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Analysis of a Lignocellulose Degrading Microbial Consortium to Enhance the Anaerobic Digestion of Rice Straws

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***Abstract*—**Rice straw is lignocellulosic biomass which can be utilized as substrate for the biogas production. However, due to the property and composition of rice straw, it is difficult to be degraded by hydrolysis enzymes. One of the pretreatment methods that modify such properties of lignocellulosic biomass is the application of lignocellulose-degrading microbial consortia. The aim of this study is to investigate the effect of microbial consortia to enhance biogas production. To select the high efficient consortium, cellulase enzymes were extracted and their activities were analyzed. The results suggested that microbial consortium culture obtained from cattle manure is the best candidate compared to decomposed wood and horse manure. A microbial consortium isolated from cattle manure was then mixed with anaerobic sludge and used as inoculum for biogas production. The optimal conditions for biogas production were investigated using response surface methodology (RSM). The tested parameters were the ratio of amount of microbial consortium isolated and amount of anaerobic sludge (MI:AS), substrate to inoculum ratio (S:I) and temperature. Here, the value of the regression coefficient R^2 = 0.7661 could be explained by the model which is high to advocate the significance of the model. The highest cumulative biogas yield was 104.6 ml/g-rice straw at optimum ratio of MI:AS, ratio of S:I, and temperature of 2.5:1, 15:1 and 44°C respectively.

***Keywords*—**Lignocellulolytic biomass, microbial consortium, cellulase, biogas, Response Surface Methodology.

I. Introduction

ANAEROBIC digestion (AD) of organic materials including agricultural residues is a process under continuous development because its capacity to degrade organic matter simultaneously into valuable biogas and into a nutrient-rich digestate with agronomic qualities. Lignocellulosic biomass obtained from agricultural residues is an important renewable resource for biofuel production to replace petro-chemical and fossil fuel [1]. Rice straw is one of the most abundance lignocellulosic biomass. In 2008, about 620 million tons of rice straw residues were produced in Asia [2]. And in Thailand, in 2013 about 29.15 million metric tons of rice straws were left in the fields, disposed off and burned in various ways [3].

Lignocellulosic biomass mainly consists of three different types of biopolymers, celluloses, hemicelluloses, and lignin [4]. In nature, lignocellulosic biomass is hydrolysed to sugars by lignocellulolytic enzyme complexes, composed of cellulolytic and hemicellulolytic enzymes, produced by a variety of microorganisms, including bacteria and fungi under aerobic and anaerobic conditions [5]. The sugars derived from lignocellulosic biomass are promptly converted to biogas or other products by microbial fermentation. However, the process of hydrolysis of lignocelluloses has been addressed as the rate-limiting step in biogas production because the components and physical properties, for examples high crystalline cellulosic structure, are barricades for the reaction [6], [7]. A solution to this problem is a pretreatment prior to the biogas production.

In general, pretreatment methods could be mainly categorized to be physical, chemical, and biological methods. Biological pretreatment offers some important advantages, such as low capital cost, and low energy use. Recently, microbial co-cultures or consortia have been demonstrated as an effective approach for lignocellulosic biomass pretreatment because it avoids the problems of feedback regulation and metabolite repression found in isolated single microbial strains [8]-[10]. Studies on the use of thermophilic cellulose degrading microbial consortium, MC1, to pretreat waste paper and cardboard showed that the methane yields were increased significantly [11]. Similarly, microbial consortia obtained from various sources have different enzyme efficiency and stability, and improve biogas production from lignocellulosic biomass differently [12].

In this study, three different lignocellulolytic microbial consortia was collected from natural environment in Thailand and maintained in media containing rice straw as sole carbon source. The lignocellulolytic activities of these microbial consortia were observed in different conditions. The highest efficient consortium was selected and then applied in biogas production experiments using response surface methodology (RSM). The process parameters on biogas production were analyzed to demonstrate the correlation with the lignocellulolytic enzyme activities, which could be applied to other biorefinery processes in the future.

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II. Material and Method

A. Culturing of Microbial Consortium

The microbial consortia in this study were obtained from samples collected from three different sources in Thailand. Cattle manure and horse manure samples were collected from local farm in Nakhonrachsrima province, located in Eastern part of Thailand. Decomposed wood sample and rice straw were collected from local farm in Ayuthdhaya province, located in Central part of Thailand. One gram of cattle manure, horse manure, and decomposed wood were separately inoculated in 50 ml of basal medium (containing 0.2% (w/v) rice straw as the carbon source, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.1% KCl, 0.05% $MgSO_4$, 0.05% yeast extract). Each culture was incubated at 40°C under static aerobic conditions. After 3 d, 1 ml of the culture was then transferred into fresh medium. The procedure was repeated for 10 times to obtain a stable microbial community capable of degrading rice straw as described in other studies [5], [13], [14]. The microbial consortia were aliquoted and stored in -40°C until use.

B. Preparation of Cellulase Enzyme from Microbial Consortium

100 µl of each microbial consortia from frozen stocks were inoculated in 10 ml basal medium containing 0.2% (w/v) of the carbon source (rice straw or carboxymethylcellulose (CMC)). The mixture was incubated at 40°C for 2 day in shaker incubator (150 rev/min) and kept as a starter culture. Then 1 L of fresh basal media was inoculated with 10% v/v of starting culture, and again the culture was incubated at 40°C for 2 day in shaker incubator (150 rev/min). Each of the cultures was centrifuged at 6,000 rpm for 15 min. The supernatants were collected and defined as the enzyme fraction 1 (F1). According to this procedure, F1 samples of cattle manure, decomposed wood and horse manure were designated as F1C, F1W and F1H, respectively

The proteins in the F1 were precipitated by the addition of ammonium sulphate to 90% saturation. The precipitate was allowed to form at 4°C for 24 h and pellet was collected by centrifugation at 6,000 rpm for 20 min at 4°C. The pellets were redissolved in 30 mM sodium phosphate buffer, pH 7 and was transferred into dialysis membrane with 10 kDa MWCO (Float-A-Lyzer, Spectrum Lab. USA) and dialysed at 4°C with 3 changes of buffer. The dialyzed protein samples were concentrated by using Vivaspin-500 column (GE Healthcare Life Science, USA) with 10 kDa MWCO. The concentrated protein samples were then called as enzyme fraction 2 (F2). Likewise, F2 samples of cattle manure, decomposed wood and horse manure were designated as F2C, F2W and F2H, respectively. Both F1 and F2 samples were aliquoted and stored in -20 °C until use for cellulase activity assay.

C. Enzyme Activity Assay

To determine the cellulase activities of F1, three parameters were studied including types of substrates (carboxymethyl cellulose (CMC) and rice straw), temperature (30°C, 37°C and 44°C), and pH (5, 6, 7 and 8). For each condition, 4 ml of F1 was mixed with 5% (w/v) rice straw substrate and incubated at different temperature and pH for 4 h. Cellulase activity were analyzed based on the amount of released reducing sugar using standard 3,5-dinitrosalisylic acid (DNS) method [15]. To test F2 enzyme activity, the mixture of F2 and rice straw (5% w/v) in 50 mM citrate buffer was incubated at different temperature (30°C, 37°C and 44°C) and different pH (5, 6, 7 and 8) for 4 h. One unit of enzyme was defined as the amount of enzyme which produced 1 µmol of equivalent glucose in 1 min. The protein concentration of F1 and F2 was determined by using Biorad protein assay (Bio-Rad Laboratories, USA) using bovine serum albumin (BSA) as standard. The specific enzyme activity was defined as the unit of enzyme activity per amount of protein.

D. Experimental Design for Biogas Production and Set Up

The total enzyme activity indicated that the microbial consortium obtained from cattle manure has highest cellulase activity. Therefore, the microbial consortium obtained from cattle manure was used to set up anaerobic digestion experiment. The anaerobic sludge used as inoculum was obtained from an anaerobic digester plant in local municipal wastewater treatment plant in Bangkok, Thailand. Batch digestion experiments for biogas production were performed in 100 ml serum bottles with a 60 ml working volume. The serum bottle was added with 3 g of substrate mixture and initial pH 7.0. The gas production was measured every day for 20 day and total biogas amount was called as cumulative biogas yield. The amount of biogas produced was recorded using water displacement method.

Optimization of condition for biogas production (Y) was carried out using Response Surface Methodology (RSM) with Box-Behnken design. Three of independent variables were studied here including 1) the ratio of amount of microbial consortium isolated and amount of anaerobic sludge (MI:AS) (X_1), 2) substrate to inoculum ratio (S:I) (X_2) and 3) temperature (X_3). For each variables, three coded levels (high = +1, mid = 0, low = -1) was selected for the optimization (Table I), with a total of 17 runs. The ranges and levels of independent input variables are shown in Table II. Experimental data were analyzed using the statistical software, Design-Expert software (version 7.0.0, STAT-EASE Inc., USA), to fit the second-order polynomial regression model:

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ii} X_i^2+\Sigma\beta_{ij} X_i X_j \quad (1)$$

where Y is the response variable (biogas production), X_i, X_j are the independent variables, β_0 is a constant, β_i is the linear coefficients, β_{ii} is the squared coefficients, and β_{ij} is the is the interaction coefficient. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination (R^2).

III. Results

A. Analysis of Cellulase Enzyme Activity Produced by Microbial Consortia

In this study, two fractions of cellulase enzyme produced

from three microbial consortia were analyzed. First crude cellulase fraction (F1) was collected from extracellular portion of culture, and second cellulase fraction was partially purified and concentrated as described in Methods and Materials.

To select the microbial consortium that has highest cellulase activity to attenuate the hydrolysis of rice straw substrate, rice straw and CMC were used as carbon sources in culturing media. The results of total cellulase activities of F1 isolated from three microbial consortia showed that rice straw was the better substrate compared to CMC to induce the total cellulase activities (Table III). The maximum total cellulase activities of F1C, F1W, and F1H produced in CMC carbon source were 3.35, 0.93, and 1.95 U/L respectively, while in rice straw carbon source are 6.60, 6.51, and 5.63 U/L respectively. Additional, from this experiment, it was demonstrated that cellulase F1 produced from microbial consortium obtained from cattle manure has the highest cellulase activity compared to horse manure, and decomposed wood.

TABLE I
CODED FACTORS AND ACTUAL VALUE OF INDEPENDENT VARIABLES

Factor	Name	Range of variables		
		Low (-1)	Mid (0)	High (+1)
X_1	MI:AS	1:5	1:1	5:1
X_2	S:I	5:1	10:1	15:1
X_3	Temperature(°C)	30	37	44

TABLE II
EXPERIMENTAL DESIGN TO TEST THE EFFECT OF INDEPENDENT VARIABLES ON CUMULATIVE BIOGAS YIELD (Y)

Run	X_1	X_2	X_3	Y (ml)
1	0	-1	-1	67.50
2	1	1	0	42.50
3	0	1	-1	75.00
4	0	1	1	442.50
5	-1	-1	0	50.00
6	0	0	0	25.00
7	0	-1	1	97.50
8	1	0	-1	30.00
9	0	0	0	55.00
10	0	0	0	45.00
11	-1	1	0	55.00
12	-1	0	1	137.50
13	-1	0	-1	52.50
14	0	0	0	40.00
15	1	-1	0	45.00
16	1	0	1	135.00
17	0	0	0	67.50

Therefore, we further tested the cellulase activity from the cellulase fraction 2 (F2) isolated from three different microbial consortia that were cultured in media containing rice straw as carbon source. Again, the cellulase F2 of cattle manure samples has the highest cellulase activity (309.8 U/L or specific activity 259 U/g-protein) compared to horse manure (275.1 U/L) and decomposed wood (227.4 U/L).

Next, the effect of temperature and pH on the cellulase activities of F2 obtained from cattle manure sample were observed. Cellulase F2 from cattle manure sample was aliquoted and mixed with 5% w/v rice straw substrate in citrate buffer that adjusted pH to 5, 6, 7, and 8. Each reaction mixtures were incubated in different temperature (30°C, 37°C and 44°C). Then the released reducing sugars were measured and analyzed to indicate cellulase activities (Fig. 1).

Based on the total enzyme activity, it showed that the temperature at 44 °C is the most suitable temperature for F2 compared to lower temperatures, which is reasonable to the nature of cellulolytic enzymes that naturally prefer to function at high temperature [16], [17]. Furthermore, the pH also has effect on the cellulase activity. Clearly, pH 7.0 is the optimal pH for F2 sample obtained from cattle manure. Altogether, we decided to use microbial consortium obtained from cattle manure sample to promote the biogas product in the next experiment.

TABLE III
ANALYSIS OF ENZYME ACTIVITIES OF CELLULASE EXTRACTED FROM MICROBIAL CONSORTIA

fraction	substrate	inoculum	Total enzyme activity (U/L)	condition	
				pH	temperature (°C)
1	CMC	Cattle	3.35	8	37
		Wood	0.93	5	30
		Horse	1.95	7	44
	Rice Straw	Cattle	6.60	8	44
		Wood	6.51	8	44
		Horse	5.63	7	30
2	Rice Straw	Cattle	309.81	7	44
		Wood	227.41	8	37
		Horse	275.13	5	44

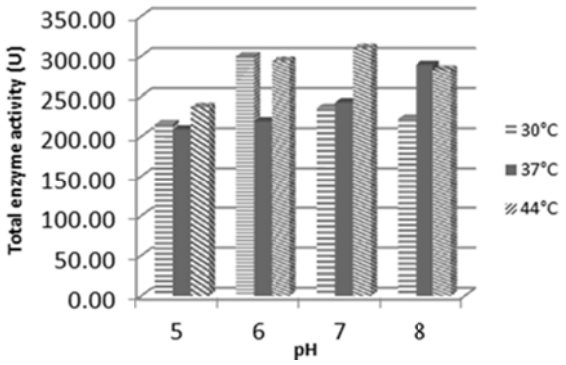


Fig. 1 Total cellulase activity of fraction 2 extracted from microbial consortium isolated from cattle manure using rice straw as substrate

B. Optimization of Biogas Production

Although we know that the optimal condition for cellulase activity of microbial consortium obtained from cattle manure is 44°C and pH 7.0. However, our goal is to apply this microbial consortium to enhance biogas production. Therefore, it is necessary to find the optimal condition to apply microbial consortia, and operation condition for biogas production. The effects of three independent variables i.e., the ratio of amount of microbial consortium and amount of anaerobic sludge (X_1), substrate to inoculum ratio (X_2) and temperature (X_3) on cumulative biogas production (Y) were explored using the Box-Behnken design of RSM. The statistic

software package Design-Expert software version 7.0.0 was used for regression analysis of experimental data and to plot response surface. One-way analysis of variance (ANOVA) was used to estimate the statistical parameters. The responses of Box-Behnken design were well fitted with the second order polynomial equation:

$$Y = 2766.24575 - 80.32143X_2 - 138.15547X_3 + 2.41071X_2X_3 + 1.68296X_3^2 \quad (2)$$

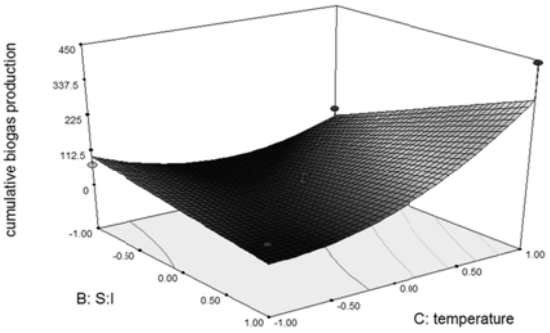
Using Design-Expert software, the statistical significance of the second order polynomial equation was evaluated by an F-test (ANOVA) (Table IV). The F-value of the model is 9.82, which implies the model is significant. There is only a 0.09% chance that a "Model F-Value" this large could occur due to noise. In addition, the ANOVA of the quadratic regression model demonstrated that the model was highly significant ($p<0.05$). The linear model terms of substrate to inoculum ratio (X_2), temperature (X_3), multiple model terms of the substrate to inoculum ratio and temperature (X_2X_3) and the quadratic model terms of the temperature (X_3) were significant ($p<0.05$), indicating that these variables had significant effect on cumulative biogas production. The coefficient of determination (R^2) of the model was 0.7661, which indicated that the model was suitable for representing the relationship among the selected variables and advocated a high significant of the model.

TABLE IV
ANOVA ANALYSIS OF THE DESIGN

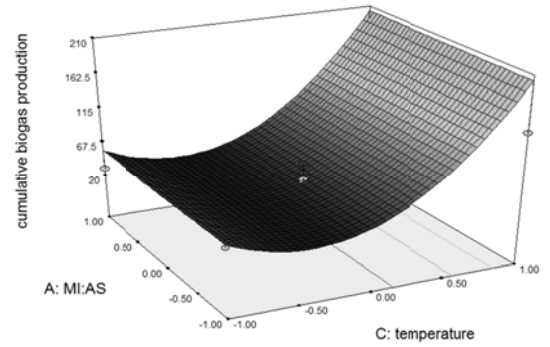
Source	Mean square	F-value	Prob>F
Model	2.90×10^4	9.82	0.0009
X_1	1.58×10^4	5.33	0.0396
X_3	4.31×10^4	14.59	0.0024
X_1X_2	2.85×10^4	9.63	0.0091
X_3^2	2.88×10^4	9.74	0.0088

Graphical representation of the 3D response surface plot between two variables at a time is helpful in understanding both the main and the interaction effects of these variables (Fig. 2). At lower temperature, it was found that cumulative biogas production increased when substrate to inoculum ratio increased, while, at higher carbon content, it decreased when the level of substrate to inoculum ratio increased (Fig. 2 (a)). Fig. 2 (b) show the effect of temperature, it was found that cumulative biogas production increased when the temperature was either high or low, but maximum cumulative biogas production was obtained at highest temperature (44°C).

The optimal condition for maximizing the cumulative biogas production calculated by using (2) were the ratio of amount of microbial consortium and amount of anaerobic sludge of 2.5:1 (X_1), substrate to inoculum ratio of 15:1 (X_2) and temperature (X_3) of 44°C. The predicted maximum cumulative biogas production under the optimum condition was 313.875 ml or 104.6 ml/g-rice straw. The amount of biogas predicted from the model in this study supported the significance of microbial consortium activity because the yield is improved, and has higher biogas production compared to other studies that used different pretreatment methods [18], [19].



(a)



(b)

Fig. 2 Response surface plots showed the effect of interaction between the independent variables to the cumulative biogas production. (a) Interaction between substrate to inoculum ratio(S:I) (X_2) and temperature (X_3); (b) interaction between ratio of amount of microbial consortium isolated and amount of anaerobic sludge(MI:AS) (X_1) and temperature (X_3)

IV. CONCLUSION

In this study, the results of enzyme activity assay of microbial consortium isolated from cattle manure has the highest efficient for production of cellulase when using rice straw as substrate. The microbial consortium from cattle manure was used as inoculum in biogas production. This experiment based on RSM focused on the optimization of three parameters including the ratio of amount of microbial consortium isolated and amount of anaerobic sludge (MI:AS), substrate to inoculum ratio (S:I) and temperature. The model of operational condition obtained from this study remarks the correlation and application of cellulolytic microbial consortium to biogas production. Further improvement of using microbial consortium could be the study of dynamic of microbial populations during biogas fermentation to better understand the interactions and reactions inside the reactor and enhance the efficiency of biogas production ultimately.

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REFERENCES

[1] L. A. Lucia, "Lignocellulosic biomass: Replace petroleum," Raleigh, N.C., 2008, pp.981-982.
[2] Data Retrieved from the link: www.irri.org
[3] Data Retrieved from the link: www.dede.go.th
[4] P. S. Nigam, N. Gupta, and A. Anthwal, "Pre-treatment of agro-industrial residues," in *Biotechnology for agro-industrial residues utilization*, 1st ed. Netherlands: Springer, 2009, pp. 13-33.
[5] S. Wongwilaiwalina, U. Rattanachomsria, T. Laothanachareona, L. Eurwilaichitra, Y. Igarashib, and V. Champredaa, "Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system," Enzyme and Microb Tech, vol. 47, 2010, pp. 283–290.
[6] V.A. Vavilina, B. Fernandezb, J. Palatsib, and X. Flotatsb, "Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview," Waste Manage, vol. 28, 2008, pp. 939-951.
[7] N. Mosiera, C. Wymanb, B.Dalec, R. Elanderd, Y.Y. Leee, M. Holtzapplef, and M. Ladischa, "Features of promising technologies for pretreatment of lignocellulosic biomass," Bioresour Technol, vol. 96, 2005, pp. 673–686.
[8] S. Soundar, and T. S. Chandra, "Cellulose degradation by a mixed bacterial culture," J Ind Microbiol, Vol. 2, 1987, pp. 257–265.
[9] M. de la Torre, and C. C. Campillo, "Isolation and characterization of a symbiotic cellulolytic mixed bacterial culture," Appl Microbiol Biotechnol, vol. 19, 1984, pp. 430–434.
[10] S. Haruta, Z. Cui, Z. Huang, M. Li, M. Ishii, and Y. Igarashi, "Construction of a stable microbial community with high cellulose-degradation ability," Appl Microbiol Biotechnol, vol. 59, 2002, pp. 529–534.
[11] X. Yuan, Y. Cao, J. Li, B. Wen, W. Zhu, X. Wang, and Z. Cui, "Effect of pretreatment by a microbial consortium on methane production of waste paper and cardboard," Bioresour Technol, vol. 118, 2012, pp. 281–288.
[12] Y. Gu, X. Chen,mj Z. Liu, X. Zhou, and Y. Zhang, "Effect of inoculum sources on the anaerobic digestion of rice straw," Bioresour Technol, vol. 158, 2014, pp. 149–155.
[13] Q. Zhang, J. He, M. Tian, Z. Maoa, L. Tang, J. Zhang, and H. Zhang, "Enhancement of methane production from cassava residues by biological pretreatment using a constructed microbial consortium," Bioresour Technol, vol. 102, 2011, pp. 8899-8906.
[14] B. Wen, X. Yuan, Y. Cao, Y. Liu, X. Wang, and Z. Cui, "Optimization of liquid fermentation of microbial consortium WSD-5 followed by saccharification and acidification of wheat straw," Bioresour Technol, vol. 118, 2012, pp. 141-149.
[15] G. L. Miller, "Use of dinitosalicylic acid reagent for determination of reducing sugas," Anal Chem, vol. 47, 1959, pp. 426-428.
[16] V. Shahhrmma, H. Vij, P. K. Singh, and S. Bhatt, "Potential Cellulase Production, Optimization and Saccharification Study by Novel Thermophilic Microbes," Sustain Biotechnol, vol. 1, 2013, pp.19-26.
[17] H.Y. Yang, H. Wu, X. Wang, Z. Cui, and Y. H. Li, "Selection and characteristic of a switch grass-colonizing microbial community to produce extracellular cellulose and xylanases," Bioresour Technol, vol. 102, 2011, pp. 3546-3550.
[18] R. Chandra, H. Takeuchi, and T. Hasegawa, "Hydrothermal pretreatment of rice straw biomass: A potential and promising method for enhanced methane production" Appli Energ, vol. 94, 2012, pp. 129-140
[19] U. G. Phutela, and N. Sahni, "Effect of *Fusarium sp.* on paddy straw digestibility and biogas production", J Adv Lab Res in Biol, vol. 3, 2012 pp. 9-12.