



Multi-Stage Optimization and Comparative Valorization of Agro-Industrial Residues for PHB/PHBV Production by *Priestia megaterium* SMI2: Structural Insights and Environmental Fate

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Abstract

Growing environmental concerns linked to conventional petroleum-based plastics have accelerated the shift toward sustainable, biodegradable materials such as polyhydroxyalkanoates (PHAs), which offer the added advantage of being biologically derived and environmentally compatible. This study comparatively evaluated the ability of *Priestia megaterium* strain SMI2 to convert two abundant agricultural residues, wheat bran and banana peel, into value-added biopolymers. A two-stage optimization strategy was employed to maximize polymer production and assess material quality and degradability. Initial screening using a One-Factor-at-a-Time (OFAT) approach established baseline physiological conditions influencing polymer accumulation, including incubation time, temperature, pH, substrate concentration, and inoculum size. Optimal general conditions were identified at 35 °C and 4 g/L substrate concentration; however, substrate-specific differences were observed. Wheat bran supported faster polymer accumulation, achieving optimal production at pH 7.0 within 48 h using a 2% inoculum, whereas banana peel required 96 h at pH 7.5 with a 1% inoculum. Subsequent multivariable optimization using Response Surface Methodology (RSM) based on Central Composite Design (CCD) significantly enhanced yields and revealed distinct performance patterns. Wheat bran produced a maximum yield of 367.47 mg/L, outperforming banana peel, which yielded 225.8 mg/L. The banana peel model was statistically significant ($F = 5.78$, $p = 0.0009$) with an R^2 of 0.84, explaining 84% of variability, while the wheat bran model was highly significant ($F = 14.92$, $p = 0.0003$; $R^2 = 0.783$) with strong agreement between predicted and experimental values. FTIR and GC–MS analyses confirmed the formation of PHB/PHBV copolymers containing both 3-hydroxybutyrate and 3-hydroxyvalerate units. Biodegradability assessment showed up to 72.5% weight loss after 60 days of soil burial, indicating substantial environmental degradability. These findings provide a commercially viable pathway for industrial-scale biopolymer production that aligns with global mandates for circular bioeconomy.

Keywords: Polyhydroxyalkanoates (PHAs), agro-industrial residues, *Priestia megaterium*, response surface methodology (RSM), PHB/PHBV copolymer, biodegradability

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Introduction

Plastic pollution has become one of the most pressing environmental challenges globally,

largely due to the extensive use of durable and inexpensive petrochemical-derived polymers. Conventional plastics such as polyethylene,

polypropylene, polyvinyl chloride, and polystyrene are highly resistant to biodegradation because of their stable molecular structures. As a result, they persist in the environment for extended periods, accumulating in terrestrial and aquatic ecosystems (Geyer *et al.*, 2017; Lebreton and Andrady, 2019). The scale of this problem continues to grow, particularly with the widespread use of single-use plastics in packaging, which represents the largest share of global plastic consumption (Geyer *et al.*, 2017; Kaza *et al.*, 2018). Beyond their persistence, plastic waste has been detected in marine, freshwater, and soil environments, where they contribute to ecological disruption and long-term environmental degradation (Jambeck *et al.*, 2015; de Souza Machado *et al.*, 2018). Their accumulation has even been suggested as a defining feature of the Anthropocene epoch (Zalasiewicz *et al.*, 2016).

These environmental concerns have stimulated increasing interest in the development of sustainable and biodegradable alternatives to conventional plastics. Among the available options, polyhydroxyalkanoates (PHAs) have received considerable attention due to their biodegradability, biocompatibility, and origin from renewable resources (Chen and Wu, 2018). Poly(3-hydroxybutyrate) (PHB), the most widely studied member of this group, exhibits material properties comparable to polypropylene, making it suitable for applications in packaging, agriculture, and biomedical fields (Yahyapour and Menciloglu, 2025; Fratini *et al.*, 2026). In addition, PHAs can be completely mineralised by microorganisms under natural conditions, thereby offering an environmentally compatible alternative to synthetic polymers. Despite these advantages, the widespread adoption of PHB has been limited by its relatively high production cost. A major contributor to this cost is the carbon substrate required for microbial fermentation, which often accounts for a significant proportion of the overall production expenses (Koller *et al.*, 2017). This has led to growing interest in the use of low-cost and renewable feedstocks as

substitutes for refined sugars. Agricultural residues such as wheat bran, banana peels, and sugarcane bagasse are particularly promising in this regard, as they are abundant, inexpensive, and do not compete with food resources (Li *et al.*, 2025; Ismail *et al.*, 2026). Their utilisation not only reduces production costs but also supports waste valorisation by converting underutilised biomass into valuable bioproducts (Adamu and Bukar, 2022; Nasruddin *et al.*, 2025; Mukhtar *et al.*, 2026a).

Previous studies have demonstrated the potential of various microorganisms to produce PHB using agro-industrial residues as carbon sources. Species belonging to genera such as *Bacillus*, *Streptomyces*, and several marine bacteria have been reported to synthesise PHB from lignocellulosic materials and fruit processing wastes (Mostafa *et al.*, 2019; Rezk *et al.*, 2020; Abidin *et al.*, 2026). However, the efficient conversion of such substrates remains challenging due to their complex composition and variability. In addition, the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), a copolymer with improved flexibility and thermal properties compared to PHB, has not been fully optimised when derived from heterogeneous waste substrates (Wang *et al.*, 2013; Ray and Kalia, 2017).

Priestia megaterium (formerly *Bacillus megaterium*) is a Gram-positive bacterium that has been widely studied for its capacity to synthesise PHAs. Its metabolic versatility and recognized safety profile make it a suitable candidate for industrial applications (Biedendieck *et al.*, 2021). While earlier work has focused largely on defined carbon sources such as glucose and glycerol, there is still limited information on its performance when grown on complex agro-industrial residues, particularly under optimised conditions. Furthermore, conventional optimisation approaches that vary one factor at a time often fail to capture interactions among process variables, which can significantly influence product yield.

To address these limitations, statistical optimisation techniques such as response surface methodology have been employed to

improve bioprocess efficiency by evaluating the combined effects of multiple variables. A sequential approach that combines preliminary screening with a more robust experimental design allows for a more reliable identification of optimal conditions.

In the present study, a locally isolated strain, *Priestia megaterium* PX567901 obtained from fadama soil in Nigeria, was investigated for its ability to produce PHB and PHBV using wheat bran and banana peel hydrolysates as carbon sources. A comparative evaluation of these substrates was carried out, followed by process optimisation using a combination of one-factor-at-a-time screening and response surface methodology based on central composite design. The resulting polymer was characterised using Fourier transform infrared spectroscopy and gas chromatography–mass spectrometry to confirm its composition, while its biodegradability was assessed under soil burial conditions. This work provides further insight into the conversion of agricultural residues into biodegradable polymers and highlights their potential for cost-effective and environmentally sustainable bioplastic production.

Materials and Methods

Bacterial strain and maintenance

Priestia megaterium SMI2 was previously isolated from dumpsite fadama soil in Kano State, Nigeria, and identified by 16S rRNA sequencing (GenBank accession number PX567901) (Mukthar *et al.*, 2026b). The strain was maintained on Nutrient Agar slants at 4 °C and subcultured monthly. For long-term storage, cultures were preserved in 20% (v/v) glycerol at –80 °C. Inoculum for fermentation was prepared by transferring a loopful of the stock culture into 50 mL of Luria–Bertani (LB) broth and incubated at 35 °C, 150 rpm for 24 h.

Preparation of agro-industrial residue hydrolysates

Wheat bran and banana peel were collected from a local market in Naibawa, Kano State. Substrates were washed thoroughly with distilled water, dried at 60 °C for 48 h, and ground to a fine powder. For both substrates, a controlled dilute-acid pretreatment was employed. The pulverised material was

suspended in a 5% (v/v) sulfuric acid (H₂SO₄) solution at a solid-to-liquid ratio of 1:10 (w/v). The mixture was sterilized in an autoclave at 121 °C (15 psi) for 30 min. After cooling, the slurry was filtered through double-layered muslin cloth, and the liquid hydrolysate was neutralised to pH 7.0 using 6 N NaOH. The neutralised fraction was centrifuged at 8,000 rpm for 10 min, and the clear supernatant was collected. The total reducing sugar concentration was determined by the dinitrosalicylic acid (DNS) method using a glucose standard curve (0.2–1.4 mg/mL, R² = 0.995). Each hydrolysate supernatant was diluted 10-fold with distilled water prior to the assay. Based on the absorbance at 620 nm (wheat bran: 1.1703 ± 0.125; banana peel: 0.9076 ± 0.108), the original wheat bran hydrolysate contained 12.5 ± 1.28 g/L of reducing sugars (glucose equivalents), and the banana peel hydrolysate contained 9.72 ± 0.98 g/L. Both hydrolysates were stored at –20 °C until use as carbon sources for *Priestia megaterium* SMI2 fermentation (Adamu & Bukar, 2022).

Mineral salt medium (MSM) composition

The basal mineral salt medium used for PHB/PHBV production contained (per litre of deionised water): KH₂PO₄ 1.5 g, Na₂HPO₄ 3.5 g, MgSO₄·7H₂O 0.2 g, CaCl₂·2H₂O 0.02 g, FeSO₄·7H₂O 0.01 g, and 1 mL of trace element solution. The trace element solution consisted of (per litre): ZnSO₄·7H₂O 0.1 g, MnCl₂·4H₂O 0.03 g, H₃BO₃ 0.3 g, CoCl₂·6H₂O 0.2 g, NiCl₂·6H₂O 0.02 g, and Na₂MoO₄·2H₂O 0.03 g. Ammonium sulfate ((NH₄)₂SO₄) was added as the nitrogen source at a final concentration of 1.0 g/L, giving an initial C/N ratio of approximately 12:1 when wheat bran hydrolysate (12.5 g/L reducing sugars) was used, and approximately 6:1 for banana peel hydrolysate (adjusted to the same sugar concentration). The hydrolysates and trace elements were filter-sterilised and added aseptically after autoclaving the other components (Adamu and Salisu, 2024).

One-Factor-at-a-Time (OFAT) optimization

OFAT experiments were conducted in triplicate to establish baseline conditions for PHB production by *Priestia megaterium*

SMI2. A series of independent parameters were varied sequentially while keeping all other factors constant at their baseline values (35 °C, pH 7.0, substrate concentration 6 g/L reducing sugars, inoculum size 2% v/v, and incubation time 72 h). The incubation temperature was first tested at 25, 30, 35, 40, and 45 °C, with 35 °C giving the highest PHB yield. The initial pH of the medium was then adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 using 1 M NaOH or 1 M HCl; optimal production occurred at pH 7.0 for wheat bran hydrolysate and at pH 7.5 for banana peel hydrolysate. Substrate concentration (as total reducing sugars from the respective hydrolysates) was varied from 2 to 10 g/L in steps of 2 g/L; the optimum was 6 g/L for both substrates, although wheat bran supported faster polymer accumulation. Inoculum size ($OD_{600} \approx 0.5$) was examined at 1, 2, 3, 4, and 5% (v/v); 2% was optimal for wheat bran, while 1% performed better for banana peel. Finally, incubation time was evaluated over 24, 48, 72, 96, and 120 h; wheat bran hydrolysate achieved peak PHB accumulation at 48 h, whereas banana peel required 96 h. All OFAT experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of mineral salt medium with the appropriate hydrolysate, incubated at 150 rpm. After each run, cells were harvested by centrifugation ($10,000 \times g$, 15 min, 4 °C), and PHB was extracted and quantified as described earlier described. Results are reported as mean $\pm$ standard deviation of triplicate measurements (Adamu and Bukar, 2022).

Response Surface Methodology (RSM) optimization

Based on OFAT results, three critical factors were selected for further optimization using a Central Composite Design (CCD): substrate concentration (X_1 , 2–8 g/L reducing sugars), incubation time (24–72 h for wheat bran; 48–96 h for banana peel based on preliminary kinetics), and initial pH (6.0–8.0). A five-level, three-factor CCD was generated using Design-Expert version 13 (Stat-Ease Inc., Minneapolis, MN, USA), consisting of 20 experimental runs including six centre points. The response variable was PHB yield (mg/L). A second-order polynomial model

was fitted. Analysis of variance (ANOVA) was used to evaluate model significance ($p < 0.05$). Optimal conditions were predicted and validated in triplicate experiments (Adamu and Salisu, 2024).

PHB extraction and quantification

After fermentation, bacterial cells were harvested by centrifugation ($10,000 \times g$, 15 min, 4 °C), washed twice with distilled water, and freeze-dried. The dried biomass was weighed to determine cell dry weight (CDW, g/L). PHB was extracted using the sodium hypochlorite/chloroform method. Briefly, 50 mg of freeze-dried cells was mixed with 5 mL of 6% (v/v) sodium hypochlorite (NaOCl) and 5 mL of chloroform ($CHCl_3$) in a screw-cap tube. The mixture was heated at 80 °C for 1 h with occasional shaking. After centrifugation ($5,000 \times g$, 10 min), the lower chloroform layer was carefully collected. PHB was precipitated by adding 10 volumes of cold methanol (1:10 methanol: chloroform, v/v) and left overnight at 4 °C. The precipitate was collected by centrifugation, dried at 60 °C to constant weight, and weighed. PHB yield was expressed as mg per litre of culture broth. PHB content (% of CDW) was calculated as (PHB concentration / CDW) $\times$ 100.

Polymer characterization (FTIR and GC-MS)

FTIR spectroscopy: Approximately 2–3 mg of extracted polymer was ground with KBr and pressed into a thin disc. FTIR spectra were recorded in the range of $4000\text{--}400\text{ cm}^{-1}$ using a Shimadzu IRTracer-100 spectrometer (Kyoto, Japan) at a resolution of 4 cm^{-1} . Characteristic absorption bands for PHB and PHBV were identified by comparison with reference spectra.

GC-MS analysis: Methanolysis was performed by adding 2 mL of chloroform, 0.3 mL of 98% sulfuric acid, and 1.7 mL of methanol to 10 mg of polymer. The mixture was heated at 100 °C for 140 min in a sealed tube. After cooling, 1 mL of distilled water was added to induce phase separation. The lower chloroform layer containing the methyl esters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) was collected. Analysis was performed using an Agilent 7890B GC system coupled to a 5977B MSD

(Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m). Helium was the carrier gas at 1 mL/min. The oven temperature programme was: 60 °C for 2 min, then increased to 280 °C at 10 °C/min. Peaks were identified by comparing retention times and mass spectra with authentic standards (Sigma-Aldrich, USA) (Adamu & Salisu, 2024; Mandragutti *et al.*, 2024).

Biodegradability assessment

Polymer films (approx. 1 cm $\times$ 1 cm, 0.2 mm thickness) were prepared by solvent casting from a 2% (w/v) polymer solution in chloroform. The films were air-dried, weighed (W_0), and buried in plastic boxes containing fadama soil (pH 6.8, organic matter content $\sim$ 2.5%) maintained at 60% water holding capacity. The boxes were kept at 30 °C for 60 days. At intervals of 7, 14, 21, 30, 45, and 60 days, films were retrieved, gently cleaned with distilled water, dried at 60 °C, and reweighed (W_1). The percentage weight loss was calculated as:

$$\text{Weight Loss (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

All experiments were performed in triplicate for each polymer type (derived from wheat bran and from banana peel) (Amir *et al.*, 2022).

Statistical analysis

All experiments were performed in triplicate, and results are presented as mean $\pm$ standard deviation (SD). RSM models were evaluated by ANOVA using Design-Expert 13. A p-value < 0.05 was considered statistically significant. Comparisons between substrate yields under optimal conditions were made using Student's t-test.

Results

OFAT Optimization

Incubation time: For wheat bran, PHB/PHBV accumulation increased rapidly, reaching a peak of 225 mg/L at 48 h, then declined significantly ($p < 0.05$) to 210 mg/L at 72 h and further to 110 mg/L at 96 h (Figure 1). Banana peel supported slower accumulation, with maximum yield (195 mg/L) at 72 h, followed by a gradual decrease.

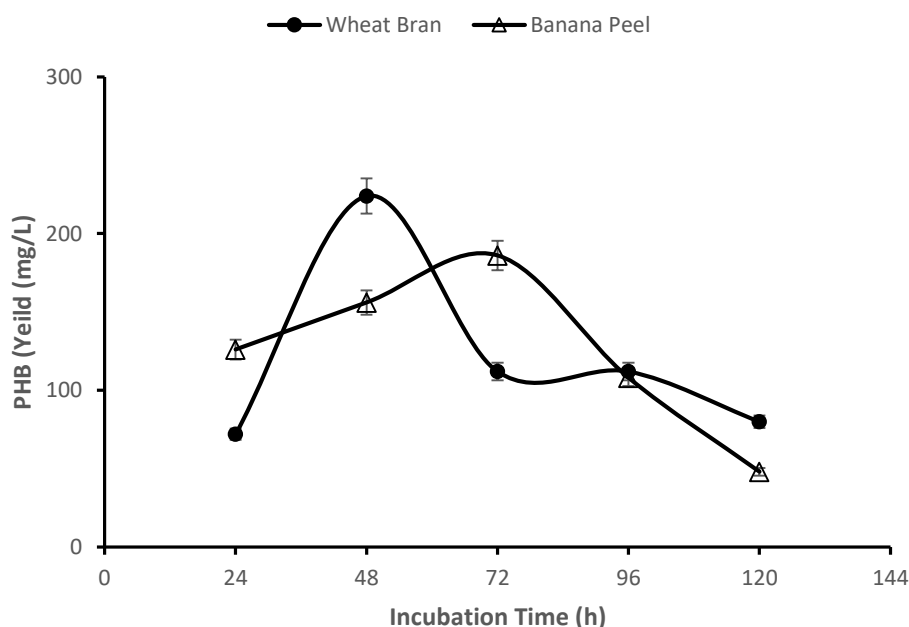


Figure 1: Effect of Incubation Time on PHA Production by *P. megaterium* Using Wheat Bran and Banana Peel Substrates (mean $\pm$ SD, n=3)

pH: The effect of pH was substrate-dependent (Figure 2): wheat bran gave the highest yield (315 mg/L) at pH 7.0, while banana peel peaked at 250 mg/L at pH 7.5. At pH 8.0 and

8.5, banana peel maintained moderate yields (230 and 200 mg/L, respectively), whereas wheat bran yields dropped more sharply.



Figure 2: Effect of pH on PHA Production by *P. megaterium* Using Wheat Bran and Banana Peel Substrates (mean $\pm$ SD, n=3)

Substrate concentration: Substrate concentration optima differed (Figure 3): wheat bran gave the highest yield (170 mg/L) at 8 g/L

reducing sugars, while banana peel peaked at 10 g/L (145 mg/L).

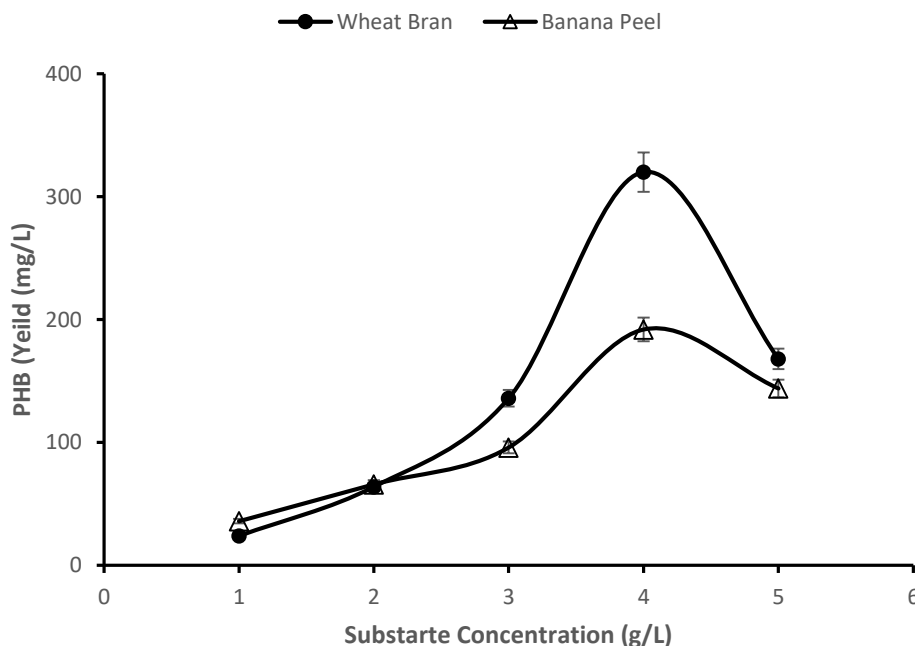


Figure 3: Effect of substrate concentration on PHA Production by *P. megaterium* Using Wheat Bran and Banana Peel Substrates (mean $\pm$ SD, n=3)

Inoculum size: Inoculum size optima also varied (Figure 4.4): 3% (v/v) was best for wheat bran (170 mg/L), whereas 2% gave the highest yield for banana peel (170 mg/L).

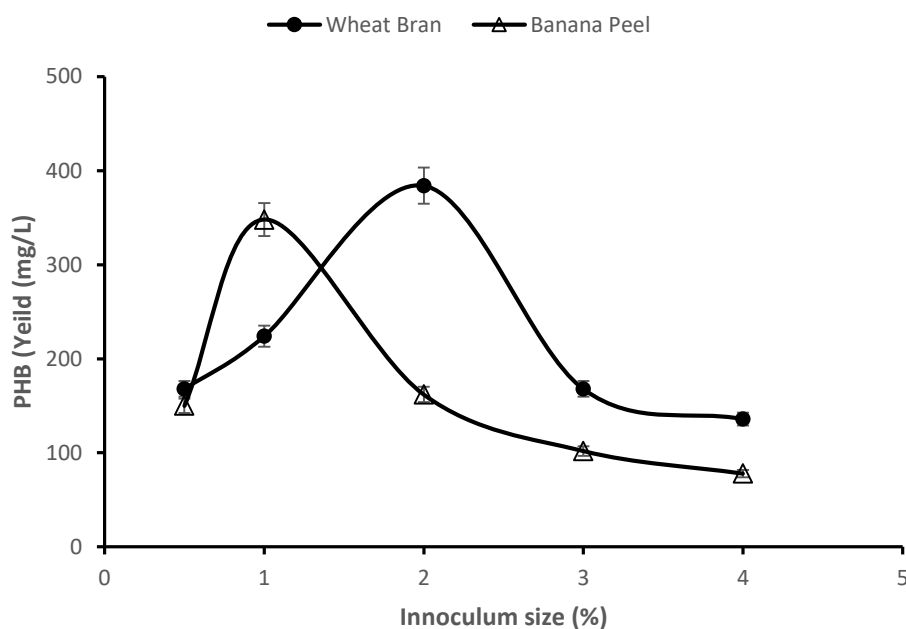


Figure 4: Effect of Inoculum size on PHA Production by *P. megaterium* Using Wheat Bran and Banana Peel Substrates (mean $\pm$ SD, n=3)

Temperature: Temperature had a strong influence on both substrates (Figure 5); the optimal temperature was 35 °C for both, with wheat bran reaching 395 mg/L and banana peel reaching 225 mg/L. At 40 °C and 45 °C, yields declined for both substrates.

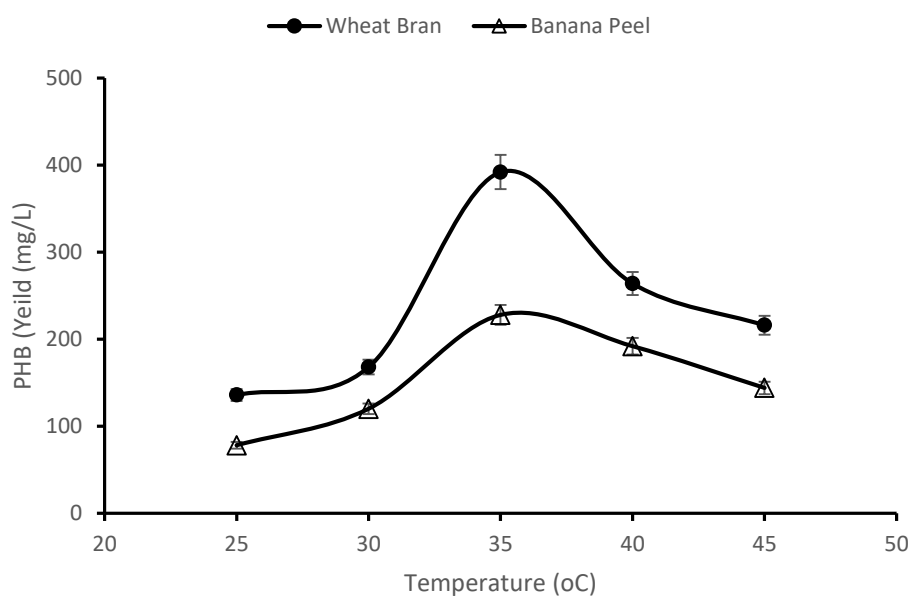


Figure 5: Effect of Temperature on PHA Production by *P. megaterium* Using Wheat Bran and Banana Peel Substrates (mean $\pm$ SD, n=3)

Response Surface Methodology (RSM) Optimization

The central composite design (CCD) matrices for banana peel (Table 1) and wheat bran (Table 3) show the experimental and predicted PHA yields under various combinations of incubation time (A), substrate concentration (B), pH (C), and inoculum size (D). For banana peel, the quadratic model was significant ($F = 5.78$, $p = 0.0009$) with an R^2 of 0.84, adequate precision of 9.01, and a non-significant lack of fit ($p = 0.1484$), confirming model adequacy (Table 2).

Substrate concentration (B) was the most influential linear term ($F = 53.7$, $p < 0.0001$). For wheat bran, the model was also highly significant ($F = 14.92$, $p = 0.0003$) with an R^2 of 0.783, adequate precision of 12.84, and non-significant lack of fit ($p = 0.4350$). pH (C) and inoculum size (D) were the dominant factors, and significant interactions (AB and BC) were identified (Table 4). Both models demonstrated good predictive ability, with close agreement between predicted and experimental yields across runs.

Table 1. Central Composite Design matrix of four process parameters showing the actual and predicted yield for PHA production by *Priestia megaterium* using Banana Peel.

Run	Factor A Incubation time (h)	Factor B Substrate conc. (g)	Factor D Inoculum size (μL)	Factor C pH	Predicted value	Experimental value
1	60	3	7.5	3	158.08	152.0
2	60	3	7.5	3	158.08	165
3	48	4	8	4	184.43	210.4
4	60	3	6.5	3	157.21	147.9
5	72	4	8	4	197.88	225.8
6	60	5	7.5	3	193.66	182.3
7	48	2	7	2	129.48	134.5
8	72	2	8	2	149.11	145.6
9	60	3	7.5	5	175.98	166.8
10	72	4	7	2	171.46	185.9
11	60	3	7.5	3	158.08	161
12	84	3	7.5	3	168.95	168.8
13	60	1	7.5	3	128.81	126.3
14	60	3	7.5	1	155.6	146.6
15	60	3	7.5	3	158.08	155.5
16	72	2	7	4	160.53	156.1
17	48	2	7	4	136.93	132.2
18	48	4	7	2	161.51	170.3
19	72	4	8	2	184.95	215.9
20	48	2	8	2	137.46	141
21	48	4	8	2	179.23	203.6
22	72	4	7	4	183.61	206.2
23	72	2	7	2	145.35	148.9
24	60	3	7.5	3	158.08	148
25	72	2	8	4	165.06	166.7
26	48	2	8	4	145.69	142.5
27	48	4	7	4	165.94	195.2
28	36	3	7.5	3	139.63	123.8
29	60	3	8.5	3	179.46	174.4
30	60	3	7.5	3	158.08	167

Table 2: Quadratic Model Analysis of variance for PHA produced by *Priestia megaterium* using Banana peel

Source	Sum of Squares	Mean Square	F- Value	Prob > F	
Model	9498.00	678.43	5.78	0.0009	significant
A	1289.20	1289.2	10.98	0.0047	
B	6308.28	6308.28	53.7	< 0.0001	
C	742.59	742.59	6.32	0.0238	
D	623.22	623.22	5.31	0.036	
A2	24.70	24.7	0.21	0.6531	
B2	17.06	17.06	1.50E-01	0.7085	
C2	180.25	180.25	1.53	0.2345	
D2	101.75	101.75	0.87	0.3667	
AB	35.11	35.11	0.3	0.5926	
AC	17.85	17.85	0.15	0.7021	
AD	59.68	59.68	0.51	0.4869	
BC	94.58	94.58	0.81	0.3837	
BD	9.15	9.15	0.078	0.784	
CD	0.60	0.6	5.11E-03	0.9439	
Residual	1761.97	117.46			
	1480.76	148.08	2.63	0.1484	not significant
Lack of Fit					
Pure Error	281.21	56.24			
Cor Total	11259.97				
Std. Dev.	10.84	R-Squared	0.84		
Mean	161.55	Adj R-Squared	0.70		
C.V.	6.71	Pred R-Squared	0.621		
PRESS	8934.14	Adeq Precision	9.01		

Table 3. Central Composite Design matrix of four process parameters showing the actual and predicted yield for PHA production by *P. megaterium* using Wheat bran.

Run	Factor A	Factor B	Factor C	Factor D	Predicted yield (mg/L)	Experimental Yield (mg/L)
	Incubation time (h)	Substrate Conc. (g)	pH	Inoculum size (μL)		
1	84	2	7.5	2	281.49	286.68
2	12	2	7.5	2	159.76	160.67
3	120	1	8.5	3	210.98	212.79
4	84	2	7.5	2	281.49	282.29
5	48	1	6.5	1	217.11	217.67
6	156	2	7.5	2	238.99	239.2
7	84	4	7.5	2	304.2	305.17
8	48	3	6.5	1	201.39	200.5
9	48	3	6.5	3	219.12	217.85
10	120	3	6.5	1	290.46	290.31
11	84	0	7.5	2	160.83	160.98
12	84	2	7.5	2	281.49	281.43
13	120	1	6.5	3	223.53	222.19
14	48	3	8.5	3	279.84	280.84

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15	48	3	8.5	1	261.61	260.9
16	84	2	7.5	2	281.49	275.6
17	84	2	5.5	2	260.24	260.38
18	120	3	8.5	1	350.86	351.22
19	84	2	7.5	2	281.49	280.51
20	84	2	7.5	0	264.11	264.54
21	84	2	7.5	4	299.76	300.46
22	48	1	6.5	3	233.55	234.11
23	120	1	8.5	1	193.06	192.29
24	120	3	6.5	3	309.17	310.38
25	48	1	8.5	3	220.82	218.93
26	84	2	9.5	2	307.91	308.89
27	120	3	8.5	3	370.07	367.47
28	48	1	8.5	1	203.88	203.59
29	120	1	6.5	1	206.11	206.04
30	84	2	7.5	2	281.49	282.44

Table 4: Quadratic Model Analysis of variance for PHA produced by *P. megatearium* using Wheat Bran

Source	Sum of Squares	Mean Square	F- Value	Prob > F	
Model	59320.45	4237.17	14.92	0.0003	significant
A	412.56	412.56	1.45	0.2470	
B	1986.34	1986.34	6.99	0.0181	
C	6245.18	6245.18	21.98	< 0.0001	
D	4872.09	4872.09	17.14	0.0006	
A2	632.48	632.48	2.23	0.1559	
B2	842.77	842.77	2.97	0.1058	
C2	1412.63	1412.63	4.98	0.0412	
D2	356.91	356.91	1.26	0.2793	
AB	5168.44	5168.44	18.20	0.0005	
AC	421.09	421.09	1.48	0.2418	
AD	389.75	389.75	1.37	0.2589	
BC	4326.18	4326.18	15.23	0.0011	
BD	278.66	278.66	0.98	0.3367	
CD	194.32	194.32	0.68	0.4210	
Residual	4267.18	284.48			
Lack of Fit	2489.61	248.96	1.03	0.4350	not significant
Pure Error	1777.57	355.51			
Cor Total	63587.63				
Std. Dev.	16.86	R-Squared	0.7830		
Mean	255.88	Adj R-Squared	0.7781		
C.V.	6.59	Pred R-Squared	0.7586		
PRESS	15367.40	Adeq Precision	12.84		

Polymer characterization (FTIR and GC-MS)

The FTIR spectra of the extracted polymers from wheat bran and banana peel hydrolysates showed characteristic absorption bands consistent with polyhydroxyalkanoates, specifically the PHBV copolymer. A strong carbonyl (C=O) stretching vibration was observed at approximately 1720 cm^{-1} , confirming the presence of ester linkages typical of PHA. Additional bands at around 2980 cm^{-1} and 2930 cm^{-1} correspond to asymmetric and symmetric C–H stretching of

methyl and methylene groups, respectively. Absorption peaks in the region of $1280\text{--}1180\text{ cm}^{-1}$ are attributed to C–O–C stretching vibrations, further confirming the polyester backbone. A band near 1380 cm^{-1} (C–H bending of CH_3 groups) and a characteristic doublet at around 980 cm^{-1} and 1050 cm^{-1} are indicative of the crystalline and amorphous phases of PHBV. The absence of significant peaks at around $1600\text{--}1650\text{ cm}^{-1}$ (C=C or amide) indicated that the polymer was free from protein or other cellular contaminants.

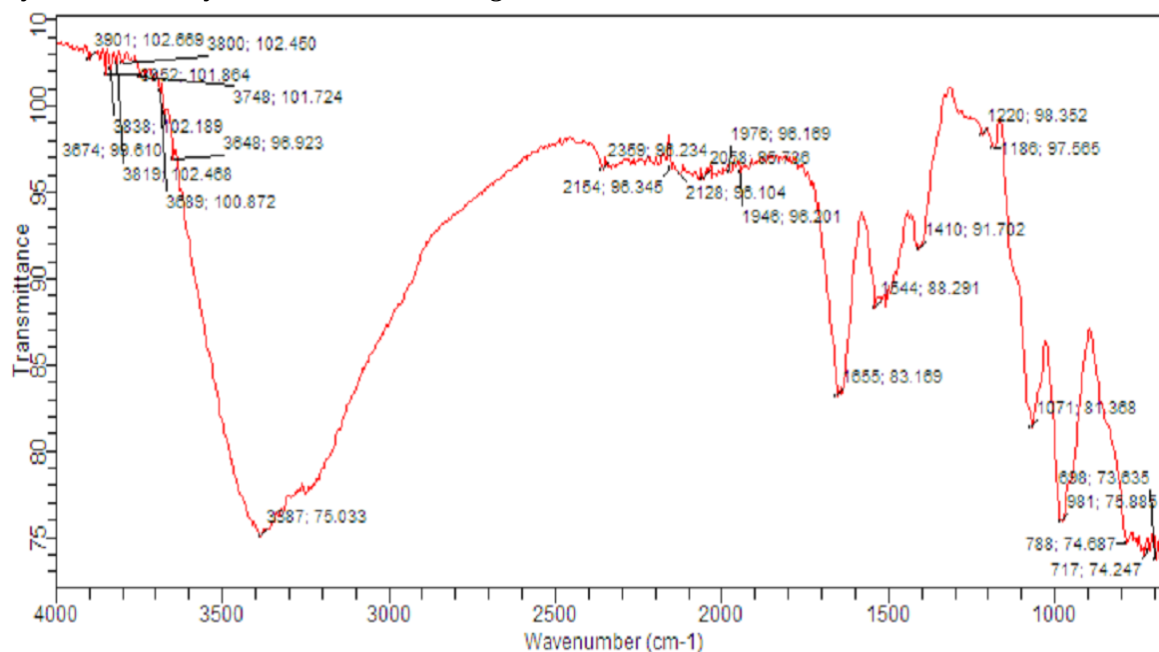


Figure 6: FTIR Spectroscopy of PHA Produced by *Prestia megaterium* using wheat bran

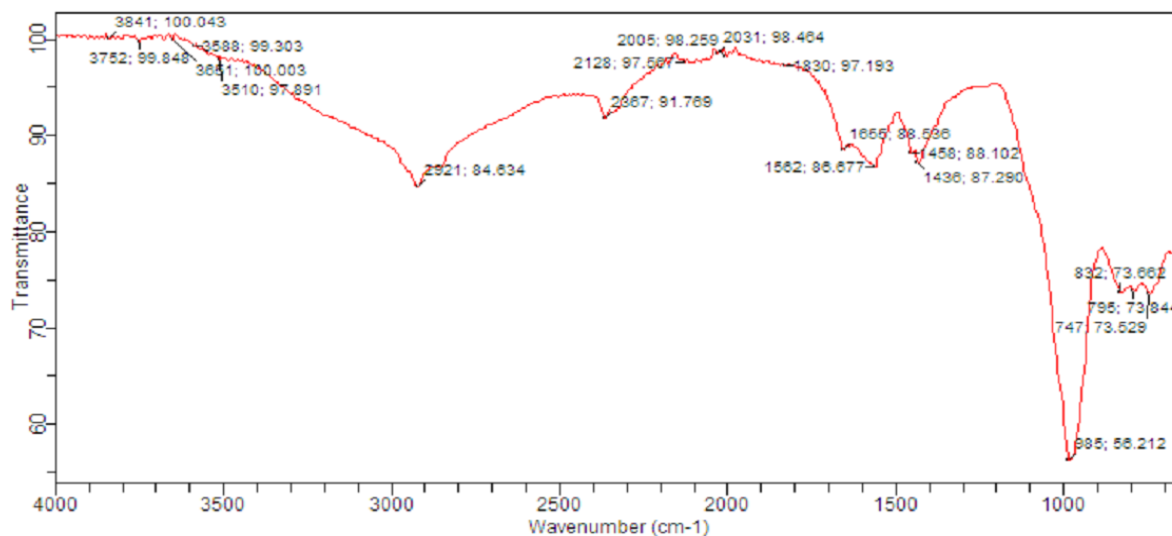


Figure 7: FTIR Spectroscopy of PHA Produced by *Prestia megaterium* using Banana Peel

Gas chromatography–mass spectrometry (GC-MS) was performed to determine the monomeric composition of the extracted polymer. The chromatogram revealed a major peak with a retention time consistent with the methyl ester of 3-hydroxybutyrate (3HB). A smaller, distinct peak corresponding to

3-hydroxyvalerate (3HV) methyl ester was also observed, confirming the presence of a PHBV-type copolymer. The mass spectra of the peaks matched the fragmentation patterns of reference standards, with characteristic ions for 3HB (m/z 43, 74, 87, 103) and for 3HV (m/z 29, 43, 55, 71, 87, 101).

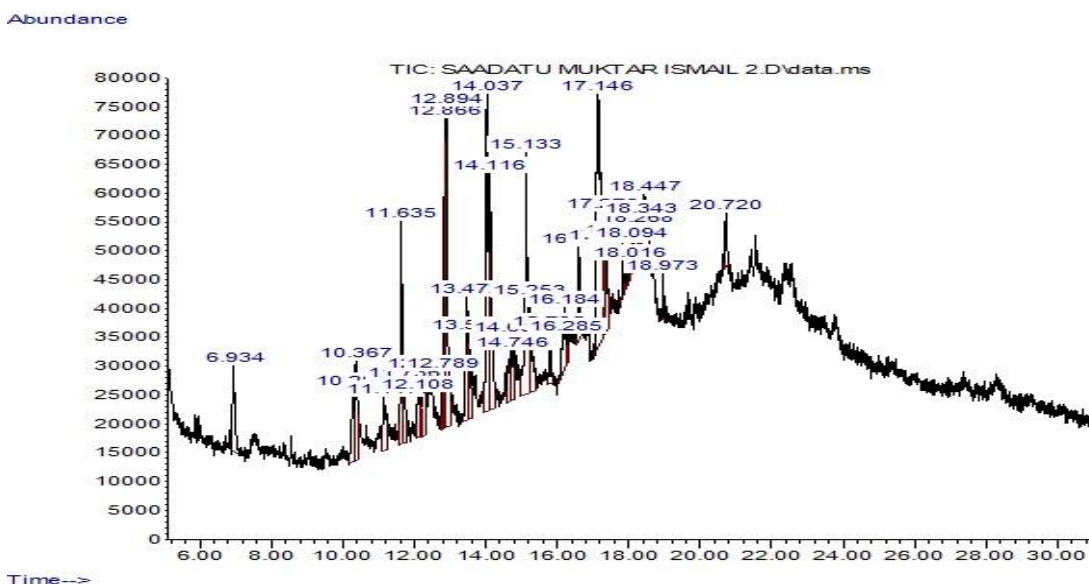


Figure 8: Gas chromatography analysis of derivatives PHB obtained from *Priestia megaterium*

Discussion

PHA production is frequently influenced by various environmental, physical, and chemical parameters. Among these, temperature, pH, substrate concentration, inoculum size, and incubation time are critical in determining microbial growth and metabolic activity, thereby affecting polymer accumulation. Careful regulation of these factors enhances both biomass formation and PHA biosynthesis (Chandani *et al.*, 2018; Możejko-Ciesielska *et al.*, 2023).

The effect of incubation time was substrate-dependent. *Priestia megaterium* SMI2 accumulated PHA faster on wheat bran hydrolysate, reaching 225 mg/L at 48 h, whereas banana peel required 72 h to attain its maximum (195 mg/L). This difference likely reflects the higher proportion of readily fermentable sugars and lower lignin content in wheat bran, which supports rapid carbon assimilation (Woiciechowski *et al.*, 2020). After the peak, yields declined significantly ($p < 0.05$) for both substrates, indicating that

the bacterium begins to mobilize stored PHA as an internal carbon and energy source once external substrate becomes depleted – a well-documented survival strategy under nutrient limitation (Ray and Kalia, 2017; Yanti *et al.*, 2021). Similar optimal periods of 48–72 h have been reported for *Bacillus megaterium* and *Ralstonia eutropha* (Patil *et al.*, 2024; Nguyen *et al.*, 2025). The slower kinetics on banana peel may be due to its higher pectin and hemicellulose content, which require longer hydrolysis before sugars become fully available (Ngaopok *et al.*, 2025). pH is a fundamental determinant of microbial physiology and metabolic activity, as it directly affects enzyme kinetics, membrane transport, and the ionisation state of nutrients. For PHA production, even minor pH deviations can shift carbon flux away from polymer biosynthesis toward stress responses (Kumar *et al.*, 2020; Singh and Mallick, 2017). In this study, wheat bran hydrolysate gave the highest yield at pH 7.0 (315 mg/L), while banana peel peaked at pH 7.5

(250 mg/L). The broader pH tolerance of banana peel (up to pH 8.5) may be attributed to the presence of mineral cations (Mg^{2+} , K^{+}) that stabilize membrane integrity under mild alkaline stress – an effect previously observed in fruit-peel hydrolysates (Pandey *et al.*, 2024). The optimal pH range (7.0–7.5) is consistent with earlier reports for *Priestia* and *Bacillus* strains (López-Cuellar *et al.*, 2011; Mohanrasu *et al.*, 2020).

The quantity of biomass used during fermentation also plays a significant role (Shehu *et al.*, 2023). The population of bacteria present in the medium influences cell adaptation and the synthesis of enzymes necessary for metabolism. Determining the appropriate inoculum volume is crucial to maximizing product accumulation. Here, the optimal inoculum size was 3 % (v/v) for wheat bran (170 mg/L) and 2 % for banana peel (170 mg/L). The denser, more nutrient-rich wheat bran hydrolysate may require a higher initial cell density to initiate effective substrate utilization, whereas the more accessible sugars in banana peel allow efficient PHA synthesis with fewer cells. Excessive inoculum (>4 %) likely leads to rapid nutrient depletion and accumulation of inhibitory metabolites, a trend observed in other *Priestia* fermentations (Getachew and Woldesenbet, 2016; Mohanrasu *et al.*, 2020).

Carbon availability plays a fundamental role in bacterial growth and PHA biosynthesis, influencing both biomass formation and polymer accumulation. While simple sugars such as glucose are widely used industrially, exploring alternative carbon sources is critical for reducing costs and improving sustainability (Adamu and Bukar, 2022). Substrate concentration optima differed: wheat bran gave the highest yield at 8 g/L reducing sugars (170 mg/L), while banana peel peaked at 10 g/L (145 mg/L). The higher reducing sugar content of wheat bran hydrolysate (12.5 g/L versus 9.72 g/L for banana peel) largely explains its superior performance. Importantly, no furfural or 5-hydroxymethylfurfural were detected in either hydrolysate, indicating that the mild dilute-acid treatment (5 % H_2SO_4 , 121 °C, 30 min) preserved sugar integrity while

effectively disrupting the lignocellulosic matrix (Ngaopok *et al.*, 2025).

Temperature is a key factor influencing microbial physiology and PHA biosynthesis, affecting both growth rate and enzyme activity (Khamkong *et al.*, 2022; Attapong *et al.*, 2023). The optimal temperature for both substrates was 35 °C, with yields of 395 mg/L (wheat bran) and 225 mg/L (banana peel). At 40 °C and 45 °C, yields declined sharply, confirming the mesophilic nature of the isolate. Similar temperature optima (31–37 °C) have been reported for *Bacillus aryabhatai* and *Bacillus* sp. C1 (Pillai *et al.*, 2017; Dash *et al.*, 2019). The drop at 45 °C likely results from denaturation of key biosynthetic enzymes, particularly β -ketothiolase and PHA synthase (Biedendieck *et al.*, 2021). The strain's ability to maintain polymer production across a broad range (25–40 °C) is advantageous for industrial fermentation where temperature fluctuations are common (Trego *et al.*, 2024). Following the OFAT experiments, a four-factor, five-level Central Composite Design was implemented to capture synergistic effects. The quadratic models for both substrates were statistically robust. For banana peel, the model was highly significant ($F = 5.78$, $p = 0.0009$) with an R^2 of 0.84; for wheat bran, $F = 14.92$, $p = 0.0003$, $R^2 = 0.783$. Non-significant lack-of-fit ($p > 0.05$) confirmed that both models adequately represent the experimental data without systematic error (Kumar *et al.*, 2020). Adequate precision ratios (9.01 for banana peel, 12.84 for wheat bran) exceeded the threshold of 4, indicating excellent signal-to-noise ratios. For wheat bran, the adjusted R^2 (0.778) and predicted R^2 (0.759) were in close agreement (difference < 0.02), satisfying RSM validation criteria and confirming that the model is not overfit (Singh and Mallick, 2017).

Regression analysis revealed that substrate concentration and pH were the most influential linear factors. Quadratic terms for these variables were significant ($p < 0.05$), indicating curvature in the response surfaces and confirming that the optima lie within the experimental domain rather than at the

boundaries. Important interactions were identified: for wheat bran, substrate concentration $\times$ incubation time ($p = 0.0005$) and substrate concentration $\times$ pH ($p = 0.0011$) were particularly strong. These interactions would have been missed by OFAT alone, highlighting the necessity of RSM for multi-factor optimisation (Kumar *et al.*, 2020; Mohanrasu *et al.*, 2020). For banana peel, the interaction between substrate concentration and pH was also significant ($p = 0.0238$) but less pronounced, possibly due to the buffering capacity of banana peel hydrolysate (Pandey *et al.*, 2024). The concave response surfaces visually confirm that yields increase up to optimal levels ($\approx 35^\circ\text{C}$, 8–10 g/L substrate), after which further increases cause decline.

Predicted optimal yields were validated experimentally: banana peel reached 225.8 mg/L (within 5 % of prediction), and wheat bran reached 367.47 mg/L. The improvement over OFAT (approximately 1.2- to 1.6-fold) is within the range commonly reported for RSM optimization of PHA production – for example, a 42.5 % increase for *Burkholderia* sp. ISTR5 (Morya *et al.*, 2021) and a 1.35-fold improvement for *B. megaterium* pPSPHAR1/1 (Pham *et al.*, 2022). These gains, while moderate, demonstrate the value of statistically capturing factor interactions and curvature.

Characterization by FTIR and GC-MS confirmed that the polymer produced on both substrates consisted of 3-hydroxybutyrate and 3-hydroxyvalerate monomers – i.e., a PHBV copolymer. The presence of 3HV is notable because PHBV has superior flexibility, impact resistance, and thermal properties compared to PHB homopolymer (Wang *et al.*, 2013; Ray and Kalia, 2017). Importantly, unlike many previous studies that required external precursors such as levulinic acid to induce 3HV incorporation (Keenan *et al.*, 2004; Wang *et al.*, 2013), *P. megaterium* SMI2 synthesized PHBV directly from both hydrolysates without supplementation. This suggests that wheat bran and banana peel naturally contain fatty acid metabolites or other precursors that can be channeled into 3HV synthesis – a strain-specific trait worthy

of further metabolic investigation (Sehgal *et al.*, 2023).

The soil burial test showed up to 72.5 % weight loss after 60 days, indicating that the produced PHBV is readily degraded by natural soil microbial communities. This rate is comparable to or faster than previously reported PHA degradation in agricultural soils (Adamu and Salisu, 2024). The degradation kinetics followed a typical two-phase pattern: an initial slower phase (0–14 days) corresponding to surface colonization, followed by a faster weight loss phase (14–45 days) due to enzymatic hydrolysis of ester bonds by microbial depolymerases (Ray and Kalia, 2017). After 60 days, the remaining film residue consisted mainly of more crystalline regions, which are less susceptible to hydrolysis.

A limitation of this study is that the optimizations were performed at shake-flask scale, and the yields (367 mg/L) remain below industrial targets (typically >10 g/L). Nevertheless, the statistical models provide a rational basis for scale-up, in which fed-batch strategies and precise pH and temperature control could substantially enhance titres (Koller *et al.*, 2017). Moreover, the exact 3HV molar ratio was not quantified; future work should determine this ratio to tailor material properties. Gene expression analysis of *phaC*, *phaB*, and *phaR* could further elucidate regulatory mechanisms. Life-cycle assessment and techno-economic analysis would also help confirm the cost-effectiveness of wheat bran versus banana peel at an industrial scale.

Conclusion

Priestia megaterium SMI2 effectively converts wheat bran and banana peel hydrolysates into the biodegradable copolymer PHBV without the need for external 3-hydroxyvalerate precursors. A two-stage optimisation approach (OFAT followed by response surface methodology) successfully identified key interactions among process variables, leading to enhanced polymer yields. The produced PHBV is readily biodegradable in soil, supporting its environmental compatibility. This work demonstrates that locally isolated *P.*

megaterium SMI2 can valorise common agricultural residues into a valuable bioplastic, offering a cost-effective and sustainable alternative to petroleum-based polymers. Future scale-up studies using bioreactors and further characterisation of polymer properties will help realise its industrial potential.

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