Media Selection for Poly(hydroxybutyrate) Production from Methanol by *Methylobacterium Extorquens* DSMZ 1340

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ABSTRACT: *Plackett-Burman design was used for selection of important media components such as carbon and nitrogen sources and minerals which affect poly(hydroxybutyrate) production and cell growth of Methylobacterium extorquens DSMZ 1340. Among the studied variables, nitrogen and phosphorus sources, $MgSO_4$ and most of the trace elements were found to be significant variables for PHB production from methanol. At best condition (based on PHB concentration), dry cell weight, PHB content and PHB concentration were 3.81 g/L, 21.23 %, and 0.809 g/L, respectively. It was also found that most of the trace elements and phosphorus sources were influential parameters on the growth of microorganism but the kind of nitrogen source was not. The experimental results showed that deficiencies of nitrogen sources (NH_4Cl and NH_4NO_3), phosphorus sources (K_2HPO_4 and Na_2HPO_4) and $MgSO_4$ in medium, increased PHB accumulation.*

KEY WORDS: *Methanol, Poly(hydroxybutyrate) (PHB), Methylobacterium extorquens, Plackett-Burman Design (PBD).*

INTRODUCTION

Poly-β-hydroxybutyrate (PHB) is an intracellular storage compound, which provides a reserve of carbon and energy in several kinds of microorganism [1-3]. Due to its biodegradability and biocompatibility, it has many applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture [4-7].

In spite of the advantages of PHB compared with petroleum-derived plastics, its use is currently limited due to their high production costs [8]. Substrate costs represent a significant proportion of total cost for PHB production, while it can be produced from relatively cheap substrates such as methanol [9], carbon dioxide [10], and agro-industrial by-products [8, 11].

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It has been argued that methanol would appear as an alternative substrate for PHB production because of several advantages including low price, complete water miscibility [12].

PHB synthesis in methylotrophs appears to be restricted to *Methylobacterium* spp., especially pink-pigmented facultative methylotrophs. Among them, *M. extorquens* DSMZ 1340 is the best choice due to high content production [13].

Several studies have been carried out with various microorganisms and fermentation processes for production of PHB from methanol [9, 13-18]. In some studies media optimization was considered and the importance of minerals for maximal biomass and/or PHB production has been recognized. [14-17]. *Suzuki* (1986) studied the effect of nitrogen and phosphorus sources concentration as well as seven trace elements on growth of *Pseudomonas* Sp. K and showed that cell growth was not dependent to the kind of cation of phosphate salts [17]. *Daneil* (1992) obtained an optimal medium for growth of *Pseudomonas* 135 and confirmed Suzuki's report. Increasing of trace elements concentration did not show any significant improvement in cell growth [16].

Also, *Bourque* (1995) reported an optimum medium for growth of *M. extorquens* ATCC 55366. He switched from medium 784 (ATCC 1985) to medium of Choi (1989) [15], and made several modification to the latter medium. The results showed high impact of trace elements concentration on growth [14].

Several investigators have studied the effect of nutrient deficiency on PHB production. Suzuki found that NH^{4+}, SO_4^{2-}, Mg^{2+}, Fe^{2+} and Mn^{2+} are crucial deficient ions for stimulation of PHB accumulation by *Pseudomonas* sp. K [17]. While critical ions for PHB accumulation *in M. organophilium* were NH_4^+, Mg^{2+}, PO_4^{3-} and K^+ [15]. *Daneil* (1992) has examined nutrient-deficient media on PHB accumulation by *Pseudomonas* 135 and showed significant stimulation by NH_4^+, Mg^{2+}or PO_4^{3-} deficient medium [16]. But these results are scattered and inconclusive. Therefore a more detailed experimental design for screening of important parameters for optimization of fermentation process in next steps is crucial.

A statistical design of experiments (DOE) has not been applied for optimization of media components. Therefore, scattered and different concentrations for media components have been reported in the literature. A well defined statistical DOE is considered to be necessary for optimization of a fermentation process, since it would be possible to get more information through conducting fewer measurements during the process. The Plackett-Burman design (PBD) has been frequently used for screening of process variables that have the greatest impact on the process [19]. There are several reports on the use of PBD in media optimization for microbial production of chemicals [20-24]. A consideration in the choice of PBD in screening studies is the ratio of the number of experiments to be conducted to the number of variables being studied. PBD and Taguchi method were used for screening of process variables in PHB production by *Ralstonia eutropha*, and it was found that PBD is more suitable [25]. To the best of our knowledge, this statistical methodology has not been applied in the screening of media components for PHB production by methylotrophic bacteria.

In this study application of PBD to assess the relative importance of medium components including carbon and nitrogen sources, phosphate and minerals for maximal biomass and biopolymer production of *M. extorquens* DSMZ 1340 by methanol was considered.

MATERIALS AND METHODS

Microorganism

Strain of *M. extorquens* DSMZ 1340 was purchased from DSMZ, Germany, and maintained at 4 °C on nutrient agar slants containing 1 % (v/v) methanol [14].

Media and culture condition

Inoculum development: A mineral salt medium consisting of : $(NH_4)2SO_4$ 1.0 g/L; KH_2PO_4 1.305 g/L; $Na_2HPO_4.7H_2O$ 4.02 g/L; $MgSO_4.7H_2O$ 0.45 g/L; $FeSO_4.7H_2O$ 1.3 mg/L; $CaCl_2.2H_2O$ 3.3 mg/L; $MnSO_4.7H_2O$ 100 µg/L; $ZnSO_4.7H_2O$ 130 µg/L; $Na_2MoO_4.2H_2O$ 40 µg/L; $CuSO_4.5H_2O$ 40 µg/L; $CoCl_2.6H_2O$ 40 µg/L; H_3BO_3 30 µg/L and 1 %(v/v) methanol was used for inoculum development [15]. The organism was cultivated at an agitation speed of 200 rpm and 30 °C for 36 h in a 250 mL Erlenmeyer flask containing 50 mL of the above mentioned medium.

PHB production Media: The media (50 mL) prepared according to the Plackett-Burman design, was taken in a 250 mL flask and inoculated with 1 % (v/v) of inoculum

while shaking at 200 rpm and 30 °C. Cell growth was estimated after 25 h [14]. After 48 h, 1 % (v/v) methanol was added to each flask and PHB was estimated in the culture broth after 90 h (unpublished data).

Analytical methods

Cell growth (optical density) was monitored spectrophotometrically at 600 nm (Cary 50 Conc., Australia) after suitable dilution of culture broth with distilled water. The dry cell weight was obtained gravimetrically after centrifugation and drying of pellets at 90 °C till constant weight reached. The PHB content of biomass was determined by gas chromatography (Philips 4410, UK) with benzoic acid as an internal standard, according to *Braunegg et al.* (1987) [26].

Plackett-Burman Design (PBD)

The first screening step was to identify which variables have significant effect on PHB production by *M. extorquens*. The variables to be evaluated include medium components and their selected levels are shown in table 1. Table 2 shows selected experimental variables and a PBD for conducting twenty experimental trials. All the trials were done in duplicate. Eighteen variables were selected for this experimental design. In every run (represented by a row) except the 20th, 10 variables are at high level and 9 are at low. The layout of the matrix shows that repetition of each variable at high and low levels is 10 times in each column.

RESULTS AND DISCUSSION

Using methylotrophic bacteria for producing PHB from methanol as main substrate has gained a renewed interest in recent years [9, 13-18]. Methanol is a cheep and renewable substrate which can be used to reduce the production cost of PHB. It is a renewable substrate since it could be derived from woody materials or from natural gas obtained after anaerobic digestion of organic substrates.

This research has focused on evaluation and screening of all medium components for growth and PHB production of *M. extorquens* DSMZ 1340 as one of the most famous candidates for PHB production from methanol [13].

Table 1 shows the independent variables and their levels used in this screening study and table 2 represents the PBD for 20 trails, which was applied for the selection of medium components for cell growth and PHB production.

Table 1: Variables to be monitored in PBD for production of PHB from methanol by Methylobacterium extorquens DSMZ 1340.

Code	Variable	Low levels (-)	High levels (+)
A	Methanol (% v/v)	0.1	1
B	$(NH_4)_2SO_4$ (g/L)	0.15	1.5
C	NH_4CL (g/L)	0.15	1.5
D	NH_4NO_3 (g/L)	0.15	1.5
E	KH_2PO_4 (g/L)	0.13	1.3
F	$K_2HPO_4.3H_2O$ (g/L)	0.23	2.3
G	$NaH_2PO_4.2H_2O$ (g/L)	0.1	1.0
H	$Na_2HPO_4.12H_2O$ (g/L)	0.5	5.0
I	$MgSO_4.7H_2O$ (g/L)	0.1	1.0
J	$FeSO_4.7H_2O$ (mg/L)	2.0	20
K	$CaCl_2.2H_2O$ (mg/L)	2.0	20
L	$MnSO_4.7H_2O$ (mg/L)	0.5	5.0
M	$ZnSO_4.7H_2O$ (mg/L)	0.3	3.0
N	$Na_2MoO_4.2H_2O$ (mg/L)	0.08	0.8
O	$CuSO_4.5H_2O$ (mg/L)	0.08	0.8
P	$CoCl_2.6H_2O$ (mg/L)	0.08	0.8
Q	H_3BO_3 (mg/L)	0.06	0.6
R	Yeast extract (g/L)	0	0.2

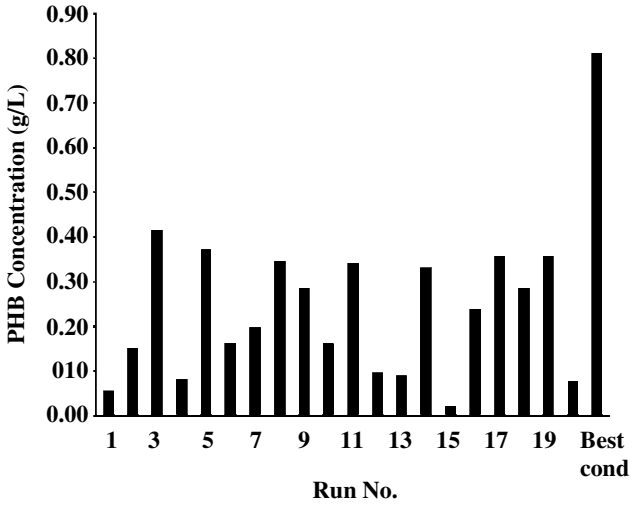

Fig. 1: The results of different PHB concentration in twenty-trials of PBD and the suitable medium.

Table 2: Twenty-trial PBD used to study eighteen factors for PHB production from methanol by Methylobacterium extorquens DSMZ 1340.

	Code setting for factors																		
Trails	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+
2	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-
3	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-
4	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+
5	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+
6	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+
7	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+
8	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-
9	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+
10	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-
11	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+
12	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-
13	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-
14	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-
15	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-
16	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+
17	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+
18	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-
19	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The results of PBD for PHB production and cell growth are given in table 3. Although PHB concentration is an important factor in total cost of PHB production, but PHB content should be considered separately. PHB concentration depends on both PHB content and cell growth, while PHB content shows the net weight of PHB in the cells. The significant variation of results of DOE and the best condition of PHB concentration are shown in Fig. 1.

The Analysis of variance was performed using Minitab 14 Software. The effect, *t-value* and *P* of each variable are shown in table 4.

If any component shows significance at or above 95 % confidence level and its effect is negative, it indicates that the component is effective in yield (cell growth, PHB content and PHB concentration) but the amount required is lower than the indicated low concentration (-) in Plackett-Burman experiment.

If the effect is positive, a higher concentration than the indicated high value (+) concentration is required for further optimization studies.

The results showed significant influence of most of the trace elements on growth. This result was similar to those reported by *Bourque* (1995) [14]. The kind of nitrogen source was not significant on the cell growth. All of the examined phosphorus sources showed significant impact on growth, which may be due to less important effect of cation type [17]. Also, our results indicated that deficiencies of nitrogen sources (NH_4Cl and NH_4NO_3), phosphorus sources (K_2HPO_4 and Na_2HPO_4) and $MgSO_4$ in medium, increase PHB accumulation. Similar results have been reported for the stimulation of PHB accumulation in different micro-organisms using methanol as carbon source [15-17]. PHB accumulation depends both on the microbial strain and the effect of the limiting nutrient on it. This is probably due to the different characteristics of the enzymes involved in PHB synthesis.

Table 3: PHB production from methanol by Methylobacterium extorquens DSMZ 1340.

Trails	Cell Concentration (g DCW/L)	Cell Growth (OD)	PHB Concentration (g/ L)	PHB Content % (w/w)
1	0.89 ± 0.09	0.6872 ± 0.0209	0.055 ± 0.011	6.400 ± 1.91
2	0.58 ± 0.06	0.2831 ± 0.0358	0.150 ± 0.025	26.565 ± 4.02
3	3.21 ± 0.31	1.4578 ± 0.0538	0.414 ± 0.026	12.960 ± 0.44
4	2.54 ± 0.10	0.8830 ± 0.0586	0.081 ± 0.020	3.205 ± 0.09
5	3.27 ± 0.27	0.3947 ± 0.0487	0.372 ± 0.049	11.325 ± 0.52
6	4.12 ± 0.04	0.1232 ± 0.0349	0.161 ± 0.012	3.915 ± 0.29
7	2.70 ± 0.50	0.2019 ± 0.0130	0.195 ± 0.007	7.445 ± 1.12
8	3.00 ± 0.02	0.5582 ± 0.0315	0.346 ± 0.0256	11.540 ± 0.85
9	2.92 ± 0.22	0.5128 ± 0.009	0.284 ± 0.016	9.730 ± 0.19
10	2.89 ± 0.35	0.3035 ± 0.0338	0.161 ± 0.001	5.640 ± 0.67
11	2.67 ± 0.19	1.3879 ± 0.0263	0.340 ± 0.017	12.755 ± 0.28
12	3.43 ± 0.17	0.5984 ± 0.003	0.095 ± 0.015	2.770 ± 0.30
13	2.85 ± 0.15	0.0948 ± 0.0176	0.088 ± 0.022	3.045 ± 0.61
14	2.89 ± 0.01	0.7698 ± 0.0172	0.330 ± 0.011	11.415 ± 0.33
15	2.47 ± 0.01	1.0902 ± 0.128	0.012 ± 0.017	0.805 ± 0.10
16	0.91 ± 0.21	0.9211 ± 0.0923	0.239 ± 0.015	27.265 ± 4.65
17	2.18 ± 0.34	0.1516 ± 0.0252	0.357 ± 0.005	16.755 ± 2.39
18	0.86 ± 0.06	0.9955 ± 0.0339	0.283 ± 0.007	33.165 ± 3.08
19	2.75 ± 0.21	0.2628 ± 0.0317	0.358 ± 0.056	12.900 ± 1.04
20	0.40 ± 0.04	0.6963 ± 0.0163	0.086 ± 0.002	18.840 ± 0.27

It should be mentioned that very different responses due to selected levels of some effective variables may mask the effect of other variables [27]. 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. At preliminary stages of this study, selected range of methanol was 0.01-0.5 % v/v which caused a very high impact on response and masked the effect of other variables (data are not shown). Therefore, the range of studied variables was modified to evaluate the effect of all variables.

Table 5 compares the effect of different components of media on cell growth, PHB concentration and PHB content with confidence level of 95 %. As is shown in this table, KH_2PO_4 and NaH_2PO_4 concentration had positive effect on cell growth because of buffer roles and microorganism requirements to K, Na, and P elements. As shown in this table, $CaCl_2$ had the most significant effect on cell growth. A possible explanation for this effect is the importance of calcium role in regulation of enzymes activity.

Suzuki (1986) [17] have also emphasized on critical role of Ca concentration on growth of *Pseudomonas* sp. K, *M. extorquens* K, as another methylotrophs. $ZnSO_4$ and H_3BO_3 also had positive effect on growth. $CaCl_2$, $FeSO_4$, $MnSO_4$ and H_3BO_3, were trace elements, which had positive effect on PHB content. It can be postulated that they are cofactor of enzymes that catalyze PHB biosynthesis pathways. $MnSO_4$ had negative effect on growth but positive effect on PHB content; so it was an insignificant variable for PHB concentration. Increased concentration of NH_4NO_3 and NH_4Cl resulted in decreased PHB content, which may be due to the necessity of nitrogen source limitation (in the presence of extra carbon source) as a signal for accumulation of PHB in most of the cells. But NH_4Cl was less effective than

Table 4: Analysis of data obtained for PHB content (SE Coef.=0.5057), PHB concentration (SE Coef.=0.0071), and cell growth (OD) (SE Coef. = 0.0176).

Variable		PHB content				PHB concentration				cell growth (OD)			
		Effect	Coef	*t-value*	P	Effect	Coef	*t-value*	P	Effect	Coef	*t-value*	P
code	Constant		11.922	23.58	0.000		0.22074	31.25	0.000		0.6185	35.05	0.000
A	Methanol	1.260	0.630	1.25	0.277	-0.00617	-0.00308	-0.44	0.667	-0.3782	-0.1891	-10.72	0.000
B	$(NH_4)_2SO_4$	2.960	1.480	2.93	0.008	0.08242	0.04121	5.83	0.000	-0.0621	-0.0310	-1.76	0.093
C	NH_4CL	-7.043	-3.522	-6.96	0.000	0.02233	0.01117	1.58	0.129	0.0100	0.0050	0.28	0.779
D	NH_4NO_3	-5.642	-2.821	-5.58	0.000	-0.06233	-0.03117	-4.41	0.000	0.0020	0.0010	0.06	0.955
E	KH_2PO_4	1.024	0.512	1.01	0.323	0.06771	0.03386	4.79	0.000	0.1128	0.0564	3.19	0.004
F	$K_2HPO_4.3H_2O$	-9.985	-4.993	-9.87	0.000	-0.05886	-0.02943	-4.17	0.000	0.0208	0.0104	0.59	0.563
G	$NaH_2PO_4.2H_2O$	0.942	0.471	0.93	0.362	0.03228	0.01614	2.29	0.033	0.1240	0.0620	3.51	0.002
H	$Na_2HPO_4.12H_2O$	-7.004	-3.502	-6.93	0.000	0.04055	0.02028	2.87	0.009	-0.3264	-0.1632	-9.25	0.000
I	$MgSO_4.7H_2O$	-2.079	-1.039	-2.06	0.052	-0.07834	-0.03917	-5.55	0.000	-0.0977	-0.0489	-2.77	0.012
J	$FeSO_4.7H_2O$	2.773	1.378	2.74	0.012	0.05743	0.02872	4.07	0.001	-0.0510	-0.0255	-1.45	0.163
K	$CaCl_2.2H_2O$	2.985	1.492	2.95	0.008	0.04533	0.02267	3.21	0.004	0.4351	0.2175	12.33	0.000
L	$MnSO_4.7H_2O$	4.364	2.182	4.31	0.000	-0.02272	-0.01136	-1.61	0.123	-0.2357	-0.1179	-6.68	0.000
M	$ZnSO_4.7H_2O$	1.428	0.714	1.41	0.173	0.11004	0.05502	7.79	0.000	0.0641	0.0320	1.82	0.084
N	$Na_2MoO_4.2H_2O$	-1.065	-0.533	-1.05	0.304	-0.06691	-0.03346	-4.74	0.000	0.1785	0.0893	5.06	0.000
O	$CuSO_4.5H_2O$	-1.548	-0.774	-1.53	0.141	-0.04439	-0.02219	-3.14	0.005	-0.0387	-0.0193	-1.10	0.286
P	$CoCl_2.6H_2O$	0.769	0.384	0.76	0.455	0.00275	0.00137	0.19	0.848	-0.0517	-0.0259	-1.47	0.157
Q	H_3BO_3	2.830	1.415	2.80	0.011	0.05581	0.02791	3.95	0.001	0.1110	0.0555	3.15	0.005
R	Yeast extract	0.700	0.350	0.69	0.496	0.04531	0.02266	3.21	0.004	0.1603	0.0802	4.54	0.000

NH_4NO_3. K_2HPO_4 had negative effect on PHB content within the cells while it did not show any effect on growth. Its negative effect on PHB content is because of the presence of potassium in it, because limitation of K in some of *Methylobacterium* sp. causes accumulation of PHB [15]. $MgSO_4$ had significant negative effect on the growth, PHB content and PHB concentration. In selected ranges of concentration, mineral nitrogen sources, $(NH_4)_2SO_4$, NH_4NO_3, and NH_4Cl, did not show significant impact on the growth. $CaCl_2$ and other trace metal ions are essential for growth because they play important role in regulation of various enzymatic reactions. However, the concentration of the ions in the medium should be at appropriate levels. The negative effects of $MgSO_4$ and $MnSO_4$ on cell growth suggest that these components inhibited the cell growth at high (tested) concentration. Yeast extract was significant for growth and subsequently for PHB concentration. Methanol concentration in selected range of the present study showed an adverse significant effect on the growth.

The composition of the selected medium (based on PHB concentration) obtained in this study is as follows: initial methanol concentration, 0.1 % v/v; $(NH_4)_2SO_4$ 1.5 g/L; NH_4Cl 1.5 g/L; NH_4NO_3 0.15 g/L; KH_2PO_4 1.3 g/L; $K_2HPO_4.3H_2O$ 0.23 g/L; $NaH_2PO_4.2H_2O$ 1.0 g/L; $Na_2HPO_4.12H_2O$ 5.0 g/L; $MgSO_4.7H_2O$ 0.1g/L; $FeSO_4.7H_2O$ 20 mg/L; $CaCl_2.2H_2O$ 20 mg/L; $MnSO_4.7H_2O$ 0.5 mg/L; $ZnSO_4.7H_2O$ 3.0 mg/L; $Na_2MoO_4.2H_2O$ 0.08 mg/L; $CuSO_4.5H_2O$ 0.08 mg/L; $CoCl_2.6H_2O$ 0.8 mg/L; H_3BO_3 0.6 mg/L; Yeast Extract 0.2 g/L. At this condition, optical density, dry cell weight, PHB content, and PHB concentration were 1.213, 3.812 g/L, 21.23 %, and 0.8093 g/L, respectively. These values can be considered as the best condition for further studies

Table 5: Comparison of the effect of media components for PHB production from methanol by Methylobacterium extorquens DSMZ 1340 for each yield.

Effect	Cell Growth (OD)	PHB (g/L)	PHB Content (% w/w)
Significant positive effect	KH_2PO_4 NaH_2PO_4 $CaCl_2$ $ZnSO_4$ Na_2MoO_4 H_3BO_3 Yeast Extract	$(NH_4)_2SO_4$ KH_2PO_4 NaH_2PO_4 Na_2HPO_4 $CaCl_2$ $FeSO_4$ $ZnSO_4$ H_3BO_3 Yeast Extract	$(NH_4)_2SO_4$ $FeSO_4$ $CaCl_2$ $MnSO_4$ H_3BO_3
Significant negative effect	Methanol $(NH_4)_2SO_4$ Na_2HPO_4 $MgSO_4$ $MnSO_4$	NH_4NO_3 K_2HPO_4 $MgSO_4$ Na_2MoO_4 $CuSO_4$	NH_4Cl NH_4NO_3 K_2HPO_4 Na_2HPO_4 $MgSO_4$
Insignificant	-$(NH_4)_2SO_4$ +NH_4Cl +NH_4NO_3 +K_2HPO_4 -$FeSO_4$ -$CuSO_4$ -$CoCl_2$	-Methanol +NH_4CL -$MnSO_4$ +$CoCl_2$	+Methanol +KH_2PO_4 +NaH_2PO_4 +$ZnSO_4$ -Na_2MoO_4 -$CuSO_4$ +$CoCl_2$ +Yeast Extract

in the development of a low cost and effective fermentation process for PHB production from methanol using *M. extorquens* DSMZ 1340.

CONCLUSIONS

PBD was used to test the relative importance of medium components on cell growth and PHB production. Among the studied variables, nitrogen and phosphorus sources, $MgSO_4$ and most of the trace elements were found to be significant variables for PHB production in terms of PHB concentration in fermentation broth. At the best condition dry cell weight, PHB content, and PHB concentration were obtained as 3.812 g/L, 21.23 %, and 0.8093 g/L, respectively. It was also found that most of the trace elements have either positive or negative effect on the growth and the kind of nitrogen sources has no effect on the growth. The results also indicated that deficiency of nitrogen sources, phosphorus sources and $MgSO_4$ in medium, increased PHB accumulation. PBD was found to be a powerful tool for identifying factors which have significant effect on the cell growth and PHB production. Nevertheless, the exact optimal values of individual factors are still unknown but can be determined by the more complete design, e.g. surface response methodology.

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