Optimization of polyhydroxybutyrate production by *Bacillus* sp. CFR 256 with corn steep liquor as a nitrogen source

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Abstract Polyhydroxyalkanoates (PHAs), the eco-friendly biopolymers produced by many bacteria, are gaining importance in curtailing the environmental pollution by replacing the non-biodegradable plastics derived from petroleum. The present study was carried out to economize the polyhydroxybutyrate (PHB) production by optimizing the fermentation medium using corn steep liquor (CSL), a by-product of starch processing industry, as a cheap nitrogen source, by *Bacillus* sp. CFR 256. Response surface methodology (RSM) was used to optimize the fermentation medium using the variables such as corn steep liquor (5–25 g l⁻¹), Na₂HPO₄ · 2H₂O (2.2–6.2 g l⁻¹), KH₂PO₄ (0.5–2.5 g l⁻¹), sucrose (5–55 g l⁻¹) and inoculum concentration (1–25 ml l⁻¹). Central composite rotatable design (CCRD) experiments were carried out to study the complex interactions of the variables.

The optimum conditions for maximum PHB production were (g l⁻¹): CSL – 25, Na₂HPO₄ · 2H₂O – 2.2, KH₂PO₄ – 0.5, sucrose – 55 and inoculum – 10 (ml l⁻¹). After 72 h of fermentation, the amount of PHA produced was 8.20 g l⁻¹ (51.20 % of dry cell biomass). It is the first report on optimization of fermentation medium using CSL as a nitrogen source, for PHB production by *Bacillus* sp.

Keywords *Bacillus* · Corn steep liquor · Central composite rotatable design · Optimization · Polyhydroxybutyrate · Response surface methodology

Introduction

Polyhydroxyalkanoates (PHAs) are intracellular carbon and energy reserves that are accumulated by various bacteria when surplus carbon is available, under certain nutrient limitation conditions for growth. A number of bacteria including *Alcaligenes*, *Azotobacter*, *Pseudomonas*, recombinant *E. coli* have been found producing PHAs in the range of 32 to 164 g/l in 40–60 h with a productivity of around 2 g/l/h¹. PHAs have similar material properties to conventional plastics and are completely biodegradable. However, one of the problems facing the development of PHAs as substitutes for conventional plastics is their high price compared to petroleum based synthetic plastics¹. Therefore, efforts are being made to use alternate less expensive carbon, nitrogen sources, besides making alterations in the fermentation strategies to control the cost of the polymer. Use of various industrial wastes, as a carbon source, such as alcoholic distillery effluent² and potato processing waste³ for the production of PHA by microorganisms were reported. Corn steep liquor (CSL), a by product of starch industry, can be used as a nitrogen source to replace the much expensive pure chemicals such as ammonium sulphate, ammonium chloride, ammonium nitrate etc. CSL was used as a carbon source to produce PHB from *Azotobacter vinelandii* UWD⁴. When many factors and interactions affect desired response, response surface methodology (RSM) can be used effectively for optimizing the fermentation process parameters^{5–6}.

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Table 1 Variables and their levels for Central composite rotatable design.

Variables	Symbols	Levels				
		−2	−1	0	1	2
Corn Liquor (g l ^{−1})	X ₁	5.0	10.0	15.0	20.0	25.0
Na ₂ HPO ₄ (g l ^{−1})	X ₂	2.2	3.2	4.2	5.2	6.2
KH ₂ PO ₄ (g l ^{−1})	X ₃	0.5	1.0	1.5	2.0	2.5
Sucrose (g l ^{−1})	X ₄	5.0	17.5	30.0	42.5	55.0
Inoculum (% v/v)	X ₅	1.0	7.0	13.0	19.0	25.0

Table 2 Treatment schedule for five factor Central composite rotatable design and the response for polyhydroxybutyrate production by *Bacillus* sp. CFR 256.

Exp. No.	X ₁	X ₂	X ₃	X ₄	X ₅	Biomass yield (g l ^{−1}) (Y ₁)	PHB yield (g l ^{−1}) (Y ₂)
1	−1	−1	−1	−1	−1	4.92	1.97
2	1	−1	−1	−1	−1	7.37	2.33
3	−1	1	−1	−1	−1	5.97	2.90
4	1	1	−1	−1	−1	7.56	0.62
5	−1	−1	1	−1	−1	5.73	2.59
6	1	−1	1	−1	−1	6.88	1.32
7	−1	1	1	−1	1	6.12	2.34
8	1	1	1	−1	1	7.35	1.29
9	−1	−1	−1	1	−1	4.57	2.21
10	1	−1	−1	1	1	11.11	4.89
11	−1	1	−1	1	1	6.90	3.23
12	1	1	−1	1	1	10.69	5.24
13	−1	−1	1	1	1	4.22	2.05
14	1	−1	1	1	−1	9.81	4.69
15	−1	1	1	1	−1	6.47	3.32
16	1	1	1	1	1	5.51	2.08
17	−2	0	0	0	0	2.50	1.70
18	2	0	0	0	0	7.50	2.89
19	0	−2	0	0	0	6.76	3.19
20	0	2	0	0	0	7.20	3.13
21	0	0	−2	0	0	7.14	3.32
22	0	0	2	0	0	6.08	2.84
23	0	0	0	−2	0	4.50	0.28
24	0	0	0	2	0	6.87	2.72
25	0	0	0	0	−2	8.25	3.91
26	0	0	0	0	2	7.11	2.84
27	0	0	0	0	0	6.42	2.79
28	0	0	0	0	0	6.20	2.80
29	0	0	0	0	0	6.71	3.05
30	0	0	0	0	0	6.82	2.88
31	0	0	0	0	0	6.62	3.02
32	0	0	0	0	0	6.81	2.85

X₁: Corn Liquor concentration (g l^{−1}); X₂: Na₂HPO₄ (g l^{−1}); X₃: KH₂PO₄ (g l^{−1})X₄: Sucrose (g l^{−1}); X₅: Inoculum concentration (% v/v).

Central composite rotatable design (CCRD) is used to fit first or second order polynomial by a least squares technique. RSM has been used to optimize the process parameters

for the production of many metabolites including PHA⁷. Very recently optimization of PHB production by pure and mixed cultures was reviewed⁸. The objective of the present

Fig. 1 Response surfaces showing the effect of Corn steep liquor (CSL) and Inoculum concentration (a and b); CSL and sucrose (c and d); CSL and Na₂HPO₄ (e and f) on biomass (a, c and e) and PHA yields (b, d and f). Levels as shown in Table 1.

Table 3 Estimated coefficients of the fitted second order polynomial representing the relationship between the response and the process variables for biomass and polyhydroxybutyrate production by *Bacillus* sp. CFR 256.

	Estimated coefficient for Biomass yield (g l ⁻¹)	Estimated coefficient for PHB yield (g l ⁻¹)
a ₀	6.486 ^a	2.887 ^a
a ₁	1.308 ^a	0.176 ^a
a ₂	0.118 ^{ns}	-0.048 ^{ns}
a ₃	-0.380 ^a	-0.195 ^a
a ₄	0.505 ^a	0.718 ^a
a ₅	-0.293 ^b	-0.342 ^a
a ₁₁	-0.288 ^b	-0.139 ^b
a ₂₂	0.207 ^c	0.077 ^c
a ₃₃	0.114 ^{ns}	0.057 ^{ns}
a ₄₄	-0.117 ^{ns}	-0.338 ^a
a ₅₅	0.382 ^a	0.131 ^b
a ₁₂	-0.630 ^a	-0.436 ^a
a ₁₃	-0.460 ^a	-0.231 ^a
a ₁₄	0.534 ^a	0.646 ^a
a ₁₅	-0.224 ^c	-0.201 ^b
a ₂₃	-0.271 ^c	-0.138 ^c
a ₂₄	-0.140 ^{ns}	0.068 ^{ns}
a ₂₅	-0.253 ^c	-0.181 ^b
a ₃₄	-0.470 ^a	-0.197 ^b
a ₃₅	-0.533 ^a	-0.133 ^c
a ₄₅	-0.179 ^{ns}	-0.022 ^{ns}

^aSignificant at 0.1% level, ^bSignificant at 1.0% level,^cSignificant at 5.0% level, ^{ns} Not significant.

work was to optimize PHB production by *Bacillus* sp. CFR 256 by using RSM and to study the effect of medium components on the yield of the biomass and the polymer.

Materials and Methods

Bacillus sp. CFR 256 (MTCC 5149) was isolated from local soil sample and maintained on nutrient agar slants at 4°C. Inoculum was prepared by transferring the growth of a 24 h old slant culture, which was incubated at 30°C, into 100 ml medium containing (g l⁻¹): Na₂HPO₄·2H₂O – 4.4; KH₂PO₄ – 1.5; (NH₄)₂SO₄ – 1.0; MgSO₄·7H₂O – 0.2; sucrose – 10; pH 7.0. The inoculated flasks were incubated at 30°C and 250 rpm for 24 h and it had 2 × 10³ viable cells/ml.

For PHB production by RSM, CSL (N-source), sucrose (C-source) along with Na₂HPO₄·2H₂O, KH₂PO₄, (NH₄)₂SO₄ and inoculum were considered for optimization of medium components. MgSO₄·7H₂O (0.2 g l⁻¹) was used as a common component throughout the studies. A central rotatable composite design (CCRD)⁹ with 5 variables mentioned above, at 5 levels was used to study the response pattern and optimum combination of the variables used (Table 1). The CCRD was arranged to allow the fitting of the regression model using multiple regression program (Table 2). Optimum conditions were deduced from the surface plots, which allowed visualization of the relationship between the response and the experimental levels of each factor used in the experiments. Six replicates (treatments from 27–32) were included for estimation of a pure error of sum of squares. A

Table 4 Analysis of variance for the fitted second order polynomial model and lack of fit for biomass yield as per Central composite rotatable design.

Source of Variation	df	Biomass Yield			PHA yield		
		Sum of squares	Mean sum of squares	F- value	Sum of squares	Mean sum of squares	F- value
<i>Regression</i>							
1 st order terms	5	53.005 ^a	10.601	143.250	16.888 ^a	3.378	259.846
2 nd order terms	15	35.555 ^a	2.370	32.027	18.040 ^a	1.203	95.538
Total	20	88.560			34.927		
<i>Residual</i>							
Lack of fit	6	1.837 ^{ns}	0.306	4.135	0.480 ^{ns}	0.080	6.154
Pure error	5	0.371	0.074		0.063	0.013	
Total error	11	2.208			0.542		
Grand Total	31	90.769			35.470		
Coefficient of Determination (R ²)			0.976			0.984	

^a Significant at 5.0% level, ^{ns} Not significant at 5.0% level.

Table 5 Feasible optimum conditions and predicted value of response at optimum conditions for biomass and polyhydroxybutyrate production by *Bacillus* sp. CFR 256.

Optimum conditions		For Biomass (Y ₁)	For PHB (Y ₂)
Corn Liquor (g l ⁻¹)	x ₁	25.0	25.0
Na ₂ HPO ₄ (g l ⁻¹)	x ₂	2.2	2.2
KH ₂ PO ₄ (g l ⁻¹)	x ₃	0.5	0.5
Sucrose (g l ⁻¹)	x ₄	55.0	55.0
Inoculum (ml l ⁻¹)	x ₅	250.0	10.0
Responses for biomass:			
Predicted value (g l ⁻¹)		20.67	9.20 (44.5)*
Experimental Value (g l ⁻¹)		18.00	7.20 (40.0)
Responses for PHA:			
Predicted value (g l ⁻¹)		18.73	9.84 (52.5)
Experimental Value (g l ⁻¹)		16.00	8.20 (51.2)

*Values in the parenthesis are the PHB yield for the biomass produced.

second order polynomial equation was used to fit the experimental data given in Table 3. The model proposed for the responses (Y₁ and Y₂) was $Y_i = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{44}x_4^2 + a_{55}x_5^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{14}x_1x_4 + a_{15}x_1x_5 + a_{23}x_2x_3 + a_{24}x_2x_4 + a_{25}x_2x_5 + a_{34}x_3x_4 + a_{35}x_3x_5 + a_{45}x_4x_5 + \epsilon$, where Y_i (i = 1 and 2) is the predicted response for biomass and PHB, respectively, a₀ is the value of the fitted response at the center point of the design, a_i, a_{ii}, a_{ij} the linear, quadratic and cross point terms, respectively. Optimization of fitted polynomials for cell biomass and PHB yield was performed by a non-linear mathematical optimization procedure of the Quattro Pro software package (Quattro pro, Version 4.0, Borland International Inc. USA)¹⁰. The optimum conditions were monitored and the results were compared with predicted values.

For the measurement of biomass, 10 ml of culture broth was centrifuged (6000 × g for 15 min) and the sediment was washed thoroughly with distilled water and the cells were dried to a constant weight at 70°C in airflow dryer. The PHB content from the biomass was determined after sodium hypochlorite hydrolysis and chloroform extraction^{11–12}. Extracted PHB sample was characterized by gas chromatography analysis by converting it to methyl esters of monomers as described earlier⁷.

Results and Discussion

The estimated coefficients representing the relationship between the response (biomass (Y₁) and PHB (Y₂)) and

the process variables are given in Table 3. The responses obtained under different combinations of variables and defined experimental design (Tables 1 and 2) were analyzed by analysis of variance (Table 4) appropriate to the experimental design. The first and second (quadratic and cross product) order terms were found to be significant (p > 0.5) and lack of fit was not significant (p > 0.5). The values of coefficient of determination (R²) vary from 0.976 to 0.984, which suggest that the models are in good fit. The conditions optimized by the RSM for the production of biomass and PHB are listed in Table 5. Although the amount of biomass and PHB produced using the optimized conditions is less than the predicted values, the yield of PHB was comparable to that of the predicted values.

The effect of CSL concentration, sucrose concentration, inoculum level, buffering salts on responses such as biomass and PHB yields are reported by the coefficients of second order polynomials. Some of the response surfaces based on these coefficients are shown in Figure 1. At optimum level of Na₂HPO₄ (2.20 g l⁻¹), KH₂PO₄ (0.50 g l⁻¹) and sucrose (55.00 g l⁻¹), maximum biomass yield (20.67 g l⁻¹) was obtained at the highest corn steep liquor concentration (25 g l⁻¹) as well as highest inoculum concentration (250 ml l⁻¹). Highest concentration of inoculum (250 ml l⁻¹) was found to favour increased biomass content, whereas, at the same concentration of CSL and minimum inoculum concentration (10 ml l⁻¹), increased PHB yield and decreased biomass content were obtained (Fig. 1 a, b). Similar results were reported for *Rhizobium meliloti*⁷. When sucrose was used at optimum level (55 g l⁻¹), maximum quantities of biomass and PHB yields were found at highest concentration of CSL (25 g l⁻¹) (Fig. 1 c, d). As can be seen in the present study, many bacteria synthesize PHAs intracellularly under certain nutrient depleted conditions, presence of excess carbon and less nitrogen source, as PHA formation is favoured by conditions that led to high NADPH, high acetyl CoA and low free coenzyme A concentrations inside the cells. The β-ketothiolase, a key enzyme in PHA synthesis, is completely inhibited by high concentrations of COASH, when grown in nutrient rich medium with out any limitation of carbon and nitrogen. Under unbalanced growth conditions, acetyl-CoA concentrations remain high but free CoA is low, thus avoid inhibition of β-ketothiolase activity¹³.

At optimum level of KH₂PO₄ (0.5 g l⁻¹), inoculum concentration (10 ml l⁻¹) and sucrose (55.00 g l⁻¹), the maximum biomass and PHA yields were obtained at lowest Na₂HPO₄ concentration (2.2 g l⁻¹) and highest CSL concentration (25 g l⁻¹) (Fig. 1 e, f). Although there is a reduction in PHB yield with increase in the concentration of KH₂PO₄, the biomass yield was dropped by two folds, when the con-

centration was raised to 2.0 g l^{-1} . The data indicated that the organism also requires phosphate limitation in addition to nitrogen limitation for optimum production of PHB as higher concentration of phosphate levels led to a drop in PHB content. This is in accordance with the observation made by Williams and Munro¹³, who opined that besides nitrogen, level of phosphorous or sulfur can also influence rate of PHA production. Similar observation was made by Kshama *et al*⁷, wherein; 8% increase of PHA concentration when the initial level of phosphate in the medium is reduced from 0.78 g l^{-1} to 0.33 g l^{-1} was reported. Addition of nitrogen source (NH_4Cl) to the fermentation medium during PHB synthesis caused *Alcaligenes eutrophus* H16 to cease PHB accumulation, indicating that synthesis of PHB is regulated by N assimilation¹⁴. Sucrose, besides glucose and fructose was found to be more suitable for cell growth as well as PHB accumulation (69.4 % dry cell weight) by *Bacillus mycoides* RLJ B-017 and accumulation of PHB was observed to be growth associated¹⁵. The gas chromatography analysis of the PHA produced from corn steep liquor medium showed (data not shown) that the polymer was pure PHB. Additional co-carbon substrates can lead to production of PHA co-polymers. A strain of *Rhizobium meliloti* produced only PHB when grown on fructose¹⁶, however, it synthesized 58% PHA of cell biomass, containing up to 22% of β -hydroxyvalerate, when grown with succinate as main carbon source and propionate as co-substrate¹⁷.

Conclusion

The response surfaces indicated a complex interaction among all the variables tested. The low PHB yield (42.5%) obtained with CSL than the yield with ammonium sulphate (62.0 %) may not be significant, when the cost of these substrates is compared. The PHB yields (51.2%) obtained in the optimized conditions are comparable to the values predicted by using RSM technique (52.5%). Further studies in this direction can give more information for the production of PHA in large- scale level.

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