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Full Length Research Paper

Novel approach for optimization of fermentative condition for polyhydroxybutyrate (PHB) production by *Alcaligenes* sp. using Taguchi (DOE) methodology

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Polyhydroxybutyrate (PHB) is a biodegradable thermoplastic polymer which is accumulated as energy reserve material by large number of microorganisms including bacteria, fungus and yeast under nutrient stress condition. In this study, efforts have been made to optimize PHB production by *Alcaligenes* sp. NCIM 5085 using Taguchi (DOE) methodology. This approach facilitates the study of interaction of a large number of variables spanned by factors and their settings with a smaller number of experiments, leading to considerable savings in time and cost for process optimization. Eight factors at two levels with an OA layout of L-12 were selected for proposed experimental design. PHB concentration was increased from 5.20 to 6.58 g/L under optimal cultural condition. Result validation showed 95% resemblance with the expected value.

Key words: Polyhydroxybutyrate, *Alcaligenes* sp. Taguchi (DOE), orthogonal arrays layout.

INTRODUCTION

Polyhydroxybutyrate (PHB) is a natural, biodegradable polymer, which is accumulated in the form of intracellular granules by large number of bacteria. These granules acts as energy reserve materials when nutrients such as nitrogen and phosphorus sources are available in limiting concentrations in the presence of excess carbon sources (Byrom, 1987; Anderson and Dawes, 1990). These can be completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in soil, sea, sewage and other fresh water sources (Lee, 1996).

Currently, the main concern associated with the widespread application of PHB and its copolymers is its relatively high cost as compared to polypropylene. Raw materials cost and downstream processing makes PHB to be expensive in comparison with other petroleum derived plastics. Optimization of fermentation process and gene cloning had been extensively used for number of measurements to get the greatest amount of information. Taguchi method of orthogonal array (OA) experimental design (DOE) involves the study of any given system by a set of independent variables (factors) over a specific region of interest (levels) (Grothe et al., 1999; Ross, 1996). This approach not only helps in considerable saving in item and cost but also leads to more fully developed process by providing systematic, simple and efficient methodology for the optimization of the near optimum design parameters with only a few well defined experimental sets (Mohan et al., 2005). It has been applied for the optimization of a few biochemical techniques and bioprocess applications (Venil and Lakshmanaperumalsamy, 2009). Taguchi approach was previously used for optimization of tannase production by *Bacillus licheniformis* KBR6 (Mohapotra et al., 2009) and chitinase production by *Serratia marcescens* B4A (Mandana et al., 2010).

The objective of this study was to optimize the submerged culture conditions for PHB production by *Alcaligenes* sp. NCIM 5085 using cane molasses and urea as carbon and nitrogen source. Taguchi (DOE) was selected as software tool for optimization process.

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Abbreviations: **OA**, Orthogonal arrays; **DOE**, design of experiment; **PHB**, polyhydroxybutyrate.

Table 1. Factor and level descriptions.

S/N	Factor	Level 1	Level 2
1	A Cane molasses	1	4
2	B Urea	1	3
3	C KH_2PO_4	0.5	3
4	D $MgSO_4.7H_2O$	0.1	1
5	E Na_2HPO_4	0.5	2
6	F Yeast Extract	0.6	4
7	G $CaCl_2$	0.01	0.03
8	H Trace metal solution	5	15
	Column unused	*Unused*	-
10	Column unused	*Unused*	-
11	Column unused	*Unused*	-

MATERIALS AND METHODS

Alcaligenes sp. NCIM 5085 was used for fermentative PHB production. The culture was maintained on nutrient agar slants at 5°C and subcultured monthly.

Taguchi methodology

Optimization methodology adopted in this study was divided into three phases viz., experiment designing, prediction of performance by analysis of experimental data and process validation. Taguchi method of DOE involves establishment of large number of experimental situation described as OAs (orthogonal arrays) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments. Each phase had separate objective, inter connected in sequence wise to achieve the overall optimization process.

Experiments designing (phase 1)

The first step in phase 1 is to determine the various factors to be optimized in the culture medium that have critical effect on the PHB yield. The normal practice is to experiment with the feasible range, so that the variation inherent in the process does not mark the factor effect. Factors were selected and the ranges were further assigned based on the group consensus consisting of design engineers, scientists and technicians with relevant experience data analysis procedure. Submerged fermentation experiments were carried out in cotton plugged 250 ml Erlenmeyer flasks containing 100 ml of production medium [(g/1000 ml of distilled water): Cane molasses (10, 40); urea (1, 3), KH_2PO_4 (0.5, 3); $MgSO_4.7H_2O$ (0.1, 1); CSL (0.5, 2); Na_2HPO_4 (0.6, 4); $CaCl_2$ (0.01, 0.03) and trace metal solution (5 ml/L), 15 ml/L)] (Table 1). Cane molasses, CSL and salt solutions were sterilized separately at 121°C and then aseptically reconstituted at room temperature prior to inoculation. The pH of resulting broth was adjusted to 7.0 with 2 N NaOH/2 N HCl.

The appropriate OAs for the control parameters which is suitable for a specific study was selected. Taguchi provides many standard OAs and corresponding linear graphs for this purpose. In this study, twelve set of experiments were designed with eight factors for PHB yield (carbon, nitrogen, KH_2PO_4, $MgSO_4.7H_2O$, Na_2HPO_4, CSL, $CaCl_2$ and trace metal solutions) at two levels with OA layout of L-12 (Table 2). Bacterial growth was initially carried out as given in literature (Raje et al., 1998).

The organism was cultivated at an agitation speed of 150 rpm and temperature of 30°C for 24 h in a 250 ml Erlenmeyer flask containing 50 ml of medium described earlier. For production of PHB, 100 ml of media (containing 40 g/L cane molasses) was taken in a 500 ml flask and inoculated with 5 ml of inoculum. The flask was kept under shaking condition for 48 h at 150 rpm and 30°C. Culture broth was centrifuged at 10,000 rpm for 10 min at 4°C and cell pellet obtained was dried at 90°C for 24 h. Dried cell mass was then used for PHB analysis by Law and Splecky (1961) method.

Prediction of performance by analysis of experimental data (phase 2)

The obtained experimental data was processed in the Qualitek-4 software with bigger and better quality characteristics for the determination of the optimum culture conditions for fermentation, to identify individual factors influence on the PHB production and to estimate the performance (fermentation) at the optimum conditions. In Taguchi's method, quality is measured by the deviation of a characteristic from its target value and a loss function [L(y)] is developed for the deviation as represented by $L(y) = k \times (y-m)^2$, where k denotes the proportionality constant, m represents the target value and y is the experimental value obtained for each trail. In the case of bigger is better quality characteristics, the loss function can be written as $L(y) = k \times (1/y^2)$ and the expected loss function can be represented as:

$$E\left[L(y)\right] = k \times E\left(\frac{1}{y^2}\right) \quad (1)$$

Where, $E(1/y^2)$ can be estimated from a sample of *n* as:

$$\frac{\sum \left[1/Y_i^2\right]}{n}$$

The results obtained from the data processing are shown in Tables 2 to 6.

Validation (phase 3)

In order to validate the methodology, fermentation experiments were further performed for PHB production using the obtained optimized culture condition.

Software

Qualitek-4 software (Nutek Inc., MI) for automatic design of experiments using Taguchi approach was used in this study. Qualitek-4 software is equipped to use L-4 to L-64 arrays along with selection of 2 to 63 factors with two, three and four levels for each factor. The automatic design option allows Qualitek-4 to select the array used and assign factors to appropriate columns.

RESULTS AND DISCUSSION

Effect of individual factor on PHB production

Submerged fermentation studies with the designed experimental conditions showed significant variation in

Table 2. Design of experiment.

Trial	Column											
	1	2	3	4	5	6	7	8	9	10	11	PHB (g/l)
Trial 1	1	2	1	2	1	2	2	2	0	0	0	0.10±0.1
Trial 2	2	2	1	1	1	2	1	2	0	0	0	3.28±0.3
Trial 3	2	2	1	1	2	1	2	1	0	0	0	1.05±0.2
Trial 4	1	1	1	2	2	2	1	1	0	0	0	0.60±0.3
Trial 5	2	1	2	1	1	2	2	1	0	0	0	3.67±0.6
Trial 6	1	1	2	1	2	2	2	2	0	0	0	0.40±0.4
Trial 7	2	2	2	2	2	2	1	1	0	0	0	4.6±0.3
Trial 8	1	2	2	2	2	1	2	1	0	0	0	0.81±0.2
Trial 9	2	1	2	2	1	1	1	2	0	0	0	5.20±0.5
Trial 10	2	1	1	2	2	1	2	2	0	0	0	4.75±0.4
Trial 11	1	1	1	1	1	1	1	1	0	0	0	0.34±0.3
Trial 12	1	2	2	1	2	1	1	2	0	0	0	0.03±0.2

the PHB produced (Table 1). Twelve (12) set ofex-periments were designed by Qualitek-4 software at two levels of each factor (Table 2). From Table 2, it can be clearly deduced that trial no.12 gave minimum PHB production (0.03 g/L) and trial no. 9 gave maximum PHB production of 5.20 g/L. Similarly, trial no 10, 7 and 5 gave PHB concentration of 4.75 ± 0.4, 4.60 ± 0.3 and 3.67 ± 0.6 g/L, respectively where cane molasses concentration was kept at level 1. On the other hand, trial no 2 and 3 gave PHB concentration of 3.28 ± 0.3 and 1.05 ± 0.2 g/L, respectively. These finding demonstrate that high concentration of cane molasses and lower concentration of urea favors the PHB production. The average effect of the factors together with their interactions at assigned levels on the PHB production indicates that individuals at level stage, cane molasses (Factor A), KH_2PO_4 (Factor B), $MgSO_4.7H_2O$ (Factor 4) and trace metal solution (Factor H) showed significant effect at their higher level (Table 3) which is in correlation with previous findings of Khanna and Srivastava (2005). However, nitrogen source (urea) showed its significant effect at level 1, on PHB concentration. The difference between level 2 and l (L_2-L_1) of each factor indicates the relative influence of different factors on PHB production (Table 3). The larger the difference value, the stronger is the influence of that factor on PHB produced. Mahmudi et al. (2010) under similar cultural condition reported PHB production of 1.6 g/L which is lower than the current findings. Trace metal solution showed significant effect ($p < 0.05$) on PHB production since these are needed for regulation of various enzymatic reactions during PHB synthesis (Shojaosadati et al., 2009).

Na_2HPO_4, $CaCl_2$ and yeast extract showed negative influence ($p < 0.05$) on PHB production, since these are needed for growth of cell biomass and not for PHB production.

Effect of factors interaction on PHB production

Taguchi (DOE) provides an opportunity to understand the interaction between two factors which are essential for overall process analysis. Presence of different factors creates the possibility of the presence of number of interactions. Estimated interaction severity index (SI) of the factors under study helps to show the influence of two individual factors at various levels of interaction (Table 4). In Table 4, the 'columns' represent the locations to which the interesting factors are assigned. Interactions SI presents 100% of SI for 90° angle between the lines and 0% SI for parallel lines. 'Reserved column' represents the column that should be reserved if this interaction effect has to be studied. "Levels" indicate the factor levels desirable for optimum conditions. From Table 4, it can be pooled that Na_2HPO_4 and trace metal solution (at levels 1 and 2; column 14) interaction showed higher SI value (84.03%) followed by $CaCl_2$ and trace metal solutions interactions (at level 1 and 2; column 15) with SI value of 72.85%. It is interesting to note that Na_2HPO_4 with less impact factor (-0.66%) showed higher severity index in combination with trace metal solution (55.68%). On the contrary, the SI between cane molasses (most significant factor) and trace metal solution was significantly low (15.65%). This indicates that the influence of individual factor on PHB yield varies in combination with other factors. Increasing trace metal solution concentration enhances PHB production since certain divalent ions such as copper and zinc acts as activator of PHB synthase (Mokhtari et al., 2010). Similarly, urea (least impact factor) and Na_2HPO_4 interaction showed high SI (58.2%) in comparison with other higher impact factors which depicts that urea and KH_2PO_4 at their low concentration showed significant effect on PHB concentration. KH_2PO_4 and NaH_2PO_4

Table 3. Main effects of selected factors.

S/N	Factor	Level 1	Level 2	L2 – L1
1	A Cane molasses	0.258	4.228	3.969
2	B Urea	2.879	1.606	-1.273
3	C KH_2PO_4	1.798	2.688	0.89
4	D $MgSO_4.7H_2O$	1.783	2.703	0.919
5	E Na_2HPO_4	2.573	1.913	-0.66
6	F Yeast Extract	2.386	2.1	-0.28
7	G $CaCl_2$	2.365	2.121	-0.245
8	H Trace metal solution	2.016	2.469	0.452

Table 4. Estimated interaction of severity index for different factors.

S/N	Interacting pair	Column	SI (%)	COI	Opt
1	F x H	6 x 8	84.03	14	[1, 2]
2	G x H	7 x 8	72.85	15	[1, 2]
3	D x F	4 x 6	70.88	2	[2, 1]
4	C x E	3 x 5	68.43	6	[2, 2]
5	D x H	4 x 8	65.92	12	[1, 1]
6	E x G	5 x 7	64.83	2	[2, 1]
7	D x G	4 x 7	62.13	3	[2, 1]
8	F x G	6 x 7	60.73	1	[2, 1]
9	C x G	3 x 7	59.84	4	[2, 2]
10	C x H	3 x 8	59.66	11	[1, 1]
11	D x E	4 x 5	59.53	1	[1, 2]
12	B x F	2 x 6	58.20	4	[1, 2]
13	E x H	5 x 8	55.60	13	[1, 1]
14	B x H	2 x 8	54.84	10	[2, 2]
15	B x E	2 x 5	52.95	7	[2, 2]
16	C x F	3 x 6	52.32	5	[2, 1]
17	C x D	3 x 4	51.32	7	[2, 2]
18	B x G	2 x 7	48.86	5	[1, 2]
19	B x D	2 x 4	47.38	6	[1, 2]
20	B x C	2 x 3	47.31	1	[1, 2]
21	E x F	5 x 6	45.00	3	[1, 2]
22	A x B	1 x 2	22.18	3	[2, 1]
23	A x C	1 x 3	18.10	2	[2, 2]
24	A x D	1 x 4	16.70	5	[2, 2]
25	A x H	1 x 8	15.65	9	[2, 2]
26	A x E	1 x 5	12.16	4	[2, 1]
27	A x -	1 x 7	6.58	6	[2, 1]
28	A x F	1 x 6	5.09	7	[2, 1]

concentration stimulates the cell growth because microorganism requires K, Na and P elements for their optimal growth. $MgSO_4.7H_2O$ and KH_2PO_4 showed significantly higher SI (68.45 and 70.88%) with other factors. In contrast, the strongest impact factor (cane molasses) with L_2-L_1 value of 3.961 showed very low severity index with other factors. However, it can be clearly seen from Table 3 that cane molasses gave higher SI (22.18%) on interaction with urea (least impact factor) at its higher level, indicating that C : N ratio play significant role in PHB production.

ANOVA of fermentation process

In Taguchi approach, analysis of variance (ANOVA) is

Table 5. Analysis of variance.

S/N	Factor	Df	Sum of square	Variance	F-ratio	Pure sum	Percentage contribution
1	A Fructose	1	47.282	47.282	476.563	47.183	79.024
2	B Urea	1	4.864	4.864	49.025	4.764	7.980
3	C KH_2PO_4	1	2.376	2.376	23.950	2.277	3.813
4	D $MgSO_4.7H_2O$	1	2.539	2.539	25.592	2.439	4.086
5	E Na_2HPO_4	1	1.306	1.306	13.171	1.207	2.022
6	F Yeast Extract	1	0.246	0.246	2.484	0.147	0.246
7	G $CaCl_2$	1	0.177	0.177	1.790	0.078	0.131
8	H Trace metal solution	1	0.616	0.616	6.214	0.517	0.866
	Other/error	3	0.297	0.099			1.832
	Total	11	59.707				100.00%

Table 6. Optimum culture conditions and performance.

S/N	Factor	Level dec.	Level	Contribution
1	A	4	2	1.98
2	B	1	1	0.64
3	C	3	2	0.45
4	D	1	2	0.46
5	E	0.5	1	0.33
6	F	0.6	1	0.14
7	G	0.01	1	0.12
8	H	15	2	0.27
Total contribution from all factors				4.33
Current grand average of performance				6.58
Expected result at optimum condition				7.46

used to analyze the results of the OAs experiment and to determine how much variation each factor has contributed. By studying the main effects of each factor, the process can be characterized. ANOVA with the percentage contribution of each factor with interaction depicts that cane molasses (79.02% contribution) is the most significant factor for PHB production followed by urea (7.98% contribution) and $MgSO_4.7H_2O$ (3.81% contribution). Yeast extract, $CaCl_2$ and trace metal solution showed less contribution due to factors interaction effect. Mercan et al. (2002) investigated reduced PHB production in two strains of *Rhizobium* sp. when grown on yeast extract mannitol (YEM) broth which is in correlation with the current finding. However, SI data (Table 3) revealed that calcium chloride and yeast extract interaction showed high SI value (60.73) which indicates that $CaCl_2$ supplementation in the presence of yeast extract had positive influence on PHB production. Reusch et al. (1986) reported that Ca^{2+} ions triggers PHA synthase activity.

Optimum cultural conditions

Taguchi DOE design, used for the optimization of culture condition for PHB production by *Alcaligenes* sp. NCIM 5085 revealed the effect of interaction of different factors on PHB concentration. Taguchi (DOE) requires lesser number of trials for process optimization in comparison with other statistical software tools such as central composite design (CCD) and box benhken design. Optimum condition and their performance in terms of contribution for achieving higher PHB concentration depicts that the cane molasses and $MgSO_4.7H_2O$ had strong influence on the PHB production, while urea showed least significant influence. Higher C : N ratio of the feed substrate favors PHB production (Lafferty et al., 1988). Under unbalanced condition, acetyl CoA cannot enter the tricarboxylic acid (TCA) cycle to obtain energy for cells due to high concentration of NADH and changes the TCA cycle to PHB synthesis pathway (Doi et al., 1990). In interaction studies, Na_2HPO_4 with trace metal solution showed highest interaction activity (SI). However, Na_2HPO_4 and trace metal solution are not very strong impact factors when considered individually. It is shown in the results that individual factors have varied influence on PHB production, while in combination of factors, PHB concentration varies. The higher levels of PHB production can be achieved with obtained optimization culture condition (g/L): Cane molasses 4; urea 1;

KH_2PO_4 3; $MgSO_4.7H_2O$ 1; yeast extract 0.5; Na_2HPO_4 0.6; $CaCl_2$ 0.01 and trace metal solution 15 (ml/l). The expected result at optimum condition is 7.46 g/L with total contribution from all the factors being 4.34 g/L. It is evident from Table 6 that upon consideration of the optimum culture condition from the experiments designed, the PHB concentration can be increased from 5.20 to 6.58 g/L, that is, overall 26.54% enhancement in the PHB yield can be achieved. Furthermore, to validate the proposed experimental methodology, experiments were performed for PHB production by employing the obtained optimized culture conditions (Table 6). The experimental data showed PHB concentration of 6.25 g/L under modified culture conditions which represents 95% validation of the expected result. PHB production can be further scaled up in larger fermentor under optimized cultural conditions.

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