Polyhydroxyalkanoate (PHA) synthesis by newly bacterial isolates using non-detoxified rice husk hydrolysate

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Abstract

Rice husk, a low cost carbon sources from agricultural waste was used for PHAs production. However, the structural complexity of lignocellulosic materials in rice husk requires a pretreatment step prior used as carbon source. The effect of the main operating factors including sulfuric acid concentration and hydrolysis temperature on fermentable sugar production from rice husk were investigated by response surface methodology. The results indicated that total sugar and reducing sugar concentration ranged between 12.36 and 30.12 g/L, and 0.28 and 6.66 g/L, respectively. The satisfactory condition of 9.20 % w/v sulfuric acid at 107.86 ^{0}C was established for reducing sugar and total sugar productions. Furthermore, the non-detoxified rice husk hydrolysate was then used as a carbon source for 10 isolate microbial strains to test PHA production capability. It was found that the highest biomass was observed as 1.01g/l and PHA content as 11.2 %.
Keywords : *PHA, Rice husk, RSM.*

1. Introduction

Rice husk have always played an important part in the economic and social lives in Thailand which represent approximately 20% dry weight of the harvest, being an abundant lignocellulosic residues (1). Rice husk is a cheap raw material consisting of the complex structure of cellulose (36-40%) and hemicellulose (12-19%) (2) being unsuitable feed stocks for microbial biotransformation. Pretreatment step of rice husk is thus necessary in order to gain the monosaccharide for fermentation. The chemical and biological pretreatment of rice husk were widely used to produce bioethanol (2), xylitol (3) and other value added products. According to our knowledge, however, the PHA productions by using rice husk as carbon sources have a few reported (4, 5).

Hence, the aim of this study was to investigate for the experimental hydrolysis conditions by using response surface methodology (RSM) leading to the obtained maximum total sugar and reducing sugar concentration released from rice husk using

sulfuric acid as hydrolysis solution. In addition, PHA production from rice husk hydrolysate was investigated and evaluated to achieve potential economic value of PHA.

2. Materials and methods

2.1 Material

Rice husk was collected from local rice mill industry in Nakhon Ratchasima province, Thailand. Rice husk were ground to small size and stored at room temperature until used for hydrolysis processes.

2.2 Experiment design

The experiment was conducted using a five-level two-factor central composite design (CCD) with three assays at the central point. The influenced factors studied were sulfuric acid concentration (2.76 – 11.24 %w/v) and pre-treatment temperature (65.68 – 129.32 ^{0}C). Both factor variations can be seen in Table 1. The test runs (Table 2) were performed by using Expert design version 11.0 (Trial version). The responses of this experiment were total sugar and reducing sugar concentration.

Optimization of rice husk hydrolysis experiment was carried out to determine the effect of variation factors (Table 1) on the sugar production. In brief, dried rice husk power (5 g) was added into the flask containing 100 mL of diluted sulfuric acid solution (2.76 – 11.24 %w/v). The mixture was heated in an autoclave (Hirayama Manufacturing Corp., Tokyo, Japan) at varying pre-treatment temperatures (65.68 - 129.32 ^{0}C) for 20 min, then cooled down to room temperature. After filtration of these samples with double cheesecloth, the supernatant was obtained by centrifugation at 10,000 rpm for 5 min, and kept at 4 ^{0}C prior sugar analysis.

Table 1. Factor levels for central composite design (CCD) of effected factors for rice husk hydrolysis.

Factors	Code level				
	-α(-1.414)	-1	0	+1	+ α(+1.414)
Sulfuric acid concentration (%w/v)	2.76	4.00	7.00	10.00	11.24
Temperature (^{0}C)	65.68	75.00	97.50	120.00	129.32

2.3 Microorganism and growth condition

To test the rice husk hydrolysate ability for PHA production, PHA-producing bacteria were previously isolated and screened from soil sources by using Nile red stained (data not showed). The 10 bacterial strains were selected according to growth ability in xylose media (6). Then, those were maintained on NA slants. The inoculums were cultured in NB medium at 30 ^{0}C and 200 rpm for 24 h. the seed culture (5% v/v) was transferred into mineral salt medium (MSM) for PHA production which consisted of $(NH_4)_2SO_4$ (1.50 g/L), KH_2PO_4 (1.50 g/L), Na_2HPO_4 (3.57 g/L), $MgSO_4.7H_2O$ (0.20 g/L), rice husk hydrolysate at the optimal conditions (100 mL) and trace element solution (1.00 mL/L) (7). In order to determine production of PHA from rice husk hydrolysate as a sole carbon source, the isolated bacterial strains were cultured in 100 mL of MSM, in 500 mL Erlenmeyer flask, and incubated on orbital shaker with 200 rpm at 30 ^{0}C for 3 days.

2.4 Analytical methods

Reducing sugar was determined by 3, 5 – dinitrosalicylic acid method (8) and total sugar was analyzed by phenol sulfuric acid method (9).

PHAs concentration were determined by modification method of Law and Slepeky (10) and dry cell weight were determined by modified gravimetric method of Tanamool et. al., 2013 (7).

3. Results and discussion

3.1 Optimization of rice husk hydrolysis for reducing and total sugar production from rice husk.

In order to achieve the most effective procedure for hydrolysis of rice husk, optimization of the crucial factor was examined base on CCD. The experimental runs comprises of 13 trials as shown in Table 2. The results showed that total sugar and reducing sugar concentration were obtained in range of 12.36 – 30.12 g/L and 0.28 - 6.66 g/L, respectively. Polynomial equations were generated by Design Expert software to predict in term of reducing sugar (eq. 1) and total sugar (eq. 2) concentrations. Models proposed for both responses were as follows:

$$\text{Reducing sugar} = 6.25 + 1.09A + 1.79B + 0.10AB - 1.20A^2 - 1.93B^2 \qquad \text{(eq. 1)}$$

$$\text{Total sugar} = 23.59 + 5.29A + 3.81B - 0.62AB - 2.06A^2 - 1.12B^2 \qquad \text{(eq. 2)}$$

Where A is sulfuric acid concentration and B is pre-treatment temperature.

Then the results were calculated by analysis of variance (ANOVA) to test a significant model and revealed that both models of reducing sugar and total sugar concentrations with a p-value < 0.0003 and p-value < 0.0001 were statistical significance (Table 3). The coefficient of determination (R^2) in reducing sugar and total sugar productions models were 0.9461 and 0.9560. They showed in a good adjustment of the model to the experimental data. The R^2 almost reach to 1.0, being a stronger model and better predicted responses (5). Moreover, the lack of fit of reducing sugar model was 0.0983 and total sugar model was 0.5939 which indicated the goodness of fit model.

Table 2. Central composite design (CCD) for the optimization of reducing sugar and total sugar production from rice husk.
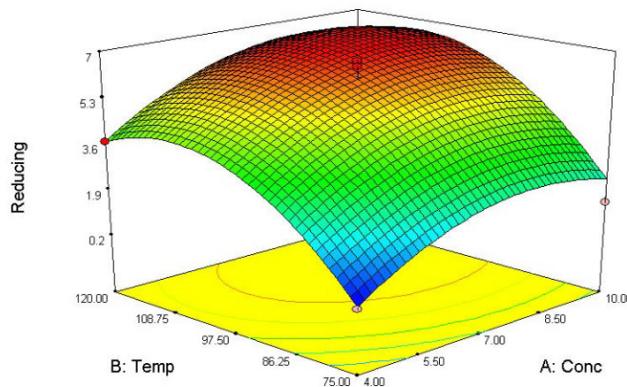
Run	Sulfuric acid concentration (%w/v)	Temperature (^{0}C)	Reducing sugar (g/L)	Total sugar (g/L)
1	10.00	120.00	5.29	30.12
2	7.00	97.50	5.96	24.72
3	7.00	97.50	6.66	25.49
4	7.00	97.50	5.56	21.18
5	2.76	97.50	2.19	12.36
6	7.00	97.50	6.46	23.71
7	4.00	75.00	0.28	9.66
8	7.00	129.32	5.31	25.51
9	7.00	65.68	0.36	16.98
10	10.00	75.00	1.43	22.16
11	11.24	97.50	6.42	26.38
12	4.00	120.00	3.72	20.11
13	7.00	97.50	6.62	22.83

Table 3. ANOVA analysis of RSM model for the reducing sugar and total sugar concentration from rice husk.

Sources	Reducing sugar			Total sugar	
	F-Value	p-value		F-Value	p-value
Models	24.58478	0.0003		30.44	0.0001
A-Acid concentration	17.29202	0.0043		90.641	< 0.0001
B-Temperature	46.67804	0.0002		46.961	0.0002
AB	0.079422	0.7862		0.6211	0.4565
A^2	18.22167	0.0037		11.898	0.0107
B^2	47.31052	0.0002		3.5313	0.1023
Lack of Fit	4.2400	0.0983		0.7118	0.5939

The relationships between the dependent variables are illustrated by response surface plot in three dimensions. Fig. 1 depicts the response surface describing the influences of the reaction temperature and sulfuric acid concentration on reducing sugar concentration. The plot showed that reducing sugar concentration increased significantly with the increasing of reaction temperature and sulfuric acid concentration. However, when the reaction temperature increased above 97.50 ^{0}C, the reducing sugar concentration began decreasing due to reducing sugar degradation to hydroxymethyl furfural (HMF) (11). Consistently, increase of sulfuric acid concentration leads to enhancement of the reducing sugar production. However, the higher concentration above 10 % (w/v) may cause degradation of sugar to other by-products (8).

Figure 1. Response surface plot showing the effect of pre-treatment temperature and sulfuric acid concentration on reducing sugar production.

Similarly, the higher total sugar could be obtained by pretreatment with increasing of temperature and sulfuric acid concentration (Fig. 2). This was due to the complete hydrolysis of the hemicellulose and charring of the remaining cellulose in the pretreatment step. On the other hand, by-products particularly being as inhibitors for microbial growth would be subsequently generated (12).
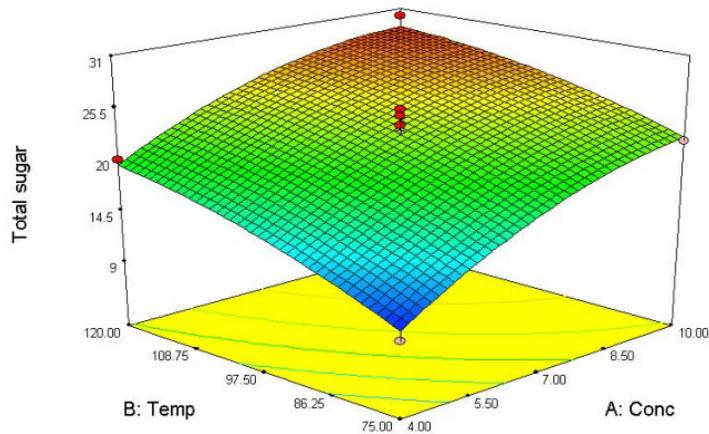
Figure 2. Response surface plot showing the effect of pre-treatment temperature and sulfuric acid concentration on aqueous extraction of total sugar.

3.2 Validation of the RSM results.

Pretreatment condition was predicted by RSM with using 9.20 % (w/v) of sulfuric acid and temperature at 107.86 °C for 20 min. The reducing sugar and total sugar concentrations obtained from this condition were 6.93 and 26.80 g/L, respectively. The validations of these experiments were verified and performed under the optimized conditions. The results showed that reducing sugar and total sugar concentration were 7.98 and 30.80 g/L. Hence, the validations of the predicted models were achieved at 115.15 % and 114.92 % for reducing sugar and total sugar production. These values were indicated an acceptable agreement between experimental and predicted values and implied that the mathematical model is clearly reliable for both reducing sugar and total sugar productions.

3.3 Potential PHA production from rice husk by 10 PHA-producing isolates.

To demonsrtate the possibility of using rice husk as an alternative bio-refinery feed stock for production of PHA, 10 PHA producing strains were cultured in MSM containing rice husk hydrolysate (without non-detoxification step) as a sole carbon source. The results revealed that these isolates could utilize the hydrolysate as a sole carbon source with cell dry weight about 0.53-1.01 g/L and could accumulate PHA 4.32-11.20 %(w/w) (Table 4). Among them, the isolate NK13 showed the highest PHB production.

Table 4. PHA production from 10 different isolates bacteria.

Isolate No.	DCW (g/L)	PHA content (%w/w)
NK4	0.78	8.53
NK11	0.89	10.21
NK12	0.90	9.84
NK13	1.01	11.20
NK14	0.97	9.88
NK15	0.53	10.55
NK16	0.67	9.67
NK17	0.68	8.38
NK18	0.96	4.32
NK19	0.97	9.58

Previously, Patel (2014) (4) reported that PHAs accumulation in *Pseudomonas aeruginosa* by using rice husk as carbon source was obtained at 0.43 % w/w. While Gowda and Shivakumar (2014) (5) had reported *Bacillus thuringiensis* IAM 12077 could accumulate higher PHA (24.40 % w/w) in their cell. Using hydrolysate of rice husk directly might suppress bacterial growth cause by several inhibitors. In addition, it would be more severe in case of the hydrolysate having low sugar content thus providing high sugar concentration hydrolysate would be more advantageous.

4. Conclusion

The different hydrolysis conditions for reducing sugar and total sugar production revealed that these variables markedly affect the fermentable sugars from rice husk. These variables can be related to the hydrolysis conditions by second order polynomials. Optimum hydrolysis condition was obtained by RSM. Non-detoxified rice husk hydrolysate could be used as carbon source for PHA production by isolate strain NK13. However, bacterial growth and PHA content from non-detoxified rice husk hydrolysate still low due to low concentration of reducing sugar was obtained in fermentation media. The evaporating system should be applied to concentrate the fermentable sugar from rice husk hydrolysate.

However, this study explored the potential of isolate strain NK13 to produce cost-effective PHA from alternative carbon source obtained from agro-wastes hydrolysate without non-detoxification step, which lead to reduce the cost of PHA production. In addition, physiological characteristics of this newly isolate strain would be carried out further to improve the strain more effective on PHA production.

5. Acknowledgment

6. References

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