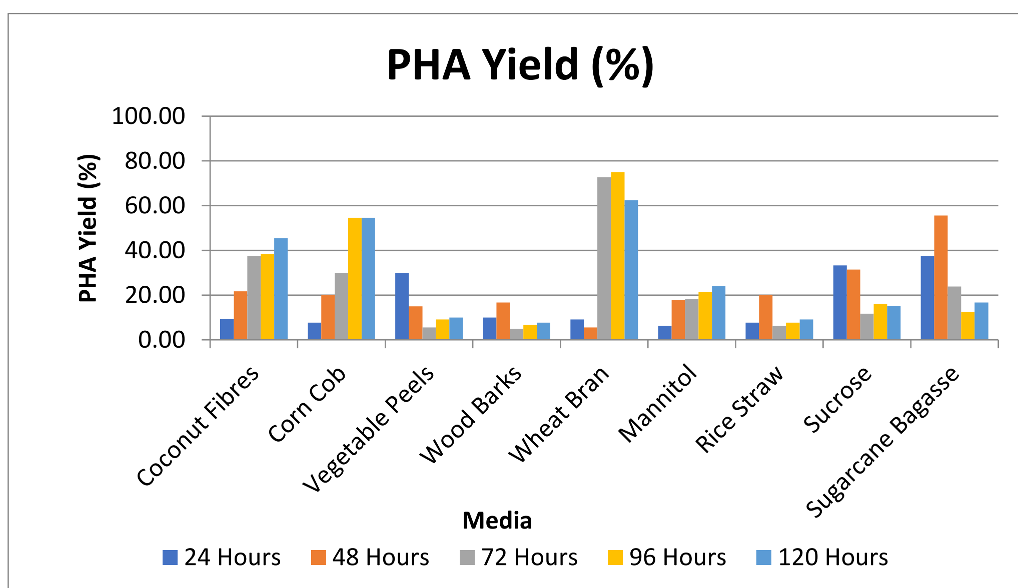



**Figure 7. Effect of Different Carbon Sources on PHA Yield of PS2 Strain**

The present study demonstrated that both substrate type and incubation period significantly influenced PHA biosynthesis and accumulation. Among all tested substrates, Wheat Bran exhibited the highest PHA yield (75.00%) at 96 h, indicating its superior efficiency as a substrate for biopolymer production. The enhanced PHA accumulation observed with Wheat Bran may be attributed to its rich nutritional composition, including fermentable carbohydrates, proteins, vitamins, and

minerals, which support both bacterial growth and intracellular polymer synthesis. Similar findings were reported by Koller et al. (2010), who observed enhanced PHA production using agro-industrial residues rich in lignocellulosic and nutrient components. The high yield obtained in the present study suggests that Wheat Bran provided favorable nutrient-limiting and carbon-excess conditions required for efficient PHA accumulation.[15]

Sugarcane Bagasse also exhibited considerable PHA production, reaching a maximum yield of 55.56% at 48 h. Compared with Wheat Bran, Sugarcane Bagasse supported faster polymer accumulation within a shorter incubation period, indicating rapid utilization of fermentable sugars released after lignocellulosic degradation. Similar observations were reported by Khardenavis et al. (2007), who demonstrated efficient PHA biosynthesis using sugarcane bagasse hydrolysates as low-cost carbon substrates. The present findings are in agreement with these reports and further confirm the industrial relevance of Sugarcane Bagasse because of its abundance, low cost, and easy availability as an agro-industrial by-product.[37]

Corn Cob and Coconut Fibres also supported moderate to high PHA accumulation during extended incubation periods. The gradual increase in PHA yield observed in these substrates may be associated with the slow hydrolysis of complex lignocellulosic polymers, resulting in sustained carbon release during fermentation. In contrast, Sucrose promoted higher biomass formation but comparatively lower PHA accumulation during later incubation stages, suggesting that readily metabolizable sugars favored cellular growth rather than intracellular polymer storage.

The decline in PHA accumulation observed in some substrates after prolonged incubation may be associated with nutrient depletion and intracellular degradation of stored PHA, which is commonly reported under stress conditions when bacterial cells utilize accumulated polymer as an alternative energy source.

The present findings indicate that lignocellulosic agro-industrial wastes are effective and economical substrates for sustainable PHA production. Although Wheat Bran exhibited the highest PHA yield, Sugarcane Bagasse showed comparatively high PHA production at an earlier incubation period, indicating its rapid substrate utilization and efficient polymer accumulation. Due to its low cost, wide availability, and abundant generation as an agro-industrial residue, Sugarcane Bagasse appears to be a promising substrate for large-scale and economically feasible bioplastic production.

### 3.5 Effect of Different pH & Temperature for PHA Production by *Priestia megaterium*

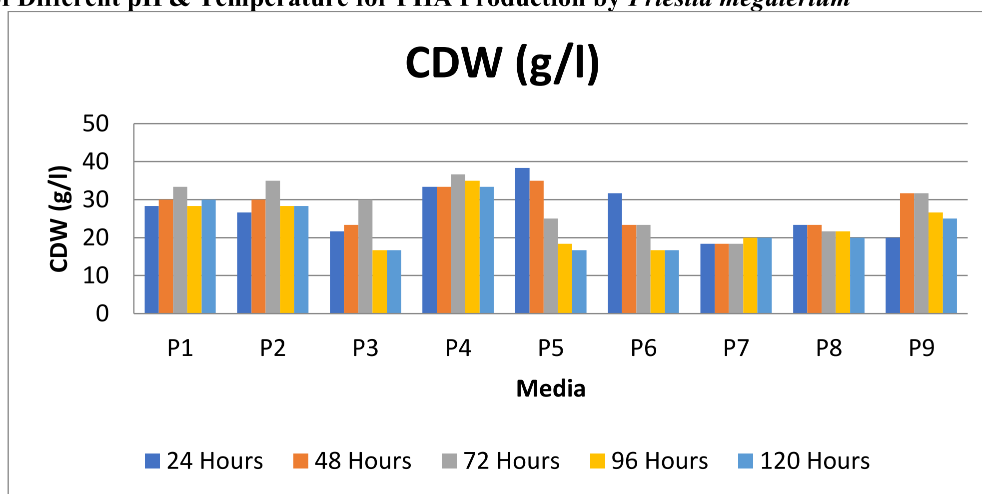



**Figure 8. Effect of Different pH on Cell Dry Weight of PS2 Strain**

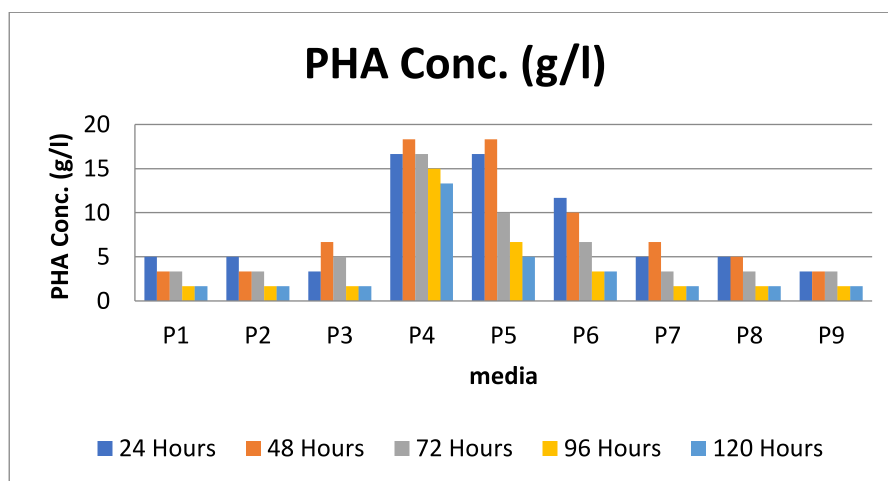



**Figure 9. Effect of Different pH on PHA Concentration of PS2 Strain**

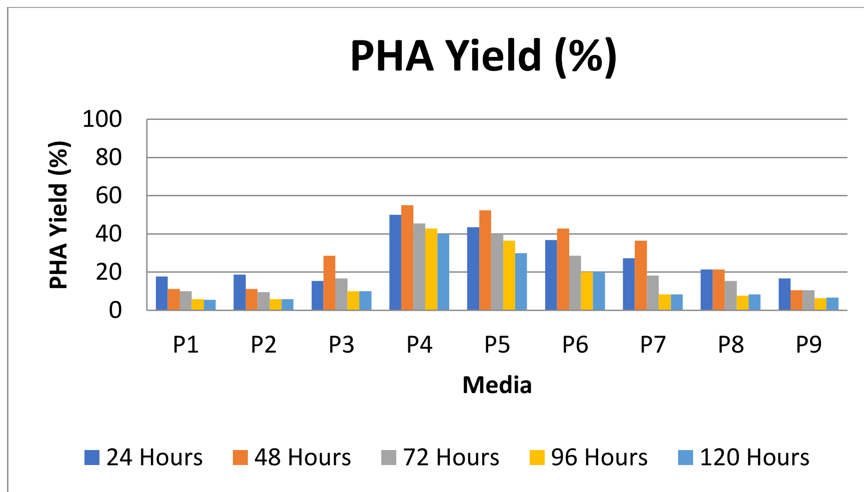



**Figure 10. Effect of Different pH on PHA Yield of PS2 Strain**

The effect of initial pH and incubation time on biomass production and polyhydroxyalkanoate (PHA) accumulation is presented in Table X. Both parameters significantly influenced bacterial growth and polymer synthesis. At 24 h, PHA concentrations ranged from 0.44 to 2.22 g/L, with the highest PHA yield (50.0%) observed at pH 6.5. PHA production increased further after 48 h of cultivation, reaching a maximum concentration of 2.44 g/L and a yield of 55.0% at pH 6.5, while a similar concentration was obtained at pH 7.0 with a yield of 52.38%. Biomass production was also highest within the pH range of 6.5–7.0, indicating that slightly acidic to neutral conditions favored both cell growth and intracellular polymer accumulation.[15]

Beyond 48 h, a gradual decline in PHA concentration and yield was observed across all pH levels. At pH 6.5, PHA concentration decreased from 2.44 g/L at 48 h to 2.22, 2.00, and 1.78 g/L after 72, 96, and 120 h, respectively. Similarly, PHA yield declined from 55.0% to 45.45%, 42.86%, and 40.0% over the same period. This reduction suggests that the accumulated polymer was utilized as an intracellular carbon and energy reserve during the stationary phase when nutrients became limiting. In contrast, cultures maintained at highly acidic (pH 5.0–5.5) or alkaline (pH 8.5–9.0) conditions showed lower biomass and PHA production throughout the study, demonstrating the sensitivity of PHA biosynthesis to environmental pH.[38]

The results obtained in this study are consistent with previous reports indicating that near-neutral pH conditions are optimal for PHA production. Studies on *Cupriavidus necator* and other PHA-producing bacteria have shown maximum polymer accumulation at pH values between 6.5 and 7.0, with PHA contents ranging from 50–60% of cell dry weight. The highest PHA yield achieved in the present study (55.0% at pH 6.5 after 48 h) falls within this reported range and compares favorably with values reported by Rondʼošová et al. (54.7%) and other researchers. Furthermore, the decline in PHA content after prolonged cultivation agrees with literature findings that stored PHA is metabolized by bacterial cells once external carbon sources become depleted. Therefore, pH 6.5 and 48 h incubation can be considered the optimum conditions for maximizing PHA production under the tested experimental conditions.[15,41]

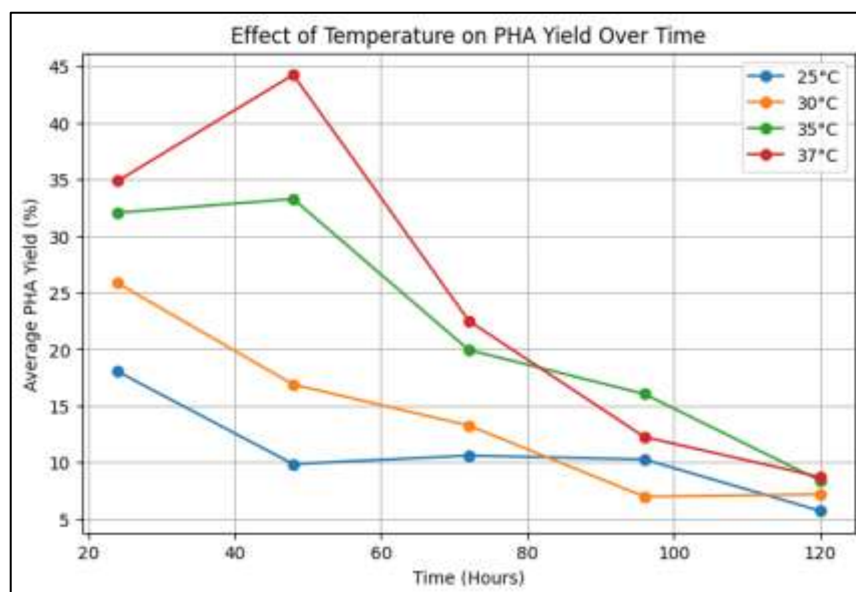



**Figure 11. Evaluation of PHA Yield of PS2 Strain at Different Temperatures**

The effect of temperature on PHA production clearly demonstrated that temperature plays a crucial role in regulating both bacterial growth and intracellular PHA accumulation over time. Among the tested temperatures, 37°C showed the highest PHA yield during the early incubation period, reaching approximately 44% at 48 hours. This indicates that the bacterial strain exhibited maximum metabolic activity and efficient carbon conversion into PHA under moderately higher temperature conditions during the exponential growth phase.

At 35°C, a comparatively high PHA yield was also observed, with values around 32–33% at 24–48 hours, suggesting that this temperature range was also favorable for polymer biosynthesis. In contrast, lower temperatures such as 25°C and 30°C resulted in significantly lower PHA accumulation throughout the incubation period. Reduced metabolic rate and slower enzymatic activity at lower temperatures may have limited substrate utilization and polymer synthesis.

A gradual decline in PHA yield was observed at all temperatures after 48 hours. The decrease became more prominent after 72 hours and continued until 120 hours. This reduction may be attributed to nutrient depletion, exhaustion of carbon sources, and possible intracellular degradation of accumulated PHA by the bacterial cells as an alternative energy source during the stationary phase. The sharp decline at 37°C after 48 hours suggests that although higher temperature initially enhanced PHA synthesis, prolonged incubation under these conditions may have accelerated metabolic stress and polymer consumption.

By the end of the incubation period (120 hours), PHA yields at all temperatures converged to relatively low values (approximately 6–9%), indicating reduced polymer stability and accumulation over extended cultivation time. Overall, the results suggest that 37°C and 48 hours represent the optimum conditions for maximum PHA production in the studied bacterial system, while prolonged incubation negatively affected PHA accumulation irrespective of temperature.

**3.6 16S rRNA Gene Sequence Analysis and Bacterial Isolate Phylogenetic Identification**

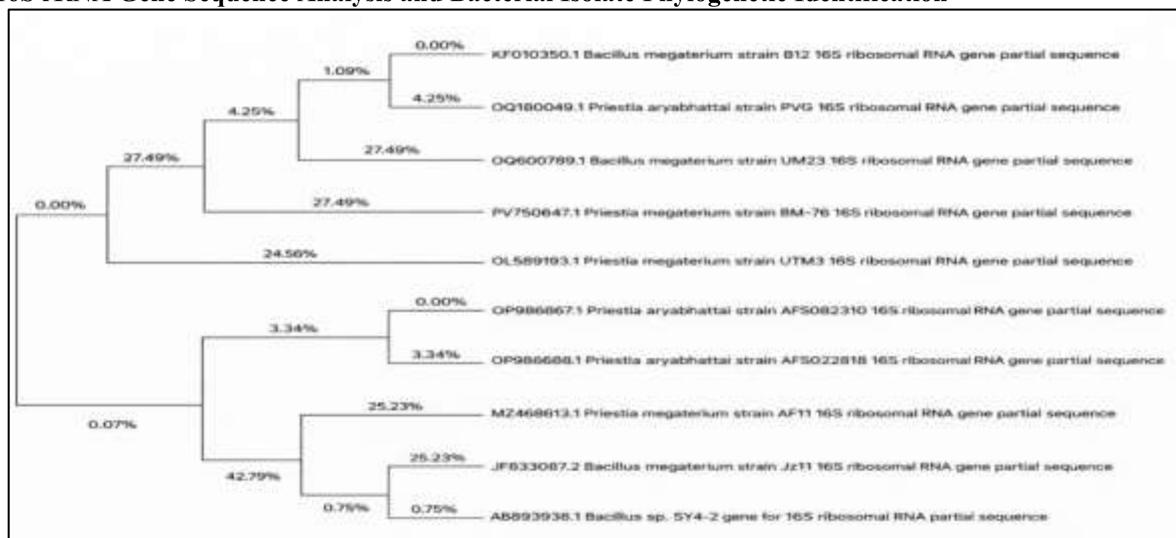



**Figure 12. Phylogenetic relationship based on 16sRNA of the isolate PS2**

One of the most popular molecular markers for identifying bacteria is the 16S rRNA gene, which has both conserved and variable regions that enable accurate taxonomic differentiation. In this study, amplification of an approximately 1500 bp fragment of the 16S rRNA gene confirmed successful targeting of the nearly full-length region commonly used for phylogenetic and taxonomic analyses. BLASTn sequence similarity analysis showed a very high identity (≈99–100%) with Priestia megaterium, particularly strain BM-76. A sequence similarity of ≥99% is generally considered sufficient for species-level identification when compared with type strain sequences. [27]

Phylogenetic analysis further supported the BLAST results by clustering the isolate with reference strains of P. megaterium, clearly separating it from other related taxa within the family *Bacillacea*e. Combining sequence similarity analysis with phylogenetic reconstruction is a well-established approach for confirming bacterial taxonomy because phylogenetic relationships provide evolutionary context beyond simple pairwise comparisons.

Historically*, P. megaterium* was classified as Bacillus megaterium. However, phylogenomic analyses of the genus *Bacillus* revealed significant taxonomic heterogeneity, leading to the reclassification of several species into new genera, including *Priestia.* [29] The updated taxonomy is also recognized in the List of Prokaryotic names with Standing in Nomenclature (LPSN) database. [28]

Members of P. megaterium are Gram-positive, aerobic, endospore-forming bacteria commonly found in soil and environmental habitats. The species is known for its metabolic versatility and ecological adaptability. Genomic studies have also reported its potential involvement in environmental processes such as sulfur metabolism and biodegradation.[27]

**Figure 13.** ***Priestia megaterium*****-derived PHA biofilm.**

Although 16S rRNA gene sequencing provides strong evidence for bacterial identification, closely related species may sometimes share highly similar sequences. Therefore, additional approaches such as multilocus sequence analysis (MLSA) or whole-genome sequencing may be required for higher taxonomic resolution. Nevertheless, the high sequence similarity and consistent phylogenetic clustering observed in this study strongly support the identification of the isolate as *Priestia megaterium*. Overall, the combination of PCR amplification, Sanger sequencing, BLAST analysis, and phylogenetic reconstruction provides a reliable framework for molecular identification and confirms the taxonomic placement of the isolate *within P. megaterium.*

### 3.2.5 Characterization of Polyhydroxyalkanoate (PHA) Using Fourier Transform Infrared Spectroscopy (FTIR)

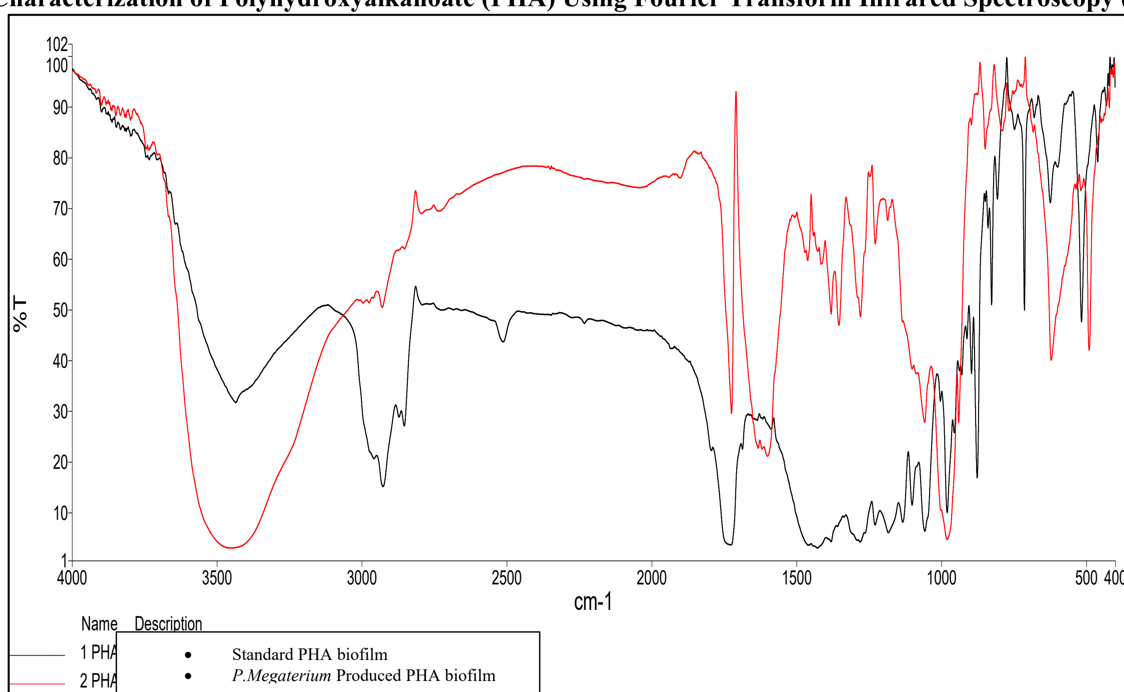



**Figure 14. FTIR Spectra of Standard and Sample Polyhydroxyalkanoates (PHA)**

Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize the extracted polymer and to compare the spectral features of the sample PHA with those of standard PHA. The FTIR spectra of both the standard and sample PHA showed similar characteristic absorption bands, indicating that the extracted polymer possesses structural features typical of polyhydroxyalkanoates. The similarity in the spectral pattern between the two samples confirms that the polymer produced by the bacterial isolate corresponds to PHA.

A strong absorption peak observed at 1724.48 $cm^{-1}$ corresponds to the ester carbonyl (C=O) stretching vibration, which is considered the most diagnostic band for PHA polymers. This peak generally appears in the range of 1720–1730 $cm^{-1}$ in standard PHA and polyhydroxybutyrate (PHB), representing the ester linkage that forms the backbone of the polymer. The presence of this prominent band in both the standard and sample spectra confirms the polyester nature of the extracted polymer and indicates successful biosynthesis of PHA [30]

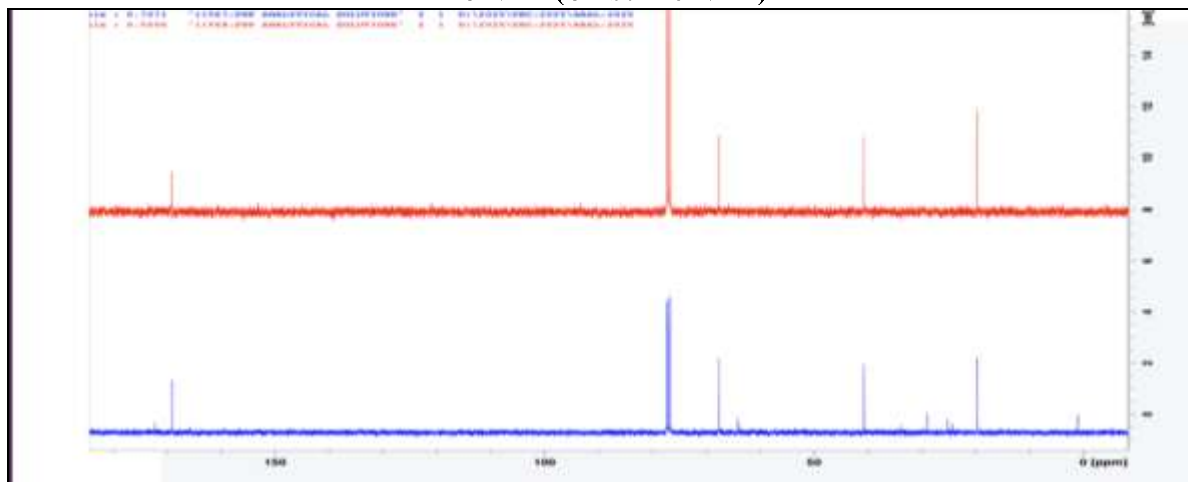
The sample spectrum also exhibited peaks at 2931.23 $cm^{-1}$ and 2794.52 $cm^{-1}$, which correspond to aliphatic C–H stretching vibrations of methyl and methylene groups. These peaks are characteristic of the long hydrocarbon chains present in PHA polymers and are also observed in the standard PHA spectrum. Their presence in both spectra suggests that the extracted polymer possesses a similar aliphatic structure to the reference polymer.[31,32]

Further peaks observed at 1461.50 $cm^{-1}$, 1415.52 $cm^{-1}$, and 1380.53 $cm^{-1}$ are attributed to bending vibrations of methyl and methylene groups, which are commonly reported in PHA polymers. In addition, peaks detected at 1279.36 $cm^{-1}$,

1228.17 cm⁻¹, and 1185.17 cm⁻¹ correspond to C–O–C stretching vibrations of ester groups, while the band at 1057.84 cm⁻¹ represents C–O stretching in the polyester backbone. These absorption bands further confirm the presence of ester functional groups characteristic of PHA [31,32] A broad band observed around 3455.99 cm⁻¹ may be attributed to O–H stretching vibrations, which could arise from residual moisture or minor impurities remaining after the extraction process. Such bands are often reported in biologically synthesized PHA samples and may be related to hydrogen bonding interactions or incomplete purification .[30]Overall, the FTIR spectral profile of the sample PHA closely resembles that of the standard PHA, particularly in the presence of the strong ester carbonyl peak (~1724 cm⁻¹), aliphatic C–H stretching bands (~2930 cm⁻¹), and characteristic ester-related peaks in the fingerprint region. The similarity of these absorption bands confirms that the polymer extracted from the bacterial isolate is structurally consistent with polyhydroxyalkanoate.

### 3.2.6 Characterization of Polyhydroxyalkanoate (PHA) Using $^{1}H$ NMR and $^{13}C$ NMR Spectroscopy

**$^{13}C$ NMR (Carbon-13 NMR)**



[1] **Figure 15 . $^{3}C$ NMR Spectrum of Polyhydroxyalkanoate (PHA) Samples**

**$^{1}H$ NMR** (Proton NMR)

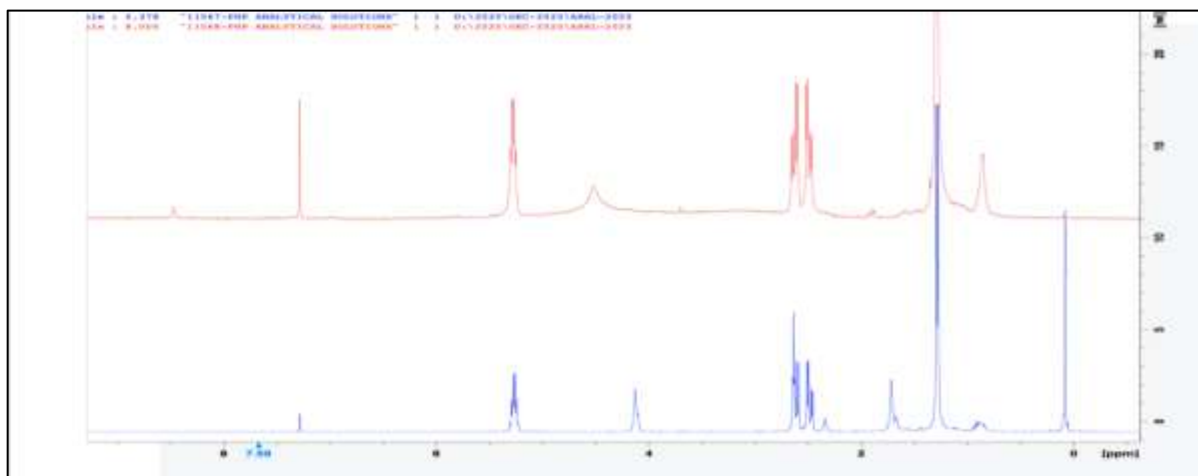


**Figure 16 . $^{1}H$ NMR Spectrum of Polyhydroxyalkanoate (PHA) Sample**

The structural confirmation of the produced polymer was further evaluated using $^{13}C$ Nuclear Magnetic Resonance ($^{13}C$ NMR) spectroscopy. The obtained spectrum was compared with a standard polyhydroxyalkanoate (PHA) spectrum to verify the chemical structure of the synthesized polymer. In the spectrum, several characteristic carbon signals corresponding to the PHA backbone were observed. The most prominent peak appeared around ~169–172 ppm, which corresponds to the carbonyl carbon (C=O) of the ester group, a key structural feature of polyhydroxyalkanoates. The presence of this strong signal confirms the polyester nature of the polymer. Additional signals were detected in the region of ~63–68 ppm, attributed to the methine carbon (–CH–) adjacent to the ester oxygen, while peaks between ~40–45 ppm correspond to methylene carbons ($–CH_2–$) in the polymer backbone. Furthermore, signals appearing near ~19–22 ppm are associated with methyl carbons ($–CH_3$) of the side chain. These peaks collectively represent the typical carbon environments present in poly(3-hydroxybutyrate) (PHB), the most common form of PHA. When comparing the sample spectrum with the standard PHA spectrum, the peak positions and relative intensities were found to be highly consistent, indicating that the polymer produced in this study possesses a chemical structure similar to that of standard PHA. Minor variations in peak intensity may be attributed to differences in polymer purity, molecular weight distribution, or residual cellular components present in the extracted polymer. Overall, the $^{13}C$ NMR analysis confirms the successful biosynthesis of polyhydroxyalkanoate by the bacterial isolate, as evidenced by the presence of characteristic ester carbonyl, methine, methylene, and methyl carbon signals typical of PHB polymers. These findings are consistent with previously reported NMR profiles of microbial PHAs and further validate the polymer identity. [17,30] Successful polymer isolation and

structural conformity are demonstrated by the close agreement in chemical shift patterns that are typical of polyhydroxyalkanoates found in the comparative ^1H NMR analysis of the isolated PHA sample and the standard PHA. The backbone methine protons (–CH–) next to ester linkages in the polymer chain are indicated by chemical shifts in the region δ 5.30–5.24 ppm in both the sample and standard spectra. The methine protons of short-chain-length PHAs, like poly(3-hydroxybutyrate) (PHB), fall in the 5.2–5.3 ppm range in $CDCl_3$, which is a diagnostic feature for PHA identification. This observation is in line with previously reported PHA structural assignments. Resonances observed around δ 4.52 ppm (sample) and δ 4.13–4.10 ppm (standard) are attributable to methylene protons adjacent to the ester oxygen ($–CH_2–O–$), a key structural element influencing polymer flexibility and conformation. Similarly, the β-methylene resonances (δ 2.66–2.48 ppm) and methyl protons (δ 1.35–1.25 ppm) reflect expected chemical environments of PHA backbone chains and terminal substituents. These chemical shifts align with typical ^1H NMR assignments for PHB and PHA co-polymers, reaffirming the structural similarity between sample and standard materials observed in recent polymer studies. Notably, the standard PHA spectrum includes additional peaks in the aliphatic region (e.g., δ 1.71–2.34 ppm), which may indicate the presence of minor comonomer units such as 3-hydroxyvalerate (3HV) or other co-monomer sequences, consistent with heteropolymer spectra reported in literature. Detailed spectral deconvolution and quantitative integration can provide monomer distribution and comonomer content, which are critical for correlating microstructural variations with material properties in biopolymer studies. Taken together, the strong similarity in chemical shift trends between the sample and standard PHA spectra reinforces the conclusion that the isolated polymer is structurally consistent with short-chain PHA, while subtle differences in chemical shift and peak distribution may reflect compositional heterogeneity or differing monomer ratios. Future work integrating quantitative ^1H NMR with complementary techniques such as ^13C NMR and size-exclusion chromatography could further elucidate monomer composition, polymer tacticity, and molecular weight distribution, providing deeper insights into polymer quality and performance. [33,34]

## 4. CONCLUSION

The present study demonstrated the successful production of polyhydroxyalkanoate (PHA) by the bacterial isolate PS2, which was identified as *Priestia megaterium* through 16S rRNA gene sequence analysis. Optimization studies revealed that cultivation conditions significantly influenced PHA biosynthesis, with maximum polymer accumulation achieved at 96 h of incubation. Among the tested nutritional parameters, ammonium chloride proved to be the most suitable nitrogen source, while wheat bran supported the highest PHA yield, highlighting the potential of low-cost agro-industrial residues as sustainable substrates for biopolymer production. Furthermore, alkali pretreatment was found to be the most effective method for enhancing fermentable sugar release from lignocellulosic biomass, thereby improving substrate availability for microbial fermentation. Characterization of the extracted polymer using FTIR, $^{1}H$ NMR, and $^{13}C$ NMR analyses confirmed the presence of characteristic structural features of PHA, validating the successful biosynthesis of the biopolymer by *P. megaterium*. The ability of the isolate to efficiently utilize inexpensive agricultural wastes and accumulate substantial amounts of PHA demonstrates its potential as a promising candidate for environmentally sustainable bioplastic production. These findings contribute to the development of cost-effective and eco-friendly strategies for PHA production and support the utilization of renewable biomass resources as alternatives to conventional petroleum-based plastics.

**Acknowledgment**The present study was supported by Knowledge consortium of Gujarat (KCG).

**Declarations-**

**Conflict of interest** The authors declare no competing interests.

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