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Optimization of Process Parameters for Production of Poly(3-hydroxybutyrate) by *Bacillus pumilus* AHSD 04, a Seed Borne Endophyte of Oleaginous Plant Arachis hypogaea L.

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Abstract: Endophytic bacteria, which thrive inside the plants, synthesize and accumulate intracellular biopolymer polyhydroxyalkanoates (PHAs) to strive against the hostile plant environment. The present study is focused on enhancing the production of poly(3-hydroxybutyrate) [P(3HB)], the most common PHA by the seed endophytic bacterium Bacillus pumilus AHSD 04 (GenBank accession number KY038573; MCC accession number 3573) isolated from the oleaginous plant Arachis hypogaea L. Interactions among the three most important influencing variables, glucose, tryptose, and initial pH affecting growth and P(3HB) production were studied using the central composite design (CCD) of response surface methodology (RSM). A 3.94-fold enhancement of P(3HB) production (5.36 g/L) was achieved over that of the 'one variable at a time' method. The isolate also produced co-polymers of 3hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) [P(3HB-co-3HV)] with the incorporation of 4.98 mol% 3HV during dual-step cultivation using glucose and valeric acid as co-substrate. The polymers, P(3HB) and P(3HB-co-3HV), so produced have been validated and characterized by Fourier transform infrared (FTIR) and ¹H nuclear magnetic resonance (¹H NMR) spectroscopic analysis. It is apparent that RSM has been successfully established as a promising tool to increase P(3HB) yield by the seed endophyte B. pumilus AHSD 04, which can further be utilized to scale up the production of these biodegradable polymers of industrial importance.

Keywords: Poly(3-hydroxybutyrate); poly(3-hydroxybutyrate-co-3-hydroxyvalerate); response surface methodology; *Bacillus pumilus*; *Arachis hypogaea* L.

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1. Introduction

Plants as multicellular hosts provide distinct ecological niches for diverse microorganisms. Endophytes inhabiting the internal tissues of the plants are an essential component of the plant microbiota [1]. Being present inside the plants, endophytes influence the development of the host plant significantly [2]. These endogenously associated microorganisms have also been recognized as chemical synthesizers inside the plants and critically evaluated for their ability to produce an innumerable number of unique chemical

compounds [3, 4]. Polyhydroxyalkanoates (PHAs), the polyesters of *R*-hydroxyalkanoic acids, are often synthesized and accumulated intracellularly by a wide variety of bacteria and archaea. These intracellular biopolymers serve as carbon and energy reserves and contribute to bacteria's survival in competitive and stressful environments [5]. The endosphere-associated bacterial communities have also been well explored for biosynthesis and intracellular accumulation of PHAs [6, 7]. The PHA biopolymers contribute to colonization and endow survival of the endophytic bacterial communities in the hostile microenvironment inside the host plants [8, 9]. Recently, Alves *et al.* [10] have established that the plant growth promotion by the diazotrophic endophytic bacterium *Herbaspirillum seropedicae* is dependent on PHA metabolism. Moreover, the lack of PHAs in mutant strains of *H. seropedicae* altered the redox balance and increased oxidative stress that significantly affected the expression of several genes [11].

That the internal environments of oleaginous plants provide unique ecological habitats for the growth and sustenance of PHA-accumulating bacteria [12] has been well justified from our laboratory [13, 14]. The peanut plant (*Arachis hypogaea* L.), the fourth most important edible oil source of the world [15] has been investigated quite extensively and recognized as a treasured microbial repository [16, 17]. The seed endophytes, in particular, have gained significant interest as they are transmitted vertically and ensure their presence in progeny plants [18, 19]. While Sobolev *et al.* [20] have demonstrated the predominance of members of the genus *Bacillus* as peanut seed endophytes, the antagonistic activity of *Bacillus velezensis*, a peanut seed borne endophyte, have been documented by Chen *et al.* [21].

Despite the fact that the endophytic *Bacillus* spp. are excellent plant growth promoters [22, 23] and biocontrol agents [24], their efficacy towards biosynthesis and accumulation of intracellular PHAs have rarely been explored [25]. On the contrary, non-endophytic strains of Bacillus spp. have been recognized as efficient PHA producers [26, 27]. The PHAs produced by Bacillus spp., in general, are free from endotoxins and hence preferred for biomedical applications. However, until now, commercial production of PHAs by *Bacillus* spp. has met with limited success mainly due to the negative impact of the process of sporulation [28]. While a few Bacillus spp. accumulate poly(3-hydroxybutyrate) [P(3HB)], one of the most common PHAs during the exponential phase of growth; others accumulate the polymer during the late exponential to stationary phase [29]. The P(3HB) so accumulated was found to be degraded and utilized at the onset of sporulation [14]. Valappil et al. [30] showed that acidic pH of the medium was responsible for the repression of sporogenesis in *Bacillus cereus* SPV and thereby enhanced biosynthesis and accumulation of P(3HB). Hence, critical evaluation and optimization of fermentation medium and process parameters might promote P(3HB) production even at the instance of sporogenesis in Bacillus spp. The parameters such as carbon, nitrogen source, and pH of the medium have been reported to exert remarkable influence on the production and accumulation of P(3HB) [31]. Response surface methodology (RSM) has been established as an efficient statistical tool for analyzing the interactive effect of process parameters involved in fermentation seeking optimized conditions for improved product yield [32, 33].

The endophytic existence of *Bacillus pumilus* is not new and has already been documented in various plant species [23, 34, 35]; however, intracellular accumulation of P(3HB) in such endogenously associated *B. pumilus* strains is of rare occurrence [36]. We, from this laboratory, have reported the isolation of a new endophytic strain designated as *Bacillus pumilus* AHSD 04 from seeds of oleaginous plant *Arachis hypogaea* L., and have documented the biosynthesis and intracellular agglomeration of P(3HB) by this strain under

batch cultivation [37]. The present study is an attempt to optimize the physicochemical process parameters and nutritional factors for P(3HB) production by the endophytic B. pumilus AHSD 04 following response surface methodology (RSM). Furthermore, the production of a copolymer of 3-hydroxybutyrate and 3-hydroxy valerate [P(3HB-co-3HV)] by the strain has also been achieved following the dual-step cultivation process.

2. Materials and Methods

2.1. Bacterial strain and maintenance.

Bacillus pumilus AHSD 04 (GenBank accession number KY038573; MCC accession number 3573), a seed-borne endophyte of oleaginous plant Arachis hypogaea L., isolated and reported from this laboratory [37] was used throughout the present study. The endophytic isolate was grown on the slopes of tryptic soy agar at 32 °C for 24 h and maintained on the same medium by sub-culturing at a regular interval of one month.

2.2. Culture condition.

The endophytic bacterial isolate AHSD 04 was grown in modified mineral salts (MS) medium [38] supplemented with tryptose for growth and P(3HB) production. The medium (20 mL/100 mL flask) was inoculated (at 1% level) with freshly grown culture and incubated at 32 °C on a rotary shaker (120 rpm) for 72 h.

2.3. Estimation of growth.

For estimation of growth, the cell mass from the growing culture was harvested by centrifugation (12000 $\times g$ for 10 min), washed thrice with deionized water, and subsequently, the dry weight was determined following drying to a constant weight at 80 °C.

2.4. Quantification of P(3HB).

The intracellular P(3HB) was extracted from the dried cell mass (2 mg) with warm chloroform (40-45 °C) and quantified according to the crotonic acid assay method [39]. The polymer extracted from the dried cell mass was transformed to crotonic acid succeeding treatment with H₂SO₄ in a boiling water bath for 10 min, cooled to room temperature, and subjected to UV spectral analysis. The absorbance of the treated polymer was scanned in the range of 200-300 nm in a UV-VIS spectrophotometer (Jenway, Model 6505) using analytical grade chloroform as the blank, and the absorbance at 235 nm was determined. The P(3HB) content of the cell mass was determined from the calibration curve prepared according to the same method using the original P(3HB) from Sigma-Aldrich (USA); the total PHA was determined by the gravimetric method.

2.5. Extraction and purification of the polymer.

The intracellularly accumulated polymer was extracted from the thoroughly washed and dried cell mass with chloroform at 40-45 °C for 2-4 h, and the process was repeated thrice. The chloroform extracts were pooled, filtered through glass wool, concentrated under reduced pressure, and precipitated the crude polymer with double volumes of pre-chilled diethyl ether. The precipitate was collected by centrifugation $(16,000 \times g \text{ for } 12 \text{ min})$, washed with acetone and ethanol, re-dissolved in chloroform, and the pure polymer was recovered following https://biointerfaceresearch.com/

precipitation with chilled diethyl ether. Finally, the precipitate was obtained by centrifugation $(16,000 \times g \text{ for } 12 \text{ min})$, air-dried, and the white powdery mass was stored in a desiccator for further use.

2.6. Process optimization for P(3HB) production.

2.6.1. Experimental design.

The Central composite design (CCD) of response surface methodology (RSM) was principally followed to determine the optimum levels and elucidate the interaction among the independent variables viz. carbon source (glucose), nitrogen source (tryptose), and initial pH of the medium, which significantly influence the growth and P(3HB) production. The values of the remaining variables (incubation time, temperature, inoculum volume, agitation, etc.) were maintained at a constant level. The CCD experiments were formulated in 20 trial runs using the Design-Expert software (version 13.0.1.0) (Stat-Ease, Inc. MN, USA). The three independent variables (glucose, tryptose and pH) were used at five coded levels (- α , -1, 0, +1, + α) (α = 1.682) including six axial points, eight factorial points and six central points. All the experiments based on the CCD were performed in triplicate.

Real levels of the independent variables were coded according to the following equation:

$$Z = (Z_i - Z_0) / \Delta Z \tag{1}$$

where, Z is the dimensionless coded value, and Zi is the real value of the independent variable; Z_0 indicates the value of the independent variable at the central point, and ΔZ represents the step change value.

The data obtained from the design were analyzed by multiple regression analysis using the following second-order polynomial equation (2) for two response variables [growth and P(3HB) production]:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$
(2)

where, Y represents the predictive response variable, X_1 , X_2 , X_3 are the coded values of three independent variables, i.e., glucose, tryptose, and pH, respectively, β_1 , β_2 , and β_3 are linear coefficients, β_{12} , β_{13} , and β_{23} are interactive cross-product coefficients, β_{11} , β_{22} , and β_{33} are quadratic coefficients, and β_0 is the constant.

2.6.2. Statistical analysis.

Interaction between the independent variables and their effect on growth (Y₁) as well as PHA production (Y₂) were inferred from the three-dimensional response surface plots. These plots were prepared by plotting the individual responses [growth and P(3HB) production] on the Z-axis against any two selected independent variables within their experimental range while holding the other independent variables at a constant level. Validation of the statistical model was executed using the point prediction tool of RSM. To validate the model and rationalize the predicted values of the response variables, a test in triplicate was performed using the optimum estimates of independent variables. Statistical analysis and consistency of the model were evaluated by analysis of variance (ANOVA). The statistical significance of the model equation and the model terms were determined by Fischer's F test. The F-value was checked to determine the significance of the fitted equations and terms at a 5% level and represented as a p-value (p < 0.05). The adequacy of the model was checked by the coefficient of determination https://biointerfaceresearch.com/ (R^2) as well as adjusted R^2 . The statistical accuracy of the model was resolved through the lack of fit (LOF) assessment.

2.7. Dual-step cultivation for co-polymer production.

A dual-step cultivation methodology [6] has opted for the synthesis of co-polymers. Initially, the endophytic isolate was grown in a modified mineral salts medium containing glucose (37.7 g/L) and tryptose (4.3 g/L) under continuous shaking at 32 °C. Cells from actively growing culture (24 h old) were collected aseptically by centrifugation $(12,000 \times g$ for 10 min) at 4 °C, washed with sterile normal saline, and immediately transferred to the same fresh medium containing valeric acid (0.05 - 0.5%, w/v) as co-substrate and incubated under continuous shaking (120 rpm) at 32 °C for 48 h.

2.8. Characterization of polymers.

2.8.1. Fourier transform infrared spectroscopy.

The extracted and purified homo- and co-polymers (5 mg) were thoroughly mixed with spectroscopic grade KBr (100 mg) individually and pelletized. The functional groups of the isolated polymers were analyzed by Fourier transform infrared (FTIR) spectroscopy using a Bruker FTIR spectrophotometer in the range of 4000 to 400 cm⁻¹.

2.8.2. Nuclear magnetic resonance spectroscopy.

The chemical structure of the isolated homo- and co-polymers was interpreted based on proton nuclear magnetic resonance (¹H NMR) spectroscopic analysis. The polymer (5 mg) samples were dissolved in x deuterated chloroform (1 mL) and subjected to analysis in a Bruker ¹H NMR (300 MHz) spectrophotometer. A multinucleate probe head at a 30-degree flip angle was used. The chemical shifts were represented on δ scale [parts per million (ppm)], and tetramethylsilane (Me₄Si) was used as the internal standard.

3. Results and Discussion

The seed-borne endophytic bacterium *B. pumilus* AHSD 04 produced P(3HB), accounting 52.3% of its cell dry weight (CDW) when grown under batch cultivation in glucose (2%, w/v) containing mineral salts medium. Production of P(3HB) was further enhanced (55.2%) by supplementation of tryptose (0.2%, w/v) and maintenance of the growth medium at pH 7.2 [37]. During the present optimization study, the influencing factors such as carbon (glucose), nitrogen source (tryptose) as well as pH of the medium have been prudently chosen, keeping the values of remaining factors (incubation time, temperature, inoculum volume, agitation, etc.) at a constant level.

3.1. Optimization of P(3HB) production by response surface methodology.

3.1.1. Fitting the model.

A three-variable-five-level design of CCD was employed to determine the optimum conditions of independent variables and their interactive effect for growth (Y_1) as well as P(3HB) production (Y_2) . Glucose (X_1) , tryptose (X_2) and initial pH (X_3) of the medium were

selected as the independent variables and used at five coded levels $(-\alpha, -1, 0, +1, +\alpha)$ ($\alpha = 1.682$) (Table 1).

Table 1. Independent variables and their corresponding coded levels for growth and P(3HB) production by *B.pumilus* AHSD 04 based on central composite design (CCD) of response surface methodology (RSM).

Independent variable	Symbol	Coded levels				
		-α	-1	0	+1	+α
Glucose (g/L)	X_1	13.18	20	30	40	46.82
Tryptose (g/L)	X ₂	0.64	2	4	6	7.36
pH	X ₃	5.7	6	6.5	7	7.3

The experimental results of growth and P(3HB) production based on CCD model of RSM were presented in Table 2. Coefficients of the polynomial equation were computed from the experimental data to predict the values of the response variables (Y_1 and Y_2). Regression equations for each response variable obtained from RSM are as follows:

$$Y_{1} = 6.664 + 0.843X_{1} + 0.794X_{2} + 0.716X_{3} - 0.35X_{1}X_{2} + 0.15X_{1}X_{3} + 0.3X_{2}X_{3} - 0.821X_{1}^{2} - 0.927X_{2}^{2} - 0.645X_{3}^{2}$$
(3)

$$Y_{2} = 74.77 + 11.415X_{1} + 2.593X_{2} + 1.719X_{3} + 0.638X_{1}X_{2} - 1.463X_{1}X_{3} - 2.838X_{2}X_{3} - 9.776X_{1}^{2} - 7.813X_{2}^{2} - 3.695X_{3}^{2}$$
(4)

where, X_1 , X_2 , X_3 are the coded values of three independent variables glucose, tryptose, and pH, respectively.

The predicted values for growth as well as P(3HB) production were calculated using the regression analysis and correlated with the experimental data, which revealed that the actual response values were in good agreement with the predicted response values. The predicted versus actuals plots for both the responses (Y₁ and Y₂) are represented in Figure 1a and 1b, respectively.



Figure 1. Predicted vs actual plot for (a) growth and (b) P(3HB) production by B. pumilus AHSD 04.

 Table 2. Experimental design based on central composite design (CCD) of response surface methodology (RSM) and observed responses for growth and P(3HB) production by *B. pumilus* AHSD 04.

Run order	Independent variables			Responses		
	Glucose (g/L)	Tryptose (g/L)	рН	Growth, CDW, g/L	P(3HB), % CDW	
1	40	6	6	4.2 ± 0.3	69.5 ± 2.1	
2	30	0.64	6.5	2.5 ± 0.2	46.7 ± 1.3	
3	40	2	7	5.1 ±0.2	63.2 ± 1.8	
4	20	6	7	5.4 ± 0.2	42.9 ± 0.8	
5	30	4	6.5	6.2 ± 0.4	68.5 ± 1.7	
6	20	2	6	2.2 ± 0.2	35.4 ± 2.2	
7	46.82	4	6.5	6 ± 0.1	67.7 ± 0.8	
8	30	7.36	6.5	5.5 ± 0.1	58.3 ± 2.4	
9	30	4	5.7	3.5 ± 0.3	59.4 ± 0.5	

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Run order	Independent variables			Responses		
	Glucose (g/L)	Tryptose (g/L)	pH	Growth, CDW, g/L	P(3HB), % CDW	
10	13.18	4	6.5	2.6 ± 0.4	26.2 ± 0.8	
11	40	2	6	4.2 ± 0.1	60.3 ± 1.2	
12	20	2	7	2.8 ± 0.1	47.6 ± 1.6	
13	20	6	6	3.9 ± 0.5	45.5 ± 1.1	
14	40	6	7	6.6 ± 0.4	64.5 ± 0.9	
15	30	4	7.3	6.1 ± 0.2	68.9 ± 1.0	
16	30	4	6.5	6.8 ± 0.1	76.8 ± 0.4	
17	30	4	6.5	6.6 ± 0.2	76.2 ± 0.8	
18	30	4	6.5	6.9 ± 0.1	74.9 ± 1.1	
19	30	4	6.5	6.7 ± 0.1	75.8 ± 1.2	
20	30	4	6.5	6.8 ± 0.1	76.5 ± 0.5	

Each experimental value represents the mean of 3 replicates \pm SD.

The significance of the model for growth as well as P(3HB) production was evaluated following the execution of analysis of variance (ANOVA) (Table 3). The ANOVA of both the regression models confirmed their significance ($F_{model} > 1$, significant) [40] for analyzing the interactive effect of independent variables for biomass formation (Y₁) and P(3HB) production (Y₂). The *F* model values were 73.31 and 55.22 for growth and PHA production, respectively, as calculated from the Fisher's *F*-test. The lack of fit was non-significant ($p \le 0.05$) relative to pure error for both the response variables (Y₁ and Y₂), which indicated that the models were statistically accurate and sufficient to predict the response variables under any combination of values.

The coefficient of determination (\mathbb{R}^2) for Y_1 and Y_2 were recorded as 0.9851 and 0.9803, respectively. The \mathbb{R}^2 is the measure of goodness of fit, and the value varies between 0 and 1. The closer the \mathbb{R}^2 to unity, the stronger the model and better it predicts the response [41]. On the other end, lower \mathbb{R}^2 values indicate that the response variables are not appropriate to explain the variation. The \mathbb{R}^2 values in the present study demonstrated that both the models could explain more than 98% variability in biomass (Y_1) and P(3HB) (Y_2) production. The adjusted \mathbb{R}^2 , which corrects the \mathbb{R}^2 value for the sample size and the number of terms, were 0.9716 and 0.9625 for Y_1 and Y_2 , respectively.

Source	Sum of squares	Degree of	Mean square	<i>F</i> -value	<i>p</i> -value	
		freedom				
Model	50.81	9	5.65	73.31	< 0.0001***	
X_1	9.71	1	9.71	126.14	< 0.0001***	
X_2	8.61	1	8.61	111.83	< 0.0001***	
X_3	6.99	1	6.99	90.81	< 0.0001***	
$X_1 X_2$	0.9800	1	0.9800	12.73	0.0051***	
X1 X3	0.1800	1	0.1800	2.34	0.1573	
$X_2 X_3$	0.7200	1	0.7200	9.35	0.0121**	
X_1^2	9.72	1	9.72	126.25	< 0.0001***	
X_2^2	12.40	1	12.40	160.96	< 0.0001***	
X_{3}^{2}	5.99	1	5.99	77.75	< 0.0001***	
Residual	0.7701	10	0.0770			
^a Lack of fit	0.4568	5	0.0914	1.46	0.3446	
Pure error	0.3133	5	0.0627			
Cor Total	51.58	19				
P(3HB) production (%, CDW) (Y ₂)						
Model	4120.27	9	457.81	55.22	< 0.0001***	
X1	1779.55	1	1779.55	214.66	< 0.0001***	
X_2	91.81	1	91.81	11.07	0.0076***	
X ₃	40.36	1	40.36	4.87	0.0519	
$X_1 X_2$	3.25	1	3.25	0.3922	0.5452	
X ₁ X ₃	17.11	1	17.11	2.06	0.1813	

Table 3. Analysis of variance (ANOVA) based on quadratic polynomial model. **Growth** $CDW(q/L)(Y_1)$

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Growth, CDW (g/L) (Y ₁)					
Source	Sum of squares	Degree of freedom	Mean square	<i>F</i> -value	<i>p</i> -value
$X_2 X_3$	64.41	1	64.41	7.77	0.0192**
X_{1}^{2}	1377.20	1	1377.20	166.13	< 0.0001***
X_2^2	879.81	1	879.81	106.13	< 0.0001***
X_3^2	196.71	1	196.71	23.73	0.0007***
Residual	82.90	10	8.29		
^b Lack of fit	33.35	5	6.67	0.6731	0.6627
Pure error	49.55	5	9.91		
Cor Total	4203.17	19			

X₁, X₂, X₃ are the three independent variables glucose, tryptose, and pH, respectively. *** Highly significant; ** Significant at 95% probability level a, bThe lack of fit F-value of 1.46 and 0.67 for Y₁ and Y₂, respectively,

implies the lack of fit is not significant relative to the pure error. The coefficient of determination (R^2) values for Y_1 and Y_2 are 0.9851 and 0.9803, respectively. The adjusted R^2 (Adj R^2) are 0.9716 and 0.9625 for Y_1 and Y_2 ,

respectively.

3.1.2. Effect of independent variables on response variables.

The *p* values indicate the significance of each coefficient, and the corresponding coefficient is considered to be more significant if the inferred magnitude of *p* is small. The model indicated that the linear (X₁, X₂, X₃), interactive (X₁X₂, X₂X₃), and quadratic (X₁², X₂², X₃²) terms are significant (*p* < 0.05) for growth (Y₁). Similarly, for P(3HB) production, the linear (X₁, X₂), interactive (X₂X₃), and quadratic (X₁², X₂², X₃²) terms imparted significant effect (Table 3).

To determine the optimal levels of the interacting variables (glucose, tryptose and pH) for maximum response, three-dimensional response surface plots (Figure 2) were constructed by plotting the individual responses [growth (Y_1) and P(3HB) production (Y_2)] on the Z-axis against any two selected independent variables within their experimental range while the other variables were kept at a constant level. These 3D surface plots illustrated complex interactions among the independent variables. The interactive effect of glucose and tryptose (Figure 2a) showed the most significant influence on growth as compared to the interactions between glucose and pH (Figure 2b). Response plots (Figure 2c) representing the interactive effect of the pH and tryptose also positively impacted the biomass formation. Conversely, the interactive effect of the pH and tryptose (Figure 2f) imparted the most positive impact on P(3HB) production as compared to the interactive effect of glucose and tryptose (Figure 2d) as well as glucose and pH (Figure 2e). Response plot (Figure 2f) representing the interactive effect of the pH and tryptose clearly indicated that polymer accumulation was maximum with 4.3 g/L tryptose and an initial pH of 6.8 and further increase of tryptose exerted a negative impact on P(3HB) production, which is represented by the convergence of the curve towards the boundary of the 3D plot.

3.1.3. Optimization of independent variables.

Optimization of independent variables was executed by desirability function approach using the Design Expert Software. The desirability value (*d*) varies in the interval $0 \le d \le 1$ and increases as the desirability of the corresponding response increases. Combined optimized conditions for growth as well as P(3HB) production were recorded as 37.7 g/L for glucose, 4.3 g/L for tryptose, and an initial pH of 6.8.



Figure 2. Three-dimensional response surface plots showing the interactive effect of (a) glucose and tryptose, (b) glucose and pH, (c) tryptose and pH on growth as well as (d) glucose and tryptose, (e) glucose and pH, (f) tryptose and pH on P(3HB) production by *B. pumilus* AHSD 04.

3.1.4. Validation and confirmation of the model.

Validation of the statistical model was executed using the point prediction tool of RSM where the optimum values of all three independent variables, i.e., glucose (37.7 g/L), tryptose (4.3 g/L), and initial pH (6.8), were employed for experimentation (Table 4). The experimental values for growth (Y₁) and P(3HB) production (Y₂) (mean of 3 replicates) were compared with the corresponding predicted values. The actual biomass production (7.0 g/L) under optimized conditions was very close to the predicted value (7.2 g/L). Likewise, the experimental and predicted values for P(3HB) production were 76.5% and 77.2% of CDW, respectively. Such closeness of values indicated both validity and suitability of the model.

'One variable at a time' (OVAT) method of optimization involves testing a single variable at a time, keeping the other variables constant. Following OVAT methodology, the isolate AHSD 04 produced biomass of 2.55 g/L with P(3HB) content of 53.2%, CDW when glucose and tryptose were used as carbon and nitrogen sources, respectively [37]. The conventional single factor system of analysis is time-consuming, especially for many variables, and does not ensure the determination of exact optimal conditions. As an alternative, experimental designs based on statistical models have gained importance as economical and practical solutions for process optimization of various biomaterial productions. The response surface methodology (RSM) has been implemented as a powerful device to improve the production of P(3HB) by a wide variety of bacteria [27, 41]. In this study, the central composite design (CCD) was applied for determining the optimum levels of three independent variables glucose, tryptose, and initial pH of the medium for growth and P(3HB) production. The maximum growth (7.0 g/L) and P(3HB) production (76.5%, CDW) were achieved under the optimized conditions of glucose (37.7 g/L), tryptose (4.3 g/L), and at initial pH of 6.8. It was apparent that statistical method-based experimental design has enhanced the P(3HB) yield by 3.94 fold (1.36 to 5.36 g/L) as compared to conventional OVAT methodology [37].

Independent variable	Optimum conditions	Optimum conditions			
	Coded level	Actual Level			
Glucose (g/L)	0.39	37.7			
Tryptose (g/L)	0.08	4.3			
рН	0.3	6.8			
Response	Predicted value	Experimental value			
Growth, CDW (g/L)	7.2	7.0 ± 0.1			
P(3HB), % CDW	77.2	76.5 ± 1.4			

 Table 4. Optimum values of the independent variables and confirmatory trials for the predicted responses under optimal conditions.

Each experimental value represents the mean of 3 replicates \pm SD

The coded levels of the independent variables were calculated as: $Z=(Z_i - Z_0)/\Delta Z$, where Z is the coded value and Zi is the real value; Z_0 indicates the value of the independent variable at the central point, and ΔZ represents the step change value

The intracellular accumulation of P(3HB) by the isolate *B. pumilus* AHSD 04 supported earlier observations of biopolymer accumulation by diverse endophytic bacterial species derived from multiple plant sources [42-44]. While reports on P(3HB) production by endophytic *B. pumilus* strains appear to be rare, the potential of non-endophytic *B. pumilus* strains to produce the biopolymer has been well recognized [45, 46]. The results obtained from this study appeared to be quite satisfactory when the P(3HB) yield (5.36 g/L) by endophytic *B. pumilus* AHSD 04 was compared with other non-endophytic strains of *B. pumilus*. The bacterium *B. pumilus* H9, isolated from municipal wastes, accumulated 2.47 g/L of P(3HB)

following optimization by a four-factor central composite design of RSM [45]. Similarly, *B. pumilus* E10 derived from the urban wastewater yielded approximately 6 mg of P(3HB) following consumption of 2.5 g/L glucose [46]. Furthermore, these findings also corroborated the P(3HB) accumulation in *B. megaterium* [47] and *B. mycoides* [26], where organic nitrogen source (protease peptone) was found to enhance the intracellular biopolymer production.

3.2. Production of co-polymers.

As against the production of P(3HB) by Bacillus spp., synthesis and accumulation of PHA co-polymers are restricted to few selective species only [48, 49]. It is well established that the addition of alkanoic acids as co-substrate during dual step cultivation triggers the synthesis of co-polymers [6]. In this study, a dual-step cultivation method was adopted for the production of co-polymers by the endophytic isolate B. pumilus AHSD 04. Cells from exponentially growing culture of AHSD 04 in mineral salts medium containing glucose (37.7 g/L) and tryptose (4.3 g/L), were harvested aseptically, washed in sterile saline, and used to inoculate the same fresh medium with additional supplementation of valeric acid as cosubstrate. After 48 h of growth, the intracellular accumulation of polymers showed incorporation of 3-hydroxyvalerate (3HV) in the PHA chain leading to the production of copolymers of 3HB and 3HV [P(3HB-co-3HV)]. Variation in concentration of valeric acid (0.05-0.5% w/v) revealed maximum biomass (6.4 g/L) and PHA production (76.1%, CDW) at the lowest concentration of valeric acid (0.05%) tested. The P(3HB-co-3HV) so produced showed incorporation of only 2.52 mol% 3HV (Table 5). On the contrary, growth (5 g/L), as well as co-polymer production (65.6%, CDW), were comparatively low at 0.3% valeric acid, but the 3HV incorporation in the co-polymer appeared to be maximum (4.98 mol%). Further increase in valeric acid concentration showed a prominent negative impact on growth, PHA accumulation as well as 3HV incorporation. As far as we are aware, this appears to be the first report of co-polymer accumulation by *B. pumilus* strain.

Valeric acid,	Growth,	РНА,	PHA compos	ition, mol % ^d
% (₩/v) ^a	CDW (g/L) ^b	% CDW ^c	3HB	3HV
0	6.9 ± 0.2	76.1 ± 2.4	100	0
0.05	6.4 ± 0.7	74.5 ± 1.2	97.48	2.52
0.1	5.7 ± 0.4	69.8 ± 1.9	96.90	3.10
0.2	5.2 ± 0.2	68.3 ± 0.8	95.80	4.20
0.3	5.0 ± 0.3	65.6 ± 1.7	95.02	4.98
0.4	3.8 ± 0.6	39.5 ± 1.1	98.50	1.50
0.5	1.7 ± 0.5	17.4 ± 3.2	98.80	1.20

 Table 5. Influence of valeric acid as co-substrate on growth, production and composition of PHA accumulated by the endophytic bacterium *B. pumilus* AHSD 04 during dual-step cultivation.

^aValeric acid of different concentrations was added individually to the glucose (37.7 g/L) and tryptose (4.3 g/L) containing MS medium under dual-step cultivation

^bGrowth was determined by measuring the cell dry weight (CDW)

^cP(3HB) was determined by Law and Slepecky [39] method, and PHA content was quantified gravimetrically ^dThe mol % of monomers were calculated following ¹H NMR analysis of the purified samples Results represent mean of triplicate readings \pm SD

3.3. Spectroscopic analysis of accumulated polymers.

3.3.1. Fourier transform infrared spectroscopic analysis.

The FTIR spectra of the purified P(3HB) (Figure 3a) revealed distinct absorption spectra for esters; -OH bending at 3429 cm⁻¹, C-H stretching at 2930-2980 cm⁻¹, the absorption

band of aliphatic carbonyl C=O at 1729 cm⁻¹ and –CH group of the aliphatic compound at 1228-1383 cm⁻¹. Likewise, the usual banding patterns at 1728 cm⁻¹ and 1286 cm⁻¹ for copolymer P(3HB-co-3HV) correspond to C=O and C-O, respectively (Figure 3b). The other typical bands appearing at 3432 cm⁻¹, 2930-2980 cm⁻¹, and 1228-1386 cm⁻¹, were attributed to the –OH bending, C-H stretching, and –CH group, respectively. However, the existence of HV in the co-polymer could not be definitively distinguished from HB by FTIR spectroscopic analysis [50].



Figure 3. FTIR spectral analysis of purified (a) P(3HB) and (b) P(3HB-co-3HV) isolated from *B. pumilus* AHSD 04.

3.3.2. Nuclear magnetic resonance spectroscopic analysis.

The monomeric composition of the polymers produced by *B. pumilus* AHSD 04 was resolved by ¹H NMR (at 300 MHz) spectral analysis. The characteristic spectrum (Figure 4a) of the purified P(3HB) exhibited distinct chemical shifts (δ) at 1.2, 2.4- 2.6, and 5.2 ppm, which corresponds to the methyl group (CH₃) coupled to one proton, methylene group (CH₂) adjacent to an asymmetric carbon atom bearing a single proton and methylene group (CH), respectively and confirmed the chemical structure of homopolymeric P(3HB) [46]. On the other hand, the typical peaks at 1.2 and 0.9 ppm as displayed in ¹H NMR spectrum of P(3HB-co-3HV) confirmed the presence of the methyl (CH₃) group of 3HB (95 mol%) and 3HV (4.98 mol%)

monomers, respectively (Figure 4b). That the valerate was a monomeric constituent of the copolymer was confirmed by the existence of triplet at 0.9 ppm [49].



Figure 4. ¹H NMR spectra of (a) purified P(3HB) and (b) P(3HB-co-3HV) isolated from *B. pumilus* AHSD 04.

4. Conclusions

This study has unveiled the potential for PHA accumulation by the seed endophytic bacterium *B. pumilus* AHSD 04. It showed efficient biomass as well as P(3HB) production when the process variables were optimized following response surface methodology. Compared to the single factor system of analysis, optimization using statistical method (RSM) enhanced the P(3HB) yield by 3.94-fold. Furthermore, the ability of the endophytic isolate to accumulate [P(3HB-co-3HV)] co-polymer during dual-step cultivation appeared to be advantageous in terms of its material properties. A more detailed analysis of the accumulated polymers is essentially warranted for fruitful utilization of this endophytic microbial resource in the future.

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Conflicts of Interest

The authors declare no conflict of interest.

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