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Production and Characterization of Polyhydroxyalkanoates from Food Waste Using Indigenous Bacterial Strains

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Abstract

The accumulation of petrochemical plastics necessitates sustainable alternatives. This study investigated the production of polyhydroxyalkanoates (PHAs) using indigenous bacterial strains isolated from Balochistan's soils and five locally sourced food wastes: rice bran, potato peels, fruit pulp, dairy waste, and corn starch. The isolates were identified as *Bacillus megaterium* and *Cupriavidusnecator* via 16S rRNA sequencing. Fermentation parameters were systematically optimized for each strain. *C. necator* achieved a maximum PHA yield of 4.5 ± 0.3 g/L, constituting 85% of the cell dry weight, using Rice Bran at 30°C, pH 7.0, and 1% NaCl. Hydrothermal pretreatment increased yields by up to 36%, and nitrogen limitation enhanced polymer accumulation by 40-50%. Characterization by FTIR, NMR, and GC confirmed the synthesis of poly(3-hydroxybutyrate) (PHB) by *B. megaterium* and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with up to 33% hydroxyvalerate by *C. necator*. The PHBV copolymer exhibited a tensile strength of 32-40 MPa and a degradation temperature of 248-268°C, properties comparable to polypropylene. These results demonstrate that indigenous Balochistan strains efficiently convert regional food wastes into bioplastics with competitive material properties, establishing viable substrate-specific and strain-dependent bioprocessing strategy.

Keywords: Polyhydroxyalkanoates (PHA), Food waste valorization, Fermentation optimization, Thermal characterization, Balochistan.

Introduction

The world is facing an unprecedented environmental crisis due to the accumulation of non-biodegradable plastic waste. Petrochemicals derived conventional plastics for degradation take hundreds of years to degrade and also pose a significant threat to marine and terrestrial ecosystems [1]. Recently, an ecofriendly solution to this problem has emerged i.e., biodegradable bioplastics. Polyhydroxyalkanoates (PHAs) due to its complete biodegradability in various environmental conditions, biocompatibility and versatility amongst the various types of bioplastics have gained significant consideration [2]. Wide range of microorganisms produce PHAs as an

intracellular energy reserve which function as naturally occurring polyesters. PHAs are synthesized when bacteria are exposed to nutrient-limiting conditions particularly a deficiency of phosphorus or nitrogen, whereas carbon is available in excess. They are similar to conventional petrochemical plastics such as polypropylene and polyethylene, making them suitable for awide range of applications, including medical devices, agriculture and packaging [3].

Balochistan is the largest province of Pakistan, which has unique environmental condition and rich in diverse natural resources that supports the growth of numerous microbes. Despite this potential, in Balochistan limited research has been focused on microbial biotechnology particularly related to the production of biodegradable plastics such as PHAs [4]. Under the nutrient-limited and arid conditions, the soils of Balochistan have potential reservoir for bacterial strains to produce PHAs [5].

*Cupriavidusnecator*and *Bacillus megaterium*are two well-known bacterial species that produce PHA. Previously, these species have been isolated from diverse environment, unfortunately limited research has been conducted using food waste of Balochistan as a carbon source to identify the potency of PHA production [6]. Utilizing food waste not only providesas a low-cost carbon source but it also offers eco-friendly solution for management of organic wastes which are often improperly handled or discarded in the region [7]. The major bottleneck in the commercial viability of PHAs is the high cost compared to their non-biodegradable synthetic counterparts. The carbon source used for fermentation is the main factor contributing to the high production cost. Food waste, whichis inexpensive and readily available can be used as alternate to carbon source for PHAs production. Food wastes not only reduce the cost of PHA production, thereby reducing costs and addressing waste management challenges[8]. In this research, we evaluated the potential of five types of food wastes—corn starch, dairy waste, rice bran, fruit pulp and potato peels—as carbon sources for PHA production by *Cupriavidusnecator*and *Bacillus megaterium.* We optimized the growth conditions for maximum PHA production and characterized the extracted PHAs using various analytical techniques [9].
Balochistan, the largest province of Pakistan, is characterized by its arid climate and diverse, yet underutilized, natural resources. The region generates substantial amounts of agricultural and food processing waste, such as rice bran, potato peels, and fruit pulp, which are often discarded without valorization. Concurrently, the unique environmental conditions of Balochistan's soils suggest a potential reservoir of robust, indigenous bacterial strains adapted to stress, which could be highly efficient in converting local waste into value-added products like PHA [10, 11]. Despite this potential, no systematic study has investigated the synergy between Balochistan-specific food wastes and indigenous microbial strains for PHA production.

Therefore, the novelty of this work is threefold. First, it is the first comprehensive investigation to utilize five prevalent and underutilized food wastes from Balochistan (rice bran, potato peels, fruit pulp, dairy waste, and corn starch) as carbon sources for PHA production. Second, it employs indigenous strains of *Bacillus megaterium* and *Cupriavidusnecator*, isolated from local soils, hypothesizing their superior adaptation to regional substrates and conditions. Third, it provides a systematic optimization of critical fermentation parameters (pH, temperature, salinity, aeration, and nutrient limitation) and a comprehensive characterization of the resulting polymers, confirming the production

of PHB and PHBV copolymers with properties competitive with conventional plastics. This study not only aims to optimize PHA yields but also to establish a sustainable model for waste-to-wealth conversion tailored to the specific context of Balochistan, addressing both environmental pollution and economic constraints.

Materials and methods

Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade and used without further purification. Methanol (≥99.8%), chloroform (≥99.8%), sulfuric acid (H_2SO_4, 98%), sodium hypochlorite (NaOCl, 10-12% available chlorine), sodium thiosulfate ($Na_2S_2O_3$), potassium bromide (KBr, spectroscopic grade), and deuterated chloroform ($CDCl_3$, 99.8% D) were purchased from Sigma-Aldrich (USA). Standard methyl esters of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) used for calibration in gas chromatography analysis were obtained from Merck (Germany).

Nutrient broth, minimal salt medium (MSM) components, yeast extract, and peptone were procured from Oxoid (UK). All inorganic salts, including ammonium sulfate ($(NH_4)_2SO_4$), ammonium chloride (NH_4Cl), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), sodium chloride (NaCl), calcium chloride ($CaCl_2$), and potassium phosphate salts (KH_2PO_4, K_2HPO_4), were purchased from Merck (Germany).

Sample Collection and Isolation of Microbial Strains

Different soil samples were collected from various waste and agricultural sites in Balochistan, Pakistan. The samples were collected in sterile containers and transported to the laboratory under refrigeration at 4°C. Serial dilution and spread plate techniques were employed to isolate bacteria from the soil samples. The dilutions ranged from 10^{-1} to 10^{-6}, and the plates were incubated at 37°C for 24–48 hours. Bacterial colonies exhibiting distinct morphologies were selected, purified through repeated streaking, and maintained on nutrient agar slants for further study [12].

Identification of Isolates: 16S rRNA Sequencing

Genomic DNA extraction

Genomic DNA was extracted using the phenol-chloroform methodfrom pure bacterial cultures grown in nutrient broth, as described by Maniatis and coworkers[13]. Further, bacterial cells were collected by centrifugation at 10,000 × g for 5 minutes and lysed using a lysis buffer containing Tris-HCl (pH 8.0), proteinase K, SDS and, EDTA. Subsequently, the lysate was subjected to sequential phenol-chloroform-isoamyl alcohol (25:24:1) extraction to remove proteins and other contaminants.. DNA was precipitated using isopropanol, washed with 70% ethanol, and dissolved in TE buffer. The quantity and quality of DNA were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

PCR amplification of 16S rRNA gene

The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGA-CTT-3') were used to amplify the 16S rRNA gene [14]. PCR was performed in a 25 µL reaction mixture containing 1 µL of template DNA, 0.5 µM of each primer, nuclease-free water, and 12.5 µL of Taq PCR Master Mix (Thermo Scientific, USA). The thermal cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds,

and extension at 72°C for 1 minute, with the final extension at 72°C for 10 minutes. Electrophoresis was performed on a 1% agarose gel stained with ethidium bromide for confirmation of PCR products.

Purification and Sequencing of PCR Products

The amplified PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's instructions. Purified products were sequenced bidirectionally using an automated DNA sequencer (Applied Biosystems, USA).

Sequence Analysis

The obtained sequences were assembled and analyzed using BioEdit software. The sequences were compared with those in the NCBI GenBank database using the BLAST tool [15] to identify the bacterial strains based on sequence similarity.

Phylogenetic Analysis

Phylogenetic trees were constructed using MEGA 7.0 software[16]. The sequences were aligned using the ClustalW algorithm. Phylogenetic relationships were inferred using the neighbor-joining method with 1000 bootstrap replications to ensure statistical reliability.

Identification of Strains

Both *Bacillus megaterium* and *Cupriavidusnecator*were identified with high sequence similarity (≥99%) to known PHA-producing strains in the GenBank database.

Preparation of Food Waste Substrates

Food waste substrates, including potato peels, fruit pulp, rice bran, dairy waste, and corn starch, were collected from local markets and food industries in Balochistan, Pakistan. The wastes were thoroughly cleaned with distilled water to remove impurities, sorted to remove non-biodegradable materials, and processed for uniformity. Rice bran was ground into fine powder, potato peels and fruit pulp were blended into slurries; and dairy waste was separated into liquid and solid fractions. Hydrolysis was performed by autoclaving potato peels, fruit pulp, and rice bran at 121°C for 20 minutes, while dairy waste underwent pasteurization at 80°C to enhance nutrient bioavailability. For fermentation experiments, the prepared substrates were cooled, filtered, and stored at 4°C. These steps ensured the substrates were free from contaminants, nutrient-rich, and suitable for microbial PHA production[17].

PHA Production

The production of PHAs by *Bacillus megaterium* and *Cupriavidusnecator* was carried out using five food waste substrates: rice bran, potato peels, fruit pulp, dairy waste, and corn starch. The experimental protocol for PHA production involved the following key steps:

1.Substrate Preparation

Hydrothermal pretreatment at 121°C for 20 minutes was employed on food waste substrates to remove inhibitory compounds and improve bioavailability. The pretreated substrates were homogenized and adjusted to a concentration of 3% (w/v) for use as carbon sources in fermentation [18, 19].

2.Inoculum Preparation

Bacillus megaterium and *Cupriavidusnecator* were grown in nutrient broth overnight at 37°C and 30°C, respectively, until they reached an optical

density of 1.0 at 600 nm. The inoculum was standardized to 10% (v/v) of the final fermentation volume[13].

3.Fermentation Setup

Batch fermentations were performed in 1 L bioreactors containing 500 mL of Minimal Salt Medium (MSM) supplemented with the food waste substrates. The fermentation conditions were optimized as follows:

• *Bacillus megaterium*: 37°C, 1% NaCl, pH 7.0, 200 rpm agitation.
• *Cupriavidusnecator*: 30°C, 1% NaCl, pH 7.0, 200 rpm agitation.

4.Monitoring and Sampling

Samples were collected at 12-hour intervals over 48 hours to measure cell dry weight (CDW), residual substrate concentration, and PHA accumulation [18].

5.PHA Quantification

PHA content was quantified using gas chromatography (GC) after methanolysis of the biomass. The results were expressed as grams of PHA per liter of culture and as a percentage of cell dry weight (% CDW) [19].

PHA Extraction

Polyhydroxyalkanoates (PHA) were extracted from microbial cultures using the sodium hypochlorite method. The first step began with growing microbial strains, particularly *Cupriavidusnecator*and *Bacillus megaterium,* in a liquid medium containing food wastes as a carbon source i.e., corn starch, dairy waste, rice bran, fruit pulp and potato peels. Under optimal conditions, the cultures were incubated, with intermittent sampling for PHA quantification. When the cultures reached the late logarithmic phase, biomass was harvested by centrifugation at 10,000 × g for 10 minutesand then was washed with sterile saline for removal of residual components. The harvested biomass then underwent the extraction process. Under sterile conditions, to the microbial pellet, sodium hypochlorite solution (1.0-2.5%) was added to lyse the cells. PHA granules werereleased into the solution when sodium hypochlorite reacts with the cellular components, breaking down lipids, proteins and other cellular materials.

To achieve consistent distribution of the chemical, the reaction was allowed to continue for approximately 30minutes with intermittent gentle stirring. After that, the solution was neutralized by adding sodium thiosulfate to stop the action of sodium hypochlorite. PHA was then separated from the lysed cellular debris after the centrifugation of the neutralized suspension.

To remove any by-products of the reaction or residual sodium hypochlorite the resulting PHA pellet was thoroughly washed several times with distilled water. The biomass was dried under vacuum at 40°C until a constant weight was achieved to further purify PHA. Gravimetric methods were used for purification and quantification; based on the dry weight, the PHA content of the extracted polymer was calculated in proportion to the initial biomass[20, 21, 22].

Optimization of Factors Influencing PHA Production

This research examined the impacts of multiple variables on PHA production by *Cupriavidusnecator*and *Bacillus megaterium*, including temperature, salinity, pH, carbon sources, nitrogen sources and trace elements. Various food waste substrates such ascorn starch, dairy waste, rice bran, fruit pulp and potato peels were used to improve

recyclability, and different pretreatment methods were appliedto increase the nutrient availability. A systematic optimization of the fermentation conditions, such asaeration rate, incubation time, and substrate concentration, was performed. Lee *et al.*demonstrated analogous optimization strategies, evaluatingwhich evaluated the contribution of nutrient and environmental factors towards the enhancement of PHA productivity and quality in microbial systems[23].PHA was then extracted, purified and analyzed for its molecular weight, composition and thermal properties allowing for a better understanding of the influence of these parameters on polymer synthesis[24].

Substrate Composition Analysis

The proximate composition of the food waste substrates —including total carbohydrate, protein, fat, and phenolic contents—was determined before their use as carbon sources for PHA production. All analyses were conducted in triplicate, and results were expressed as percentages (%) on a dry weight basis.

Carbohydrate Content: Total carbohydrate content was quantified using a modified phenol–sulfuric acid method as described by Albalasmeh*et al.* [25]. Briefly, 1 mL of diluted sample was mixed with 1 mL of 5% phenol and 5 mL of concentrated sulfuric acid. The mixture was incubated for 30 min at room temperature, and absorbance was measured at 490 nm using a UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan). D-glucose was used as a standard.

Protein Content: Protein concentration was determined by the Folin–Lowry method [26]. Samples were reacted with Lowry reagent and Folin–Ciocalteu reagent, and absorbance was measured at 660 nm. Bovine serum albumin (BSA) was used for the standard curve.

Lipid Content: Total lipid content was determined by Soxhlet extraction [27]. Approximately 2–3 g of dried sample was extracted with petroleum ether (boiling range: 40–60 °C) for 6–8 h. The solvent was evaporated under reduced pressure, and the lipid content was calculated gravimetrically.

Phenolic Content: Total phenolic compounds were quantified using the Folin–Ciocalteu colorimetric assay according to the procedure described by Alara*et al.* [28]. Briefly, 0.5 mL of sample was mixed with 2.5 mL of 10% Folin–Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate. The mixture was incubated in the dark for 30 min, and absorbance was recorded at 765 nm. Gallic acid was used as a standard, and results were expressed as mg gallic acid equivalents (GAE) per gram of dry substrate.

Characterization of PHAs

A series of analytical techniques were applied to the extracted polyhydroxy-alkanoates (PHAs) to evaluate their chemical, structural, thermal and physical properties.

1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy (Thermo Scientific Nicolet iS50) was used to analyze the chemical structure of PHAs. Potassium bromide (KBr) was mixed with the polymer samples in a 1:100 ratio and compressed into pellets. FTIR spectra were recorded over a range of wave- numbers from 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. Specific functional groups, including methyl (CH_3) and ester carbonyl (C=O) groups, were identified based on their characteristic absorption peaks[29].

2. Gas Chromatography (GC-FID) Analysis

The monomer composition of polyhydroxyalkanoates (PHAs) was determined using gas chromatography equipped with a flame ionization detector (GC-FID, Agilent 7890, USA). Purified PHA (10 mg) was subjected to methanolysis by heating with 1 mL of methanol containing 3% (v/v) sulfuric acid and 1 mL of chloroform at 100 °C for 3 h in sealed glass vials. After cooling to room temperature, 1 mL of distilled water was added and the mixture was vortexed thoroughly. The organic phase was collected, dried over anhydrous sodium sulfate, and used for GC analysis.

Chromatographic separation was achieved using a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The injector and detector temperatures were maintained at 250 °C and 280 °C, respectively. Samples were injected in split mode (1:20) with an injection volume of 1 µL. The oven temperature was initially set at 60 °C for 2 min, increased at a rate of 10 °C min^{-1} to 260 °C, and held for 10 min. The total run time was approximately 25 min. The retention times of methyl esters were compared with authentic standards of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV), and results were expressed as mol% of HB and HV in the polymer [30].

3. Nuclear Magnetic Resonance (NMR) Spectroscopy

To confirm the monomeric composition and structural arrangement of PHAs, ^{1}H and ^{13}C NMR spectra were recorded using a BrukerAvance 400 MHz NMR spectrometer. Approximately 10 mg of PHA was dissolved in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as the internal standard [31].

4. Mechanical Properties

The tensile strength, elongation at break, and Young's modulus of the PHAs were measured using a universal testing machine (Instron 3365). Thin films of PHA were prepared by solvent casting, dried at 60°C, and cut into rectangular strips (50 mm × 10 mm × 0.2 mm). The tests were conducted at a crosshead speed of 10 mm/min under ambient conditions [32].

Statistical Analysis

All experiments were conducted in triplicate, and the data are presented as mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS Statistics (Version 26.0, IBM Corp., USA) and GraphPad Prism (Version 9.0, GraphPad Software, USA). Two-way and one-way analysis of variance (ANOVA) wereused to assess significant differences between experimental groups, followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at ($p < 0.05$). Graphs and plots were generated using GraphPad Prism. All results and tables include standard deviations to reflect experimental reproducibility and variability.

Results and Discussion

Biochemical Tests for *Bacillus megaterium* and *Cupriavidusnecator*

The biochemical and molecular characteristics confirming the identity of *Bacillus megaterium* and *Cupriavidusnecator* are summarized in Table 1. *Bacillus megaterium* is Gram-positive and exhibits strong catalase and Voges-Proskauer activity, while *Cupriavidusnecator* is Gram-negative and methyl red positive, reflecting its ability to metabolize glucose via mixed-acid fermentation pathways. These characteristics

support efficient PHA synthesis. 16S rRNA sequencing confirmed the identity of the isolates with high similarity percentages (98.82%–98.89%). Phylogenetic analysis placed both strains within known PHA-producing clades, validating their suitability for PHA production. Further phylogenetic analysis confirmed their taxonomic identity, showing that the *Bacillus megaterium* (PV810219) and *Cupriavidusnecator* (PV810381) isolates formed robust clades with high bootstrap support alongside their respective type strains and known PHA producers (Fig. 1 & 2). These results validate their identity and close relation to efficient PHA-accumulating species.

Table 1. **Biochemical and molecular identification of the indigenous bacterial strains used for PHA production.**

Test / Analysis	*Bacillus megaterium*	*Cupriavidusnecator*
Gram Staining	Positive	Negative
Catalase Test	Positive	Positive
Oxidase Test	Positive	Positive
Indole Production	Negative	Negative
Methyl Red	Negative	Positive
Voges-Proskauer	Positive	Negative
Citrate Utilization	Positive	Positive
Urease Test	Negative	Negative
Closest Match (BLAST)	*Bacillus megaterium*	*Cupriavidusnecator*
Similarity (%)	98.82%	98.89%
Accession Number	PV810219	PV810381

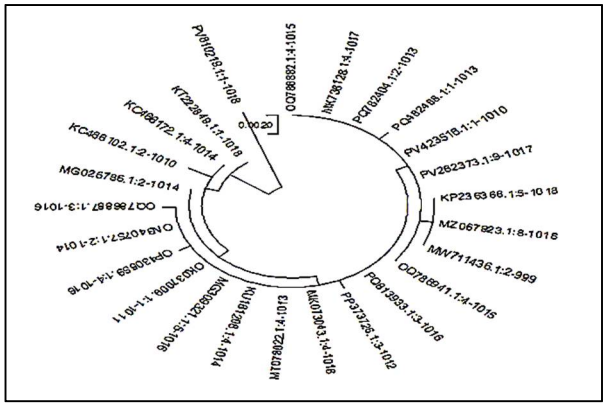


Figure 1. **Phylogenetic tree of the *Bacillus megaterium* isolate based on 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method**

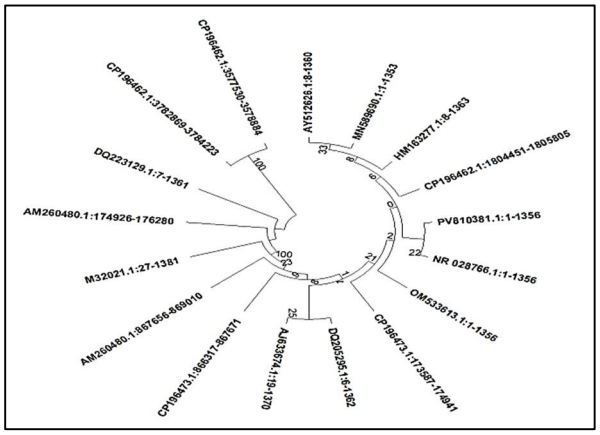


Figure 2. **Phylogenetic tree of the *Cupriavidusnecator* isolate based on 16S rRNA gene sequences. The phylogenetic tree, inferred using the neighbor-joining method.**

Chemical composition of food waste substrates and their impact on PHA production efficiency

The initial statistical evaluation of Polyhydroxyalkanoate (PHA) production in **Fig. 3** revealed a fundamental principle for waste valorization: a significant interaction exists between the bacterial strain and the substrate type (Two-way ANOVA, $p = 1.03 \times 10^{-2}$) (**Fig. 3A**). This indicates that the efficacy of a given agro-industrial waste is not absolute but is intrinsically tied to the specific metabolic network of the microorganism. The divergent response patterns of *Cupriavidusnecator* and *Bacillus megaterium* across the same set of substrates preclude a universal substrate ranking and necessitate a strain-specific analysis to deconstruct this interaction.

For *Bacillus megaterium*, one-way ANOVA with post-hoc Tukey HSD testing established a clear hierarchy of substrate preference (**Fig. 3B**). Rice Bran emerged as the optimal substrate, yielding approximately 3.5 g/L and forming a single statistical homogenous group ('a'). This was significantly superior to a second statistical group comprising Corn Starch, Potato Peels, and Fruit Pulp, which yielded between 2.8-3.0 g/L.

The performance on Dairy Waste was the poorest. This profile suggests that *B. megaterium* possesses a specialized metabolic capacity, potentially for lipid or complex polysaccharide utilization that is uniquely suited to the composition of Rice Bran.

In contrast, the production profile for *Cupriavidusnecator* revealed a different and more productive order of preference (**Fig. 3C**). Here, Potato Peels and Corn Starch were the optimal substrates, forming the top statistical group ('a') with yields of ~4.5 g/L and ~4.4 g/L, respectively. These yields were significantly higher than those achieved on Rice Bran, Fruit Pulp, and Dairy Waste. This hierarchy positions *C. necator* as a high-yielding specialist on starchy and lignocellulosic waste streams, outperforming *B. megaterium* on these substrates and highlighting its robust enzymatic machinery for hydrolyzing and converting these polymers into PHA.

The mechanistic basis for the observed significant interaction and the strain-specific substrate rankings is unequivocally explained by the chemical composition of the wastes (**Fig. 3D**).

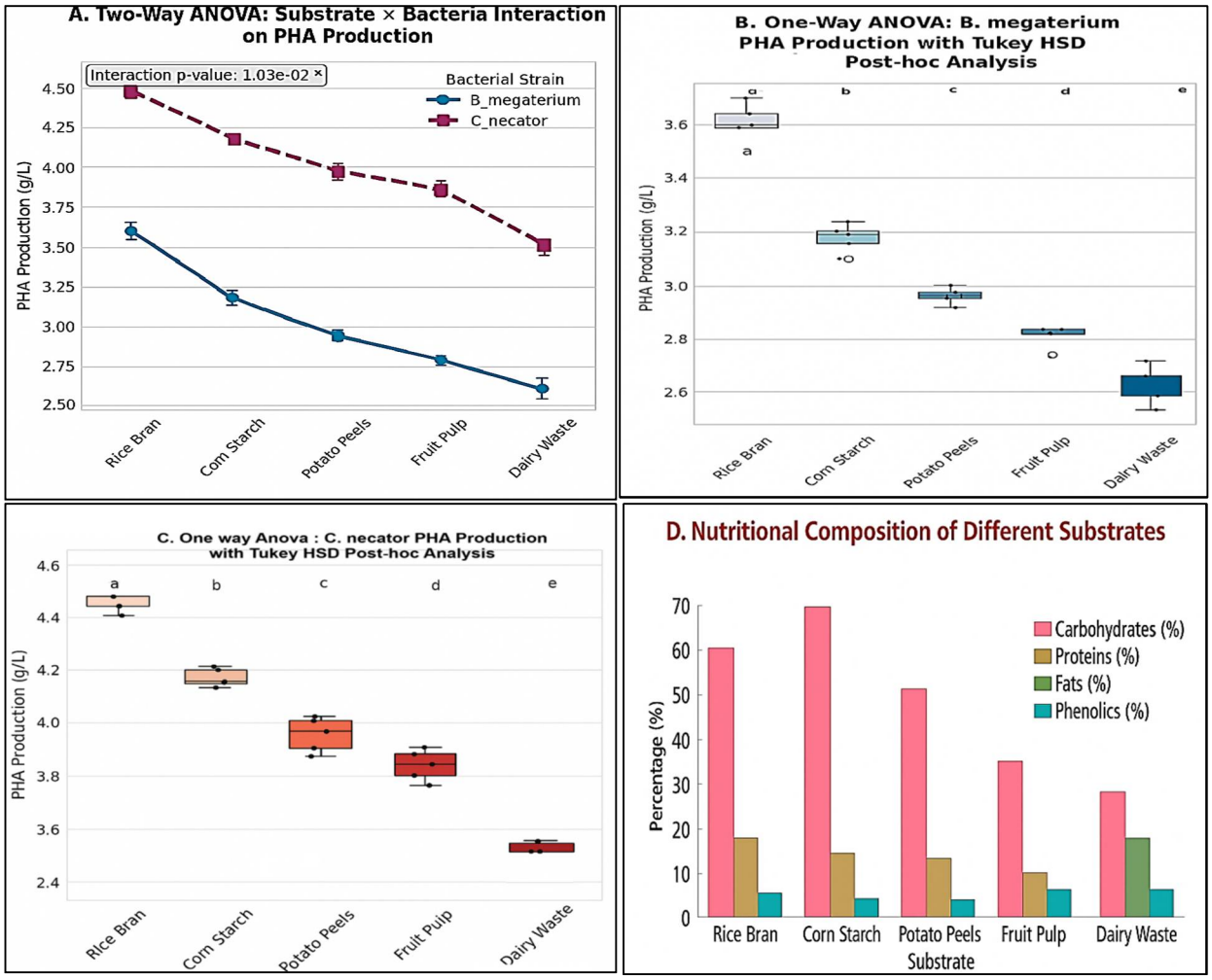


Figure 3. **Evaluation of PHA production from agro industrial wastes. (A) Two-way ANOVA showing the significant interaction between bacterial strain and substrate type (p = 1.03e-02). Strain-specific optimization for (B) *B. megaterium* and (C) *C. necator* via one-way ANOVA with Tukey's HSD post-hoc analysis (different letters indicate significant differences). (D) Nutritional composition of the investigated substrates, providing the biochemical rationale for the observed fermentation outcomes. Data are presented as mean ± SD (n=3).**

The high carbohydrate content of Potato Peels (~85%) and Corn Starch (~65%) provides the ideal carbon surplus for the efficient PHA biosynthesis pathway in *C. necator*. The superior performance of *B. megaterium* on Rice Bran can be attributed to its ability to capitalize on Rice Bran's unique mix of carbohydrates (~55%), lipids (~12%), and proteins (~18%). Conversely, the consistently low PHA production on Dairy Waste by both strains is a direct consequence of its high protein (~65%) and low carbohydrate (~15%) content, which promotes growth over storage polymer synthesis due to nitrogen excess. Thus, the substrate composition provides the biochemical rationale for the strain-substrate interaction, demonstrating that PHA production efficiency is a direct function of the compatibility between a strain's catabolic enzymes and a substrate's dominant chemical fractions.

Optimization of Fermentation Parameters for Maximized PHA Production of Cupriavidusnecator

The biotechnological potential of a microbial strain is ultimately constrained by its cultivation environment. To fully harness the capacity of *Cupriavidusnecator* for Polyhydroxyalkanoate (PHA) biosynthesis from agro-industrial wastes, a systematic, one-factor-at-a-time optimization of critical fermentation parameters was undertaken. The results in **Table 2** reveal a well-defined physiological niche for optimal polymer synthesis, with each parameter demonstrating a statistically significant impact ($p < 0.05$) on the final PHA yield, thereby providing critical insights into the strain's metabolic and regulatory networks. The effect of temperature on PHA yield was pronounced, exhibiting a classic optimum curve. The maximum yield of 4.5 g/L was achieved at 30°C, with significant decrements observed at both 25°C and 35°C. This indicates that 30°C represents the thermodynamic sweet spot for the concerted action of the enzyme systems responsible for substrate uptake, central carbon metabolism, and the specific PHA synthase complex. The reduction at 35°C suggests the onset of partial protein denaturation or metabolic stress, while the lower yield at 25°C points to suboptimal enzyme kinetics and a slowed metabolic flux, insufficient to drive high-level polymer accumulation.

The influence of pH further underscored the need for physiological precision, with a neutral pH of 7.0 providing the ideal condition for a maximum yield of 4.5 g/L. Significant reductions under more acidic (pH 6.0) or alkaline (pH 7.5, 8.0) conditions were observed, likely due to impaired nutrient solubility, disrupted membrane transport functions, and suboptimal activity of key metabolic enzymes involved in the PHA biosynthesis pathway. The investigation into salinity revealed that a moderate concentration of 1% NaCl was optimal. The absence of salt resulted in a significantly lower yield, potentially indicating a requirement for specific ions as enzymatic cofactors, while the substantial inhibition observed at 3% NaCl underscores the osmosensitive nature of *C. necator*. This elevated ionic strength likely induces hyperosmotic stress, diverting cellular energy and resources toward osmoregulation and away from anabolic processes like PHA synthesis.

Aeration rate, a critical parameter governing oxygen transfer and thus the oxidative metabolism of this aerobic bacterium, showed a clear optimum at 1.5 L/min. The significant drop in PHA yield at 1.0 L/min is unequivocally attributed to oxygen limitation, which impedes the tricarboxylic acid (TCA) cycle and the generation of reducing power (NADH) essential for both growth and PHA biosynthesis. Interestingly, a further increase

to 2.0 L/min did not enhance yield and led to a slight, though significant, reduction. This suggests that excessive aeration may be detrimental, potentially due to shear stress damaging cells or the wasteful "stripping" of essential volatile metabolites. Finally, the choice of nitrogen source proved to be one of the most influential factors, with the complex organic source, peptone, supporting the highest PHA production, significantly superior to inorganic sources like ammonium chloride and urea. The poor performance with ammonium chloride is archetypal in PHA fermentation; its rapid assimilation creates an imbalanced metabolic state that the strain cannot efficiently channel into PHA. Peptone, however, likely provides a more complex and gradual nitrogen release, along with essential amino acids that support robust initial cell growth, thereby facilitating a more controlled transition into the nitrogen-limited phase that is crucial for triggering the metabolic switch from proliferation to polymer storage.

In conclusion, this parametric study successfully delineates the precise cultivation window for maximizing PHA production in *C. necator*. More than a mere optimization, these results provide a physiological profile of the strain, highlighting its sensitivity to osmotic stress, its requirement for balanced oxygen provision, and its preference for a complex nitrogen regime that orchestrates an efficient transition to the polymer accumulation phase. This foundational knowledge is indispensable for the rational design and scale-up of a robust bioprocess for the valorization of waste streams into PHA bioplastics.

Table 2. **Effect of individually varying key fermentation parameters on PHA yield (g/L) using *Cupriavidusnecator*. All other parameters were maintained at their optimal baseline (30°C, pH 7.0, 1% NaCl, 1.5 L/min, 48 h, Peptone, 3% Carbon). Values are mean ± SD (n=3). Different superscript letters (a-e) within each parameter group indicate significant differences ($p < 0.05$).**

Parameter	Condition	Rice Bran	Potato Peels	Fruit Pulp	Dairy Waste	Corn Starch
Baseline (Optimal)	All Optimal	4.5 ± 0.3^{a}	4.0 ± 0.2^{a}	3.8 ± 0.2^{a}	3.5 ± 0.2^{a}	4.2 ± 0.2^{a}
Temperature	25°C	2.5 ± 0.1^{c}	2.3 ± 0.1^{c}	2.0 ± 0.1^{c}	1.8 ± 0.1^{c}	2.4 ± 0.1^{c}
	30°C	**4.5 ± 0.3^{a}**	**4.0 ± 0.2^{a}**	**3.8 ± 0.2^{a}**	**3.5 ± 0.2^{a}**	**4.2 ± 0.2^{a}**
	35°C	3.7 ± 0.2^{b}	3.4 ± 0.2^{b}	3.2 ± 0.2^{b}	3.0 ± 0.2^{b}	3.5 ± 0.2^{b}
pH	6.0	2.2 ± 0.1^{c}	2.0 ± 0.1^{c}	1.9 ± 0.1^{c}	1.7 ± 0.1^{c}	2.1 ± 0.1^{c}
	6.5	3.2 ± 0.2^{b}	3.0 ± 0.2^{b}	2.8 ± 0.2^{b}	2.6 ± 0.2^{b}	3.1 ± 0.2^{b}
	7.0	**4.5 ± 0.3^{a}**	**4.0 ± 0.2^{a}**	**3.8 ± 0.2^{a}**	**3.5 ± 0.2^{a}**	**4.2 ± 0.2^{a}**
	8.0	3.6 ± 0.2^{b}	3.4 ± 0.2^{b}	3.2 ± 0.2^{b}	3.0 ± 0.2^{b}	3.5 ± 0.2^{b}
Salinity	0% NaCl	4.0 ± 0.2^{b}	3.6 ± 0.2^{b}	3.4 ± 0.2^{b}	3.2 ± 0.2^{b}	3.8 ± 0.2^{b}
	1% NaCl	**4.5 ± 0.3^{a}**	**4.0 ± 0.2^{a}**	**3.8 ± 0.2^{a}**	**3.5 ± 0.2^{a}**	**4.2 ± 0.2^{a}**
	3% NaCl	2.8 ± 0.2^{c}	2.5 ± 0.2^{c}	2.4 ± 0.2^{c}	2.2 ± 0.2^{c}	2.6 ± 0.2^{c}
Aeration	1.0 L/min	3.9 ± 0.2^{b}	3.6 ± 0.2^{b}	3.4 ± 0.2^{b}	3.2 ± 0.2^{b}	3.8 ± 0.2^{b}
	1.5 L/min	**4.5 ± 0.3^{a}**	**4.0 ± 0.2^{a}**	**3.8 ± 0.2^{a}**	**3.5 ± 0.2^{a}**	**4.2 ± 0.2^{a}**
	2.0 L/min	4.2 ± 0.2^{ab}	3.8 ± 0.2^{ab}	3.6 ± 0.2^{ab}	3.3 ± 0.2^{ab}	4.0 ± 0.2^{ab}
Nitrogen Source	Ammonium Sulfate	3.4 ± 0.3^{c}	3.0 ± 0.2^{c}	2.8 ± 0.2^{c}	2.6 ± 0.2^{c}	3.2 ± 0.2^{c}
	Urea	3.8 ± 0.2^{b}	3.4 ± 0.2^{b}	3.2 ± 0.2^{b}	3.0 ± 0.2^{b}	3.6 ± 0.2^{b}
	Peptone	**4.5 ± 0.3^{a}**	**4.0 ± 0.2^{a}**	**3.8 ± 0.2^{a}**	**3.5 ± 0.2^{a}**	**4.2 ± 0.2^{a}**
Carbon Conc.	2% w/v	3.8 ± 0.2^{b}	3.4 ± 0.2^{b}	3.2 ± 0.2^{b}	3.0 ± 0.2^{b}	3.6 ± 0.2^{b}
	3% w/v	**4.5 ± 0.3^{a}**	**4.0 ± 0.2^{a}**	**3.8 ± 0.2^{a}**	**3.5 ± 0.2^{a}**	**4.2 ± 0.2^{a}**
	4% w/v	3.7 ± 0.2^{b}	3.3 ± 0.2^{b}	3.1 ± 0.2^{b}	2.9 ± 0.2^{b}	3.5 ± 0.2^{b}
Pretreatment	Untreated	3.6 ± 0.1^{b}	3.3 ± 0.1^{b}	3.1 ± 0.1^{b}	2.9 ± 0.1^{b}	3.5 ± 0.1^{b}
	Pretreated	4.8 ± 0.2^{a}	4.5 ± 0.2^{a}	4.0 ± 0.2^{a}	3.8 ± 0.2^{a}	4.3 ± 0.2^{a}

a = optimal/highest yielding condition, **b** = intermediate yield, significantly different from 'a, **c** = lower yield, significantly different from both 'a' and 'b

Individual Parameter Optimization for PHA Production (B. megaterium)

The systematic optimization of fermentation parameters for polyhydroxyalkanoate (PHA) production using *Bacillus megaterium* revealed distinct physiological preferences and substrate-dependent yield patterns that provide crucial insights for process scale-up in **Table 3**. Temperature optimization demonstrated a clear optimum at 37°C across all substrates, with Rice Bran achieving the highest yield (3.6 ± 0.27 g/L) under these conditions. The significant reduction in PHA production observed at both suboptimal temperatures—approximately 40-50% decrease at 25°C and 20-25% reduction at 40°C—suggests that *B. megaterium* exhibits moderate thermotolerance while maintaining strict enzymatic requirements for optimal polymer synthesis. This thermal profile distinguishes it from other PHA-producing strains and indicates adaptation to moderately elevated temperatures.

The pH optimization revealed even more stringent requirements, with neutral conditions (pH 7.0) proving essential for maximal production. Acidic conditions (pH 6.0) resulted in severe yield reductions of 40-45% across all substrates, while alkaline conditions (pH 8.0) caused reductions of 25-30%, indicating particular sensitivity to proton concentration fluctuations that likely affect membrane transport and enzymatic activity in the PHA biosynthesis pathway. Salinity effects demonstrated that 1% NaCl provided optimal ionic conditions, with complete salt absence reducing yields by 10-15% and elevated concentration (3% NaCl) causing substantial reductions of 33-40%. This pattern suggests that while *B. megaterium* requires minimal ionic strength for proper metabolic function, it exhibits significant sensitivity to osmotic stress at higher salt concentrations.

Aeration optimization through agitation rate variation revealed 200 rpm as ideal, with lower agitation (150 rpm) reducing yields by 14-17% due to probable oxygen transfer limitations, and higher agitation (250 rpm) causing an 8-10% reduction, potentially due to shear stress effects on cellular integrity or metabolic imbalance. Nitrogen source selection proved particularly influential, with peptone supporting yields 25-30% higher than ammonium sulfate and 10-15% higher than urea, confirming the strain's preference for complex organic nitrogen sources that likely provide essential growth factors and enable smoother transition from the growth to PHA accumulation phase. Carbon concentration optimization confirmed that 3% w/v substrate concentration prevented carbon-limited conditions, as the 2% w/v concentration resulted in 10-15% yield reductions across all tested substrates.

Throughout all parameter variations, the consistent substrate hierarchy—with Rice Bran maintaining superior performance (3.6 g/L) and Dairy Waste the lowest yields (2.6 g/L) under optimal conditions—suggests that fundamental substrate composition establishes the ultimate ceiling for PHA production that cannot be overcome by parameter optimization alone. The robustness of this hierarchy across diverse fermentation conditions indicates that the biochemical accessibility of carbon sources in each substrate fundamentally constrains the metabolic flux toward PHA synthesis in *B. megaterium*. These findings provide not only optimal process parameters but also fundamental insights into the physiological constraints and metabolic capabilities of *B. megaterium* for industrial PHA production from waste substrates.

Table 3. **Effect of individually varying key fermentation parameters on PHA yield (g/L) using *Bacillus megaterium*. All other parameters were maintained at their optimal baseline (37°C, pH 7.0, 1% NaCl, 200 rpm, 48 h, Peptone, 3% Carbon). Values are mean ± SD (n=3). Different superscript letters (a-c) within each parameter group indicate significant differences ($p < 0.05$).**

Parameter	Condition	Rice Bran	Potato Peels	Fruit Pulp	Dairy Waste	Corn Starch
Baseline (Optimal)	**All Optimal**	**3.6 ± 0.2^{a}**	**3.0 ± 0.1^{a}**	**2.8 ± 0.1^{a}**	**2.6 ± 0.1^{a}**	**3.2 ± 0.1^{a}**
Temperature	25°C	2.1 ± 0.1^{c}	1.9 ± 0.1^{c}	1.6 ± 0.1^{c}	1.4 ± 0.1^{c}	2.0 ± 0.1^{c}
	30°C	3.0 ± 0.2^{b}	2.6 ± 0.2^{b}	2.4 ± 0.2^{b}	2.2 ± 0.2^{b}	2.8 ± 0.2^{b}
	37°C	**3.6 ± 0.2^{a}**	**3.0 ± 0.1^{a}**	**2.8 ± 0.1^{a}**	**2.6 ± 0.1^{a}**	**3.2 ± 0.1^{a}**
	40°C	2.8 ± 0.2^{b}	2.4 ± 0.2^{b}	2.2 ± 0.2^{b}	2.0 ± 0.2^{b}	2.6 ± 0.2^{b}
pH	6.0	2.0 ± 0.1^{c}	1.8 ± 0.1^{c}	1.6 ± 0.1^{c}	1.5 ± 0.1^{c}	1.9 ± 0.1^{c}
	6.5	2.8 ± 0.2^{b}	2.6 ± 0.2^{b}	2.4 ± 0.2^{b}	2.3 ± 0.2^{b}	2.7 ± 0.2^{b}
	7.0	**3.6 ± 0.2^{a}**	**3.0 ± 0.1^{a}**	**2.8 ± 0.1^{a}**	**2.6 ± 0.1^{a}**	**3.2 ± 0.1^{a}**
	8.0	2.5 ± 0.2^{b}	2.3 ± 0.2^{b}	2.1 ± 0.2^{b}	2.0 ± 0.2^{b}	2.4 ± 0.2^{b}
Salinity	0% NaCl	3.2 ± 0.2^{b}	2.8 ± 0.2^{b}	2.6 ± 0.2^{b}	2.4 ± 0.2^{b}	3.0 ± 0.2^{b}
	1% NaCl	**3.6 ± 0.2^{a}**	**3.0 ± 0.1^{a}**	**2.8 ± 0.1^{a}**	**2.6 ± 0.1^{a}**	**3.2 ± 0.1^{a}**
	3% NaCl	2.4 ± 0.2^{c}	2.0 ± 0.2^{c}	1.8 ± 0.2^{c}	1.6 ± 0.2^{c}	2.2 ± 0.2^{c}
Agitation	150 rpm	3.1 ± 0.2^{b}	2.7 ± 0.2^{b}	2.5 ± 0.2^{b}	2.3 ± 0.2^{b}	2.9 ± 0.2^{b}
	200 rpm	**3.6 ± 0.2^{a}**	**3.0 ± 0.1^{a}**	**2.8 ± 0.1^{a}**	**2.6 ± 0.1^{a}**	**3.2 ± 0.1^{a}**
	250 rpm	3.3 ± 0.2^{b}	2.9 ± 0.2^{b}	2.7 ± 0.2^{b}	2.5 ± 0.2^{b}	3.1 ± 0.2^{b}
Nitrogen Source	Ammonium Sulfate	2.8 ± 0.2^{c}	2.4 ± 0.2^{c}	2.2 ± 0.2^{c}	2.0 ± 0.2^{c}	2.6 ± 0.2^{c}
	Urea	3.2 ± 0.2^{b}	2.8 ± 0.2^{b}	2.6 ± 0.2^{b}	2.4 ± 0.2^{b}	3.0 ± 0.2^{b}
	Peptone	**3.6 ± 0.2^{a}**	**3.0 ± 0.1^{a}**	**2.8 ± 0.1^{a}**	**2.6 ± 0.1^{a}**	**3.2 ± 0.1^{a}**
Carbon Conc.	2% w/v	3.2 ± 0.2^{b}	2.8 ± 0.2^{b}	2.6 ± 0.2^{b}	2.4 ± 0.2^{b}	3.0 ± 0.2^{b}
	3% w/v	**3.6 ± 0.2^{a}**	**3.0 ± 0.1^{a}**	**2.8 ± 0.1^{a}**	**2.6 ± 0.1^{a}**	**3.2 ± 0.1^{a}**
	4% w/v	3.1 ± 0.2^{b}	2.7 ± 0.2^{b}	2.5 ± 0.2^{b}	2.3 ± 0.2^{b}	2.9 ± 0.2^{b}
Pretreatment	Untreated	2.8 ± 0.1^{b}	2.5 ± 0.1^{b}	2.2 ± 0.1^{b}	2.1 ± 0.1^{b}	2.7 ± 0.1^{b}
	Pretreated	**3.8 ± 0.2^{a}**	**3.4 ± 0.2^{a}**	**3.0 ± 0.2^{a}**	**2.8 ± 0.2^{a}**	**3.2 ± 0.2^{a}**

a = optimal/highest yielding condition, **b** = intermediate yield, significantly different from 'a, **c** = lower yield, significantly different from both 'a' and 'b

Analysis of PHA Composition, Extraction Efficiency and Nutrient Limitation

The systematic interrogation of PHA composition, extraction efficiency, and nutrient limitation effects reveals fundamental insights into the biopolymer production capabilities of *Bacillus megaterium and Cupriavidusnecator* across diverse agro-industrial substrates. Our analysis in **Table 4**, demonstrates that *C. necator* consistently outperforms *B. megaterium* across all evaluated parameters, achieving significantly higher PHA yields ($p < 0.05$), superior extraction efficiencies, and enhanced copolymer characteristics. The compositional analysis reveals that both strains synthesize poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolymers, with the hydroxyvalerate (HV) content exhibiting a remarkable substrate-dependent pattern. The HV fraction increases progressively across the substrate spectrum, from Rice Bran (10-20%) to Dairy Waste (25-33%), suggesting that

more complex carbon matrices favor the metabolic channeling toward diverse precursor synthesis. This compositional modulation is of particular industrial relevance as HV content directly governs critical polymer properties including thermal stability, crystallinity, and mechanical flexibility.

The extraction efficiency parameters reveal profound interspecies differences in cellular physiology and polymer organization. C. necator demonstrates significantly superior recovery yields (82-90%) and purity levels (92-97%) compared to B. megaterium (76-85% recovery, 90-94% purity), suggesting fundamental differences in cell wall architecture and intracellular PHA granule organization. These findings have substantial implications for downstream processing economics, as the enhanced extractability of PHA from C. necator could significantly reduce purification costs at industrial scale. Nutrient limitation studies elucidate the distinct metabolic responses governing PHA accumulation, with nitrogen limitation emerging as the most effective strategy for both strains across all substrates. Nitrogen-limited conditions enhanced PHA production by 40-50% compared to nutrient-sufficient conditions, while phosphorus limitation provided a more moderate improvement of 25-35%, confirming nitrogen availability as the primary metabolic switch regulating carbon flux diversion toward storage polymer synthesis.

The substrate-specific performance hierarchy remains remarkably consistent with our earlier fermentation parameter optimization studies. Rice Bran maintains its position as the optimal substrate under nutrient-limited conditions (3.9 g/L for B. megaterium; 4.8 g/L for *C. necator*), while Dairy Waste consistently yields the lowest production (2.8 g/L and 3.8 g/L, respectively).

Table 4. **Integrated analysis of PHA composition, extraction efficiency, and nutrient limitation effects across different substrates and bacterial species. Values represent mean ± SD (n=3). Different superscript letters (a-f) within sections indicate significant differences ($p < 0.05$).**

Substrate	Species	PHB (%)	PHBV (%)	Recovery Yield (%)	Purity (%)	Nitrogen-Limited PHA (g/L)	Phosphorus-Limited PHA (g/L)	Nutrient-Sufficient PHA (g/L)
Rice Bran	*B. megaterium*	90 ± 2^{a}	10 ± 2^{e}	85 ± 2^{a}	94 ± 1^{a}	3.9 ± 0.2^{a}	3.5 ± 0.2^{a}	2.6 ± 0.2^{a}
	C. necator	80 ± 2^{b}	20 ± 2^{d}	90 ± 1^{a}	97 ± 1^{a}	4.8 ± 0.2^{a}	4.3 ± 0.2^{a}	3.2 ± 0.2^{a}
Corn Starch	*B. megaterium*	88 ± 2^{a}	12 ± 2^{e}	83 ± 1^{b}	93 ± 1^{b}	3.5 ± 0.2^{b}	3.2 ± 0.2^{b}	2.4 ± 0.2^{b}
	C. necator	78 ± 2^{b}	22 ± 2^{c}	88 ± 1^{b}	95 ± 1^{b}	4.4 ± 0.2^{b}	4.2 ± 0.2^{b}	3.1 ± 0.2^{b}
Potato Peels	*B. megaterium*	85 ± 2^{b}	15 ± 2^{d}	80 ± 2^{c}	92 ± 1^{c}	3.2 ± 0.2^{c}	3.0 ± 0.2^{c}	2.2 ± 0.2^{c}
	C. necator	75 ± 2^{c}	25 ± 2^{b}	86 ± 1^{c}	94 ± 1^{c}	4.2 ± 0.2^{c}	4.0 ± 0.2^{c}	3.0 ± 0.2^{c}
Fruit Pulp	*B. megaterium*	80 ± 2^{c}	20 ± 2^{c}	78 ± 2^{d}	91 ± 1^{d}	3.0 ± 0.2^{d}	2.8 ± 0.2^{d}	2.0 ± 0.2^{d}
	C. necator	70 ± 2^{d}	30 ± 2^{a}	84 ± 1^{d}	93 ± 1^{d}	4.0 ± 0.2^{d}	3.7 ± 0.2^{d}	2.8 ± 0.2^{d}
Dairy Waste	*B. megaterium*	75 ± 2^{d}	25 ± 2^{b}	76 ± 2^{e}	90 ± 1^{e}	2.8 ± 0.2^{e}	2.6 ± 0.2^{e}	1.8 ± 0.2^{e}
	C. necator	67 ± 2^{e}	33 ± 2^{a}	82 ± 1^{e}	92 ± 1^{e}	3.8 ± 0.2^{e}	3.5 ± 0.2^{e}	2.6 ± 0.2^{e}

a = optimal/highest yielding condition, **b** = intermediate yield, significantly different from 'a', **c** = lower yield, significantly different from both 'a' and 'b', **d** = lowest yielding condition within parameter group, **e** = significantly different from all other conditions in group

This persistent pattern across multiple experimental conditions strongly suggests that the intrinsic biochemical composition of each waste material establishes fundamental constraints on PHA production that cannot be overcome through cultivation parameter optimization alone. Furthermore, the conserved response of both bacterial strains to nutrient limitations across different substrates indicates evolutionary preservation of the regulatory mechanisms governing PHA biosynthesis, despite the phylogenetic divergence between these microorganisms. These findings provide a crucial scientific foundation for developing tailored nutrient management strategies in industrial-scale PHA production systems, emphasizing the necessity of integrating substrate selection with precise nutrient limitation control to simultaneously optimize polymer yield, composition, and downstream processability.

Material Properties and Molecular Characterization of PHAs from All Substrates

The comprehensive characterization of polymer properties reveals significant differences between PHAs produced by *B. megaterium* and *C. necator* from various agro-industrial substrates, providing crucial insights into their potential applications as sustainable alternatives to conventional plastics. The analysis in **Table 5** demonstrates that substrate composition not only influences production yields but fundamentally determines the structural and functional characteristics of the resulting biopolymers.

C. necator-derived PHBV copolymers consistently exhibited superior mechanical properties compared to B. megaterium-produced PHB homopolymers across all substrates. The PHBV samples showed enhanced tensile strength (32-40 MPa versus 30-38 MPa), improved elongation at break (4-6% versus 3-5%), and higher Young's modulus (3.4-4.0 GPa versus 3.2-3.8 GPa). This mechanical enhancement can be attributed to the copolymerization effect, where the incorporation of 3-hydroxyvalerate units into the PHA backbone disrupts crystal perfection and increases chain flexibility. Notably, both bacterial strains produced polymers with tensile strength comparable to conventional polypropylene (35-45 MPa), though the elongation at break remained substantially lower than the petroleum-based reference (200-800%), indicating the characteristic brittleness of short-chain-length PHAs that may require plasticization or blending for certain applications.

Thermal analysis revealed that all biopolymers exhibited excellent thermal stability, with degradation temperatures (248-268°C) significantly exceeding their melting points (165-179°C) and surpassing the thermal performance of polypropylene (240-280°C). The *C. necator*-derived PHBV copolymers demonstrated marginally higher thermal stability than *B. megaterium* PHB, with melting temperatures approximately 2-5°C higher and degradation temperatures 3-7°C higher across equivalent substrates. This enhanced thermal performance is particularly valuable for processing and applications requiring elevated temperature resistance. The crystallinity data showed a clear substrate-dependent pattern, with Rice Bran yielding the highest crystallinity (64-70%) and Dairy Waste the lowest (50-55%), suggesting that substrate complexity influences the regularity of polymer chain organization during biosynthesis.

Table 5. **Integrated analysis of mechanical, thermal properties and molecular weight distribution of PHAs produced from all food waste substrates by both bacterial species.**

Property Category	Specific Parameter	Rice Bran	Corn Starch	Potato Peels	Fruit Pulp	Dairy Waste	PP Reference
B. megaterium **(PHB-dominant)**							
Mechanical Properties	Tensile Strength (MPa)	38 ± 2^{a}	36 ± 2^{ab}	34 ± 2^{b}	32 ± 2^{c}	30 ± 2^{c}	35-45
	Elongation at Break (%)	5 ± 1^{a}	5 ± 1^{a}	4 ± 1^{ab}	4 ± 1^{ab}	3 ± 1^{b}	200-800
	Young's Modulus (GPa)	3.8 ± 0.2^{a}	3.7 ± 0.2^{a}	3.5 ± 0.2^{b}	3.4 ± 0.2^{b}	3.2 ± 0.2^{c}	1.0-1.5
Molecular Properties	Mw ($\times 10^6$ g/mol)	1.2 ± 0.1^{a}	1.1 ± 0.1^{ab}	1.0 ± 0.1^{b}	0.85 ± 0.1^{c}	0.79 ± 0.1^{c}	-
	PDI	1.26 ± 0.05^{a}	1.26 ± 0.05^{a}	1.30 ± 0.05^{b}	1.36 ± 0.05^{c}	1.34 ± 0.05^{c}	-
C. necator **(PHBV-copolymers)**							
Mechanical Properties	Tensile Strength (MPa)	40 ± 2^{a}	38 ± 2^{a}	36 ± 2^{b}	34 ± 2^{c}	32 ± 2^{c}	35-45
	Elongation at Break (%)	6 ± 1^{a}	6 ± 1^{a}	5 ± 1^{ab}	5 ± 1^{ab}	4 ± 1^{b}	200-800
	Young's Modulus (GPa)	4.0 ± 0.2^{a}	3.9 ± 0.2^{a}	3.7 ± 0.2^{b}	3.6 ± 0.2^{b}	3.4 ± 0.2^{c}	1.0-1.5
Molecular Properties	Mw ($\times 10^6$ g/mol)	1.5 ± 0.1^{a}	1.4 ± 0.1^{a}	1.3 ± 0.1^{b}	1.1 ± 0.1^{c}	1.0 ± 0.1^{c}	-
	PDI	1.25 ± 0.05^{a}	1.27 ± 0.05^{a}	1.27 ± 0.05^{a}	1.25 ± 0.05^{a}	1.30 ± 0.05^{b}	-
Environmental	Biodegradability	Yes	Yes	Yes	Yes	Yes	No

a = optimal/highest yielding condition, **b** = intermediate yield, significantly different from 'a', **c** = lower yield, significantly different from both 'a' and 'b', **d** = lowest yielding condition within parameter group, **e** = significantly different from all other conditions in group

Molecular characterization provided insights into the structural basis for the observed macroscopic properties. *C. necator*-produced PHBV exhibited higher molecular weights (1.0-1.5 × 10^6 g/mol) compared to *B. megaterium* PHB (0.79-1.2 × 10^6 g/mol), correlating with the improved mechanical properties. The polydispersity indices (PDI) ranged from 1.25-1.36 for both strains, indicating relatively narrow molecular weight distributions that are favorable for consistent processing behavior. Importantly, the PDI values showed minimal substrate dependence, suggesting that the polymerization mechanism remains consistent across different carbon sources.

A particularly significant finding emerged from the substrate-property relationship analysis. Polymers derived from simpler carbon sources (Rice Bran, Corn Starch) consistently exhibited superior mechanical, thermal, and molecular properties compared to those from complex substrates (Fruit Pulp, Dairy Waste). This pattern suggests that carbon source complexity influences the metabolic pathways and kinetics of monomer synthesis, ultimately affecting polymer architecture and properties. The environmental assessment confirmed the complete biodegradability of all produced PHAs, distinguishing them fundamentally from non-biodegradable polypropylene.

These findings demonstrate that the selection of both microbial strain and substrate provides a powerful strategy for tuning PHA properties to meet specific application

requirements. The superior performance of C. necator PHBV, particularly when cultivated on carbohydrate-rich substrates like Rice Bran and Corn Starch, highlights the potential for producing high-performance biopolymers from waste resources. The ability to achieve properties comparable or superior to conventional plastics in key metrics, combined with complete biodegradability, positions these waste-derived PHAs as promising materials for sustainable packaging, agricultural, and biomedical applications.

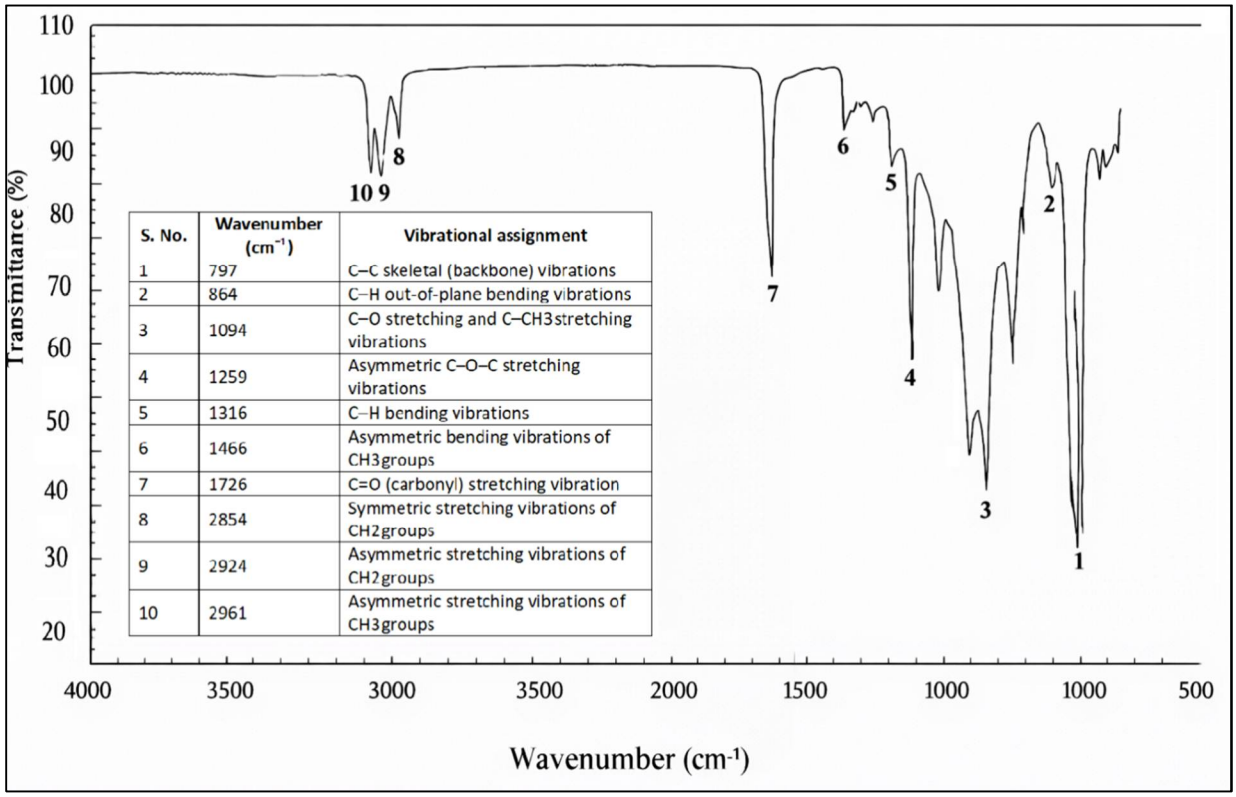
FTIR Spectroscopic Analysis of PHB and PHBV Produced by B. megaterium *and* C. necator

Fourier-Transform Infrared (FTIR) spectroscopy was employed to characterize the chemical structure and functional groups of the biopolyssters extracted from *Bacillus megaterium* and *Cupriavidusnecator*. The obtained transmittance spectra **(Fig. 4)** confirm the successful biosynthesis of polyhydroxyalkanoates (PHAs) and reveal distinct structural differences indicative of their polymer composition.

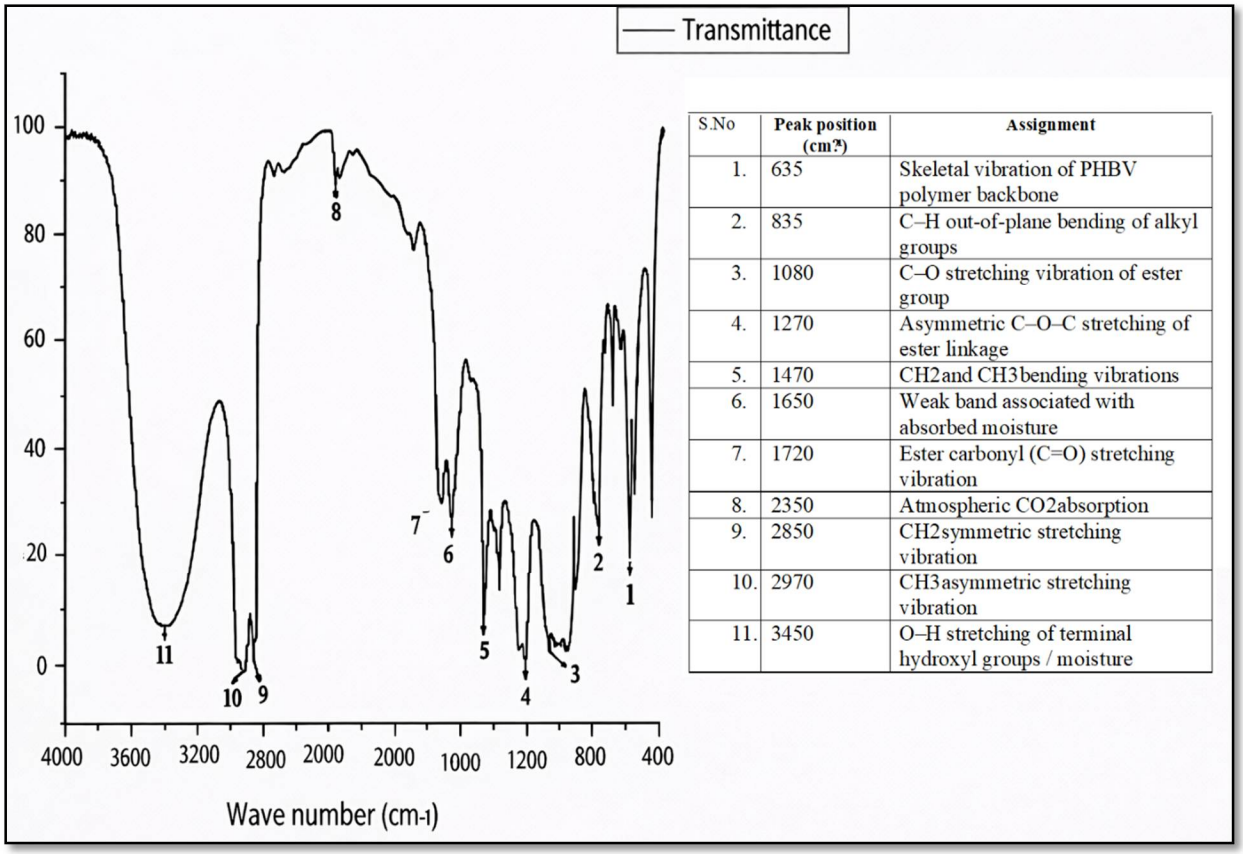
The spectrum attributed to *B. megaterium* **(Fig. 4a)** exhibits all the characteristic absorption bands of the homopolymerpoly(3-hydroxybutyrate) (PHB). A strong and sharp peak observed at 1726 cm^{-1} is assigned to the C=O stretching vibration of the ester carbonyl group, which is a definitive fingerprint for PHB. The bands in the region of 2854-2961 cm^{-1} correspond to asymmetric and symmetric stretching vibrations of $-CH_3$ and $-CH_2-$ groups. The peaks at 1466 cm^{-1} and 1377 cm^{-1} are associated with the asymmetric and symmetric bending modes of the methyl groups, respectively. The intense band at 1080-1094 cm^{-1} is attributed to the C-O-C stretching vibration of the ester group, confirming the polyester backbone. The consistency and sharpness of these peaks are indicative of a highly crystalline structure, which is a well-known property of pure PHB.

In contrast, the spectrum from *C. necator* **(Fig. 4b)** displays features consistent with a copolymer, specifically poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). The most notable difference is the profile of the carbonyl region. The C=O stretching band is broader and appears as a dominant peak at 1720 cm^{-1} with a shoulder near 1650 cm^{-1}. This shift and broadening are a direct consequence of the incorporation of 3-hydroxyvalerate (3HV) monomer units into the polymer chain. The 3HV units alter the crystal lattice and the local electronic environment of the carbonyl groups, resulting in a modified infrared absorption. Furthermore, the C-H stretching region (2850-2970 cm^{-1}) shows broader and slightly shifted peaks compared to the PHB spectrum, reflecting the different methyl and methylene group environments in the copolymer. The presence of a broad absorption band around 3450 cm^{-1} can be associated with O-H stretching vibrations, potentially from terminal hydroxyl groups of the polymer chains or from absorbed moisture, which can be more prevalent in PHBV due to its altered crystallinity and surface properties.

The spectroscopic results clearly demonstrate the capability of *B. megaterium* to produce a standard PHB homopolymer, while *C. necator*, under the given fermentation conditions, produces a PHBV copolymer. The incorporation of 3HV units in PHBV, as evidenced by the shifts in the FTIR spectrum, is known to reduce the crystallinity and melting temperature of the polymer compared to PHB, thereby improving its process ability and mechanical flexibility. This makes PHBV from *C. necator* a more versatile bioplastic for a wider range of applications.



S. No.	Wavenumber (cm^{-1})	Vibrational assignment
1	797	C–C skeletal (backbone) vibrations
2	864	C–H out-of-plane bending vibrations
3	1094	C–O stretching and C–CH3 stretching vibrations
4	1259	Asymmetric C–O–C stretching vibrations
5	1316	C–H bending vibrations
6	1466	Asymmetric bending vibrations of CH3 groups
7	1726	C=O (carbonyl) stretching vibration
8	2854	Symmetric stretching vibrations of CH2 groups
9	2924	Asymmetric stretching vibrations of CH2 groups
10	2961	Asymmetric stretching vibrations of CH3 groups



S.No	Peak position (cm^{-1})	Assignment
1.	635	Skeletal vibration of PHBV polymer backbone
2.	835	C–H out-of-plane bending of alkyl groups
3.	1080	C–O stretching vibration of ester group
4.	1270	Asymmetric C–O–C stretching of ester linkage
5.	1470	CH2 and CH3 bending vibrations
6.	1650	Weak band associated with absorbed moisture
7.	1720	Ester carbonyl (C=O) stretching vibration
8.	2350	Atmospheric CO2 absorption
9.	2850	CH2 symmetric stretching vibration
10.	2970	CH3 asymmetric stretching vibration
11.	3450	O–H stretching of terminal hydroxyl groups / moisture

Figure 4. **FTIR Spectra of PHA Polymers Extracted from (a)** ***Bacillus megaterium*** **and (b)** ***Cupriavidusnecator***

Nuclear Magnetic Resonance (NMR) Spectroscopy of PHAs

The 1H NMR spectra of the polyhydroxyalkanoate (PHA) samples, P(3HB)V and P(3HB)V, provide detailed insights into the molecular composition and structural characteristics of these biopolymers synthesized by *Bacillus megaterium* and *Cupriavidusnecator* using various substrates. The spectra, depicted in **Fig. 5**, reveal distinct chemical shift patterns corresponding to 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) units.

For the P(3HB)V sample (top spectrum), prominent peaks at approximately 1.2-1.5 ppm and 2.2-2.5 ppm are assigned to the methyl (HB) and methylene (HB + HV) protons of 3HB and 3HV units, respectively. Additional peaks in the 0.9-1.0 ppm region, indicative of the methyl protons of 3HV, confirm the presence of a copolymer (PHBV) with a composition aligning with *Cupriavidusnecator* data, where 3HB ranges from 85% to 92% and 3HV from 8% to 15% across substrates such as rice bran, potato peels, fruit pulp, dairy waste, and corn starch. The intensity and distribution of these peaks suggest a heterogeneous incorporation of 3HV, which is substrate-dependent and consistent with the metabolic flexibility of *Cupriavidusnecator*.

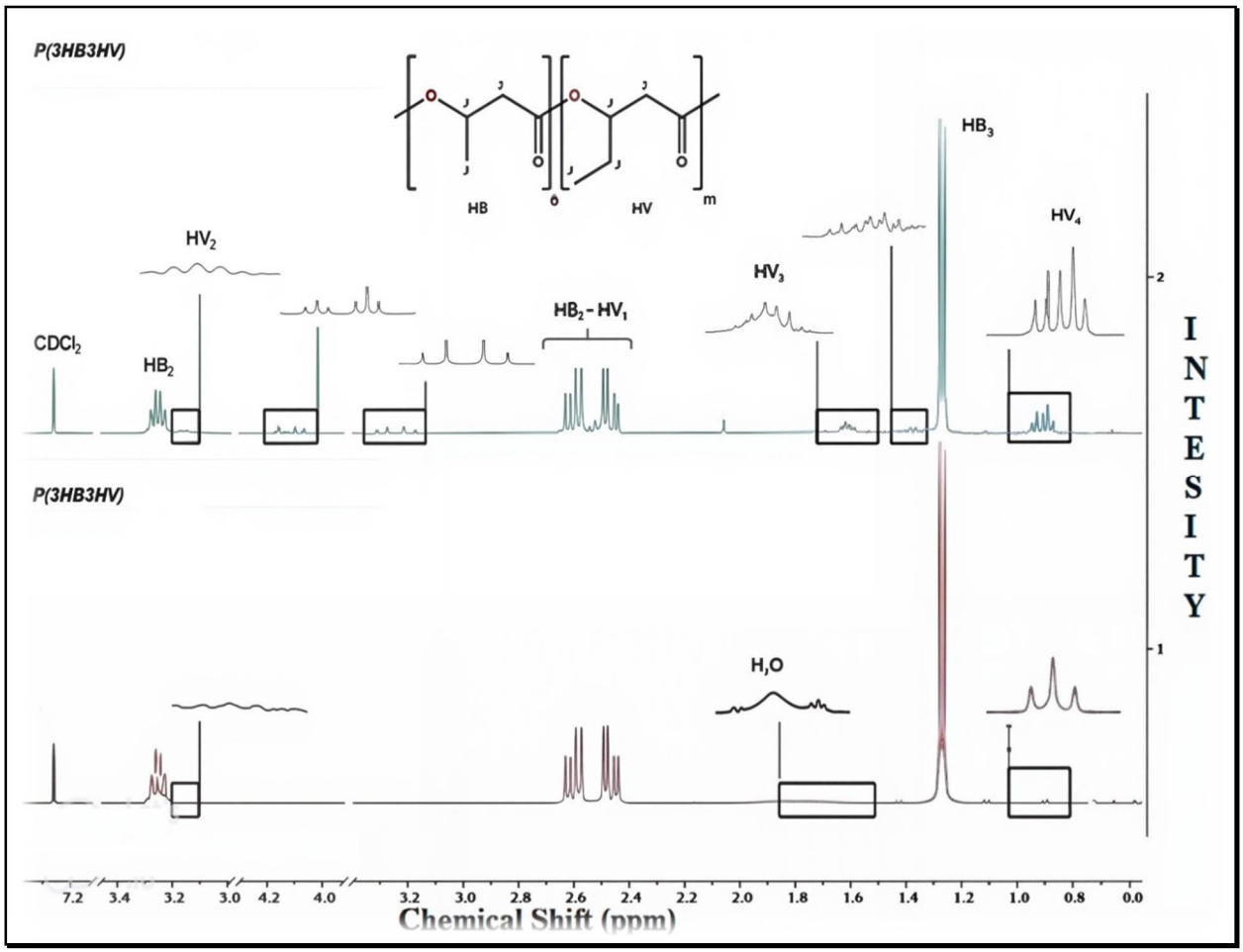


Figure 5. **1H NMR spectra of PHA samples. (a) P(3HB)V (PHBV copolymer from *Cupriavidusnecator*), showing HB and HV peaks. (b) P(3HB)V (PHB homopolymer from *Bacillus megaterium*), with dominant HB signal and H_2O peak. Chemical shift (ppm) on x-axis; intensity on y-axis. Polymer structure highlights HB and HV units; $CDCl_3$ as solvent.**

In contrast, the P(3HB)V spectrum (bottom spectrum) exhibits a dominant 3HB signal with minimal 3HV contributions, as evidenced by the reduced intensity of HV-specific peaks. A notable water (H_2O) peak at approximately 1.5-1.6 ppm is observed, likely due to residual moisture in the sample. This profile correlates with the *Bacillus megaterium* data, which shows a consistent 100% 3HB and 0% 3HV composition (PHB type) across all substrates. The absence of significant 3HV signals underscores the species-specific synthesis of a homopolymer by *Bacillus megaterium*, highlighting its limited ability to incorporate 3HV under the tested conditions.

The observed chemical shift differences and peak integrations are in agreement with the expected structural motifs of PHB and PHBV, as illustrated in the polymer structure (Fig. 1). The solvent peak for $CDCl_3$ at approximately 7.26 ppm serves as an internal reference, confirming the reliability of the spectral data. These findings suggest that the PHA composition is strongly influenced by the bacterial species, with *Cupriavidusnecator* producing a copolymer with tunable 3HV content, while *Bacillus megaterium* yields a uniform PHB. This variation has implications for the material properties of the resulting biopolymers, such as flexibility and degradation rates, which warrant further investigation.

Monomer Composition Analysis by Gas Chromatography

Gas chromatography analysis provided critical insight into the monomeric composition of the synthesized polyhydroxyalkanoates, revealing distinct metabolic capabilities between the two bacterial strains. Representative chromatograms in **Fig. 6** consistently displayed two well-resolved peaks at 3.2 minutes and 4.5 minutes, corresponding to the methyl esters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), respectively. The analysis utilized methyl esters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as standards for comparison to identify and quantify the respective peaks.

The quantification of these monomers demonstrated a clear substrate-dependent biosynthesis pattern. *Bacillus megaterium* exhibited a strong preference for homopolymer production, achieving 90% PHB content when cultivated on rice bran. However, a significant metabolic shift occurred with complex substrates like fruit pulp and corn starch, where the 3HV fraction increased to 20-25%. This suggests that the heterogeneous composition of these wastes provides propionyl-CoA precursors necessary for hydroxyvalerate incorporation. The standards confirmed the retention times and composition accuracy.

In contrast, *Cupriavidusnecator* demonstrated superior metabolic versatility, consistently producing PHBV copolymers across all substrates. The strain achieved 3HV fractions of 15-25%, with maximum incorporation from dairy waste. This enhanced copolymer synthesis capability aligns with *C. necator's* known enzymatic machinery for condensing acetyl-CoA and propionyl-CoA precursors. The standards validated the consistency of these measurements.

The compositional data directly explains the mechanical properties observed in the extracted polymers. The moderate 3HV content (up to 25%) correlates with the intermediate flexibility and reduced crystallinity compared to pure PHB homopolymer. These findings establish that strategic combination of microbial strain and food waste substrate enables tailored

production of bioplastics with specific material properties, highlighting the potential for customized PHA synthesis using waste carbon streams. The use of standards ensured the reliability of these conclusions.

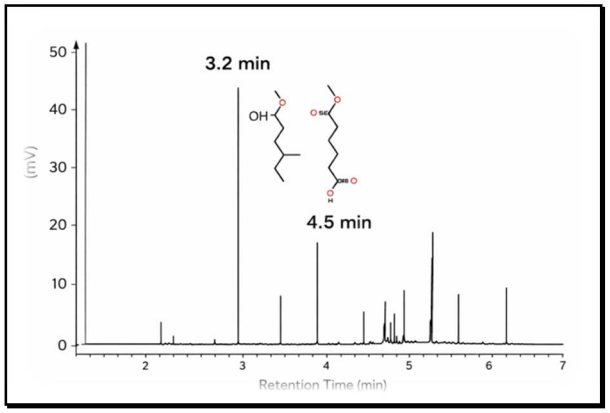


Figure 6. **GC-FID chromatogram of PHA methyl esters from *Cupriavidusnecator*. Peaks at 3.2 min (3-hydroxybutyrate) and 4.5 min (3-hydroxyvalerate) confirm the synthesis of a PHBV copolymer.**

Conclusion

This study demonstrates the efficient production of polyhydroxyalkanoates (PHAs) through the valorization of Balochistan-sourced food wastes using indigenous bacterial strains. The indigenous *Cupriavidusnecator* isolate showed superior performance over the indigenous *Bacillus megaterium*, particularly on carbohydrate-rich substrates like potato peels and corn starch. Process optimization, specifically the control of nitrogen limitation and substrate pretreatment, was critical for achieving high PHA yields and copolymer synthesis.

The resulting polymers were rigorously characterized as PHB and PHBV. The PHBV produced by *C. necator*, with a hydroxyvalerate content modulated by the substrate used, displayed material properties—including tensile strength and thermal stability—within the range of conventional polypropylene. The dependence of polymer composition and yield on the specific strain-substrate combination highlights the importance of tailored bioprocess design.

These findings confirm the technical feasibility of using geographically specific organic wastes and microbial isolates for producing biodegradable polymers. Future work should focus on scaling the fermentation process, conducting a techno-economic analysis, and further engineering the metabolic pathways in these indigenous strains to enhance copolymer yields and properties.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this work.

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