Application of the Taguchi Design for Production of Poly(β-hydroxybutyrate) by *Ralstonia eutropha*

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ABSTRACT: *The Taguchi design of experiments was used to test the relative importance of medium components and environmental factors on poly(β-hydroxybutyrate)(PHB) production by Ralstonia eutropha. The optimum condition was obtained as: fructose concentration, 15 g/L; C/N ratio, 7.4; agitation speed 200 rpm; culture time, 40 h; temperature, 25 °C; seed age, 15 h. At optimum condition the yield of PHB production was found to be 92.36%.*

KEY WORDS: *Poly (β-hydroxybutyrate), Taguchi experimental design, Ralstionia eutropha, Biodegradable*

INTRODUCTION

Poly (β-hydroxybutyrate)(PHB) is an intracellular storage compound, which provides a reserve of carbon and energy in microorganisms [1]. It accumulates as distinct inclusions in the cell and comprises up to 80% of cell dry weight for strains of *Ralstonia eutropha*, under conditions of nitrogen and phosphate limitation and excess of carbon source [2]. PHB, which is a biodegradable, biocompatible thermoplastic, has broadly similar physical properties to polypropylene. It has many applications in medicine, veterinary practice, and agriculture due to its biodegradability [3]. Currently the main problem, which limits the widespread use of PHB and its copolymers, is its relatively high cost compared to polypropylene. The fermentation process, substrates and product recovery are the major costs [4]. Research has focussed on reducing these costs by optimizing fermentation process and gene cloning [5]. A good experimental design to be used for optimization employs the fewest number of measurements to get the greatest amount of information. The Taguchi experimental design has been frequently used for screening process variables that make the greatest impact on a process [6, 7]. Recent reports on the use of Taguchi design of experiments in biotechnology research include its application toward improved citric acid production from apple pomace in flask [8] and multiylayer packed bed solid state bioreactor [9]. This design which allows for the study of k = (N-1)/(L-1) factors (each with L levels) with N experimental trials, involves the construction of a special set of orthogonal array (OA) to lay out the experiments.

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In this study the application of Taguchi method to test the relative importance of medium components (i.e., carbon and nitrogen source) and environmental factors (i.e., temperature, culture time, seed age, and agitation speed) on the PHB production is presented. The effects of these variables at two levels were studied using an L8 orthogonal array of Taguchi method on PHB production.

EXPERIMENTAL

Organism and growth conditions

Ralstonia eutropha (ACM 1296) was obtained from the Australian Collection of Microorganisms. It is derived from strain ATCC 17699, which has been used for studies involving single cell protein and PHB production [10]. Cells were maintained on PYEA (peptone yeast extract agar), medium, which consists of (g/L): peptone, 10; yeast extract, 5; sodium chloride, 5; agar, 15. The cultures were subcultured every 3 weeks to ensure the availability of sufficient stock culture for experimental purpose. Bradford medium was modified to provide a source of carbon, nitrogen and trace elements in a phosphate buffer and to simplify medium formulation and sterilization [11].

The chemical composition of seed culture medium were (g/L): Na_2HPO_4, 3.5; KH_2PO_4, 1.5, Fructose, 9.5; $(NH_4)_2SO_4$, 1.35 and filtered trace elements solution (10%, v/v). Fermentation medium was similar to seed medium, except fructose concentration.

Analytical methods

Cell dry weight was determined gravimetrically. 10-mL culture samples were centrifuged at 5000 rpm for 15 minutes and the pellets were washed with distilled water twice. The cells were transferred to a dry pre-weighted petry dish and dried to a constant weight at 110 °C.

The PHB content of biomass was determined by gas chromatography [12, 13]. To determine the PHB concentration, 2 mL of solution was subjected to the methanolysis in the presence of 3% (v/v) sulphuric acid according to Brand1 method [13]. Gas chromatography of the resulting methylester of the constituent 3-hydroxybutyric acid was performed with a Philips Scientific Model 44100 chromatograph equipped with flame ionization detector (FID) and on-column injector. Stock solution of PHB (1000 $\mu g.cm^{-3}$) was prepared by dissolving appropriate amount of the solid sample in chloroform. A set of standard solutions was then prepared by appropriate dilution of the stock solution .The calibration curve was used to establish the concentration of PHB in each flask. The nitrogen flow rate through the PEG-20M packed column was 30 $mL.min^{-1}$. The flame ionization detector was supplied with 330 $mL.min^{-1}$ of air, 30 $mL.min^{-1}$ of hydrogen. The injection and detector temperatures were 180 and 200 °C, respectively. The initial column temperature was 90 °C (maintained for 1 min), which raised at a rate of 8 $°C.min^{-1}$ to a final temperature of 150 °C (maintained for 5 min). The internal and external standards were benzoic acid and 3-hydroxybutyric acid. The retention times of the methylesters of 3-hydroxybutyric (HB) acid and benzoic acid were 3.7 and 7.2 min, respectively.

Experimental Design

The first optimization step was to identify which variables have significant effects on PHB production by *R. eutropha*. The selection of these factors was based on our prior experience about growing the PHB producing microorganism, and choice of settings reflected a wide but reasonable numerical range [14-15]. Also some changes in the response (PHB yield) were expected for each factor over the range of settings selected. To choose factor settings for any two-level screening design, one should consider the following criteria: (i) the factor range ideally should contain the optimum response for that factor, ii) the range should be wide enough for any effect or trend to be exposed, and iii) the range should avoid combinations of low and high factor settings which are likely to produce an outright process failure. Factor level selection can be a difficult part of the experimental process. Experience gained by prior experimentation, and the literature can be valuable resources for choosing factor setting [14, 15]. The variables to be evaluated are listed in Table 1, that include some medium components (i.e., carbon and nitrogen source) and environmental factors (i.e., temperature, time, seed condition, agitation).

The Taguchi Design

Degree of Freedom (DOF): Each of the six factors as given in Table 1 was at two levels, so each factor has a DOF of 1. The DOF for the interaction is computed by multiplying the DOF of each of the interacting factors. Thus, the DOF for A X C =1. Our prior experience

shows that there is no any significant interaction between other variables, so the total DOF for the 6 factors and interactions in this case is 7. The appropriate Taguchi array can not have a DOF less than the total DOF of the experiment. An L_8 orthogonal array has seven columns and seven DOF. Each row represents a trial condition with factor levels indicated by numbers in the row [6,7].

Column assignment: In designing experiments with interaction, the column for interaction must be identified first. To determine whether the interaction is present, a proper interpretation of the results is necessary. The general approach is to separate the influence of an interacting member from the influence of the other. The trick is to select position for A x C, such that there are free columns for each of the factors, A and C. This can be done by using the triangular table for a 2 level orthogonal array or the corresponding linear graphs [7].

Table 2 shows selected experimental variables and an L_8 Taguchi design for selected experimental variables. The signs, + (high level) and - (low level) represent the two different levels of the independent variables examined. Any two columns of an L_8 (2^7) have the same number of combinations of (-,-), (-,+), (+,-), (+,+). Thus, all seven columns of an L_8 are orthogonal to each other. This table also includes the response of each trial in terms of yield (%).

RESULTS AND DISCUSSION

The analysis of data including interactions follows the same steps as are taken when there is no interaction. The objectives are the same: (1) determine the optimum condition, (2) identify the individual influence of each factor, and (3) estimate the performance at the optimum condition. In order to determine the optimum condition, the main effect and interaction effects should be calculated.

Main effect: The average effect of level 1 of the factor in column 1 (the effect of temperature) was computed by adding the first 4 trial results of Table 2 and dividing the sum by 4. Thus each of these trial runs contains the effect of factor A at level 1. The notation A is used for this value. The results of calculations for each factor and level are summarized in Table 3(a). The difference between the average value of each factor at levels 2 and 1 indicates the relative influence of that factor. The larger the difference, the stronger the influence. Table 3(a) shows an improvement at level 2 for all factors except A and D.

Among the variables, culture age and initial fructose concentration were found to be the most significant factors (Table3). Also Table 3 shows that PHB production was stimulated in the presence of low levels of C/N. Both temperature and C/N in their low levels stimulated PHB production.

PHB production decreased in the condition of low level agitation speed and enhanced with increase of culture time until 40h.

Results in this study demonstrated that C/N ratio in low level is essential for PHB production of *R.eutropha.* The C/N requirement for PHB production varies from species to species and from strain to strain and the importance of it has been reported for *R.eutropha* [11]. Initial fructose concentration may also be important for PHB production. It's role in PHB production has been reported as stimulator on related enzyme [1]. Therefore, PHB production was examined with different initial concentration of it. Fructose plays an important role in cell growth and metabolic pathway, so it is not surprising that it has a stimulatory effect on biomass production and growth related metabolite.

Table 3 shows that optimum temperature, in the studied range was 25 ºC for PHB production. The results also show an increase in PHB production when a low level agitation was used. In fact the lack of oxygen in flask culture is one of the most probable cause of PHB accumulation during growth phase. The key feature of this control is the fate of acetyl-CoA, which may be oxidized via tricarboxilic acid (TCA) cycle or can serve as a substrate for PHB synthesis. In oxygen limitation when NADH/NAD ratio increases citrate synthase and isocitrate dehydrogenase are inhibited by NADH, and in consequence, acetyl-CoA no longer enters the TCA cycle at the same rate and instead it is converted to acetoacetyl-CoA by 3-ketothiolase (the first enzyme of PHB biosynthesis) which is inhibited by CoA. There is therefore a greatly decreased flux of carbon through the TCA cycle under these conditions [1].

Although inoculum age was found to be one of the most significant variables, however any response to each variable depends on the selected range of it. In fact in Taguchi design or other screening designs, dummy or null variables may occur if the difference between the

Table 1: Variables to be screened in Taguchi design for production of PHB

Variables		Unit	Low level (-)	High level (+)
A	Temperature	°C	25	30
B	Fructose	g/L	10	15
C	Agitation	rpm	150	200
D	C/N	----	7.40	11.11
E	Culture Time	h	20	40
F	Inoculum age	h	10	15

Table 2: Eight-trial Taguchi design used to study six factors in PHB production[a]

Coded setting for six factors							
Trials	A	B	C	D	E	F	Yield (%) p/x
1	-	-	-	-	-	-	25.02
2	-	-	-	+	+	+	96.97
3	-	+	+	-	-	+	53.25
4	-	+	+	+	+	-	32.31
5	+	-	+	-	+	-	38.85
6	+	-	+	+	-	+	35.25
7	+	+	-	-	+	+	18.08
8	+	+	-	+	-	-	97.36

[a] *Factors A through F refer to those in Table*

Table 3: (a) Main Effects

Variables			Level 1	Level 2	Difference (2-1)
1	A	Temperature	51.77	47.38	4.39
2	C	Agitation	49.02	50.25	1.23
3	A X C	1 X 2	59.35	39.83	-19.52
4	B	Fructose	33.80	65.47	31.67
5	D	C/N	52.72	46.55	-6.17
6	E	Time	48.38	50.88	2.50
7	F	Inoculum age	27.66	71.60	43.94

Table 3: (b) ANOVA

Variables		DF	Sum of Squares	Variance	F	Percent
A	Temperature	1	40.27	40.27	6.73	0.59
C	Agitation	1	3.02	3.02	0.5	0.04
A X C	1 X 2	1	762.06	762.06	127.43	11.26
B	Fructose	1	2005.9	2005.9	335.43	29.64
D	C/N	1	76.13	76.13	12.73	1.12
E	Culture Time	1	12.52	12.52	2.09	0.18
F	Inoculum age	1	3860.56	3860.56	645.57	57.70
Error		1	5.98	5.98		

Total	7	6765.98			100.0

high and low levels of each variable is not large enough to ensure a measurable response. Some sensitive variables on the other hand may have their high and low levels chosen such that the size of their differential response is so great as to mask the effect of other variables. Since the Taguchi design is typically used as a preliminary optimization technique, more accurate quantitative analysis of the effect of these variables for PHB production is required

Interaction Effects: To determine whether the interaction is present, a proper interpretation of the results is necessary. The general approach is to separate the influence of an interacting member from the influence of the other. So, the A_1C_1 which is found from the result that contains both A_1 and C_1 is not the average value in level 1 for interaction A x C, and comes from results of trial runs 1 and 2 from Table2. So, the obtained value for A_1C_1, A_2C_1, A_1C_2 and A_2C_2 were 60.99, 57.72, 64.64 and 37.05, respectively. These results can be easily visualized by inspection of Figure 2. The intersecting lines represent the interaction between A and C. Recall that in Table 3(a), the average influence of interaction (A x C) assigned to column 3 was 59.35. Further analysis for the significance of this influence is made possibly by the analysis of variance (ANOVA).

Analysis of variance (ANOVA)

ANOVA establishes the relative significance of the individual factors and the interaction effects. Table 3(b) shows the result of ANOVA in this research. F value at n_1 (DOF of factor) = 1 and n_2 (DOF of error term) = 1 at a 99% confidence limit is 5.59. As F_c and F_E are smaller than the F value, so these factors with selected levels have no significant effect on the yield, ignoring the interaction effect.

Table 3(b) and Fig. 1 show an improvement at level 1 only for factors A and D while level 1 effects for B, C, E, and F cause a decrease in the yield. Hence the optimum levels for the factors based on the data are $A_1B_2C_2D_1E_2F_2$. Now to reexamine the optimum condition determined only from the main effect, we see from Figure 2 that A_1C_1 has a higher value than A_2C_1, A_1C_2 and A_2C_2. Table 3(b) shows that percent calculation of interaction A x C is 11.26% compared to the individual main effects of fructose equal to 29.64% and inoculum age equal to 57.7%, etc. Although the optimum levels for the factors should be based on the interaction effect (A X C) (because it is more powerful than individual factors), the new optimum condition would be satisfied with previous one (includes level A_1 and C_1).

However, the performance at the optimum condition should be determined. Using T = average result of 8 runs (Table 3 (a) = 49.59), the optimum performance was calculated by Eq. (1):

$$Y_{opt} = T + (A_1-T) + (B_2-T) + (C_2-T) + (D_1-T) + (E_2-T) + (F_2-T) \quad (1)$$

and Y_{opt} = 94.85.

But the optimum is not one of the trial runs so this projection should be verified by running a confirmation test(s). Confirmation test is a necessary and important step in the Taguchi method as it is direct proof of the methodology. Therefore a run with $A_1B_2C_2D_1E_2F_2$ was done in duplication. The experimental yield was equal to 92.36. This result from the confirmation test agrees with the optimum performance (Y_{opt}) estimated by the analysis.

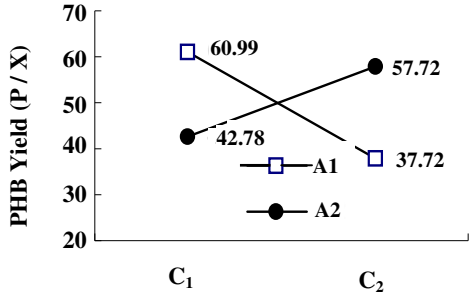

Fig. 1 (A):. Test of interaction

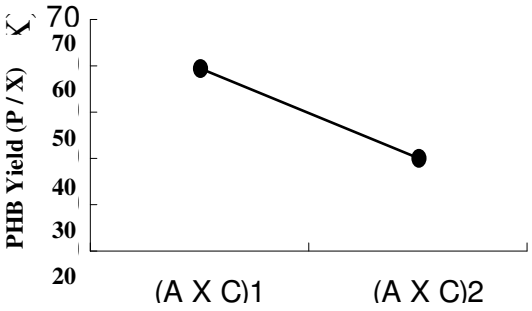

Fig. 1 (B): Interaction effect

CONCLUSIONS

The Taguchi design has been successfully used to test the relative importance of medium components and environmental factors on PHB production. The selected orthogonal array was L_8 and optimum condition for PHB production was estimated to be $A_1B_2C_2D_1E_2F_2$ which, was verified by running a confirmation test. The optimum condition was obtained as: fructose concentration, 15 g/L; C/N ratio, 7.4; agitation speed, 200 rpm; culture time, 40 h; temperature, 25 °C, seed age, 15 h. At optimum condition the yield of PHB production was found to be 92.36%.

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