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Research Article

OPTIMIZATION OF THE HYDROLYSIS PROCESS OF MICROALGAE *PORPHYRIDIUM CRUENTUM* BIOMASS WITH VARIATIONS OF HYDROCHLORIC ACID (HCl) CONCENTRATION, TEMPERATURE, AND TIME USING RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT

Microalgae *Porphyridium cruentum* has potential as a bioethanol feedstock because it has a high carbohydrate content. These carbohydrates can be broken down into reducing sugars through the hydrolysis process. The reducing sugar obtained will be used as a substrate in the bioethanol production. This study aimed to produce a substrate with the best-reducing sugar indicator and to determine the optimum hydrolysis conditions of *P. cruentum* biomass following the Box-Behnken statistical experimental design, using Response Surface Methodology (RSM). The parameters of the optimized hydrolysis process were HCl concentration (2 - 0.2 N), temperature (60 -120 ˚C), and hydrolysis time (30-180 minutes). The optimum conditions that have been achieved using RSM were a HCl concentration of 1.91 N, a temperature of 60 °C, and a hydrolysis time of 180 minutes. The prediction of maximum total reducing sugar was 810 mg/L by RSM at optimum conditions. The actual total reducing sugar obtained at optimum conditions was 895 mg/L. Therefore, it was well close to the predicted value and verifying of the model appropriateness.

Keywords: bioethanol, microalgae, *Porphyridium cruentum*, optimization

1. Introduction

The demand for energy in the industrial sector, transportation and household needs has increased significantly. This is accompanied by the decreasing ability of domestic petroleum production that occurs naturally. As a result, there is an energy crisis, depletion of fossil fuel reserves, and increasing world fuel oil prices. In recent years, research has been conducted to obtain alternative fuels from renewable natural resources such as biodiesel, bioethanol and biogas.

Microalgae had potential as one of the promising renewable feedstocks in the future for biofuel production, because microalgae have high biomass productivity and can be cultured in a limited area, and have the ability to grow in a relatively short time depending on the species and environmental conditions [1]*Porphyridium cruentum* microalgae has a fairly high carbohydrate content with levels of 40% - 57% (w/w) [2]. Mutmainnah *et al*. in 2018 also stated that the dry biomass of *P. cruentum* microalgae contains as much as 32.1% (w/w) carbohydrates [3]. Various types of carbohydrates in *P. cruentum* can be found as stored floridean starch, cell wall lipopolysaccharides and extracellular polysaccharides [4]. Carbohydrates from microalgae can be broken down into reducing sugars through hydrolysis. The absence of lignin in *P. cruentum* microalgae cells causes the hydrolysis process to be easier.

Hydrolysis is a reaction of breaking large molecules (polymers) into smaller parts (monomers) through the addition of water molecules (H_2O) and catalyst assistance to accelerate the reaction. Breaking the polymer chain can be done by several methods, namely enzymatically, physically, and chemically. Chemical hydrolysis usually uses sulfuric acid (H_2SO_4)

or hydrochloric acid (HCl). Chemical hydrolysis with acid can convert polysaccharides into monosaccharides randomly where no certain pattern in the breaking of glycosidic bonds in polysaccharides, while enzymatic hydrolysis converts polysaccharides specifically depending on the type of enzyme used.

The quality and quantity of reducing sugars production influenced by the several factors of hydrolysis, such as acid concentration, temperature of hydrolysis and time used. High acid concentration, temperature condition, and hydrolysis time can cause by-product components formation, such as phenolic compounds, furan derivatives, and weak acids which can be inhibitors during the fermentation process [5]. Therefore, this study examined the optimal conditions of HCl concentration, temperature and time of hydrolysis for reducing sugar production as a fermentation medium in the manufacture of bioethanol using Response Surface Methodology (RSM).

2. Materials and Methods

This research was performed from May to August 2022 in the Industrial Chemistry Laboratory Politeknik Negeri Lampung. The research method used is the *Box-Behnken* statistical experimental design, using *Response Surface Methodology* (RSM), Minitab var.18. Microalgae *Porphyridium cruentum* as the object of research were collected from Ugo Plankton, Yogyakarta, Indonesia.

2.1 Experimental design by Response Surface Methodology

Determination of optimum conditions in the hydrolysis process of *P. cruentum* microalgae biomass follows the *Box-Behnken* statistical experimental design, using *Response Surface Methodology* (RSM) available in Minitab software version 18. *Box-Behnken design* is a design plan used for experimental design that can only be applied to experiments with at least 3 treatment factors.

The 3 treatment factors in this study include HCl concentration, temperature, and hydrolysis time (Table.1). The minimum and maximum limit values were entered into the Minitab ver.18 program and then randomized according to the *Box-Behnken* experimental design. After randomizing the combination, 15 treatments were obtained to be analyzed (Table. 3) and the response to be measured was reducing sugar concentration after the hydrolysis process.

Tabel 1. Actual and coded values of optimization process factors

Factors	Unit	Symbol	Coded value	
			Low	High
Concentration of HCl	N	A	0,2	2
Temperature	˚C	B	60	120
Time	min	C	30	180

2.2 Hydrolysis of Microalgae Biomass P .cruentum

Hydrolysis of *P. cruentum* microalgae use hydrochloric acid (HCl) with concentration, temperature, and process time based on *Box-Behnken* experimental design in Table 3. Microalgae *P. cruentum* samples before the hydrolysis process were first measured for pH, % Brix and reducing sugar analysis. Hydrolysis took place using a series of hydrolysis equipment as shown in Figure 1.

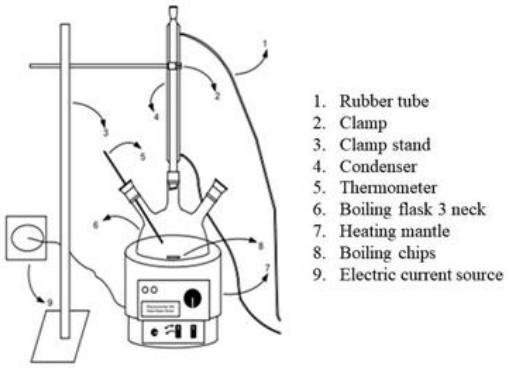

Figure 1. Hydrolysis Apparatus

2.3 Reducing Sugar Analysis

Quantitative measurement of reducing sugar uses the Dinitrosalicylate (DNS) method. The method of making DNS reagent is 1 gram of 3,5-dinitrosalicylic acid and 1.6 gram of NaOH were dissolved in 40 mL of distilled water (Solution

A)[6][7][8]. A total of 30 gram of sodium potassium tartarate being dissolved in 40 mL of distilled water (Solution B). Solution A and solution B were mixed, and then calibrated in a measuring flask with distilled water until the final volume became 100 mL, then stirred with a magnetic stirrer until homogeneous. The next step is to make a standard curve of glucose solution by preparing a standard glucose solution with concentrations of 0, 200, 400, 600, 800, 1000 ppm, respectively. One milliliter of each solution was taken and then was added 2 mL of DNS reagent. Then, each solution was vortexed and heated in a 100°C water bath for 10 minutes. After it gets cold, the solution was diluted by adding 10 mL of distilled water and was vortexed again. The absorbance was measured using spectrophotometer at 520 nm, then a linear equation was used as a standard curve. Measurement of reducing sugar content in the samples was carried out in the same way as in the glucose standard solution, then the measurement values obtained were plotted on the standard curve.

2.4 Validation of Model

The validity of the model was performed in three replicate experiments for each variable of the optimum conditions. The average actual value at optimum conditions was compared with the predicted value for verifying the appropriateness of the model.

3. Result and Discussion

3.1. Characteristics of microalgae before the hydrolysis process

Microalgae *P. cruentum* is a type of red microalgae (Rhodophyta). It has a relatively rapid growth rate and is easy to regulate its growth [4]. The red color is due to the presence of the dominant phycoerythrin pigment [3]. The pH measurement results in *P. cruentum* microalgae culture was 8.6, where the pH was the optimum pH (pH 8 - 9) in the growth of *P. cruentum* microalgae to produce the highest biomass content in the form of polysaccharides, lipid pigments, and other bioactive substances [9]. Brix levels were measured using a refractometer through calibration standards using 1 gram of pure glucose in 100 mL deionized water for 1% brix. The levels of brix and reducing sugars in microalgae *P. cruentum* culture before the hydrolysis process were 3.1% and 299.1 mg/L, this was because *P. cruentum* microalgae were able to produce extracellular polysaccharides. Patel *et al.* (2013) reported that extracellular polysaccharide of *P. cruentum* contains four monosaccharides, namely 40% galactose, 30% xylose, 20% glucose, and 10% glucuronic acid [10].

Table 2. Initial characteristics of *P.cruentum* microalgae before the hydrolysis process

Characteristic	Microalgae *P. cruentum* before hydrolysis.
Color	Red
pH	8,6
Brix (%)	3,1
Reducing sugar (mg/L)	299,1

The addition of an acid solution in the hydrolysis process with a concentration of 0.2 N, 1.1 N, and 2 N hydrochloric acid resulted in a decrease in pH, respectively, by 0.9, 0.5, to 0.1 and an increase in the brix content of 4.2%, 8% to 11.3% (Figure 2). The increase in brix content indicates the amount of dissolved solids in the filtrate which represents an estimate of the total sugar content that has been hydrolyzed.

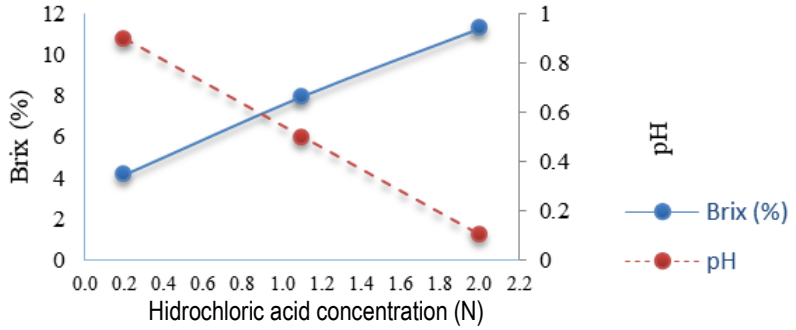

Figure 2. Brix and pH values after hydrolysis using hydrochloric acid.

3.2. Optimization of P.cruentum microalgae hydrolysis process with Response Surface Methodology

The main purpose of this optimization stage was to determine how much influence HCl concentration, temperature and hydrolysis time have on reducing sugar concentration. The produced reducing sugar can be used as a fermentation medium in the manufacture of bioethanol.

Based on the measurement results of reducing sugar according to the hydrolysis conditions suggested by the Box-Behnken experimental design (Table 3), it showed that the response value of reducing sugar concentration is 58.5 mg/L to 773 mg/L. Determination of reducing sugar concentration was calculated based on standard curve of glucose standard using linear regression. The linear regression equation of glucose standard was y = 0.001x - 0.0002 with an R^2 value of 0.9974 using the Dinitrosalicylate (DNS) method.

Table 3. Measurement of reducing sugar concentration after hydrolysis process

Formula	Concentration of HCl (N)	Temperature (˚C)	Hydrolysis time (minutes)	Reducing Sugar Concentration (mg/L)
1	0.2	60	105	233.5
2	2.0	60	105	674.0
3	0.2	120	105	509.0
4	2.0	120	105	702.0
5	0.2	90	30	58.5
6	2.0	90	30	634.0
7	0.2	90	180	405.5
8	2.0	90	180	756.0
9	1.1	60	30	345.5
10	1.1	120	30	704.0
11	1.1	60	180	696.5
12	1.1	120	180	725.0
13	1.1	90	105	773.0
14	1.1	90	105	486.5
15	1.1	90	105	529.7

The results of ANOVA Table. 4 showed that HCl concentration and hydrolysis time had significant effect ($P < 0.05$) on the concentration of reducing sugar, while the temperature treatment showed no significant effect ($P>0.05$). This indicates that different temperature treatments do not show significantly different reducing sugar levels.

Table 4. Analysis of variance of regression model for reducing sugar concentration response

Source of Diversity	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	563603	62623	5,18	0,042
Linear	3	452014	150671	12,46	0,009
HCl Concentration (N)	1	304005	304005	25,15	0,004
Temperature(°C)	1	59599	59599	4,93	0,077
Time (Minutes)	1	88410	88410	7,31	0,043
Square	3	56393	18798	1,55	0,310
HCl Concentration (N)*HCl Concentration (N)	1	45101	45101	3,73	0,111
Temperature (°C)* Temperature (°C)	1	7061	7061	0,58	0,479
Time (Minutes) * Time (Minutes)	1	1852	1852	0,15	0,712
2-Way Interaction	3	55195	18398	1,52	0,317
HCl Concentration (N)* Temperature (°C)	1	15314	15314	1,27	0,311
HCl Concentration (N)* Time (Minutes)	1	12656	12656	1,05	0,353
Temperature (°C))* Time (Minutes)	1	27225	27225	2,25	0,194
Error	5	60450	12090		
Lack-of-Fit	3	12742	4247	0,18	0,903
Pure Error	2	47708	23854		
Total	14	624053			
R-sq = 90,31%	R-sq (adj) = 72,88%	R-sq (pred) = 50,13%			

The determinant coefficient (R-sq) and standard deviation were used to evaluate the developed model. If the R-sq value is close to 100%, the standard deviation (SD) is smaller and the model is getting better at predicting the response. Data on Table 4 showed that the quadratic model has a relatively high R-sq value of 0.9031. This results means that 90.31% of the experimental data are relevant and only 9.69% of the total variation that cannot be explained by the model. Lack of

Fit from the response of reducing sugar concentration with P value > 0.05 (0.903) indicates an insignificant Lack of Fit. Lack of Fit value which is not significant is a requirement for a good model because it shows the suitability of the model with reducing sugar concentration response data [11].

The regression equation from RSM for optimization of HCl concentration (A), temperature (B), hydrolysis time (C) to the response of reducing sugar concentration (Y) is:

$$Y = -533 + 811A + 0{,}5B + 6{,}45C - 136{,}4A^2 + 0{,}0040C^2 - 2{,}29AB - 0{,}833AC - 0{,}0367BC \quad (1)$$

The above equation shows that the concentration of reducing sugar increased with an increase in the concentration component of HCl, temperature, and hydrolysis time which is indicated by a positive value constant and gives a synergistic effect on the hydrolysis process carried out [12]. The release of glycosidic bonds in polysaccharides into monosaccharide molecules will be more effective with an increase in acid concentration, temperature and hydrolysis time. This results are in line with the research of Miranda *et al* 2012, where the higher concentration of acid used, the higher the level of reducing sugar produced [13]. This study also observed the treatment of acids (H_2SO_4 and HCl) with a concentration of 2 N which has achieved efficiency in the conversion of reducing sugars. Excessive acid concentrations can reduce reducing sugar levels due to the unwanted by-products formation, such as phenolic compounds, furan derivatives, and weak acids which can become inhibitors during the fermentation process [14][15][16].

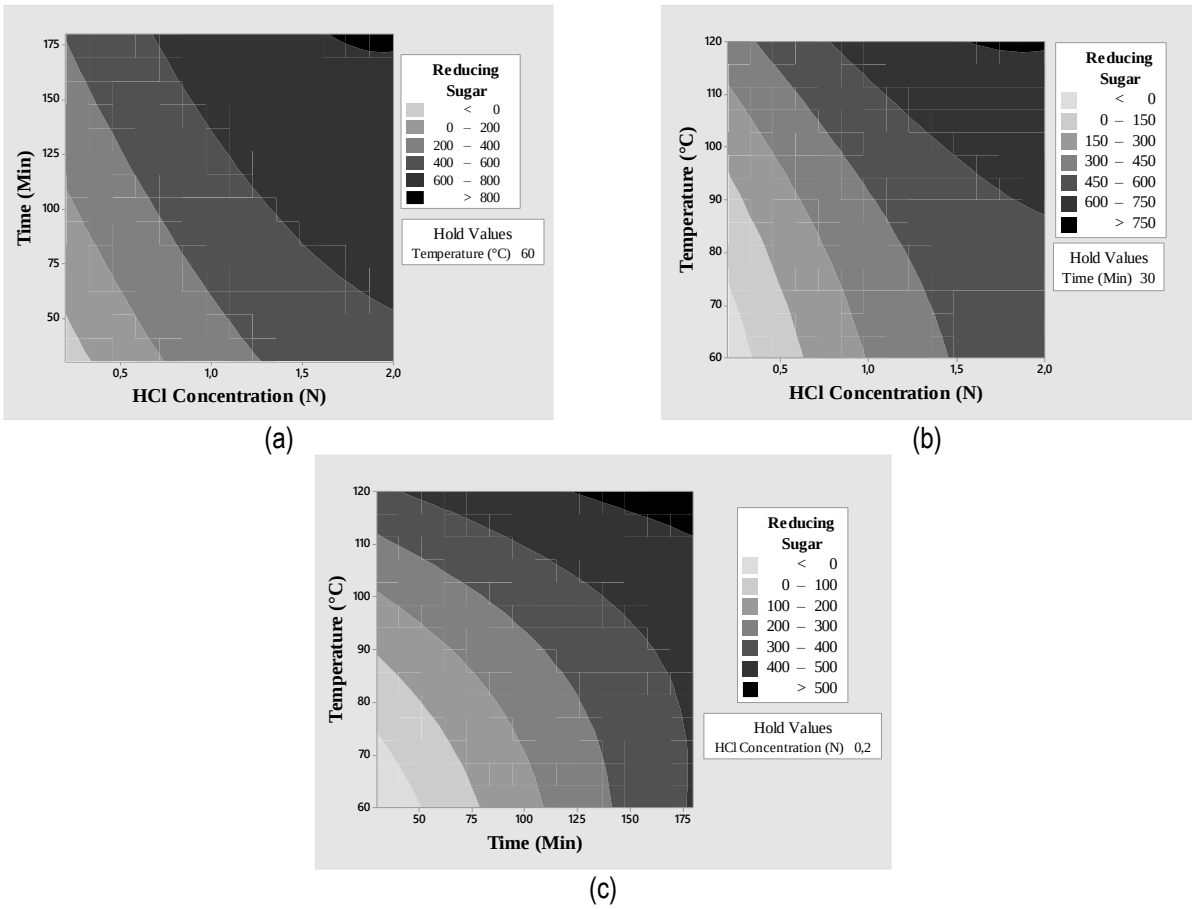

Figure 3. Contour plot of reducing sugar concentration against HCl concentration factors, temperature, and hydrolysis time. (a) at 60 ˚C (b) at 30 minutes (c) at 0.2 N HCl concentration.

After optimization using the *Response Surface Method*, a contour plot is obtained as Figure 2, where the HCl concentration for the hydrolysis process to produce reducing sugars greater than 800 mg/L is between 1.65-2 N and the hydrolysis time is between 170-180 minutes using the lowest temperature of 60 ˚C (Figure 2a).

3.3. Validation of Optimum Formula Solution

The optimum conditions recommended by Minitab 18 software with the *Response Surface Method* to obtain the maximum reducing sugar concentration were presented in Table 5. The prediction of maximum reducing sugar concentration was 810.045 mg/L. To verify the validity of the model equation obtained, three replicate experiments were conducted in determining the reducing sugar concentration at the optimum condition. The average reducing sugar from experiment was of 895 mg/L. This result had an accuracy value of 90.5%.

Table 5. Prediction and validation results of reducing sugar response values with the optimum formula generated from Minitab 18 software.

Optimum Process Parameters			Reducing sugar concentration (mg/L)		Composite Desirability
Concentration (N)	Temperature (˚C)	Time (min)	Prediction	Validation	
1,91	60	180	810,045	895	1

4. Conclusion

The optimum conditions achieved using Response Surface Methodology (RSM) with Box-Behnken design are HCl concentration of 1.91 N, temperature of 60˚C, and hydrolysis time of 180 minutes with a maximum reducing sugar content of 810 mg/L (predicted). The actual reducing sugar at the optimum condition was 895 mg/L, which is close to the prediction and can verify the model appropriateness.

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