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A Statistical Approach for Optimization of Polyhydroxybutyrate Production by *Bacillus sphaericus* NCIM 5149 under Submerged Fermentation Using Central Composite Design

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Abstract The aim of this work was to statistically optimize the cultural and nutritional parameters for the production of polyhydroxybutyrate (PHB) under submerged fermentation using jackfruit seed hydrolysate as the sole carbon source. On the basis of results obtained from "one variable at a time" experiment, inoculum age, jackfruit seed hydrolysate concentration, and pH were selected for response surface methodology studies. A central composite design (CCD) was employed to get the optimum level of these three factors to maximize the PHB production. The CCD results predicted that jackfruit seed hydrolysates containing 2.5% reducing sugar, inoculum age of 18 h, and initial medium pH 6 could enhance the production of PHB to reach 49% of the biomass (biomass 4.5 g/l and PHB concentration 2.2 g/l). Analysis of variance exhibited a high coefficient of determination (R^2) value of 0.910 and 0.928 for biomass and PHB concentration, respectively, and ensured that the quadratic model with the experimental data was a satisfactory one. This is the first report on PHB production by *Bacillus sphaericus* using statistical experimental design and RSM in submerged fermentation with jackfruit seed hydrolysate as the sole source of carbon.

Keywords Central composite design · Response surface methodology · *Bacillus sphaericus* · Polyhydroxybutyrate · Jackfruit seed hydrolysate · Submerged fermentation

Abbreviations

PHB	Polyhydroxybutyrate
SmF	Submerged fermentation
CCD	Central composite design
RSM	Response surface methodology

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Introduction

Nowadays, the issue of plastic pollution is gaining much more attention by researchers as well as common people. Bioplastics such as polyhydroxyalkonates (PHAs) have come up to a solution of this problem because of their degradation property when exposed to environment. PHAs are a class of natural polyesters produced and accumulated by many bacterial genera of Gram-positive and Gram-negative. These polymers are isotactic, highly crystalline, and stiff; their transition temperature (Tg), melting temperature (Tm), Young's modulus, and tensile strengths, etc. can be compared to polypropylene [1]. Other properties like resistance to humidity, biocompatibility, piezoelectricity, and optical purity make these polymers suitable for specific applications [2]. Polyhydroxybutyrate (PHB) was first isolated and characterized by French microbiologist Maurice Lemoigne in 1923 [3]. These polymers are accumulated as intracellular granules, carbon and energy reserves of microorganisms, when the cell's surroundings contain a high carbon to nitrogen or phosphorous ratio. These granules function as energy supplier for sporulation in *Bacillus* [4] and as a part of bacterial Ca^{2+} channels [5]. Since PHAs are of bacterial origin, these polyesters are natural material, and thus many microorganisms have evolved the ability to degrade these macromolecules to harmless products. Bacteria and fungi have been reported in the biodegradation process; they acquire precursors for cell components and energy for their own biological requirements from this break down. PHAs can be completely degraded to carbon dioxide and water under aerobic bacterial action [6, 7].

However, higher production cost makes these bioplastics unrealistic to mankind. Commercially available bioplastic named Biopol, bacterial product from *Ralstonia eutropha*, is marketing about 17 times the price of synthetic plastics [8]. The PHB production cost evaluation has reported that the cost of carbon substrate (up to 50%) is the major contributor to the overall cost [9]. Hence, it is important to exploit cheap carbon sources to increase the PHB content and productivity.

The production cost of any biotechnological process can be considerably reduced by optimization of the process [10]. Optimization has a very old history and typical method involves changing one factor or varying several factors at the same time. The method of "one variable at a time" approach allows resolving the specific requirements for growth and product formation by systematically adding or deleting components from the medium, with minimal complicated medium interactions [11]. The use of organized statistical approach like response surface methodology (RSM), which use sequential experimental methods, are more reliable than unplanned experiments [12]. Central composite design (CCD) is the most accepted design among several classes of RSM, and it offers information into a great extent and reveals overall experiment error in a least number of runs [13]. RSM is a statistical method that uses quantitative data from appropriate experiments and simultaneously determines and solves multivariate equations. Unfortunately, statistical experiments are not widely used in the optimization of PHB production [14]. Some works have been reported for the use of RSM by different bacterial strains like *Rhodobacter sphaeroides* and *R. eutropha* for increased PHB production [10, 15]. Optimization of the fermentation medium for *Bacillus* sp. using corn steep liquor as a nitrogen source is reported earlier [16]. Increased PHB production using industrial byproducts by *Azotobacter beijerinckii* has been investigated through RSM [17]. The applications of these biodegradable materials have widened to almost all the fields including medicine, agriculture, and industry [18]. The resistance to water and ultraviolet radiation and impermeability to oxygen suits them for food packaging [19].

Various natural substrates, viz. wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, etc. have been exploited for PHB production [20, 21]. *Bacillus* sp. has been reported to synthesize PHB from different agro industrial residues like soy molasses oligosaccharides, sugarcane molasses, and corn steep liquor [22, 23]. However, no investigations have been carried out on the utilization of jackfruit seed as a substrate for PHB production. Jackfruit is popular in several tropical countries and is available in large part of the year at the places where produced. In South India, the annual production of jackfruit is next to mango and banana [24]. In some places, it is not used as food material and discarded as waste and also go waste from the fallen fruits. In the present study, jackfruit seed hydrolysate containing reducing sugar was used as carbon substrate in the production medium. In order to utilize the reducing sugar present in the jackfruit seed effectively by the microorganism, it is essential to perform the enzymatic hydrolysis of the substrate to release easily metabolize sugars. Since the enzymatic hydrolysis does not produce any toxic compounds that inhibit the biomass accumulation, this process is reported to be biocompatible [20].The present study indented to optimize the production of PHB using jackfruit seed as substrate by *Bacillus sphaericus* NCIM 5149 under submerged fermentation (SmF) using a statistical approach.

Materials and Methods

B. sphaericus NCIM 5149 was obtained from NCIM (Pune, India) maintained on agar slants and petri dishes containing Luria-Bertani agar media. The inoculum was prepared in Luria-Bertani medium in 250 ml Erlenmeyer flask containing 50 ml of sterile medium (autoclaved at 121.5 °C for 15 min) and inoculated from the stock culture [25].

After 16 h incubation at 30 °C and 200 rotation m^{-1}, 2 ml (8×10^8 CFU/ml) of culture was taken to inoculate the flask containing 100 ml of sterile cultivation medium, which contained (g/l): $(NH_4)_2SO_4$, 2; KH_2PO_4, 2; Na_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; Yeast extract, 0.2; jackfruit seed hydrolysate (with reducing sugar concentration of 10 g/l), and 1 ml trace element solution. The trace element solution contained (g/l) H_3BO_3, 0.01; $MnSO_4.H_2O$, 0.02; $CuSO_4$, 01; $ZnSO_4.7H_2O$, 0.1; and $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.02. Trace element solution was autoclaved separately. The initial medium pH was set at 7.0.

Agro Industrial Residue as Carbon Substrate

The enzymatic hydrolysis was performed as follows: gelatinization at 100 °C for 15 min, followed by liquefaction with alpha amylase, 5,000 IU/ml (Novo Termamyl) at 85 °C (pH 5.0) for 30 min, and saccharification with glucoamylase, 2,000 IU/ml (Novo AMG) at 60 °C for 70 min [26]. The hydrolysate obtained was filtered through muslin cloth, and the clear hydrolysate containing reducing sugar was used as the sole carbon source for PHB production. The concentration of reducing sugar in the hydrolysates was adjusted as needed. The reducing sugar in the hydrolysate was estimated by dinitrosalicylic acid method using glucose as standard [27].

Submerged Fermentation

SmF was carried out in 250 ml Erlenmeyer flask containing 100 ml of production medium, sterilized at 121.5 °C for 20 min, inoculated with 2% (8×10^8 CFU/ml) inoculum and

incubated at 30 °C under shaking conditions (200 rotation m^{-1}) for 48 h. After fermentation, the samples were withdrawn as whole flasks in triplicate and centrifuged at 8,000×g for 20 min; then, the pellet was collected and lyophilized.

Determination of PHB

The lyophilized pellet was digested with 30% sodium hypochlorite solution at 37 °C for 20 min, and the residue was separated by centrifugation at 8,000×g for 20 min. It was washed following a series of steps using water, acetone, and finally ethanol. The residue was dissolved in chloroform and kept at 30 °C for complete evaporation. Then 5 ml of concentrated H_2SO_4 was added and heated for 40 min at 100 °C in a water bath. The resultant crotonic acid was measured at 235 nm against H_2SO_4 as blank in a spectrophotometer (Shimadzu 361A, Japan) following the method of Slepecky and Law [28].

Experimental Design

Several cultural parameters were evaluated to determine their effect on biomass accumulation and PHB production in SmF (Table 1). The optimized value for each parameter was selected and kept constant for further experiments. The time course (0–72 h) and temperature (25–40 °C) studies with jackfruit seed hydrolysates containing 1% reducing sugar were carried out to determine the optimal incubation period and temperature to maximize PHB production. Effect of initial pH, inoculum age, and size and supplementation of additional nitrogen sources and different measures of trace elements solution were evaluated. The optimization of inoculum age ranging from 12 to 24 h with an interval of 4 h was carried out. Inoculum of different age was prepared by transferring a loop full of inoculum to Luria-Bertani medium and kept at 30 °C at 200 rotation m^{-1}, and 2 ml of this inoculum was used to inoculate the production medium. The effect of inoculum size on PHB production was studied by using different volumes (1, 2, 3, 4, and 5 ml) of 16 h culture. To study the influence of initial pH of the medium on PHB production, the initial pH of the medium was set at 4.0–8.0 using 1 N NaOH or HCl. The effect of different inorganic nitrogen sources (2 g/l; ammonium chloride and urea) were determined and compared with ammonium sulfate (control) and complex sources (corn steep liquor) peptone and beef extract at a concentration of 2 g/l were studied by replacing yeast extract from the medium. Influence of trace element solution was determined by varying the measure of solution (1 to 10 ml/l) in the fermentation medium. Finally, studies were conducted to determine the effect of concentration of the reducing sugar present in jackfruit seed hydrolysate for PHB production (10 to 40 g/l).

Central Composite Design (CCD)

After identifying the potential factors that influence the bioprocess by "one variable at a time" approach, CCD was selected to resolve their optimum combination [13, 29]. In the present work, the selected variables were inoculum age, pH, and reducing sugar concentration present in jackfruit seed hydrolysate. The CCD of 20 runs with five levels was set using the Design Expert software. All the experiments were done in triplicate, and the average of biomass and PHB production obtained was taken as the dependent variable or response (Y). The second order polynomial coefficients were calculated and analyzed using the "Design Expert" software (6.0, Stat-Ease Inc., Minneapolis, MN, USA)

Table 1 Effect of single process parameter on polyhydroxybutyrate production by *Bacillus sphaericus* under submerged fermentation.

Parameter	Biomass (g/l)	Amount of PHB (g/l)
Incubation time (h)		
0	0.50±0.11	0.001±0.002
12	2.31±0.17	0.05±0.02
24	2.89±0.26	0.78±0.028
36	3.74±0.28	1.36±0.02
48	3.80±0.251	1.68±0.28
60	3.75±0.024	1.64±0.10
72	3.70±0.152	1.61±0.30
Incubation temperature (°C)		
25	0.60±0.21	0.12±0.02
30	3.32±0.21	1.4±0.07
35	3.33±0.2	1.40±0.2
40	2.26±0.3	0.89±0.08
Initial pH		
4	1.61±0.11	0.4±0.02
5	2.62±0.15	0.86±0.01
6	3.91±0.15	1.38±0.15
7	4.11±0.15	1.82±0.15
8	1.81±0.2	0.4±0.10
Inoculum age (h)		
12	1.90±0.12	0.59±0.11
16	3.90±0.11	1.70±0.11
20	1.96±0.15	0.84±0.12
24	1.83±0.15	0.41±0.01
Inoculum size (8×10^8 CFU/ml)		
1	0.96±0.047	0.4±0.11
2	3.53±0.24	1.63±0.31
3	3.00±0.08	1.2±0.26
4	2.13±0.09	0.59±0.08
5	2.13±0.12	0.426±0.02
Nitrogen source (2 g/l)		
Ammonium chloride	4.40±0.11	0.73±0.06
Urea	2.96±0.15	0.34±0.05
Ammonium sulfate (control)	3.89±0.11	1.71±0.11
Complex nutrient source (2 g/l)		
Beef extract	4.83±0.25	1.82±0.21
Corn steep liquor (CSL)	3.26±0.25	0.51±0.12
Peptone	3.43±0.31	1.36±0.11
Yeast Extract (control)	3.90±0.12	1.69±0.13
Trace element solution (ml/l)		
1	4.21±0.25	1.80±0.15
2	4.21±0.34	1.76±0.15
4	3.91±0.10	1.66±0.15

Table 1 (continued).

Parameter	Biomass (g/l)	Amount of PHB (g/l)
6	3.62±0.15	1.50±0.05
8	3.71±0.11	1.46±0.15
10	3.10±0.25	1.20±0.21
Jackfruit seed hydrolysate reducing sugar concentration (g/l)		
10	3.98±0.16	1.79±0.11
20	4.20±0.15	1.98±0.24
30	2.90±0.10	1.13±0.15
40	2.40±0.17	0.63±0.15

statistical package. The predicted response can be calculated from the second-degree polynomials, Eq. 1, which includes all interaction terms.

$$Y = \beta_{0+} \sum \beta i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X j \quad (1)$$

where Y stands for the response variable, β_0 is the offset term, βi represents the coefficient of the linear effect, βii, the coefficient of quadratic effect, and βij the ijth interaction coefficient effect; $X_i Xj$ are input variables which influence the response variable Y; βi is the ith linear coefficient. Other parameters which have no effect on PHB production were kept constant. The experimental design is shown in Table 2. The experimentation was conducted in 250 ml

Table 2 Experimental design for central composite design of response surface methodology.

Run	X_1, inoculum age (h)	X_2, pH	X_3, substrate (%)
1	12	4	1
2	24	8	1
3	18	6	2.5
4	28	6	2.5
5	12	4	4
6	18	6	2.5
7	18	6	2.5
8	18	6	5
9	12	8	4
10	18	6	2.5
11	18	6	2.5
12	18	6	2.5
13	18	2	2.5
14	18	6	0
15	12	8	1
16	24	8	4
17	24	4	1
18	24	4	4
19	18	9	2.5
20	7	6	2.5

Erlenmeyer flask containing 100 ml of sterile production media prepared as per the design. The flasks were kept for incubation in an incubator shaker maintained at 30 °C and 200 rotation m^{-1}. Incubation time was ceased at 48 h, and the responses studied were biomass (g/l) and PHB (g/l). All the experiments were done in triplicates, and the statistical and numerical analyses of the model were executed by means of the analysis of variance (ANOVA) This analysis included the Fisher's *F*-test, its associated probability p(F), correlation coefficient *R*, R^2 which explains the quality of polynomial model. For each variable, the quadratic models were represented as contour plots (3D), and response surface curves were generated.

Results and Discussion

The "one variable at a time" optimization studies were employed as the first step to confirm the significant factors that influenced the PHB production by *B. sphaericus* 5149, and the results are shown in Table 1. The result of time course (0–72 h) study revealed the maximum cell density, and PHB production was at 48 h (Table 1), and this was selected as the fermentation time for further studies. The data obtained from the effect of incubation temperature indicated the range of 30–35 °C is suitable for both biomass and PHB accumulation. It has been reported that initial pH has a significant influence on PHB production [19]. Effect of initial pH studies showed that as the pH in the medium increases, PHB production increases up to pH 7.0. *B. sphaericus* showed maximum PHB production at pH 7.0, and beyond that, it was found to be decreased (Table 1). Palleroni and Palleroni have reported that pH in the range of 6.0–7.5 was best for microbial growth [30]. The maximum growth and PHB production of *Alcaligenes eutrophus* was reported at optimum pH of 6.9, and the growth declined at pH below 5.4 [30]. Inoculum age of 16 h gave maximum PHB production. Earlier growth studies have shown that log phase of *B. sphaericus* ranges from 12 to 20 h (data not shown). When these bacterial cells were transferred from actively growing phase to the production medium, the easily assimable carbon source in the medium could facilitated the synthesis of PHB. Maximum production was obtained with 2 ml of inoculum of 16 h age, which gave about 3.53 g/l biomass and 1.63 g/l (Table 1). The influence of inoculum size study revealed the lower concentration of inoculum reduced the PHB production because amount of biomass present in the low concentration of inoculum might be insufficient to utilize the nutrients present in the production medium. The inoculum size of the culture determined was 8×10^8 CFU/ml. In the case of inorganic nitrogen sources, ammonium sulfate (control) gave 3.89 g/l biomass, and PHB yield was 1.71 g/l, which is comparable to result, obtained with *Bacillus megaterium* [16]. Even though good biomass accumulation was found with ammonium chloride, yield of PHB was not related to the increase in growth, which is contradictory to other reports [18]. Urea did not support PHB production but enhanced biomass accumulation. A low PHB production of 0.51 g/l was observed with corn steep liquor. Beef extract was found to be the best complex nutrient source since PHB yield was 1.82 g/l with biomass 4.83 g/l compared to the control (yeast extract), where the biomass and PHB production was 3.9 g/l and 1.69 g/l, respectively. The increasing amount of trace element solution negatively affected both biomass and PHB production. The analysis of growth and PHB production characteristics of the strain suggested that jackfruit seed hydrolysate with a concentration of 20 g/l reducing sugar was best for synthesizing PHB with a concentration of 1.98 g/l, and cell density was 4.2 g/l.

Central Composite Design

From the "one variable at a time" experiment, it was obvious that inoculum age, pH, and concentration of reducing sugar in the jackfruit seed hydrolysate had a positive influence on both biomass accumulation and PHB production. CCD was employed to identify the optimal level and interaction of these selected factors. The results are showed in Table 3. The run no. 7 exhibited a maximum response of PHB production of 49% (dry weight) with biomass of 4.5 g/l and PHB concentration of 2.22 g/l. The lower response was found in run no. 19. From these results, it is clear that *B. sphaericus* 5149 is able to synthesize maximum PHB at pH 6 with 2.5% substrate concentration and inoculum age of 18 h. Multiple regression analysis was used to analyze the data, and thus a polynomial equation was derived from regression analysis as follows:

$$\text{Biomass} = 4.38 + 0.0059X_1 + 0.025X_2 + 0.097X_3 - 0.588X_1^{2-}0.977X_2^2 - 0.977X_3 + 0.425X_1X_2 + 0.075X_1X_3 - 0.1X_2X_3 \quad (2)$$

$$\text{PHB} = 2.03 + 0.084X_1 - 0.045X_2 + 0.069X_3 - 0.383X_1^2 - 0.581X_2^2 - 0.565X_3^2 + 0.095X_1X_2 + 0.074X_1X_3 - 0.047X_2X_3 \quad (3)$$

Table 3 Observed and predicted responses obtained for central composite design.

Run	Biomass (g/l)		Amount of PHB (g/l)	
	Actual value	Predicted value	Actual value	Predicted value
1	2.8	2.1	0.84	0.5
2	2.2	2.2	0.55	0.5
3	4.1	4.3	1.96	2.0
4	3	2.7	1.2	1.0
5	2.7	2.3	0.783	0.6
6	3.7	4.3	1.66	2.0
7	4.5	4.3	2.22	2.0
8	3.8	3.5	0.8	0.7
9	3	2.5	0.48	0.22
10	4.6	4.3	1.97	2.0
11	4.8	4.3	2.2	2.0
12	4.7	4.3	2.25	2.0
13	1	1.5	0.2	0.46
14	1.3	1.4	0.22	0.31
15	1.5	1.5	0.37	0.32
16	2.1	2.3	0.66	0.8
17	1	1.1	0.28	0.34
18	2	1.6	0.88	0.72
19	1.8	1.6	0.30	0.31
20	2	2.7	0.42	0.8

where Y is the response variable (Biomass and PHB production), X_1 is the coded value of inoculum age, X_2 is the coded value of pH, and X_3 is the coded value of substrate concentration. The capability of the model was checked using ANOVA which was tested using Fisher's statistical analysis, and the results are showed in Tables 4 and 5. Using ANOVA, the model for response biomass was checked, and the results are represented by Table 4. The R^2 value closer to unity, significant model F value, insignificant lack-of-fit F value, and standard deviation less than 10 in all cases implies the model was significant. The calculated R^2 value of 0.910 for biomass production shows improved correlation between the observed and predicted response. The R^2 value is always between 0 and 1. The closer the R^2 is to 1, the stronger the model and the better it predicts the response [31]. The P values represents the significance of the coefficients and also helpful in understanding the pattern of the mutual interactions between the variables [32]. Value of Prob>F less than 0.05 indicate model terms are significant. ${X_1}^2$, ${X_2}^2$, and ${X_3}^2$ are significant models in this case.

ANOVA of response for PHB concentration is shown in Table 5. Here, also the F value 14.50 and values of prob>F (<0.05) indicated that the model terms are significant. The R^2 value explains that the variability in the PHB yield could be associated to the experimental factors to the extent of 92.8%. For PHB production, the coefficients of ${X_1}^2$, ${X_2}^2$, and ${X_3}^2$ are significant models.

To investigate the interactive effects of variables for biomass and PHB production, the response surface and contour plots were generated as graphical representations of the regression equation. The three-dimensional response surface and their corresponding contour plots for the biomass production and concentration of PHB against any two independent variables while keeping the other independent variable at zero levels are presented in Figs. 1 and 2. The effect of inoculum age and initial pH on biomass production is shown in Fig. 1. It was depicted that increase in inoculum age had not much influence on biomass accumulation. The alkaline pH had a deleterious effect on bacterial growth and consequently slowed down the biomass accumulation. The biomass yield was found to decrease above neutral pH. *Synechocystis* sp. was capable of

Table 4 Analysis of variance for the response of biomass.

Source	Sum of squares	DF	Mean square	F value	Prob>F
Model	29.26654	9	3.251838	10.97313	0.0004
A	0.00049	1	0.00049	0.001653	0.9684
B	0.008737	1	0.008737	0.029484	0.8671
C	0.130766	1	0.130766	0.441262	0.5215
A2	4.99625	1	4.99625	16.85954	0.0021
B2	13.77607	1	13.77607	46.4865	<0.0001
C2	13.77607	1	13.77607	46.4865	<0.0001
AB	1.445	1	1.445	4.876064	0.0517
AC	0.045	1	0.045	0.15185	0.7049
BC	0.08	1	0.08	0.269955	0.6147
Residual	2.963456	10	0.296346		
Lack of fit	2.083456	5	0.416691	2.367563	0.1830

R^2, 0.910

Table 5 Analysis of variance for the response of amount of polyhydroxybutyrate.

Source	Sum of squares	DF	Mean square	*F* value	Prob>*F*
Model	10.10981	9	1.123313	14.50913	0.0001
A	0.096636	1	0.096636	1.248182	0.2900
B	0.027679	1	0.027679	0.357509	0.5632
C	0.066819	1	0.066819	0.863061	0.3748
A2	2.12155	1	2.12155	27.40275	0.0004
B2	4.876004	1	4.876004	62.98032	<0.0001
C2	4.612916	1	4.612916	59.58218	<0.0001
AB	0.072771	1	0.072771	0.93994	0.3552
AC	0.044551	1	0.044551	0.575439	0.4656
BC	0.018336	1	0.018336	0.236836	0.6370
Residual	0.774211	10	0.077421		
Lack of fit	0.523211	5	0.104642	2.084505	0.2197

R^2, 0.928

synthesizing biopolymer maximum at pH 8.5, but the acidic pH and high alkaline pH was not suitable for this strain [1].

The interaction effect between initial pH and inoculum age is showed in Fig. 2. The 18 h inoculum and pH 6.0 was best for maximizing PHB production (2.2 g/l). It has been reported that the inoculum age of 15 h has given maximum PHB production by *R. eutropha*, and above 20 h, the productivity was found to be decreased [33]. The increase in the substrate concentration did not have a positive influence on PHB accumulation. The reduction in the polymer synthesis can be explained by the higher protein concentration in the hydrolysate. Glucose, fructose, and sucrose have been reported as the major sugar constituents in the jackfruit seed [34]. The substrate concentration and inoculum age for PHB production indicated that both inoculum age and substrate concentration had no effect on PHB synthesis beyond their optimum values. Inoculum age of 18 h (8×10^8 CFU/ml) with substrate concentration of 2.5% gave highest PHB

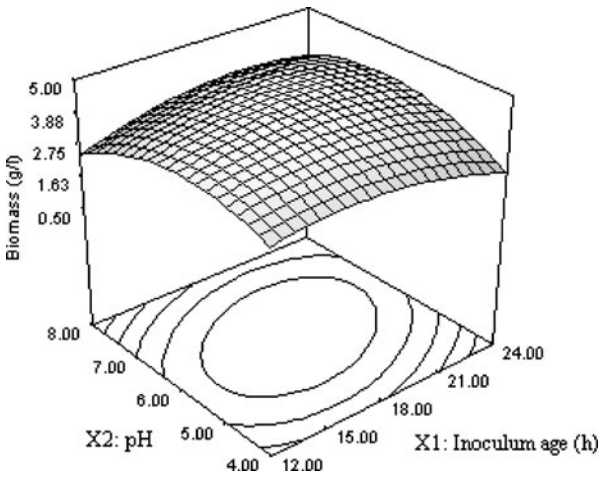

Fig. 1 Response surface graph showing interaction between inoculum age and initial pH on biomass production

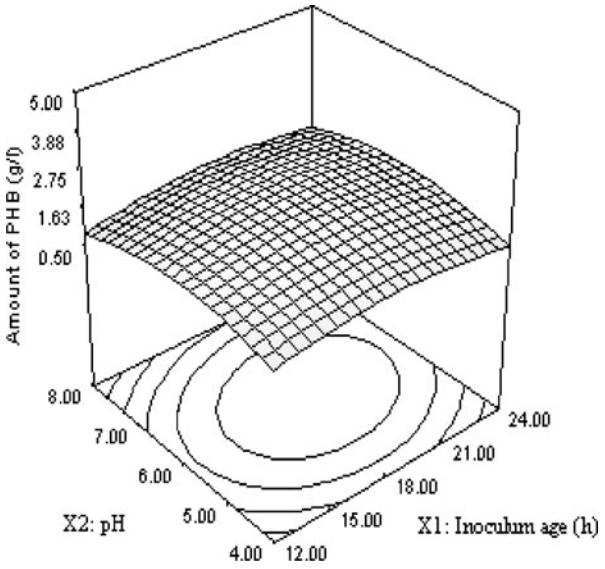

Fig. 2 Response surface graph showing interaction between inoculum age and initial pH on polyhydroxybutyrate production

concentration (data not shown). The available nitrogen from the proteins in the hydrolysate would have shifted the PHB synthesis to biomass accumulation. The results suggest that the removal of excess protein from the jackfruit seed hydrolysate would yield higher PHB.

Validation of the Model

Validation of the model was carried out in shake flasks under conditions predicted by the software. A good correlation can be seen between the experimental and the predicted values, and hence, the model was successfully validated. Validation of the statistical model and regression equation was performed by taking X_1 (18 h), X_2 (6), and X_3 (2.5%) in the experiment. The predicted and the actual (experimental) responses of biomass (4.8 and 5 g/l) and PHB production (2.4 and 2.7 g/l) were comparable.

Conclusion

To the best our knowledge, there are no reports of optimization of PHB production by *B. sphaericus* 5149 using statistical experimental design. The statistical approach showed significant results for optimizing the process parameters for maximal biomass and PHB production under SmF. This study also explains the use of cheap agro-residues as substrate for fermentation, thus contributing to the cost reduction in the bioplastic production. In this study, it is evident that various process parameters like pH and substrate concentration and inoculum age significantly influenced the PHB production. The higher similarity between the predicted and experimental results indicated the accuracy and applicability of RSM to optimize the process for PHB production. Considering the results obtained in the present study, we can conclude that the strain used in this study offers great potential for further investigation on PHB production.

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