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ORIGINAL ARTICLE

Development of a novel solid-state fermentation strategy for the production of poly-3-hydroxybutyrate using polyurethane foams by *Bacillus sphaericus* NII 0838

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Abstract The extensive use of synthetic plastics has caused serious waste disposal problems in our environment. Poly-3-hydroxybutyrates (PHB) are eco-friendly bacterial polyesters which are produced under unbalanced nutrient conditions. Few reports are available on PHB production by solid state fermentation (SSF). We have developed a novel SSF bioprocess in which polyurethane foam (PUF) is used as a physical inert support for the production of PHB by *Bacillus sphaericus* NII 0838. Media engineering for optimal PHB production was carried out using response surface methodology (RSM) adopting a Box–Behnken design. The factors optimized by RSM were inoculum size, pH and $(NH_4)_2SO_4$ concentration. Under optimized conditions—6.5 % inoculum size, 1.7 % (w/v) $(NH_4)_2SO_4$ and pH 9.0—PHB production and biomass were 0.169±0.03 and 0.4±0.002 g/g PUF, respectively. This is the first report on PHB production by SSF using PUF as an inert support. Our results demonstrate that SSF can be used as an alternative strategy for the production of PHB.

Keywords Polyurethane foam · Poly-3-hydroxybutyrate · Jackfruit seed hydrolyzate · Solid-state fermentation · Box–Behnken design

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Introduction

During the past few decades there has been an increased demand for bioplastics due to the increase in petroleum prices as well as environmental concerns regarding plastic pollution. Poly-3-hydroxybutyrate (PHB) is a biodegradable, biocompatible and microbial thermoplastic which can replace petroleum-derived thermoplastics (Tabandeh and Farahani 2003). It is synthesized as intracellular granules by microorganisms in response to nutrient stress and is stored as carbon and energy reserve (Steinbuchel 1991; Byrom 1994).

Solid state fermentation (SSF) can be used as an alternative to submerged fermentation for the production of PHB. To date, only a few reports have been published on PHB production by SSF. Oliveira et al. (2004, 2007) reported on PHB production by *Ralstonia eutropha* in a SSF system using soy cake and soy cake supplemented with 2.5 % sugarcane molasses. One of the major inherent problems associated with employing SSF for PHB production is the difficulty in retrieving bacterial cells from the solid substrate after fermentation. This can be overcome by using an inert support in SSF. The substrates used in SSF should absorb water so that the microorganism can utilize the water for growth and metabolic activities. In this context, polyurethane foam (PUF) possesses a number of advantageous characteristics, including high porosity, low density and relatively high water absorption capacity. PUF allows cell adsorption to a large extent because it enables mobilization of a large number of cells within a short period. The use of a defined liquid medium and an inert support with a homogenous physical structure improves the control and monitoring of the SSF process and the reproducibility of the fermentations (Zhu et al. 1994). It has been reported that

PUF can provide a continuous homogenous aerobic environment up to the end of incubation period (Aidoo et al. 1982).

The microorganism used in this study was *Bacillus sphaericus* NII 0838. The ability of *B. sphaericus* to produce PHB has been reported in our earlier studies (Sindhu et al. 2011; Ramadas et al. 2009, 2010). In the present study we explored the possibility of SSF using an inert support to develop a bioprocess for PHB production. A statistical approach was applied to optimize the nutritional and environmental parameters for growth of the microorganism and PHB production. The Plackett–Burman design was used to identify significant variables, and further optimization was carried out by employing a Box–Behnken design.

Materials and methods

Chemicals and reagents

Crotonic acid was obtained from Sigma-Aldrich, India. All other chemicals were reagent grade procured from local vendors.

Microorganisms and growth conditions

Bacillus sphaericus NII 0838 used in this study was maintained on nutrient agar slants. The inoculum was prepared in a 250-ml Erlenmeyer flask containing Luria–Bertani media (50 ml) and incubated at 30 °C for 16 h on a rotary shaker at 200 rpm. A 3-ml seed culture (8×10^8 CFU/ml) was transferred to 50 ml of fermentation media as and when required. The PUF was cut into 0.5-cm^3 cubes and used as the inert solid support in the SSF medium. The PUF was thoroughly washed in distilled water and dried in an oven at 85 °C overnight before being added to the SSF system.

Solid state fermentation

Solid state fermentation was carried out in 250-ml Erlenmeyer flasks, with each flask containing 1 g PUF (sterilized at 121.5 °C for 20 min). The reducing sugar concentration in the hydrolyzate was estimated by the dinitrosalicylic acid method (Miller 1959) using glucose as the standard and was adjusted to 1.0 % (w/v) using distilled water. This hydrolyzate was further enriched with a nutrient mixture containing (per liter) 2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.6 g Na_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2$, 0.2 g beef extract and 1 ml trace element solution. The trace element solution contained (per liter) 0.01 g H_3BO_3, 0.02 g $MnSO_4{\cdot}H_2O$, 0.1 g $CuSO_4$, 0.1 g $ZnSO_4{\cdot}7H_2O$, and 0.02 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. The hydrolyzate and trace element solutions were autoclaved separately and mixed with the production medium aseptically prior to use. The initial medium pH was set at 7.0. The flasks containing PUF were impregnated with 10 ml production medium, inoculated with 3 ml of inoculum (8×10^8 CFU/ml) of *B. sphaericus* NII 0838 and incubated at 30 °C for 96 h.

Biomass recovery from PUF

After fermentation, each PUF cube was agitated with 50 ml of distilled water at 250 rpm for 20 min. This process was repeated four times to ensure the maximum recovery of bacterial cells from the PUF cubes. The resulting bacterial suspension was a pool of the four filtrates that was centrifuged at 8,000 *g* for 15 min. The pellet obtained was lyophilized and used for PHB quantification and biomass dry weight determination.

PHB assay

The PHB assay was carried out using the method of Law and Slepecky (1961). The pellet was lyophilized and digested with 30 % sodium hypochlorite solution at 37 °C for 30 min. The sample was then centrifuged at 8,000 *g* for 30 min and washed sequentially with distilled water (5×), acetone (5×) and methanol (5×) before being dissolved in chloroform (5×). The chloroform was allowed to vaporize completely at room temperature, and the sample was further treated with concentrated H_2SO_4 and incubated at 100 °C for 30 min. Absorbance of the resultant solution was measured at 235 nm using a UV-visible light spectrophotometer (model UV-1601; Shimadzu, Japan) with crotonic acid as the standard.

Scanning electron microscopy analysis

Scanning electron microscopy (SEM) analyses were carried out to check the effectiveness of biomass recovery from the PUF after fermentation using a JEOL JSM-5600 scanning microscope (JEOL, Japan). The PUF cubes were cut into thin pieces and lyophilized. These samples were mounted on a double-sided conductive tape on precut brass sample stubs and sputter coated with gold palladium using a JEOL JFC-1200 fine coater. The images were acquired at an accelerating voltage of 10–15 kV and magnification of 7,000×.

One-parameter-at-a-time approach for PHB production

Wheat bran (WB), cassava bagasse (CB) and jackfruit seed (JS) powder were screened for their effect on PHB production during SSF. Prior to use, these substances were gelatinized, liquified and saccharified, as described by John et al. (2006). The reducing sugar concentration in the hydrolyzate was estimated by the dinitrosalicylic acid method using glucose as the standard. The hydrolyzate of each agro-industrial residue was further enriched with a nutrient mixture and screened individually as well as in various

combinations (1:1) at a concentration of 10 g/l (total reducing sugar). The influence of initial moisture content on PHB production was studied by adjusting the moisture content (55, 60, 65, 70, 75, 80 and 85 %) of PUF with medium without affecting the final concentration of each component. Studies were carried out to evaluate the effect of nitrogen sources on PHB production by replacing the ammonium sulfate in the medium with 0.2 % (w/v) of different inorganic nitrogen sources (ammonium nitrate, urea, glycine and ammonium chloride). The organic complexes evaluated were beef extract, yeast extract, corn steep liquor (CSL) and peptone at 0.2 % (w/v) concentration.

Statistical optimization for PHB production

Plackett–Burman experimental design

The Plackett–Burman experimental design was applied to identify significant variables that affect PHB production. The Minitab ver. 15 software program (Minitab Inc, USA) was used for creating the experimental design, and the details are shown in Table 1. The variables selected were substrate concentration (JS hydrolyzate containing reducing sugar as the carbon source), incubation period, inoculum size, inoculum age, initial medium pH, incubation temperature and concentration of beef extract and $(NH_4)_2SO_4$. The respective effect of these eight variables was studied at two different levels, and a total of 12 combinations were organized according to the Plackett–Burman design matrix. All trials were performed in triplicate, and average values were considered for the response (PHB and biomass). The effects of individual parameters on biomass and PHB production were calculated using the following equation.

$$E = (\Sigma M_+ - \Sigma M_-)/N \quad (1)$$

where E is the effect of the parameter under study, M+ and M− are the responses (biomass or PHB production) of trials at which the parameter was at its higher and lower level, respectively and N is the total number of trials. Data for both biomass and PHB production were analyzed using the general linear model (GLM). The regression coefficient, *F* values and *P* values of the factors were investigated.

Response surface methodology

The Box–Behnken (Box and Behnken 1960) design was applied to study the effect of independent variables on the response and factor interactions with different combinations of variables. The three crucial variables selected were inoculum size (X_1), $(NH_4)_2SO_4$ (X_2) and pH (X_3). The effect of these three variables was studied at three different levels, and a total of 15 runs were performed for the study. The Minitab ver. 15 software program (Minitab Inc) was used for the experimental design, data analysis and quadratic model building. The experimental setup of response surface methodology (RSM) and the results (amount of PHB and biomass) are shown in Table 2.

The other variables in the study were maintained at a constant level, which gave maximal yield in the Plackett–Burman experiments. All experiments were performed in triplicate, and the average values for biomass (cell dry

Table 1 Plackett–Burman experimental design for screening significant process variables affecting biomass and poly-3-hydroxybutyrate production

Run order	Carbon (A) (g/l)	Inoculum size (B) (ml)	Inoculum age (C) (h)	Temperature (D) (°C)	$(NH_4)_2SO_4$(E) content (%)	Beef extract content (F) (%)	pH (G)	Incubation time (H) (h)	Biomass (g/g PUF)	PHB (g/g PUF)
1	60	1	24	25	0.01	0.01	8	120	0.02	0.001
2	60	5	12	35	0.01	0.01	5	120	0.01	0.006
3	10	5	24	25	0.40	0.01	5	72	0.06	0.025
4	60	1	24	35	0.01	0.40	5	72	0.012	0.004
5	60	5	12	35	0.40	0.01	8	72	0.1015	0.048
6	60	5	24	25	0.40	0.40	5	120	0.0468	0.012
7	10	5	24	35	0.01	0.40	8	72	0.0626	0.020
8	10	1	24	35	0.40	0.01	8	120	0.0593	0.005
9	10	1	12	35	0.40	0.40	5	120	0.0412	0.007
10	60	1	12	25	0.40	0.40	8	72	0.067	0.035
11	10	5	12	25	0.01	0.40	8	120	0.0683	0.031
12	10	1	12	25	0.01	0.01	5	72	0.0247	0.011

PUF, Polyurethane foam; PHB, poly-3-hydroxybutyrate

Uppercase letters in parenthesis associated with a process variable are used as the codes in the model equation fitted by regression analysis

Table 2 Box–Behnken design for optimizing the significant variables for biomass and PHB production

Run order	Inoculum size (ml)	$(NH_4)_2SO_4$ content (%)	pH	Biomass (g/g PUF)	PHB (g/g PUF)
1	6.5	1.7	9.5	0.4	0.169
2	6.5	0.4	8.0	0.19	0.05
3	6.5	3.0	11.0	0.056	0.003
4	6.5	3.0	8.0	0.5	0.06
5	5.0	0.4	9.5	0.1	0.04
6	6.5	1.7	9.5	0.38	0.168
7	6.5	1.7	9.5	0.376	0.165
8	5.0	1.7	11.0	0.028	0.002
9	8.0	3.0	9.5	0.298	0.02
10	8.0	1.7	11.0	0.05	0.002
11	8.0	0.4	9.5	0.089	0.005
12	8.0	1.7	8.0	0.40	0.019
13	6.5	0.4	11.0	0.02	0.003
14	5.0	1.7	8.0	0.38	0.02
15	5.0	3.0	9.5	0.2	0.005

weight) and PHB production obtained were taken as the responses (Y). The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (2)$$

where Y is the dependent variables (PHB and biomass), β_0 is constant, and βi, βii and βij are constant regression coefficients estimated by the model. xi, xj indicates levels of the independent variables and they correspond to the linear, quadratic and cross-product effects of the X_1, X_2 and X_3 factors on the response, respectively. The model was evaluated for the effect of each independent variable to a response. The accuracy and general ability of the model could be evaluated by the coefficient of determination R^2.

Results and discussion

Despite PHB being an interesting alternative for synthetic plastics, the production cost is the most discouraging factor for long-term market penetration.

SEM analysis

After fermentation, we observed very good growth of *B. sphaericus* NII 0838 on both the surface and inner pores of the PUF. Since PHB is synthesized as intracellular inclusion bodies within the cytoplasm of the microorganism, it is important to be able to easily and efficiently extract the bacterial cells from the PUF. After each washing step, we observed the PUF samples, and the SEM images revealed that most of the biomass could be retrieved by repeated washing using distilled water (Fig. 1a–d).

Screening of agro-residues for PHB production

Among the various substrates (hydrolyzate of agro-industrial residues) screened for SSF, the highest production of PHB (0.013±0.001 g/g PUF, 21.6 %) and biomass (0.06±0.002 g/g PUF) was obtained using JS powder hydrolyzate, followed by WB hydrolyzate, the WB:CB combination (1:1), the JS powder:CB combination (JS:CB, 1:1), the WB:JS powder combination (1:1) and CB hydrolyzate. The details are presented in Fig. 2. These results suggest that the JS powder hydrolyzate may be an excellent carbon and nitrogen source. It contains 31.9 % protein, 66.2 % carbohydrates and 1.3 % lipids (Bobbio et al. 1978). Ramadas et al. (2009, 2010) reported the effect of JS powder hydrolyzate on PHB production by *B. sphaericus* under SSF. The main limitation of this system for the industrial production of biopolymers is the difficulty involved in synthesizing the biopolymers from inexpensive precursors and the high cost of their recovery (Byrom 1987). The cost of raw materials accounts for more than 50 % of the total production cost, of which this carbon source accounts for 70–80 % of the total raw material cost (Choi and Lee 1997). Utilization of an inexpensive renewable carbon source such as JS hydrolyzate would play a key role in reducing PHB production costs.

Effect of initial moisture content on the production of PHB

Initial moisture content plays an important role in SSF systems. In general, bacteria require a higher water activity for their growth, and moisture causes the substrate to swell, thereby facilitating better utilization of the substrate. However, increases in the moisture content have been found to lead to a reduction in product yield during SSF, likely due to a reduction in inter-particle spaces, decreased substrate degradation and impaired oxygen transfer (Ramesh and Lonsane 1990; Sandhya and Lonsane 1994). If there is insufficient water, the diffusion of solutes and gas becomes limited, and the cell metabolism slows and/or stops entirely because of a lack of substrates or an inhibitory effect of high concentrations of various metabolites in or near the cell (Gervais and Molin 2003). In our study, maximum production of biomass (0.070±0.002 g/g PUF) and PHB (0.018±0.002 g/g PUF) was observed when the substrate moisture was set at 80 % (Fig. 3).

Effect of nitrogen source on PHB production

Our analysis of the effect of inorganic (Fig. 4) and organic nitrogen (Fig. 5) sources showed that both sources gave the

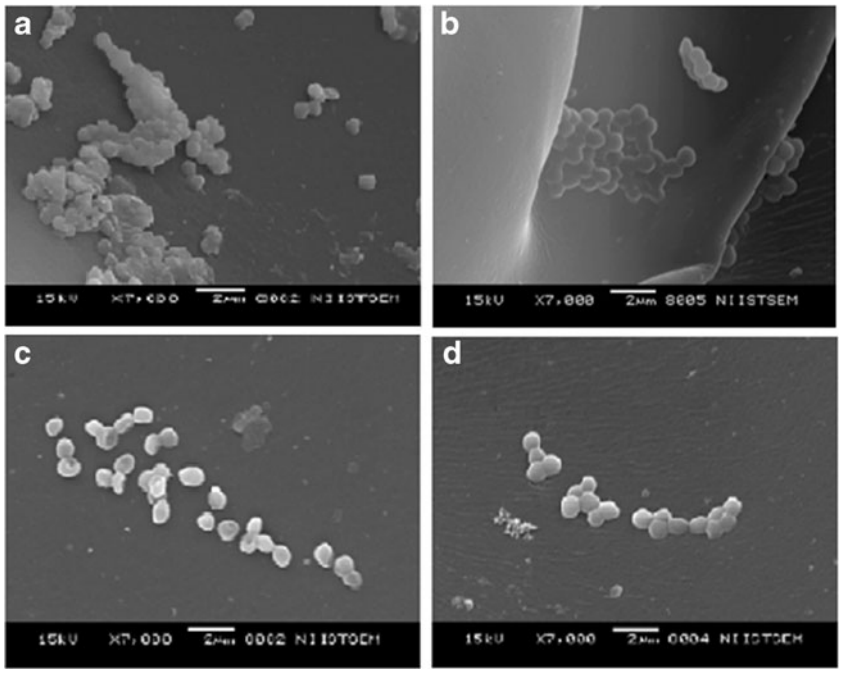

Fig. 1 Scanning electron micrographs of the polyurethane foam (PUF) cubes used as inert physical support in a solid state fermentation (SSF) system for the production of poly-3-hydroxybutyrate (PHB). **a** First washing, **b** second washing, **c** third washing, **d** fourth washing

same results for biomass and PHB production since the controls were the higher producers. $(NH_4)_2SO_4$ was the most suitable inorganic nitrogen source for higher PHB (0.019±0.003 g/g PUF) and biomass (0.07±0.002 g/g PUF) production. An identical observation was reported by Grothe et al. (1999) for PHB production by *Alcaligenes latus* where the maximum PHB production was observed in presence of inorganic nitrogen sources, such as $(NH_4)_2SO_4$ and NH_4Cl. The strain used in our study might not be able to synthesize the enzyme required to utilize urea as a nitrogen source. We also studied PHB production using a variety of complex nitrogen sources (peptone, yeast extract, beef extract, and corn steep liquor). The production of biomass (0.071±0.003 g/g PUF) and PHB (0.019±0.002 g/g PUF) was higher when beef extract (control) was used compared to peptone, corn steep liquor and yeast extract. A different observation was reported by Yuksekdag et al. (2004) for *Bacillus subtilis* and *Bacillus megaterium* where maximum PHB production was observed in the presence of peptone (78.69 % and 77 % PHB respectively).

Statistical optimization of PHB production

Screening of parameters using the Plackett–Burman design

A factorial design allows the effects of multiple variables on a response to be determined. Using the two-factorial Plackett–Burman experimental design, we identified the critical physico-chemical parameters affecting PHB production. The *F* values and *P* values of the factors investigated for biomass and PHB production are given in Tables 3 and 4, respectively. The *F* value identifies the influence of each controlled factor on the tested model and is described as the ratio of the mean square due to regression to the mean square due to error. The model equation fitted by regression analysis is given for biomass and PHB as follows:

$$\begin{aligned}\textbf{Biomass} = {} & -\mathbf{0.0045} - \mathbf{0.000196}(\mathrm{A}) + \mathbf{0.00521}(\mathrm{B}) \\ & - \mathbf{0.000722}(\mathrm{C}) - \mathbf{0.000003}(\mathrm{D}) \\ & + \mathbf{0.0762}(\mathrm{E}) + \mathbf{0.0096}(\mathrm{F}) + \mathbf{0.0102}(\mathrm{G}) \\ & - \mathbf{0.000285}(\mathrm{H}) \end{aligned} \quad (3)$$

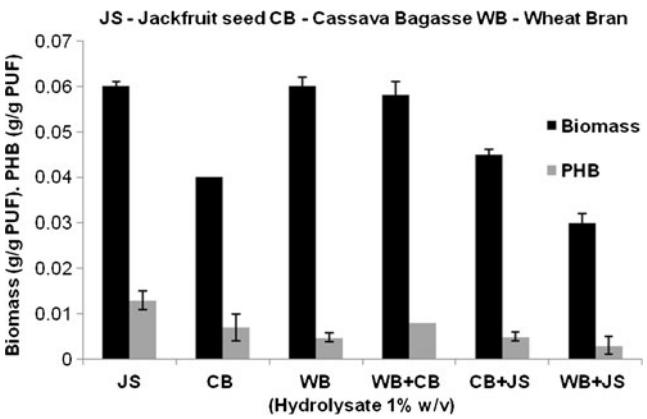

Fig. 2 Screening profile of hydrolyzates of agro-industrial residues for PHB and biomass production

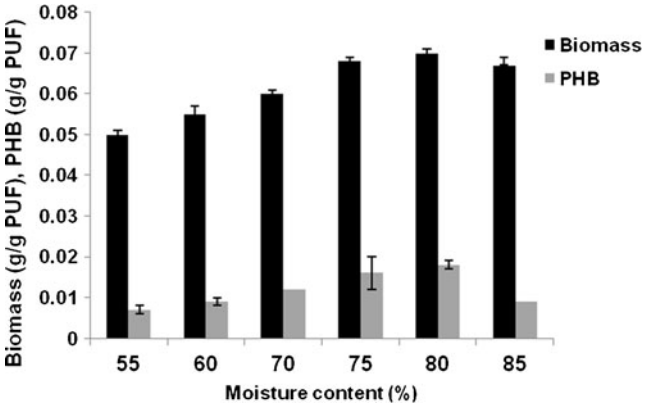

Fig. 3 Effect of initial moisture content on the production of PHB

$$\mathbf{PHB} = \mathbf{0.0303} + \mathbf{0.000023}(\mathrm{A}) + \mathbf{0.00329}(\mathrm{B}) - \mathbf{0.000986}(\mathrm{C}) - \mathbf{0.000416}(\mathrm{D}) + \mathbf{0.0252}(\mathrm{E}) + \mathbf{0.00556}(\mathrm{F}) + \mathbf{0.00417}(\mathrm{G}) - \mathbf{0.000281}(\mathrm{H}) \quad (4)$$

The codes for the variables are as given in Table 1.

The value of $P < 0.05$ indicated that the model was significant. The determination coefficient (R^2) for biomass production in SSF was 95.71 %, indicated that the statistical model with 95.71 % variability in the response and for PHB production it was 96.3 %. Maximum biomass and PHB production were 0.101±0.05 and 0.048±0.005 g/g PUF, respectively, in run no. 5, while the minimum PHB, 0.001 ±0.0 g/g PUF, was produced in run no. 1. The lowest biomass production was obtained in run no. 2 (Table 1).

However, the Plackett–Burman design is typically used a screening technique; in our study provides information on how each variable tended to affect bacterial growth and PHB production. From the Pareto charts it was evident that the order of significance of variables for biomass production were pH (highest), $(NH_4)_2SO_4$, inoculum size, beef extract,

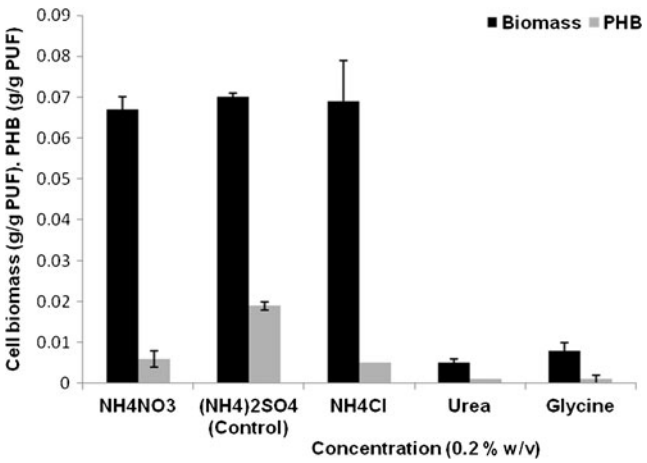

Fig. 4 Effect of inorganic nitrogen source on PHB production

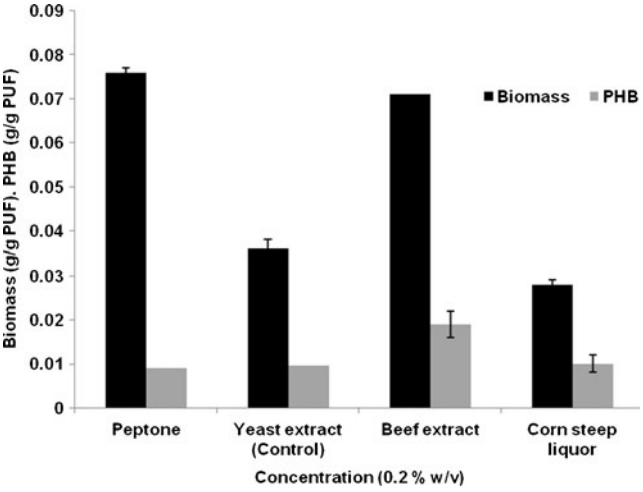

Fig. 5 Effect of organic nitrogen source on PHB production

temperature, inoculum age, incubation time and carbon source (least). For PHB production, these were inoculum size (highest), pH, $(NH_4)_2SO_4$, beef extract, temperature, carbon, inoculum age and incubation time (least) (Fig. 6a and b). Since pH, inoculum size and $(NH_4)_2SO_4$ were found to be the most significant parameters that had a positive influence on both biomass and PHB production, these were selected for subsequent optimization studies using RSM. Temperature is one of the most critical parameters that has to be controlled in a bioprocess (Chi and Zhao 2003). However, in our study, temperature did exert much influence on biomass and PHB production; hence 30 °C was selected for further studies. Our results suggest that SSF using an inert support can be used as an alternative method for PHB production. However, since the Plackett–Burman is typically only a screening technique, a more accurate quantitative analysis of the effect of variables on PHB production is required.

Box—Behnken design

The objective of the experimental design was to optimize the condition for PHB production. Since step-wise optimization of a single parameter one at a time does not allow all possible combinations of independent variables to be examined, the selection of statistical experimental design tools for optimization is important. RSM is a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximately functional relationship between a response variable and a set of design variables. RSM determines the factor levels that will simultaneously satisfy a set of desired specifications; it helps in the determination of the optimum combination of factors that yield a desired response and describes the response near the optimum. The experimental design

Table 3 Statistical analysis of the Plackett–Burman model for the biomass production

Source	*df*	Seq SS	Adj SS	Adj MS	*F*	*P*
Carbon	1	0.0002881	0.0002881	0.0002881	2.50	0.212
Inoculum size	1	0.0013021	0.0013021	0.0013021	11.28	0.044
Inoculum age	1	0.0002253	0.0002253	0.0002253	1.95	0.257
Temperature	1	0.0000000	0.0000000	0.0000000	0.00	0.996
$(NH_4)_2SO_4$	1	0.0026463	0.0026463	0.0026463	22.93	0.017
Beef extract	1	0.0000418	0.0000418	0.0000418	0.36	.590
pH	1	0.0028213	0.0028213	0.0028213	24.45	0.016
Incubation time	1	0.0005631	0.0005631	0.0005631	4.88	0.114
Error	3	0.0003462	0.0003462	0.0001154		
Total	11	0.0082342				

Determination coefficient (R^2)=95.71

and the responses (amount of PHB and biomass) are presented in Table 2. There was a considerable variation in PHB production depending on the chosen variables.

Maximum PHB production occurred in run no. 1 (0.169±0.03 g/g PUF), while the minimum PHB production occurred in runs no. 8 and 10 (0.002±0.001 g/g PUF). The optimum conditions for run no.1 were an inoculum size of 6.5 ml, 8×10^8 CFU/ml), 1.7 % (w/v) $(NH_4)_2SO_4$ and pH 9.5.

The polynomial equation for the model used is:

$$\begin{aligned}\mathbf{Y(Biomass)} = &-\mathbf{4.25298} + \mathbf{0.471871}(\mathrm{X_1}) \\ &+ \mathbf{0.101913}(\mathrm{X_2}) + \mathbf{0.602448}(\mathrm{X_3}) \\ &- \mathbf{0.0373519}\left(\mathrm{X_1^2}\right) - \mathbf{0.0389300}\left(\mathrm{X_2^2}\right) \\ &- \mathbf{0.0322407}\left(\mathrm{X_3^2}\right) \\ &- \mathbf{0.00641026}(\mathrm{X_1X_2}) \\ &+ \mathbf{0.000111111}(\mathrm{X_1X_3}) \\ &- \mathbf{0.00128205}(\mathrm{X_2X_3}) \qquad (5)\end{aligned}$$

$$\begin{aligned}\mathbf{Y(PHB)} = &-\mathbf{4.16531} + \mathbf{0.539623}(\mathrm{X_1}) \\ &+ \mathbf{0.546363}(\mathrm{X_2}) + \mathbf{0.582348}(\mathrm{X_3}) \\ &- \mathbf{0.0417963}\left(\mathrm{X_1^2}\right) - \mathbf{0.0707347}\left(\mathrm{X_2^2}\right) \\ &- \mathbf{0.0330185}\left(\mathrm{X_3^2}\right) + \mathbf{0.0139744}(\mathrm{X_1X_2}) \\ &- \mathbf{8.88889}\mathrm{E}{-}04(\mathrm{X_1X_3}) \\ &- 0.0351282(\mathrm{X_2X_3}) \qquad (6)\end{aligned}$$

Where Y indicates the responses and X_1, X_2 and X_3 are the coded values of inoculum size, $(NH_4)_2SO_4$ and pH, respectively.

The goodness of the model was checked by fitting the independent variables into the second order model equation. The adequacy of the model was evaluated using analysis of variance (ANOVA) and the results are shown in Tables 5 and 6. The determinant coefficient value (R^2=0.9953) suggested that the total variation of 99.53 % for biomass could be attributed to the independent variables and that only 0.47 % of the total variation could not be explained by the model. For PHB production, the determinant coefficient was

Table 4 Statistical analysis of the Plackett–Burman model for PHB production

Source	*df*	Seq SS	Adj SS	Adj MS	*F*	*P*
Carbon	1	0.0000041	0.0000041	0.0000041	0.14	0.735
Inoculum size	1	0.0005199	0.0005199	0.0005199	17.52	0.025
Inoculum age	1	0.0004199	0.0004199	0.0004199	14.15	0.033
Temperature	1	0.0000520	0.0000520	0.0000520	1.75	0.277
$(NH_4)_2SO_4$	1	0.0002899	0.0002899	0.0002899	9.77	0.052
Beef extract	1	0.0000141	0.0000141	0.0000141	0.48	0.540
pH	1	0.0028213	0.0028213	0.0028213	15.79	0.029
Incubation time	1	0.0005469	0.0005469	0.0005469	18.43	0.023
Error	3	0.0000890	0.0000890	0.0000297		
Total	11	0.0024046				

Determination coefficient (R^2)=96.30

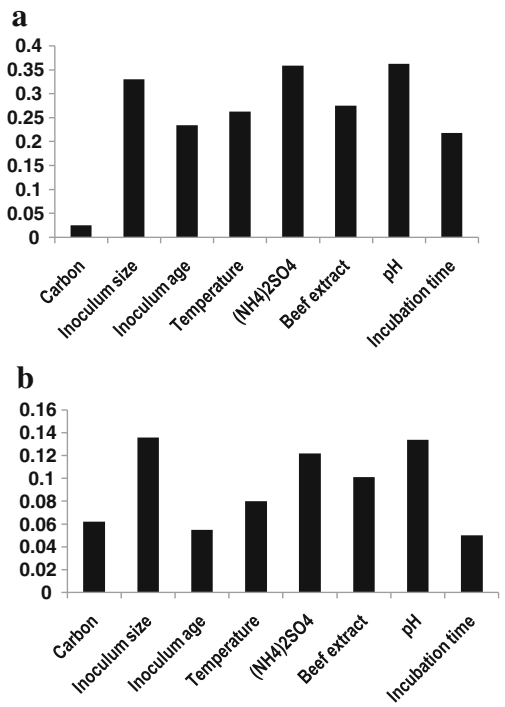

Fig. 6 Pareto chart showing significant parameters from the Plackett–Burman experimental design for biomass (**a**) and PHB production (**b**)

0.9929, and 1.33 % of the total variation could not be explained by the model. The R^2, which is the proportion of variation in the response attributed to the model rather than to random error (Henika 1972), should be above 80 % for a good fit of a model (Joglekar and May 1987). Our correlation coefficients of 0.9867 and 0.9801 for biomass and PHB, respectively, were close to 1, indicating a close agreement between the experimental results and the theoretical values predicted by the model equation. The pairs X_1X_2 and X_2X_3 showed a very good interaction, and this might be attributed to the biomass production at a significant level. In the case of PHB production, none of the pairs showed an interactive effect. The Student t distribution and the corresponding P value, along with the parameter, were estimated (data not shown). The P values verified the significance of each of the coefficients which, in turn, identified the pattern of the mutual interactions between the selected variables. The P values revealed that the PHB production was significantly influenced by the independent variables X_1 (inoculum size) and X_2 [$(NH_4)_2SO_4$]. Positive coefficients for X_1 and X_2 indicated a linear effect to increase biomass production, while the negative coefficient of X_3 (pH) revealed the opposite effect.

The interaction effects of variables on PHB as well as biomass yield were studied by plotting contour plots to determine the optimum level of each variable for maximum response. The surface plot showing interactions of a pair of factors are given in Fig. 7a–c.

Figure 7a shows the interaction between inoculum size and $(NH_4)_2SO_4$ on biomass production with the pH fixed at its middle level. The nature of the contour plots confirm that the interaction between inoculum size and $(NH_4)_2SO_4$ was significant; this significance was also evident from the P value (0.037,<0.05). The biomass production was found to increase with simultaneous increase in both factors. With increasing inoculum size, the bacteria was able to consume the available $(NH_4)_2SO_4$ and subsequently increase in biomass. At low levels of inoculum, the net biomass was not sufficient to allow utilization of all the available nutrients in the medium.

Figure 7b indicates the interaction between pH and $(NH_4)_2SO_4$ on biomass production. The shape of the contour plots indicates the absence of a positive interaction between these two factors. This study showed that PHB production was not associated to growth and that PHB was produced only when there was a higher carbon to nitrogen ratio in the medium. Therefore, a higher concentration of $(NH_4)_2SO_4$ did not enhance PHB accumulation but it did promote biomass production. These results demonstrate that a suitable concentration of $(NH_4)_2SO_4$ was required for PHB synthesis in our SSF system, otherwise excess nutrient was diverted towards biomass build-up with decreased PHB accumulation (Lakshman et al. 2004).

Figure 7c shows the effect of the interaction between $(NH_4)_2SO_4$ and pH on PHB. Here also the interactive effect was very low. There was a decrease in PHB production at high

Table 5 Analysis of variance for the quadratic polynomial model of biomass production

Source	*df*	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	0.390097	0.390097	0.043344	116.80	0.000
Linear	3	0.275828	0.275828	0.091943	247.77	0.000
Square	3	0.092514	0.092514	0.030838	83.10	0.000
Interaction	3	0.021755	0.021755	0.007252	19.54	0.003
Residual Error	5	0.001855	0.001855	0.000371		
Lack-of-fit	3	0.001525	0.001525	0.000508	3.07	0.255
Pure error	2	0.000331	0.000331	0.000165		
Total	14	0.391952				

Determination coefficient (R^2)= 99.53; Correlation coefficient (R)=98.67

Table 6 Analysis of variance for the quadratic polynomial model of PHB production

Source	*df*	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	0.053959	0.053959	0.005995	77.51	0.000
Linear	3	0.005783	0.005783	0.001928	24.92	0.002
Square	3	0.046596	0.046596	0.015532	200.80	0.000
Interaction	3	0.001580	0.001580	0.000527	6.81	0.032
Residual error	5	0.000387	0.000387	0.000077		
Lack-of-fit	3	0.000333	0.000333	0.000111	4.11	0.202
Pure Error	2	0.000054	0.000054	0.000027		
Total	14	0.054346				

Determination coefficient (R^2)= 99.29; Correlation coefficient (R)=98.01

levels of $(NH_4)_2SO_4$ and high pH. Maximum PHB production was observed at the middle level of pH (9.5). One of the most important factor that affects biomass production is the pH of the medium. In shake flask cultures PHB accumulation begins in the initial logarithmic phase and the pH of the medium decreases during growth. Kominek and Halvorson (1965) reported that a low medium pH inhibits utilization of the polymer as well as of spore formation in *Bacillus cereus*. Our study revealed that *Bacillus sphaericus* NII 0838 has a potential for producing PHB in a novel SSF system using PUF

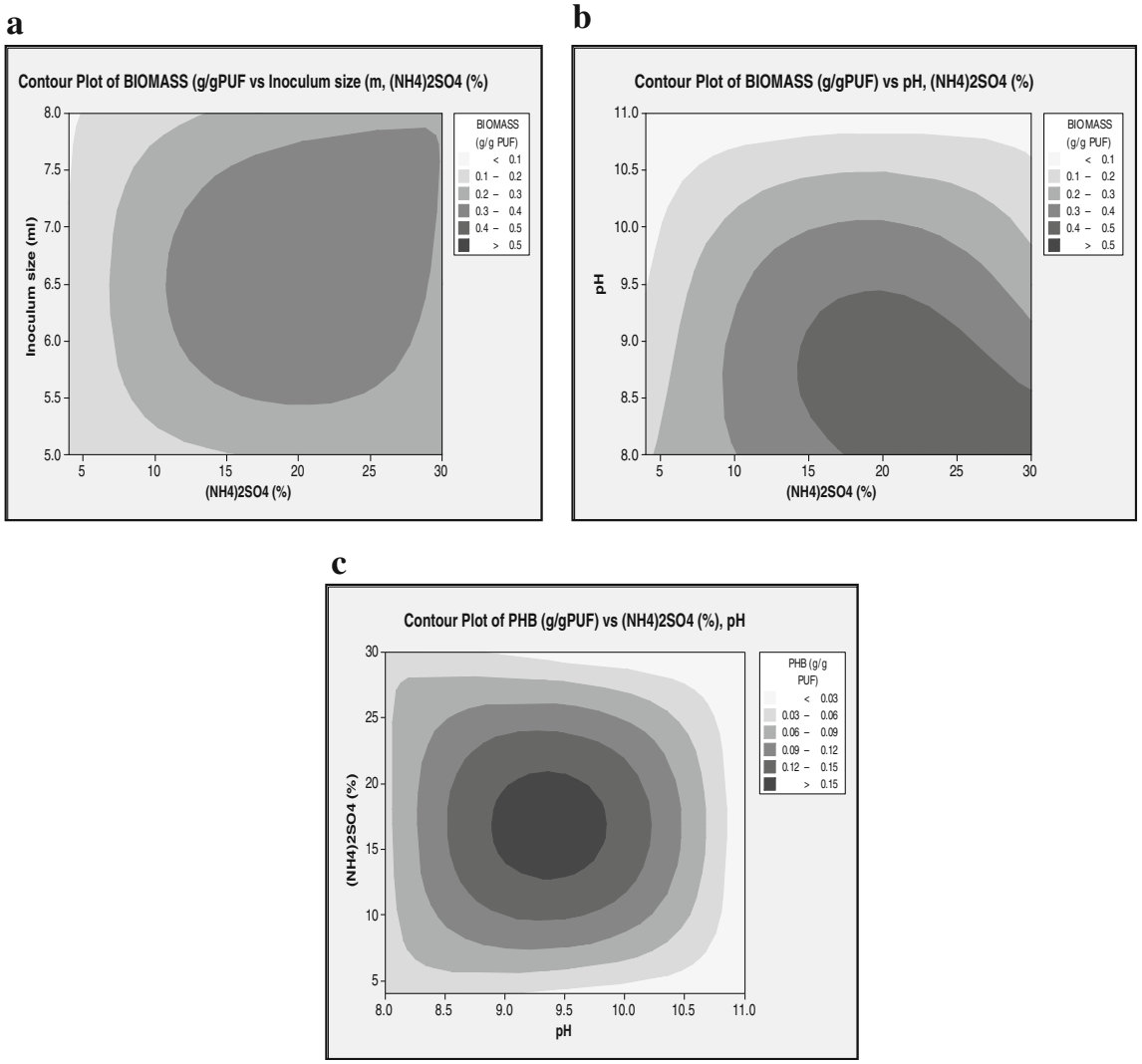

Fig. 7 Contour plot of biomass between inoculum size and $(NH_4)_2SO_4$ (**a**) and pH and $(NH_4)_2SO_4$ (**b**). **c** Contour plot of PHB between $(NH_4)_2SO_4$ and pH

supplemented with an inexpensive agro-residue, JS hydrolyzate as the sole carbon source.

Conclusions

Our results reveal the potential of a new SSF strategy using PUF as an inert support for the production of PHB by *Bacillus sphaericus* NII 0838. The major inherent problem associated with PHB production in SSF systems is the biomass retrieval of bacterial cells. This limitation can, however, be overcome by using PUF as an inert support. Media engineering for PHB as well as biomass production were carried out. The statistical optimization procedure incorporating inoculum size, pH and $(NH_4)_2SO_4$ concentration provide a useful means of trading off the interaction effects of these three variables on PHB yield. Maximum PHB yield was observed with an inoculum size of 8×10^8 CFU/ml, 1.7 % (w/v) $(NH_4)_2SO_4$ and pH 9.5. Statistical optimization resulted in a fourfold increase in PHB production. The inert nature of PUF facilitates the easy recovery of bacterial cells with fewer impurities, and the solid support can be reused in batch mode. To the best of our knowledge, this is the first report on the utilization of PUF as an inert support supplemented with minimal nutrients for the production of PHB. Based on our results, we conclude that SSF can be used as an alternative strategy for the production of PHB. However, further standardization as well as the economics of the process need to be evaluated for further scale-up of the process.

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References

Aidoo KE, Hendry R, Wood BJB (1982) Solid substrate fermentations. Adv Appl Microbiol 28:201–237

Bobbio FO, El-Dash AA, Bobbio PA, Rodrigues LR (1978) Isolation and characterization of the physicochemical properties of the starch of jackfruit seeds (*Artocarpus heterorphyllus*). Cereal Chem 55:505–511

Box GEP, Behnken DW (1960) Some new three level designs for the study of quantitative variables. Technometrics 2:455–475

Byrom D (1987) Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol 5:246–250

Byrom D (1994) Polyhydroxyalkanoates. In: Mobley DP (ed) Plastics from microbes: microbial synthesis of polymers and polymer precursors. Hanser, Munich, pp 5–33

Chi Z, Zhao S (2003) Optimization of medium and cultivation conditions for pullulan production by new pullulan-producing yeast. Enzyme Microb Technol 33:206–221

Choi J, Lee SY (1997) Process analysis and economic evaluation for Poly (3-hydroxybutyrate) production by fermentation. Bioproc Eng 17:335–342

Gervais P, Molin P (2003) The role of water in solid-state fermentation. Biochem Eng J 13:85–101

Grothe E, Moo-Young M, Chisti Y (1999) Fermentation optimization for the production of poly (β-hydroxybutyric acid) microbial thermoplastic. Enzyme Microb Technol 25:132–141

Henika RG (1972) Simple and effective system for use with response surface methodology. Cereal Sci Today 17:309–334

Joglekar AM, May AT (1987) Product excellence through design of experiments. Cereal Foods World 32:857–858

John RP, Nampoothiri KM, Pandey A (2006) Solid-state fermentation for l-lactic acid production from agrowaste using *Lactobacillus delbrueckii*. Process Biochem 41:759–763

Kominek LA, Halvorson HO (1965) Metabolism of poly-β-hydroxybutyrate and acetoin in *Bacillus cereus*. J Bacteriol 90:1251–1259

Lakshman K, Rastogi NK, Shamala TR (2004) Simultaneous comparative assessment of parent and mutant strain of *Rhizobium meliloti* for nutrient limitation and enhanced polyhydroxyalkanoate (PHA) production using optimization studies. Process Biochem 39:1977–1983

Law JH, Slepecky RA (1961) Assay of poly-β-hydroxybutyric acid. J Bacteriol 82:33–36

Miller GM (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Oliveira FC, Freire DMG, Castilho LR (2004) Production of poly (3-hydroxybutyrate) by solid-state fermentation with *Ralstonia eutropha*. Biotechnol Lett 26:1851–1855

Oliveira FC, Dias ML, Leda R, Castilho LR, Freire DMG (2007) Characterization of poly (3-hydroxybutyrate) produced by *Cupriavidus necator* in solid-state fermentation. Bioresour Technol 98:633–638

Ramadas NV, Singh SK, Soccol CR, Pandey A (2009) Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. Braz Arch Biol Technol 52:17–23

Ramadas NV, Soccol CR, Pandey A (2010) A statistical approach for optimization of polyhydroxybutyrate production by *Bacillus sphaericus* NCIM 5149 under submerged fermentation using central composite design. Appl Biochem Biotechnol 162:996–1007

Ramesh MV, Lonsane BK (1990) Critical importance of moisture content of the medium in alpha amylase production by *Bacillus licheniformis* M27 in a solid state fermentation medium. Appl Microbiol Biotechnol 33:501–505

Sandhya X, Lonsane BK (1994) Factors influencing fungal degradation of total soluble carbohydrates in sugar cane pressmud under solid state fermentation. Proc Biochem 29:295–301

Sindhu R, Ammu B, Binod P, Deepthi SK, Ramachandran KB, Soccol CR, Pandey A (2011) Production and characterization of poly-3-hydroxybutyrate from crude glycerol by *Bacillus sphaericus* NII 0838 and improving its thermal properties by blending with other polymers. Braz Arch Biol Technol 54:783–794

Steinbuchel A (1991) Polyhydroxyalkanoic acids. In: Byrom D (ed) Biomaterials: novel materials from biological sources. Stockton, New York, pp 124–213

Tabandeh F, Farahani EV (2003) Biosynthesis of poly-β-hydroxybutyrate as a biodegradable polymer. Iran Polym J 12:37–42

Yuksekdag ZN, Aslim B, Beyatli Y, Mercan N (2004) Effect of carbon and nitrogen sources and incubation times on poly-β-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium*. Afr J Biotechnol 3:63–66

Zhu Y, Smits JP, Knol WK, Bol J (1994) A novel solid-state fermentation system using polyurethane foamas inert carrier. Biotechnol Lett 16:643–648