Effect of organic acid pretreatment of water hyacinth on enzymatic hydrolysis and biogas and bioethanol production

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Abstract. Due to rapid growth and uncontrolled spreading of water hyacinth in aquatic natural and urban niches, the conversion of this weed to value-added chemicals and biofuels leads to the reduction of pollution and greenhouse effect. The rate-limiting step of the conversion process is hydrolysis reaction of lignocellulosic biomass to sugars, which are subsequently converted to biochemicals and biofuels. To improve the enzymatic hydrolysis efficiency of water hyacinth, the organic acid pretreatments were conducted in this study by using two types of organic acids, including oxalic acid and citric acid. The pretreatment parameters, including pretreatment time, pretreatment temperature, and acid concentration were optimized by using Response Surface Methodology (RSM) based on Box-Behnken design. The highest reducing sugar concentration at 66.92 mg/100 mg-biomass was obtained when using oxalic acid pretreatment. The effects of organic acids on biogas and bioethanol production were observed compared to unpretreated water hyacinth. The biogas and bioethanol yields were increased for 75.61% and 23.26% when using citric acid and oxalic acid pretreatment, respectively. The improvement of biofuel production from water hyacinth by organic acid pretreatment suggested the possibility to applications and solutions in biorefinery industry and environmental situation.

1. Introduction
Lignocellulosic material is the most abundant biomass available worldwide due to its function in plant cell wall. Currently, it is targeted to be used as potential raw materials to produce valuable biochemicals and biofuels. Due to the awareness of greenhouse effect and environmental problem, renewable energy production has been promoted by governmental and private sectors. The main sources for lignocellulose biomass for renewable energy production are agricultural residues and dedicated energy crops. Additionally, aquatic weeds are included in the list for biofuel production because, in addition to biofuel production, the uses of aquatic weeds could reduce environmental problem as well [1-2].

Water hyacinth (Eichhornia crassipes) is one type of rapid-growing aquatic weed found in tropical areas, especially in South-east Asia. In Thailand, it was firstly introduced as an alien species, and thus it lacks of natural competitors making it uncontrolled spreaded in canals and rivers. Although water hyacinth growth causes environmental problems, in the bright side, it has high potential to convert to biogas due to its high total volatile solid for 80-86% of dried biomass. For 400 m²-sized growing area
of water hyacinth, it could be converted to biomethane for 4,200 m³/yr, equivalent to 2,100 kg of LPG/yr [3]. In fact, the yields of biofuels from water hyacinth could be further increased by biomass pretreatment with various means, including chemical, physical and biological methods [4-8].

The production process of renewable energy from lignocellulosic biomass is composed of four steps as follows; namely, pretreatment, hydrolysis, fermentation and separation [1]. The bottlenecks of the whole process in terms of the operational cost are pretreatment and hydrolysis (~40-60% of total cost of the process) [8]. To enhance hydrolysis efficiency, the recalcitrant structures of cellulose fibrils should be modified and the inhibitors of hydrolysis and fermentation should be removed by an appropriated pretreatment method [1-2]. In general, diluted strong inorganic acid or alkaline, i.e. sulfuric acid and sodium hydroxide, are applied in pretreatment. Unlike organic acid pretreatment, those harsh inorganic acid pretreatment produces inhibitory by-products, i.e. 5-hydroxymethylfurfural (HMF) and furfural, to prevent enzymatic hydrolysis, fermentation as well as the growth of microorganism used in downstream process [9-12].

To reduce the downfall of strong inorganic acid pretreatment, organic acid pretreatment has been developed for various types of lignocellulosic [10-11]. In this study, the organic acid pretreatment on water hyacinth was optimized based on Response Surface Methodology (RSM) with Box-Behnken design by estimation of the level of pretreatment parameters to obtain the maximum sugar yields. In addition, the subsequent effects of the optimal pretreatment on biogas and bioethanol production were assessed to get the better knowledge on selection of an appropriate pretreatment method for water hyacinth.

2. Materials and methods

2.1. Materials and chemicals
Water hyacinth sample was collected from urban canal located in Nonthaburi province, central part of Thailand. Fresh water hyacinth was cut to approximately 1-cm-long pieces and dried in hot air oven at 60 °C for 24 h. Dried sample was milled, sieved through 20 mesh and stored in a sealed plastic bag at room temperature. CelluClast 1.5L, (produced by Trichoderma reesei ATCC 26921) and β-glucosidase (produced by Aspergillus niger) were purchased from Sigma-Aldrich (MO, USA) and Megazyme (Wicklow, Ireland), respectively. 3,5-dinitrosalicylic acid was purchased from Sigma-Aldrich (MO, USA). Other chemicals used in this study were supplied by Ajax Finechem (New South Wales, Australia).

2.2. Pretreatment and hydrolysis
The optimal pretreatment condition of water hyacinth was obtained by using Response Surface Method (RSM) with Box-Behnken design as described in previous studies [13-16]. A three levels, three factors of pretreatment parameters, including pretreatment time (X1: 30 – 90 min), pretreatment temperature (X2: 100 – 140 °C) and acid concentration (X3: 0.5 – 2% for inorganic acid and 2 – 12% for organic acid), were assigned as independent variables, where reducing sugar yield (Y) were dependent variables (Table 1). A total 17 experimental runs were designed by using Design-Expert software version 7.0.0 (Stat-Ease, Inc., MN, USA)). The regression analysis of RSM and estimation of the model coefficient of determination (R²) were conducted to assess the reliability of generated model. The second order polynomial regression model was calculated to maximize the dependent response by optimizing the three pretreatment parameters.

To pretreat biomass, dried samples were mixed with different acids, including oxalic acid, citric acid and hydrochloric acid (representing strong inorganic acid) in a glass screw-capped bottle containing different acid concentration (Table 1). Each pretreatment was carried out in a hot air oven using temperature and time conditions as designed in Table 1. After rapid cooling the pretreated sample to room temperature, solid fraction was separated by using a funnel and filter paper. The pretreated samples were washed with deionized water until neutral pH, then dried in hot-air oven at 60°C. The dried samples were subjected for downstream process and analysis. The pretreated samples were hydrolyzed by
addition of a cellulase cocktail containing 35 μl of Celluclast® 1.5L (Sigma-aldrich, USA) and 10 μl of β-glucosidase (Megazyme, USA) per 100 mg of biomass. Each hydrolysis reaction was prepared in 20 mL of 50 mM sodium citrate buffer (pH 4.7) containing 200 μl of 2 M sodium azide with 2.5% loading ratio of biomass in a screw-capped plastic tube [9-10]. The mixture was incubated in a shaking incubator at 45 °C, 200 rpm for 72 h. The concentrations of reducing sugars were measured using the 3,5-dinitrosalicylic acid (DNS) assay and glucose was used for calculation in the standard curve [17].

Table 1. Box-behnken experimental design of RSM for water hyacinth pretreatment with hydrochloric acid, oxalic acid and citric acid.

| Run | X₁ (time, min) | X₂ (temperature, °C) | X₃ (acid concentration, %w/v) | Y (reducing sugar, mg/ml) |
|-----|----------------|----------------------|-------------------------------|--------------------------|
| 1   | 60             | 100                  | 0.5                          | 9.37, 12.19, 2.60        |
| 2   | 30             | 100                  | 1.25                         | 11.52, 8.32, 1.55        |
| 3   | 90             | 100                  | 1.25                         | 15.92, 15.76, 3.74       |
| 4   | 60             | 120                  | 2                             | 13.66, 14.54, 3.12       |
| 5   | 30             | 120                  | 0.5                          | 1.71, 14.17, 3.04        |
| 6   | 90             | 120                  | 0.5                          | 2.20, 16.02, 3.66        |
| 7   | 60             | 120                  | 1.25                         | 13.74, 16.44, 3.86       |
| 8   | 60             | 120                  | 1.25                         | 11.87, 16.51, 4.00       |
| 9   | 60             | 120                  | 1.25                         | 11.00, 15.75, 3.74       |
| 10  | 60             | 120                  | 1.25                         | 15.54, 15.71, 3.82       |
| 11  | 60             | 120                  | 1.25                         | 14.60, 15.32, 3.82       |
| 12  | 30             | 120                  | 2                             | 11.22, 15.35, 3.60       |
| 13  | 90             | 120                  | 2                             | 15.39, 16.73, 4.03       |
| 14  | 60             | 140                  | 0.5                          | 7.92, 12.89, 2.70        |
| 15  | 30             | 140                  | 1.25                         | 11.70, 13.90, 2.90       |
| 16  | 90             | 140                  | 1.25                         | 6.20, 14.11, 3.02        |
| 17  | 60             | 140                  | 2                             | 12.60, 15.24, 3.82       |

2.3. Fermentation and composition analysis
To test the potential of pretreated water hyacinth in ethanol production, the hydrolysate were prepared without addition of sodium azide. One milliliter of yeast inoculum (Saccharomyces cerevisiae TISTR 5606, provided by Thailand Institute of Scientific and Technological Research (TISTR)) was added into 19 ml of culture media (containing 1% (w/v) glucose, 1% (w/v) yeast extract, and hydrolysate up to 19 ml, pH 5.0) [18]. The fermentation was set up in a shaking incubator at 32 °C, 100 rpm for 48 h. The culture supernatant was harvested by centrifugation at 8,000 rpm for 10 min. The ethanol concentration was analyzed using gas chromatography with FID detector (GC-2010, Shimadzu, Japan) equipped with DB-wax column (30 m x 0.25 mm, 0.25 μm). The oven temperature was operated at 40 °C for 4 min, ramping to 100 °C with 5 °C/min and ramping to 200 °C with 10 °C/min [18-19].

To test biogas production of pretreated water hyacinth, a set of batch anaerobic digester was set up. The fiber compositions of water hyacinth, including cellulose, hemicellulose and lignin were analyzed as described by Van Soest and Wine, 1967 [20]. The total solids (TS) and volatile solids (VS) were quantified according to the standard water and wastewater examination methods [21]. The seed inoculum was obtained from cattle manure (with %TS and %VS at 9.11% and 8.02%, respectively). After starving the seed inoculum for 48 h by incubating at 35 °C with 100 rpm agitation, each digester was set up with 200 ml of working volume containing 5% (TS) of seed inoculum, 5% of water hyacinth, 10 ml of Nutrient broth, and 15 ml of bicarbonate buffer (pH 6.0). Each digester was adjusted to pH 7.0, purged with nitrogen gas before start up, then put in waterbath at 38 °C. The production of biogas of each digester was recorded with 45-day period by using water displacement method.

3. Results and discussion
The optimal pretreatment conditions were obtained by using RSM with Box-behnken design by 
variation of the level of each pretreatment parameter, including pretreatment time (X1), pretreatment 
temperature (X2) and acid concentration (X3). In this study, hydrochloric acid was selected as a strong 
inorganic acid, whereas oxalic acid and citric acid were chosen for organic acid pretreatment. The 
experimental values of each test parameters were designed to 17 runs, and the released reducing sugars 
from enzymatic hydrolysis were measured by DNS assay and filled in the Table 1.

Table 2. ANOVA of the predicted model representing the influence of pretreatment parameters on 
reducing sugar concentrations of pretreated water hyacinth.

| Source                | Sum of Squares | df | Mean Square | F Value | p-value | Prob > F |
|-----------------------|----------------|----|-------------|---------|---------|----------|
| **Hydrochloric acid pretreatment** |                |     |             |         |         |          |
| Model                 | 241.43         | 6  | 40.24       | 7.57    | 0.0029  |          |
| A-Time                | 1.58           | 1  | 1.58        | 0.30    | 0.5977  |          |
| B-Temp               | 18.10          | 1  | 18.10       | 3.41    | 0.0947  |          |
| C-Conc               | 125.33         | 1  | 125.33      | 23.58   | 0.0007  |          |
| AB                   | 24.52          | 1  | 24.52       | 4.61    | 0.0573  |          |
| A^2                  | 28.64          | 1  | 28.64       | 5.93    | 0.0427  |          |
| C^2                  | 39.31          | 1  | 39.31       | 7.40    | 0.0216  |          |
| Residual             | 53.15          | 10 | 5.31        |         |         |          |
| Lack of Fit          | 38.96          | 6  | 6.49        | 1.83    | 0.2907  |          |
| Pure Error           | 14.19          | 4  | 3.55        |         |         |          |
| Cor Total            | 294.58         | 16 |             |         |         |          |
| Predicted model: Y = &
|                     | 33.44876 +0.85720*Time +0.17236*Temp +18.83884*Conc –4.12622*10^-3*Time*Temp – 2.89376*10^-3*Time^2 – 5.42455*Conc^2 | |

**Oxalic acid pretreatment**

| Source                | Sum of Squares | df | Mean Square | F Value | p-value | Prob > F |
|-----------------------|----------------|----|-------------|---------|---------|----------|
| Model                 | 61.28          | 5  | 12.26       | 20.36   | < 0.0001|          |
| A-Time                | 14.75          | 1  | 14.75       | 24.50   | 0.0004  |          |
| B-Temp               | 3.55           | 1  | 3.55        | 5.90    | 0.0334  |          |
| C-Conc               | 5.42           | 1  | 5.42        | 9.01    | 0.0121  |          |
| AB                   | 13.5           | 1  | 13.05       | 21.68   | 0.0007  |          |
| A^2                  | 24.51          | 1  | 24.51       | 40.72   | < 0.0001|          |
| Residual             | 6.62           | 11 | 0.60        |         |         |          |
| Lack of Fit          | 10.6556        | 7  | 0.79        | 3.00    | 0.1527  |          |
| Pure Error           | 1.06           | 4  | 0.26        |         |         |          |
| Cor Total            | 67.90          | 16 |             |         |         |          |
| Predicted model: Y = &
|                     | 100.37160 +0.40648*Time +1.65740*Temp +0.16464*Conc –3.014020*10^-3*Time*Temp – 6.01442*10^-3*Time^2 | |

**Citric acid pretreatment**

| Source                | Sum of Squares | df | Mean Square | F Value | p-value | Prob > F |
|-----------------------|----------------|----|-------------|---------|---------|----------|
| Model                 | 5.07           | 7  | 0.72        | 3.53    | 0.0412  |          |
| A-Time                | 0.13           | 1  | 0.13        | 0.63    | 0.4394  |          |
| B-Temp               | 0.19           | 1  | 0.19        | 0.91    | 0.3643  |          |
| C-Conc               | 0.43           | 1  | 0.43        | 2.08    | 0.1829  |          |
| AB                   | 1.09           | 1  | 1.09        | 5.33    | 0.0464  |          |
| AC                   | 1.31           | 1  | 1.31        | 6.36    | 0.0326  |          |
| A^2                  | 0.71           | 1  | 0.71        | 3.45    | 0.0961  |          |
| C^2                  | 1.11           | 1  | 1.11        | 5.42    | 0.0448  |          |
| Residual             | 1.85           | 9  | 0.21        |         |         |          |
| Lack of Fit          | 1.75           | 5  | 0.35        | 14.33   | 0.0116  |          |
| Pure Error           | 0.098          | 4  | 0.024       |         |         |          |
| Cor Total            | 6.92           | 16 |             |         |         |          |
| Predicted model: Y = &
|                     | -4.317798 –0.018956*Time –0.044615*Temp +0.56213*Conc +8.71089*10^-4*Time*Temp – 3.80837*10^-3*Time*Conc –4.54991*10^-4*Temp^2 – 0.202530*Conc^2 | |

The hydrolysis results showed that the concentration of reducing sugars were varied ranging from 
1.71-15.92 mg/ml, 8.32-16.73 mg/ml and 1.55-4.03 mg/ml in hydrochloric, oxalic, and citric acid 
pretreatment, respectively. Then, dependent variables, e.g. reducing sugars concentration, and 
correspondent pretreatment parameters in each run were evaluated based on RSM to generate the model
representing the correlations between independent and dependent variables. Quadratic mathematic models were suggested to represent the influence of pretreatment parameters on reducing sugar concentrations with high coefficient of determination (R²) values of 0.8366, 0.9211, and 0.7332 in the sets of hydrochloric, oxalic and citric acid pretreatment, respectively. The reliability and significance of each proposed model were determined using ANOVA and expressed as F value (Table 2). The significant model was indicated with the p-value (Prob. > F) of less than 0.05. The results showed that the models of three acid pretreatments were significant because the p-values were 0.0029, <0.0001 and 0.0412, in hydrochloric, oxalic and citric acid sets, respectively. These results of statistical analysis advocated the high reliability of generate model.

![Figure 1](attachment:image1.png)

**Figure 1.** Response surface plots representing interaction effects of the independent variables on reducing sugar yield (mg/ml) obtained from water hyacinth pretreatments. (A) Hydrochloric acid, (B) Oxalic acid, (C-D) Citric acid.

Response surface contour plots were generated to visualize the interactive effects of the pretreatment parameters on the reducing sugar concentrations (Figure 1). As observed in the plots of hydrochloric, oxalic and citric acid pretreatment, pretreatment time and pretreatment temperature influenced on the saccharification yield. While, in citric acid pretreatment, acid concentration additionally affected the yield. Theoretically, long pretreatment time and high temperature lead to further hydrolysis and oxidation of the sugars, which decrease the overall yields. However, low level of pretreatment parameters are also not sufficient to get the maximum yield [10-11, 15]. It could be observed in the contour plots that the interactive effects of temperature and time were synergistic to increase or decrease sugar yields (Figure 1A-1C). Interestingly, the maximum sugar yield was obtained when using middle
level of citric acid concentration and pretreatment time (Figure 1D). These observations suggested the differences in mechanisms of acid pretreatments by each type of acid and highlighted the importance of optimization experiments.

Based on the generated mathematical models, the predicted optimal sugar yields were 16.03 mg, 16.73 mg and 4.03 mg/ml for the hydrochloric, oxalic and citric acid pretreatment, respectively (Table 3). It could be seen that the predicted optimal reducing sugar yields obtained from hydrochloric and oxalic acid pretreatment were similar, although the hydrochloric acid concentration here was much less compared to oxalic acid. Interestingly, when pretreated water hyacinth samples were subjected for biogas and bioethanol production, the yields of biogas and bioethanol obtained from oxalic (203.8 ml/g-TS and 3.55%) and citric pretreatments (221.1 ml/g-TS and 3.36%) were similar and both were clearly higher than that of hydrochloric pretreatment (136.8 ml/g-TS and 2.78%). Additionally, the yields of biogas obtained from oxalic and citric pretreatments were increased for 61.87% and 75.62%, respectively compared to unpretreated water hyacinth. Likewise, the ethanol productions were increased by the effect of oxalic and citric pretreatments for 23.26% and 16.67%, respectively. The improvement of bioethanol and biofuel production by using organic acid pretreatment could be the result of less production of fermentation inhibitors during the pretreatment compared to the inorganic acid pretreatment. Additionally, the remaining organic acid residues could be metabolized by microbes to produce biofuels. This result was agreed well with our previous researches on pretreatments of rice straw, oil palm shell and napier grass [10-11, 15, 18]. However, hydrochloric acid pretreatment showed high improvement on enzymatic saccharification, it could not improve production of biogas and bioethanol compared to unpretreated sample. This is possibly due to the occurrences of fermentation inhibitors causing the detrimental effects on fermentation.

| Acid pretreatment     | Predicted optimal pretreatment parameters | Predicted optimal reducing sugar yield (mg/ml) | Experimental yield |
|-----------------------|-------------------------------------------|-----------------------------------------------|--------------------|
|                       | Time (min) | Temp. (℃) | Acid concentration (%) | | Accumulated biogas (ml/g-TS) | Bioethanol (%) |
| Hydrochloric          | 80.69      | 103.55    | 1.41                  | 16.03             | 136.8          | 2.78           |
| Oxalic                | 70.01      | 126.33    | 11.38                 | 16.73             | 203.8          | 3.55           |
| Citric                | 68.21      | 136.34    | 7.90                  | 4.03              | 221.1          | 3.36           |
| Unpretreated          | n.d.*      | n.d.      | n.d.                  | n.d.              | 125.9          | 2.88           |

*n.d.: not determined

4. Conclusion
Water hyacinth is aquatic weed causing various environmental problems in Thailand because it is an alien species, thus lacking of natural competitors. Attempts to convert these lignocellulose wastes to biofuels have been made, yet the technology is not readily available for actual applications. This study focused on optimization of organic acid pretreatment of water hyacinth to improve the biofuel production yields by using oxalic acid and citric acid. The results obtained from enzymatic saccharification, biogas and bioethanol production suggested that the optimal pretreatment by organic acid improved process yields. The knowledge gained from this work could benefit to development of industrial processes and mechanisms of acid pretreatments on hydrolysis and fermentation.

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