Application of the Plackett-Burman statistical design to optimize poly(β-hydroxybutyrate) production by *Ralstonia eutropha* in batch culture

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Acstract

Ralstonia eutropha accumulates poly(β-hydroxybutyrate) up to 80% of its dry weight (as a carbon and energy source) and is the best-known PHB producer. Although PHB has many potential applications in medicine, veterinary practice, agriculture and surgery, its high cost limits the widespread use of it. The recent researches have focused on reducing these costs by optimizing fermentation process. In this study the Placket Burman experimental design was used to test the relative importance of medium components and process variables on cell growth and PHB production. The optimum values of the variables, selected as the best conditions for further studies in the development of a low cost and effective fermentation process for PHB production, were as follows: initial fructose concentration, 15 g/l; C/N ratio, 7.4; shaking rate, 200 rpm; fermentation time, 40 h; temperature, 30°C; seed age, 15 h .

Keywords: *Poly (β-hydroxybutyrate) (PHB), Plackett-Burman design (PBD), Ralstonia eutropha, Batch culture*

INTRODUCTION

Poly(β-hydroxybutyrate) (PHB) is an intracellular storage compound, which provides a reserve of carbon and energy in several kinds of microorganism such as *Pseudomonas, Alcaligenes, Azetobacter* species and *Ralstonia eutropha* (Anderson and Dawes, 1990). It accumulates as distinct inclusions in the cell and comprises up to 80% of the cell dry weight for strains of *R. eutropha*, under conditions of nitrogen and phosphate limitation and excess of carbon source (Zinn *et al*., 2001; Lee, 1996). PHB, which is a biodegradable and biocompatible thermoplastic compound, has broadly similar physical pro-perties to poly(propylene). It has many applications in medicine, veterinary practice and agriculture due to its biodegradability (Ayorinde, 1998). Currently the main problem, which limits the widespread use of PHB and its copolymers, is its relatively high cost compared to poly(propylene). The fermentation process, substrates and product recovery are the major costs (Byrom, 1987). Researches have focused on reducing these costs by optimizing fermentation process and gene cloning (Poirier, 1992).

A well defined statitistical experimental design is considered to be necessary for optimization of a fermentation process, since it would be possible to get more information through conducting fewer mea-surements during the process. The Plackett–Burman design (PBD) has been frequently used for screening process variables that make the greatest impact on a process (Plackett *et al.,* 1946). It is a set of small and efficient experimental design, which is very powerful, widely applicable and especially well suited for biotechnology research and development (Haaland, 1989). Recent reports on the use of PBD in this area include its application toward improving succinic acid production (Lee *et al.,* 1999), lipase catalyzed esterification (Rao and Divakar, 2001), *Colletotrichum coccodes* spores production (Yu *et al.,* 1997), and *Saccharopolyspora spinosa* macrolide production (Strobel *et al.,* 1993). This design was also used to find conditions to increase the detection of antibiotic production by fungi isolated

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from nature (Monaghan and Koupal, 1989). A consideration in the choice of the PBD in screening studies is the ratio of the number of experiments to be conducted to the number of variables being studied. This design allows for the study of $k = (N-1)/(L-1)$ factors, each with L levels with N experimental trials. It is a kind of resolution III designs (i.e. designs which provide uncorrelated estimates of main effect) suitable for exploring $k = N-1$, 2 level factors in N trials, where N is a multiple of 4. The usefulness of the design lies in the fact that in determining the effects of one variable, the net effects of changing other variables cancel out so that the effect of each variable on the system can be independently determined.

In this study the application of PBD to assess the relative importance of medium components and process variables on cell growth and PHB production is reported.

MATERIALS AND METHODS

Microorganism and growth conditions: *Ralstonia eutropha* (ACM, 1296) was obtained from the Australian Collection of Microorganisms. It was originally derived from strain ATCC 17699, which has been used for studies involving single cell protein and PHB production (Schlegel *et al.,* 1961). Cells were maintained on PYEA (peptone yeast extract agar), this medium consists of (g/l): peptone, 10; yeast extract, 5; sodium chloride, 5 and agar, 15. Bradford medium was modified to provide a source of carbon, nitrogen and trace metals in a phosphate buffer and to simplify medium formulation and sterilization (Bradford, 1992). The chemical composition of this medium is (g/l): Na_2HPO_4, 3.5; KH_2PO_4, 1.5; fructose, 9.5; $(NH_4)_2SO_4$, 1.35 and filtered trace metals solution (10%, v/v). Metal solution was made of (g/l): $MgSO_4$. $7H_2O$, 0.2; $FeSO_4$. $7H_2O$, 0.1; $MnSO_4$. $5H_2O$, 0.1; $ZnSO_4$. $7H_2O$, 0.08; K_2SO_4, 2.2; H_3BO_3, 0.02 and $CuSO_4$. 0.08. An aliquot (100 ml) of this medium was transferred to 500 ml flask for sterilization process. Fermentation medium was similar to seed medium, except for fructose concentration, which was 15 g/l.

Analytical methods: Cell dry weight was determined gravimetrically. Culture samples in 10 ml aliquots were centrifuged at 3000 ×g for 20 min and the pellet was washed twice with distilled water. The cells were transferred to a dry pre-weighted petri dish and dried to a constant weight at 110°C.

The PHB content of biomass was determined by gas chromatography (Braunegg *et al.,* 1987). A stock solution of PHB (1 mg/ml) was prepared by dissolving in chloroform. A set of standard solutions was then prepared by appropriate dilution of the stock solution and methanolysis. The obtained calibration curve was used to determine the concentration of the PHB in the collection vial. Two ml of culture medium was subjected to methanolysis in the presence of 3% (v/v) sulfuric acid. The separation, identification and determination of the resulting methyl ester of 3-hydroxybutyrate was performed using a Philips Scientific Model 4410 gas chromatograph equipped with a flame ionization detector (FID) and a split/splitless injector, and fitted with a 25 m × 0.25 mm i.d. fused silica capillary column coated with an immobilized film of BP10. Helium was used as carrier gas at a flow rate of 1ml.min^{-1}. The injection and detector temperatures were 200 and 250°C, respectively. The samples were injected into the GC using the split mode with a split ratio of 1/20. Oven temperature was programmed to 90°C for 3 min, and then increased to a final temperature of 150°C at a rate of 8°C.min^{-1}. The retention times of the methyl esters of 3-hydroxybutyrate (HB) and benzoic acid were 5.6 and 9.2 min, respectively. The amount of resulting methyl ester detected by gas chromatography reflects the purity of recovered PHB.

Plackett-Burman Design (PBD): The first screening step was to identify the variables which have significant effects on PHB production by *R. eutropha*. Choice of these factors was based on previous experience (Hejazi, 2003) for growing the PHB producing microorganism, and selection of settings reflects a wide but reasonable numerical range. Also some changes in the response (PHB yield) were expected for each factor over the selected range. 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 cause the process failure. Selecting the factor levels can be considered as the difficult part of the experimental process. By conducting the preliminary experiments and searching the appropriate literature one could obtain valuable information regarding the selection of factor levels (Davies, 1993). The variables to be evaluated (Table 1) include some medium components (i.e., carbon and nitrogen source) and environ-

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Table 1. Variables to be monitored in PBD for production of PHB.

Variables	Low Level(-)	High Level(+)
A Fructose (g/l)	10	15
B C/N	7.4	11.1
C Temperature (°C)	25	30
D Time (h)	20	40
E Seed age (h)	10	15
F Seed Shaking (rpm)	150	200
G Culture shaking (rpm)	200	250

mental factors (i.e., temperature, time, seed age and shaking). Table 2 shows selected experimental variables and a PBD for conducting eight experimental trials. All the trials were done in triplicate.

The elements, + (high level) and - (low level) represent the two different levels of the independent variables examined. Each PBD can be easily constructed using a "generating vector", in the form of (+ + + - + - -) (Logothetis, 1989). Arranging the vector as the first column and off setting by one vector elements for each new column forms the design matrix. In other words, a new column is obtained from the previous one moving the elements of the previous column down once and placing the last element in first position. Likewise a third column is generated from the second and this procedure occurs $N - 2$ times yielding an $(N-1) \times (N-1)$ array. A row of minus signs is then added to get the last run (8th experiment) and to complete the design. This design is thus called a balanced incomplete block (Davies, 1993).

Seven variables were selected for this experimental design. In every run (represented by a row) except the 8th, 4 variables are at a high level and three are at a low level. The layout of the matrix, given in Table 2, shows that each variable is equally at a high and a low level

four times in each column.

RESULTS

The basic equation set up for the design was as follows. The coefficients for the seven variables were determined by:

$$A_i = \frac{1}{N} \sum_{i=0}^{N} X_i . K_i \qquad (1)$$

Where A_i = coefficient values, X_i = experimental yield, K_i = coded value of each variable corresponding to the respective experimental yield X_i and N = number of experiments. Table 3 gives a comparison of the experimentally determined PHB yield and biomass production to those predicted by solving the above equation, where predicted yield is given by:

$$Y_i = \sum_{i=0}^{N} A_i . K_i \qquad (2)$$

For $i = 0$, a dummy level of +1 was used and the coefficient obtained was called A_0.

The standard error was determined as the sum of the squares of the difference between the experimental and predicted yield for each run. The estimated error is given by:

$$S_b = \sqrt{\frac{S_e^2}{N}} \qquad (3)$$

The student's t-test was performed to determine the significance of each variable employed (t-value = coefficient/S_b).

Since the experiments were designed to evaluate the relative effect of each variable on response, a significant level of 0.30 is acceptable (Stowe and Mayer, 1996). However, the tabulated t-value at P< 0.02 (and

Table 2. Eight-trial PBD used to study seven factors in PHB production[a].

Trial	Coded setting for factors						
	A	B	C	D	E	F	G
1	+	+	+	-	+	-	-
2	+	+	-	+	-	-	+
3	+	-	+	-	-	+	+
4	-	+	-	-	+	+	+
5	+	-	-	+	+	+	-
6	-	-	+	+	+	-	+
7	-	+	+	+	-	+	-
8	-	-	-	-	-	-	-

[a] Factors A through G refer to those in Table 1.

Table 3. Comparison of experimental and predicted yields [a].

Run No.	PHB Production Yield (w/w%)		Biomass Production Yield (g/l)	
	Experimental	Predicted	Experimental	Predicted
1	40.99	39.16	4.30	4.31
2	29.11	27.30	5.15	5.13
3	45.36	43.54	3.36	3.35
4	30.89	32.71	3.78	3.79
5	50.18	48.36	3.75	3.73
6	35.76	37.57	2.27	2.29
7	32.33	34.13	5.27	5.25
8	28.30	30.11	3.17	3.18

[a] Each value is the average of three replication.

Table 4. Statistical data

Variables		PHB production [a]		Biomass production [b]	
		Coefficient	t-value	Coefficient	t-value
A	Fructose	2.98	4.64	0.25	47.16
B	C/N	-3.28	-5.12	0.74	139.62
C	Temperature	0.99	1.101	-0.012	-5.09
D	Time	0.23	0.35	0.22	41.51
E	Seed age	3.07	4.79	-0.35	-66.73
F	Seed shaking	2.84	4.42	0.15	28.30
G	Culture shaking	-1.33	-3.01	-0.24	-45.53

[a] $A_0 = 36.60$ (mean of the experimental yield), standard error, $S_e^2 = 3.29$, estimated error, $S_b = 0.64$, tabulated t-value at $P < 0.02$ is equal to 2.998.

[b] $A_0 = 3.88$, standard error, $S_e^2 = 0.002$, estimated error, $S_b = 0.005$, tabulated t-value at $P < 0.002$ is equal to 4.785.

degree of freedom = 7) is equal to 2.998.

Most of the coefficients of the variables exhibited t-values greater than 2.98 implying that they were significant (Table 4, column a). Column b of Table 4 shows the relative effect of each variable on biomass production. All coefficients exhibited t-values greater than 4.785 (the tabulated t-value at P<0.002) implying that with 99.8% confidence all variables had significant effect on growth. This result indicates that PHB production is not necessarily growth dependent for this strain, and selection of level for each variable should be based on the PHB production instead of growth.

DISCUSSION

Among the variables, C/N ratio was found to be the most significant (highest t-values of -5.12 and 139.62) on PHB and biomass production. Table 4 shows that, whereas PHB production was stimulated in the presence of low level of C/N ration, biomass production was inhibited by it. The C/N requirement for PHB pro-

duction varies from species to species and from strain to strain and the importance of it had been reported for *R. eutropha* (Bradford, 1992).

Table 4 shows that optimum temperature was 30ºC for growth and PHB production. These data also show an increase in PHB production under lower shaking rate. 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. Under oxygen limitation when NADH/NAD ratio increases, citrate synthase and isocitrate dehyrogenase are inhibited by NADH, and in consequence, acetyl-CoA no longer enters the TCA cycle at the same rate. Instead it is converted to acetoacetyl-CoA by 3-ketothiolase (the first enzyme of PHB biosynthesis). Therefore, under such conditions there is a greatly decreased flux of carbon through the TCA cycle (Anderson and Dawes, 1990).

The results of this study demonstrated that the initial fructose concentration was also important for biomass and PHB production by *R. eutropha* (Table 4). Its
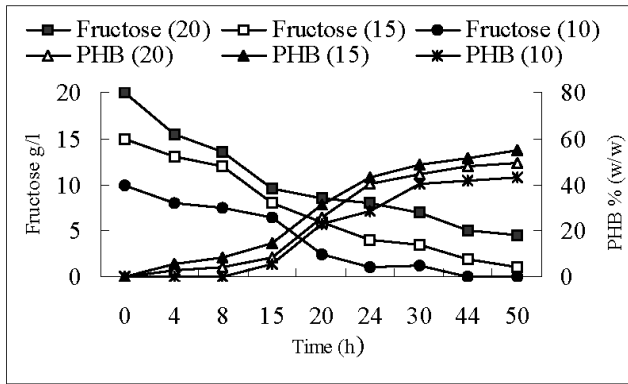
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Figure 1. Effect of initial fructose concentration (g/L) on PHB production. (Biosynthesis conditions: C/N ratio, 7.4; seed age, 15 h; shaking rate, 200 rpm).
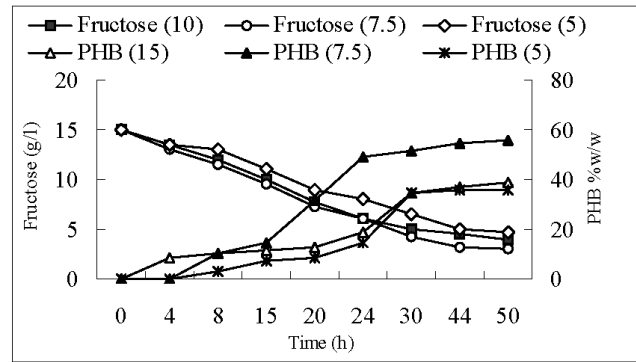

Figure 2. Effect of C/N ratio on PHB production. (Biosynthesis conditions: initial fructose concentration, 15 g/l; seed age, 15 h; shaking rate, 200 rpm).
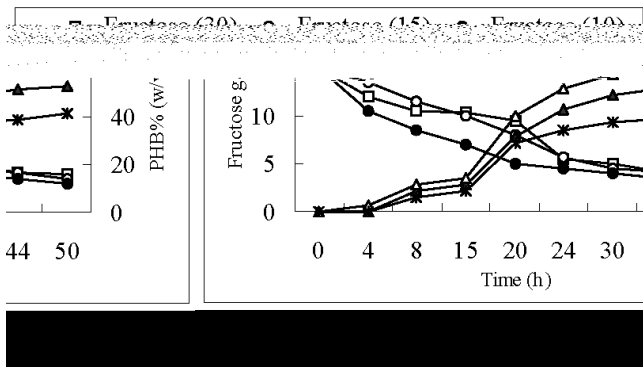

Figure 3. Effect of seed age (h) on PHB production. (Biosynthesis conditions: initial fructose concentration, 15 g/l; C/N ratio, 7.4; shaking rate, 200 rpm).
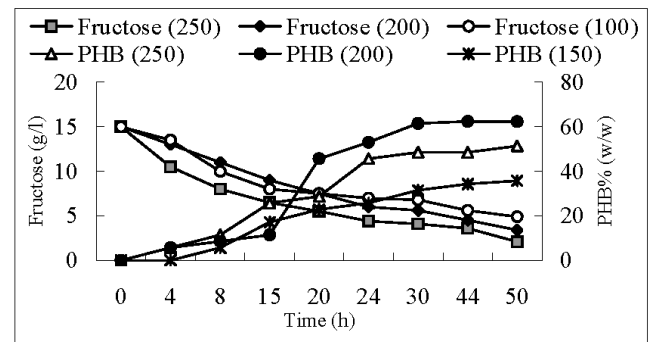

Figure 4. Effect of shaking rate (rpm) on PHB production. (Biosynthesis conditions: initial fructose concentration, 15 g/l; C/N ratio, 7.4; seed age, 15 h).

role has been reported as a stimulator on several enzymes related to PHB production (Anderson and Dawes, 1990). Fructose plays an important role in cell growth and metabolic pathways, so it is not surprising that it had a stimulatory effect on biomass production (Rusell, 1992). Table 4 also reveals that seed age and shaking rate were important for biomass and PHB production. The effect of seed age was examined to reduce culture time. As shown in Table 4, high PHB yield and low concentration of biomass were obtained with a seed age of 15 h.

Although C/N ratio was found to be the most significant variable, but any response to each variable depends on its selected range. In fact with PBD or other screening designs, dummy or null variables may occur if the difference between the 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

as the size of their differential response is so great as to mask the effect of other variables (Monaghan and Koupal, 1989). Since the PBD is typically used as a screening technique, more accurate quantitative analysis of the effect of variables on PHB production is required. This technique however, provides information on how each variable tends to affect bacterial growth and PHB production. Furthermore, independent validation experiments were carried out with initial fructose concentration, C/N ratio, seed age and shaking rate. Figures 1-4 show a more accurate analysis of these variables on PHB production. In each figure three variables were fixed at the optimized conditions of PBD and only one variable changed. The results presented in these figures satisfy the analysis of the results given in Table 4. Figure 1 shows cell growth and PHB production with different initial fructose concentrations. The PHB production increased with increasing initial fructose concentration up to 15 g/l,

but slightly decreased when 20 g/l fructose was used. This is due to fructose tolerance of *R. eutropha.* It has been reported that the viability of *Bacteroides ruminicola* rapidly also declined at the glucose concentration above 10 g/l (Rusell, 1992). Figure 2 shows a decrease in PHB yield at a high C/N ratio. These data indicate that alothough absolute concentration of carbon source is important, C/N ratio has also critical value since growth and PHB production decrease at very high or low concentration of carbon and nitrogen sources. It can be attributed to high concentration of ammonium sulphate or high C/N ratio which suppressed PHB accumulation (Mulchandani, 1989).

As shown in Figure 3, high PHB yield (57 %w/w) was obtained in a short culture time (18 h) with a seed age of 15 h . Also Figure 4 reveals that an increase in PHB production under 200 rpm shaking rate. It shows the role of critical concentration of oxygen as an stimulator on PHB production. In fact the lack of oxygen in flask culture is one of the most probable cause of PHB accumulation during growth phase.

CONCLUSION

PBD was used to test the relative importance of medium components and process variables on cell growth and PHB production. Among the variables, C/N ratio was found to be the most significant variable. The optimized values of the variables for PHB production were as follows: initial fructose concentration, 15 g/l; C/N ratio, 7.4; shaking rate, 200 rpm; fermentation time, 40 h; temperature, 30°C; and seed age, 15. These values can be selected as the best condition for further studies in the development of a low cost and effective fermentation process for PHB production.

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