Influence of Factors on Sugar Released in Acid-Hydrothermal Pretreatment of *Chlorella* sp. Biomass

อิทธิพลของปัจจัยต่อน้ำตาลที่ถูกปลดปล่อยในการปรับสภาพชีวมวล *Chlorella* sp. ด้วยไฮโดรเทอมัล ในสภาวะกรด

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ABSTRACT

There are various kinds of methods to pretreat microalgae for use as substrate in ethanol production. Our previous studies investigated different methods for pretreatment of *Chlorella* sp. biomass including hot water, hydrothermal, alkali and acid pretreatment methods. Acid pretreatment at 121ºC offered the highest sugar released when compared with other pretreatments without any enzyme addition. Amylases addition following acid pretreatment further increased glucose content in hydrolysate by 20% . In this study, factors in acid hydrothermal pretreatment of *Chlorella* sp. biomass were assessed for their influences on sugar released. Four factors were investigated using 2^k factorial design. These factors were temperature (110 and 150 ºC), time (20 and 60 min), H_2SO_4 concentration (0 and 1.5% v/v) and solid loading (5 and 20% w/v). Experiments were carried out in oil bath and timing of the reaction started after 20 min. The results showed that temperature, acid concentration and solid loading had significant effect on sugar released from microalgae biomass. Although time did not show significant effect, it had interaction with temperature. Therefore, all factors are worth for further investigation in optimization of factors in acid-hydrothermal pretreatment of *Chlorella* sp. biomass to be used in ethanol production.

บทคัดย่อ

การศึกษาก่อนหน้าได้ประเมินวิธีการปรับสภาพมวลจุลสาหร่าย *Chlorella* sp. ด้วยวิธีการต่างๆ ได้แก่ น้ำร้อน ไฮโดรเทอมัล แอลคาไลน์ และกรด การปรับสภาพด้วยกรดที่ 121 ºC ให้ปริมาณน้ำตาลทั้งหมดสูงสุดเมื่อเปรียบเทียบกับการปรับสภาพด้วยวิธีอื่น ทั้งนี้การเติมอะไมเลสและกลูโคอะไมเลสหลังการปรับสภาพด้วยกรดทำให้ปริมาณกลูโคสเพิ่มขึ้นอีกประมาณ 20% ในการศึกษานี้ได้ศึกษาถึงปัจจัยที่มีผลต่อการปรับสภาพชีวมวลของ *Chlorella* sp. ด้วยวิธีกรด-ไฮโดรเทอมัล โดยประเมินผลจากปริมาณน้ำตาลที่ถูกปลดปล่อยออกมาจากชีวมวลของ *Chlorella* sp. ในการศึกษาได้ใช้การออกแบบการทดลองแบบ 2k factorial design โดยปัจจัยที่ใช้ในการศึกษามี 4 ปัจจัย คือ อุณหภูมิ (110 และ 150 ºC), เวลา (20 และ 60 นาที), ความเข้มข้นกรดซัลฟูริก (0 และ 1.5% โดยปริมาตร) และปริมาณชีวมวลจุลสาหร่าย (5 และ 20% โดยน้ำหนักต่อปริมาตร) ในการทดลองได้ดำเนินการใน oil bath และเริ่มจับเวลาหลังจากเวลาผ่านไป 20 นาที ผลการทดลองที่ได้ชี้ให้เห็นว่าอุณหภูมิ ความเข้มข้นของกรด และปริมาณชีวมวลจุลสาหร่ายมีผลต่อน้ำตาลที่ถูกปลดปล่อยออกมาอย่างมีนัยสำคัญ เวลาไม่มีผลอย่างมีนัยสำคัญต่อน้ำตาลที่ถูกปลดปล่อยออกมา แต่มีผลร่วมกับกับอุณหภูมิ ดังนั้นควรนำทุกปัจจัยมาใช้ในการศึกษาหาสภาวะที่เหมาะสมในการปรับสภาพชีวมวลจุลสาหร่ายด้วยวิธีไฮโดรเทอมัลในสภาวะกรดเพื่อใช้ในการผลิตเอทานอลต่อไป

Keywords: Microalgae, Acid hydrothermal, 2k factorial
คำสำคัญ: จุลสาหร่าย ไฮโดรเทอมัลสภาวะกรด เชิงแฟคทอเรียล 2 ระดับ

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Introduction

The choice of biomass feedstock depends on social, environmental, economic and industrial factors, such as availability and cost of raw materials (Chen et al., 2013). Lignocellulosic biomass is cheap and plentiful to utilize. However, the cost of converting it into ethanol or other biochemical is still relatively high. In addition, their structure contains lignin and cellulose which are recalcitrant and make them difficult to degrade into sugars for further uses (Lynd, 1996). So, it is necessary to find alternative raw materials that can supplement current materials in order to secure raw materials for ethanol production (Harun et al., 2010; Ho et al., 2012).

Microalgae have been recognized as an alternative feedstock for ethanol production. It is regarded as a third generation feedstock (Chen et al., 2013). Main composition of microalgae is carbohydrates which are accumulated in cells. It also contains low lignin which makes it less recalcitrant when compared with lignocellulosic biomass. Conversion of carbohydrates in microalgae to sugar could be done via many methods such as physical pretreatment, chemical pretreatment, thermal pretreatment and enzymatic hydrolysis (Passos et al., 2014).
Our previous studies had shown that acid pretreatment offered the highest sugar released when compare to other pretreatments, which were hot water, hydrothermal and alkali pretreatments (Ngamsirisomsakul, Boonmee, 2017).

Objective of the study

This study was focused on influence of important factors on pretreatment of a microalgae, *Chlorella* sp. Information obtained from this study will be used in designing further experiments on optimization of conditions in microalgal biomass treatment for ethanol production.

Materials and Methods

Algal strain

Chlorella sp. was obtained as dried powder from College of Power Engineering, Chongqing University, China. The biomass stock was maintained at 4 ºC.

Acid-hydrothermal pretreatment

Hydrothermal pretreatment of *Chlorella* sp. biomass was carried out in high pressure reactor (100 mL Teflon Lined Hydrothermal Synthesis Autoclave Acid Digestion, High Pressure Digestion Tank 10 MPa, TOPTION Laboratory Equipment Supplies). *Chlorella* sp. powder was prepared as suspension (80 mL) in water or sulfuric acid solution at different solid loadings according to experimental design. The reactor containing microalgae suspension was placed in an oil bath, which was preset to obtain a designed reaction temperature. Timing of the reaction started after 20 min. After hydrolysis, the reactor was removed from the oil bath and immediately cooled down on ice. The pretreated suspension was centrifuged for 5 min at 10,000 rpm to obtain clear supernatant for sugar analysis. Each experimental run was performed in duplicates.

Analytical methods

Liquid fractions from pretreatments were analysed for their sugar contents which included total sugar, reducing sugar and glucose. Total sugar was determined using phenol-sulfuric method. Reducing sugar was analyzed by DNS method (Miller, 1959). Glucose concentration was determined by glucose oxidase-peroxidase method using GLUCOSE liquicolor (ETI 1210037, Human, Germany) solution.

Experimental design

Pretreatment of microalgae was carried out according to two-level (2^k) factorial design. Four factors were investigated in this study, which included temperature, time, acid concentration and solid loading. The factors and their respective levels were illustrated in Table 1. Analysis of variances was carried out for each of the sugar responses (in mg/g). Statistical analysis was done at significance level of 0.05.

Table 1 Factors and values of levels used in 2^4 factorial design.

Independent variables	Factor	Levels and values	
		-	+
Temperature (°C)	*A*	110	150
Time (min)	*B*	20	60
H_2SO_4 concentration (% v/v)	*C*	0	1.5
Solid loading (% w/v)	*D*	5	20

Results and Discussion

From previous studies, acid pretreatment of microalgae at high temperature had resulted in highest sugar released when compared with other methods (Ngamsirisomsakul, Boonmee, 2017). The result indicated that treatment of *Chlorella* sp. with acid at high temperature could disrupt cell walls and caused release of carbohydrates (Hernández et al., 2015; Harun et al., 2011). Furthermore, acid at high temperature also hydrolysed carbohydrates to glucose resulting in significant increase in glucose concentrations when compared to other pretreatments.

In the study on factors influencing the released sugar from acid-hydrothermal pretreatment, results in Table 2 showed the experimental runs and their resulting sugars obtained after each treatment. It could be seen that at high solid loading, high total sugar was likely to occur. However, the same trend was not followed in reducing sugars and glucose. There were many runs with high solid loading that resulted in high total sugar but with low reducing sugar and glucose such as in treatment *ad*, *bd* and *abd* (Table 2). In contrast, with high level of sulfuric acid, not only it resulted in high total sugar, high reducing sugar and glucose were also followed.

ANOVA shown in Table 3 indicated that temperature, acid concentration and solid loading were the factors with significant effect on sugar released. Time was the only main factor that did not show influence in the pretreatment. This could be due to a possible short reaction time required to complete the reaction. In this study, shortest reaction time was 20 min which was equivalent to total reaction time of 40 min since the timing started after placing the reactor in the oil bath for 20 min. Reaction could be completed or near completion at the end of the actual reaction period.

Although temperature had been shown to significantly affect the release of total and reducing sugars, it did not influence the release of glucose at 0.05 significance level. This result could indicate that with the presence of acid, even the low end temperature of hydrothermal range was enough to breakdown polysaccharides or oligosaccharides released from microalgae to glucose. This explanation concurred with the results from the runs with no presence of acid i.e. treatment that contained no *c* in their treatment combination coding, where very low glucose was observed.

Since 2^k factorial design could identify low level interactions between factors, 2-factor interactions were considered in this study. Some significant interactions existed between temperature and acid concentration (*AC*) and between temperature and solid loading (*AD*) in the release of total sugars (Figure 1). However, they did not interact in the releases of reducing sugar and glucose. Interaction between temperature and time (*AB*) affected both reducing sugar and glucose releases. The *AB* interaction on released reducing sugar and glucose was very strong as indicated in Figure 2

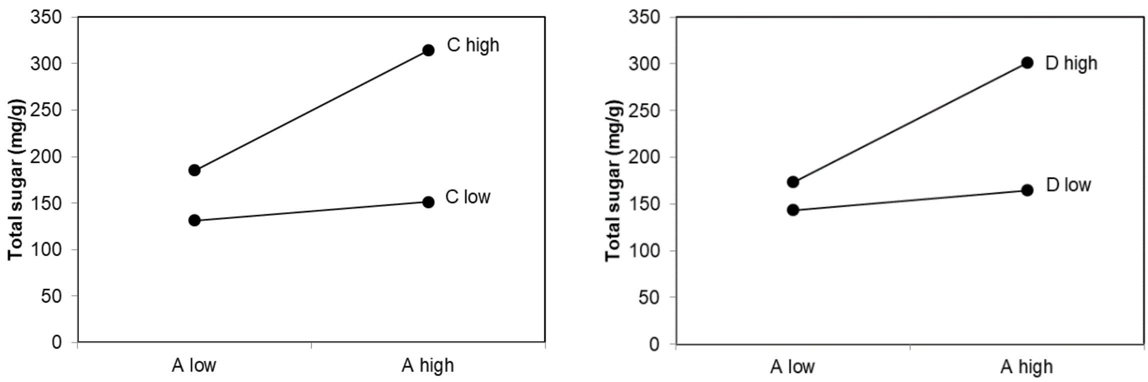
Table 2 Experimental runs based on 2^4 factorial design and their average results

Treatments	Factors and values				Resulting sugars (mg/g)		
	Temperature (*A*)	Time (*B*)	Acid concentration (*C*)	Solid loading (*D*)	Total	Reducing	Glucose
-1	-	-	-	-	114.15	13.01	0.56
a	+	-	-	-	200.31	21.22	1.01
b	-	+	-	-	129.30	13.17	0.90
ab	+	+	-	-	144.08	27.61	0.39
c	-	-	+	-	221.34	107.86	27.00
ac	+	-	+	-	295.40	233.44	189.44
bc	-	+	+	-	228.51	211.70	155.51
abc	+	+	+	-	564.02	143.31	63.42
d	-	-	-	+	123.68	7.33	0.93
ad	+	-	-	+	130.07	17.53	0.94
bd	-	+	-	+	158.80	7.70	1.73
abd	+	+	-	+	130.75	14.44	0.99
cd	-	-	+	+	157.25	80.84	10.02
acd	+	-	+	+	219.92	155.13	41.15
bcd	-	+	+	+	134.58	45.54	24.23
abcd	+	+	+	+	178.20	110.17	50.45

Table 3 ANOVA of factors and interactions influencing sugars released from *Chlorella* sp. biomass

Sources of variation	Dof	Sum of squares			Mean squares			F_0*		
		T.S.	R.S.	Glu	T.S.	R.S.	Glu	T.S.	R.S.	Glu
A = Temperature	1	44272.03	6943.95	2031.46	44272.026	6943.95	2031.46	13.329	9.611	2.558
B = Time	1	5310.53	491.74	91.92	5310.53	491.74	91.92	1.599	0.681	0.116
C = Acid concentration	1	94197.90	116637.94	38410.04	94197.89	116637.94	38410.04	28.360	161.435	48.358
D = Solid loading	1	55089.55	13830.36	11799.02	55089.54	13830.36	11799.02	16.586	19.142	14.855
AB	1	2331.62	5043.32	8560.79	2331.61	5043.32	8560.79	0.702	6.980	10.778
AC	1	23823.09	3062.57	2045.90	23823.08	3062.57	2045.90	7.172	4.239	2.576
BC	1	5868.82	619.14	77.31	5868.81	619.14	77.31	1.767	0.857	0.097
AD	1	22671.50	722.65	25.30	22671.50	722.65	25.30	6.826	1.000	0.032
ABC	1	15157.57	5324.45	8266.64	15157.57	5324.45	8266.64	4.564	7.369	10.408
ABD	1	7415.10	3811.15	7837.25	7415.09	3811.15	7837.25	2.232	5.275	9.867
ACD	1	4080.77	955.65	17.35	4080.76	955.65	17.35	1.229	1.323	0.022
BCD	1	21742.49	896.78	52.29	21742.49	896.78	52.29	6.546	1.241	0.066
ABCD	1	12597.73	4705.10	7741.07	12597.73	4705.10	7741.07	3.793	6.512	9.746
Error	16	53143.38	11560.11	12708.44	3321.46	722.51	794.28			
Total	31	367702.07	174604.92	99664.77						

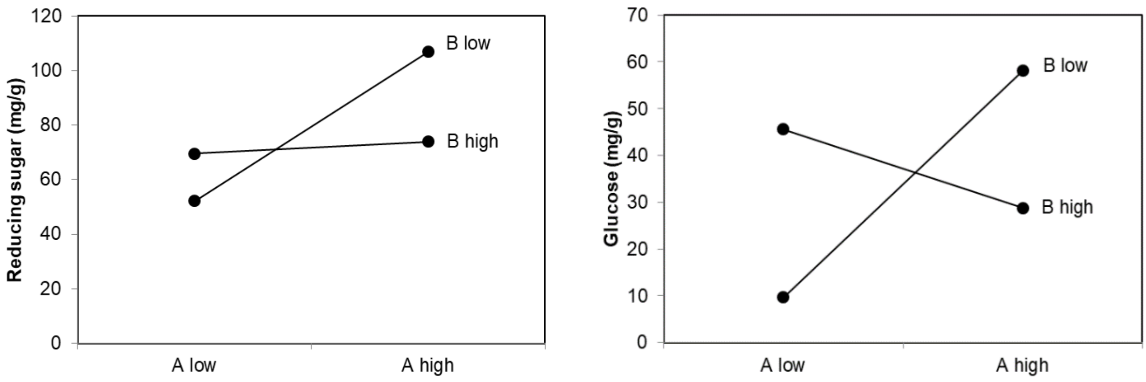
* F_0 value was compared to $F_{0.5,1,16}$ = 4.49.



(a) Temperature (*A*) and acid concentration (*C*)

(b) Temperature (*A*) and solid loading (*D*)

Figure 1 Interactions of factors on total sugar released



(a) *AB* interaction on reducing sugar released (b) *AB* interaction on glucose released

Figure 2 Interaction between temperature and reaction time (*AB*) on reducing sugar and glucose released

Conclusion

Analyses of experimental results demonstrated that factors that affected the releases of sugars from *Chlorella* sp. biomass included temperature, H_2SO_4 concentration and solid loading. Although reaction time did not show an individual effect on sugar released, its interaction with temperature was very strong on releases of reducing sugar and glucose. Therefore, all 4 factors in this study would be used in further study on optimizing acid-hydrothermal pretreatment of *Chlorella* sp. biomass.

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Reference

Chen CY, Zhao XQ, Yen HW, Ho SH, Cheng CL, Lee DJ, et al. Microalgae-based carbohydrates for biofuel production. Biochemical Engineering Journal 2013; 78: 1-10.

Harun R, Danquah MK, Forde GM. Microalgal biomass as a fermentation feedstock for bioethanol production. Journal of Chemical Technology and Biotechnology 2010; 85: 199-203.

Harun R, Jason WSY, Cherrington T, Danquah MK. Exploring alkaline pre-treatment of microalgal biomass for bioethanol production. Applied Energy 2011; 88: 3464-3467.

Hernández D, Riaño B, Coca M, García-González MC. Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. Chemical Engineering Journal 2015; 262: 939-945.

Ho SH, Chen CY, Chang JS. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresource Technology 2012; 113: 244-252.

Lynd LR. Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy. Annual Review of Energy and the Environment 1996; 21: 403–465.

Miller GL. Use of Dinitrosalycylic acid reagent for determination ofreducing sugar. Analytical Chemistry 1959; 31: 426–428.

Ngamsirisomsakul M, Boonmee M. Preliminary assessment of hot water and chemical methods on *Chlorella* sp. biomass pretreatment. The 7th International Conference on Fermentation Technology for Value Added Agricultural Products; 2017 July 25-28; Pullman Khon Kaen Raja Orchid, Khon Kaen, Thailand. Thailand: Khon Kaen University; 2017. p. 204-209.

Passos F, Uggetti E, Carrère H, Ferrer I. Pretreatment of microalgae to improve biogas production: A review. Bioresource Technology 2014; 172: 403–412.