PRODUCTION OF POLYHYDROXYALKANOATES (PHAs): EFFECT OF AGRICULTURAL MEDIUM COMPOSITION AS CARBON SOURCES BY STATISTICAL METHODOLOGY

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Abstract

This work aims to investigate the effect of agricultural medium containing dried longan, pineapple and sugarcane juices as carbon sources on PHAs production by *Bacillus* sp. SV13 via batch fermentation. Statistical methodology of Plackett-Burman design was applied for screening the variables and considering the corresponding levels of the experiment. The results revealed that all agricultural materials showed significantly effect on PHAs production at 90 % confidence interval and including the supplements of potassium, magnesium and trace elements solution.

Keywords: agricultural materials, polyhydroxyalkanoates (PHAs), carbon source, Plackett-Burman design, *Bacillus* sp.

Introduction

Polyhydroxyalkanoates (PHAs) are organic polymers, synthesized by several microorganisms such as yeast, fungi but mostly found by bacteria more than 150 species, as an intracellular product and serve as carbon and energy reserves the presence of excess carbon and limiting of nutrients such as nitrogen, phosphorous, potassium, magnesium or sulfur and also environmental stress conditions such as oxygen limiting (Ramsay *et al*. 1990; Lee *et al*. 1999; Lee 1996; Khanna and Srivastava 2005).The properties of PHAs are similar to a thermoplastic of polypropylene (PP) derived from petroleum-based plastics. However, the distinct character is a biodegradable polymer which is completely degraded to water and carbon dioxide under suitable condition by microorganisms in the environment. Hence, these desirable properties become to manage the problem of accumulation of recalcitrant plastic in the environment (Khanna and Srivastava 2005; Madison and Huisman 1999). Among monomers of PHAs, Polyhydroxybutyrate (PHB) is found and represented the best characters and also widely produced by many genera bacteria including *Bacillus* species (Kulpreecha *et al*. 2009; Pandian *et al.* 2010).

Statistical designs of experiment (DOE) provide a systematic, efficient, and strategic for investigating the effects of various parameters. It can be applied for bioprocess optimization also microbial fields (Khanna and Srivastava 2005; Dong *et al*. 2007; Sharma *et al*. 2007; Hong *et al*. 2009). Plackett-Burman design (PB design) is extensively applied to screen main effect among various factors in the experiment and also can be reduced the number of experiments whenever many factors are involved.

Despite of PHAs attractiveness than petroleum-derived polymers, products and applications of PHAs are high costs also more expensive than that plastic from petroleum-based 5-10 folds (Poirier *et al*. 1995). Therefore, in this work, the utilization of cheap agricultural materials as carbon sources by *Bacillus* sp. SV13 was studied. 3 kinds of dried longan, pineapple and sugarcane juices were chosen for PHAs production via batch fermentation. Statistical methodology was applied as a tool to screen the important factors and investigate the main effect of medium composition containing with 1, 2 mixed and 3 mixed carbon sources.

Materials and Methods

Microorganism and culture medium

A soil isolated Gram-positive bacteria of *Bacillus* sp. SV13 was employed for PHAs production. It was identified using 16SrDNA technique and found closely to *Bacillus cereus* with 99% identity. Nutrient agar was used to maintain the bacteria. The inoculum was prepared in nutrient broth and then incubated at 37 ºC for 6-8 h. The medium was withdrawn daily and measured the optical density at 600 nm approximately 1.00$\pm$0.10.

Preparation of PHAs production medium

Minimal medium contained of three agricultural raw materials of dried longan, pineapple and sugarcane juices. Before using dried longan as carbon source, it was boiled in hot water until total soluble solid was reached to 10 ºBx. Using PB Design for 9 factors; each carbon source was mixed with 1 L volume ratios of 1:1 and 1:1:1 for two and three kinds of carbon sources. The mixtures were used at 20%(v/v) and 5%(v/v) for high and low levels used as experimental values as shown in Table 1.Other components (gL^{-1}) in minimal medium consisted of $(NH_4)_2SO_4$, Na_2HPO_4, K_2HPO_4, $MgSO_4$and trace element solution (ml/L) (Modified from Handbook of microbiological media, Atlas 2004).Each carbon source was autoclaved at 110 ˚C for 20 min while other components and trace element solution were autoclaved for 15 min at 15 psi and121˚C.The initial pH was adjusted to 6.0 and 20%(v/v) inoculum was used in all experiments. The flasks were cultivated at 300 rpm, 37 ºC and for 20-24h.

Analytical methods

Sugar concentration

Initial total sugar concentration was measured by phenol-sulfuric method (Dubois *et al.* 1956).Total soluble solid was determined by hand refractometer. Sugar content was determined using HPLC (Vertical GESNH2 column; RID detector) with chromatographic conditions were: 40 ˚C; acetonitrile: water 75: 25; 1.0 $mLmin^{-1}$ flow of mobile phase.

PHAs concentration

Culture broth was harvested and centrifuged at 10,000 rpm for 10 min to obtain cell mass. Sequencing pretreatment step of cell disruption was incubated in sodium hypochlorite (1:1) at 37 ºC for 1 h. Then, intracellular biopolymer granule was extractedin hot chloroform. After that, it was evaporated at room temperature and PHAs powder was obtained. Finally, PHAs was estimated by crotonic acid method (Law and Slepecky 1960) in form of poly-β-hydroxybutyric acid (PHB). The powder was added with concentration sulfuric and boiled for 10 min before measuring the absorbance at 235 nm.

Statistical design of experiment

PHAs production was performed in 100 mL Erlenmeyer flask. The effect of medium composition on PHAs production by *Bacillus* sp. SV13 was investigated using Plackett-Burman design (PB). A 12-run design was employed to evaluate 11 factors (including dummy) and the design of experiment (DOE) was carried out as shown in Table 1. Each factor was determined at two levels high (+) and low (-) and PHAs concentration (*Y*) was analyzed as a response value.

Table 1 Plackett-Burman design and corresponding levels used in the experiments

Plackett-Burman design for 9 factors				
Code	Variables	Unit	Experimental value	
			Low (-)	High (+)
X_1	Mixed Solution A (Dried longan+Pineapple)	%(v/v)	5.0	20.0
X_2	Mixed Solution B (Dried longan+Sugarcane)	%(v/v)	5.0	20.0
X_3	Mixed Solution C (Pineapple+Sugarcane)	%(v/v)	5.0	20.0
X_4	Mixed Solution D (Dried longan+Pineapple +Sugarcane)	%(v/v)	5.0	20.0
X_5	$(NH_4)_2SO_4$	gL^{-1}	0.5	2.0
X_6	Na_2HPO_4	gL^{-1}	2.0	3.5
X_7	K_2HPO_4	gL^{-1}	0.25	1.5
X_8	$MgSO_4$	gL^{-1}	0.02	0.1
X_9	Trace elements solution	mLL^{-1}	0.0	1.0
D_1	Dummy	-	0.0	0.0
D_2	Dummy	-	0.0	0.0

The effect of individual variable for PHAs production was calculated by Equation (1):

$$E(X_i) = \frac{2(\Sigma(Y_i^+ - Y_i^-)}{N} \quad (1)$$

Where, $E(X_i)$ is the effect of the test variable (X_i),Y_i^+ and Y_i^- are responses for PHAs production of run at their high or low levels and N is the total number of runs. The experimental error was estimated by calculation of the variance between the two dummy variables using following Equation (2):

$$V_{eff} = \frac{\Sigma(E_d)^2}{n} \quad (2)$$

Where, V_{eff} is the variance of the effect, E_d is the effect for the dummy variable and n is the number of dummy variables used in the experiment. The standard error (*SE*) of the effect was the square root of V_{eff} and the significant (*p*-value) of the effect of each variable on PHAs production measured by Student's *t*-test as Equation (3):

$$t(X_i) = \frac{E(X_i)}{SE} \quad (3)$$

Results and Discussion

Characteristics and properties of carbon sources

The properties of three carbon sources of dried longan, pineapple and sugarcane juice used in this study are shown in Table 2. All of them were revealed in acidic solution (pH 3.9-5.1), however, pineapple juice showed more acidic than others and the maximum glucose concentration was found in the juice. In case of sugarcane juice, the highest total sugar concentration was found and mainly contained of sucrose. On the other hand, minimum total sugar concentration was presented in dried longan and it also contained of sucrose more than glucose same as found in sugarcane.

Table 2 Properties of agricultural materials

Agricultural materials	Properties						
	pH	Total soluble solid (ºBx)	Total sugar (gL^{-1})[a]	Total sugar (gL^{-1})[b]	Fructose (gL^{-1})	Glucose (gL^{-1})	Sucrose (gL^{-1})
Dried longan	5.1	14.8	96.60	117.03	30.92	28.00	58.17
Pineapple juice	3.9	17.0	133.22	133.22	50.27	47.77	35.18
Sugarcane juice	5.1	20.0	178.86	195.60	21.00	23.60	151.79

[a]Determined by Phenol-sulfuric method
[b]Determined by HPLC

Screening of important factors

The preliminary study based on the effect of medium components using agricultural materials as a single carbon source by PB Design (data was not shown), the 8 factors including sugarcane juice, pineapple juice, longan juice, $(NH_4)_2SO_4$, Na_2HPO_4, K_2HPO_4, $MgSO_4$ and trace element solution were selected to investigate the main effect for each factor on PHAs production. The results indicated that only pineapple juice showed a positive effect as a single carbon source and significant for PHAs concentration at 95% confidence level. Interestingly, the interaction effect among 3 carbon sources were also investigated by mixing for all carbon sources using PB Design with 9 factors. Table 3 presented response value for PHAs concentration in the 12 run number of experiments. Data analysis including effects, *t*-value, *p*-value and confidence level of Plackett-Burman design are shown in Table 4.

Table 3 Plackett-Burman design of 9 factors (code levels) for PHA concentration (gL^{-1}) as response

Run order	Factors											Response (Y)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	D_1	D_2	PHAs (gL^{-1})
1	+	-	+	-	-	-	+	+	+	-	+	0.437
2	+	+	-	+	-	-	-	+	+	+	-	0.312
3	-	+	+	-	+	-	-	-	+	+	+	0.503
4	+	-	+	+	-	+	-	-	-	+	+	0.485
5	+	+	-	+	+	-	+	-	-	-	+	0.525
6	+	+	+	-	+	+	-	+	-	-	-	0.315
7	-	+	+	+	-	+	+	-	+	-	-	0.565
8	-	-	+	+	+	-	+	+	-	+	-	0.366
9	-	-	-	+	+	+	-	+	+	-	+	0.187
10	+	-	-	-	+	+	+	-	+	+	-	0.386
11	-	+	-	-	-	+	+	+	-	+	+	0.128
12	-	-	-	-	-	-	-	-	-	-	-	0.153

Table 4 Estimated effects of factors and statistical analysis of Plackett-Burman design

Factors	PHAs concentration (gL^{-1})			Confidence level (%)
	Effect	*t*-value	*p*-value	
X_1: Mixed solution A	0.09300	4.66527	0.043	95.70
X_2: Mixed solution B	0.05567	2.79422	0.108	89.20
X_3: Mixed solution C	0.16333	8.20359	0.015	98.50
X_4: Mixed solution D	0.08633	4.34108	0.049	95.10
X_5: $(NH_4)_2SO_4$	0.03367	1.68888	0.234	76.60
X_6: Na_2HPO_4	-0.03833	-1.93280	0.193	80.70
X_7: K_2HPO_4	0.07533	3.78841	0.063	93.70
X_8: $MgSO_4$	-0.14533	-7.30511	0.018	98.20
X_9: Trace element solution	0.06967	3.50744	0.073	92.70

In Table 4, factors X_1, X_3, X_4 and X_8 were significant for PHAs concentration at the confidence level above 95%while $X_7$and X_9were also significant but in lower levels (93.7 and 92.7%). On the other hand, 3 factors of X_2, $X_5$and X_6 were not significant on PHAs concentration. Furthermore, all significant factors showed positive effects on PHAs concentration but in contrast with the cases of Na_2HPO_4 and $MgSO_4$. However, the positive or negative effect in each factor was interpreted that at higher or lower concentration level was affected on PHAs concentration.

To accumulate PHAs by this bacterial strain, deficiency of $MgSO_4$ should be considered because of the greater negative effect on PHAs concentration. Similar results for the effect of $MgSO_4$ on PHAs production using methylotrophic bacteria have been reported by Suzuki *et al* (1986); Choi *et al* (1989).The result on K_2HPO_4 effect revealed that the positive effect of $K_2HPO_4$to use as buffer was affected on pH condition. The suitable of pH condition leaded to accumulate of PHAs (Wu *et al.* 2001).The effect of trace elements solution to employ as growth factors for bacteria could be influenced on PHAs production. In case of trace elements solution had positive effect for PHAs concentration related to Grothe *et al* (1999) study who reported that a suitable complement of trace elements was essential to obtain high PHAs productivity and yield. The effect of various carbon sources implied that mixed carbon sources of agricultural materials were significant affected on PHAs concentration at above 95% confidence level except the mixed solution of dried longan and sugarcane. It might be caused from sugar content. The different amounts of sugar types leaded to mixed carbon sources that showed significantly effect for PHAs concentration.

Conclusion

Successfully, the significant main factors as $MgSO_4$, K_2HPO_4 and trace elements solution were achieved by PB design that could be employed to design the suitable condition for PHAs production. However, different carbon sources used, only pineapple juice as a sole carbon source showed an effect on PHAs concentration but mixed for 2 or 3 agricultural materials showed in different levels and also showed significantly effect on PHAs concentration. Therefore, the appropriate DOE should be selected and investigated to obtain the correct analyzed data. In addition, the interaction effect among all factors also needs to consider and make decision for improving of PHAs production optimization process. Besides, the design with high resolution should be good to apply as a tool for data collection and analysis. In addition, the use of mixed carbon sources can be applied to reduce the limitation of using carbon sources that depend on the season especially single carbon source. Moreover, the potential of use the mixed carbon sources leads to the development of PHAs production in a large scale.

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