Process Optimization for Deep Eutectic Solvent Pretreatment of Water Hyacinth for Efficient Enzymatic Saccharification

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Abstract. Water hyacinth is an invasive aquatic species that causes severe ecological disruption but can serve as a promising lignocellulosic feedstock for bioenergy production. In this study, a deep eutectic solvent (DES) system based on choline chloride and formic acid (ChCl/FA) was applied to improve pretreatment efficiency and enhance enzymatic saccharification. Process variables were optimized using response surface methodology (RSM) coupled with a Box–Behnken design (BBD), considering temperature, reaction time, and solid-to-liquid ratio. A quadratic regression model adequately described the relationship between these variables and reducing sugar yield, exhibiting a high coefficient of determination. Optimal conditions were identified at approximately 91.85 °C, 60.70 min, and an S/L ratio of 1:5.07, corresponding to a predicted sugar yield of 0.21 g/g. Experimental validation showed good agreement with model predictions. The findings confirm that ChCl/FA is an effective and environmentally benign solvent for biomass pretreatment and highlight its potential application in sustainable biorefinery processes.

1 Introduction

The growing demand for sustainable energy sources has driven interest in bioenergy as an alternative to fossil fuels. Bioenergy offers advantages such as reduced greenhouse gas emissions and improved environmental sustainability. Among potential biomass resources, water hyacinth (*Eichhornia crassipes*) is one of the most problematic invasive aquatic plants worldwide. Its rapid growth leads to blockage of waterways, reduction of dissolved oxygen levels, deterioration of water quality, and disruption of aquatic ecosystems [1,2].

Following its life cycle, submerged decomposition of dead water hyacinth biomass lowers water pH and dissolved oxygen levels while releasing greenhouse gases such as CH_4, CO_2, and hydrogen sulfide. These changes adversely affect aquatic ecosystems and alter invertebrate and fish populations. Water hyacinth exhibits an exceptionally high growth rate, reaching up to 2 tons per acre and doubling its biomass every 5–15 days, thereby severely hindering fishing activities and navigation [1]. Although physical removal is commonly

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practiced, such measures are costly, labor-intensive, and provide only temporary solutions [1]. In Thailand alone, more than 150 million baht is spent annually on mechanical, chemical, and biological control methods [2]. Consequently, the valorization of water hyacinth into value-added products represents a sustainable strategy to mitigate environmental impacts while generating economic benefits. Lignocellulosic biomass is considered a valuable renewable resource for biorefineries, enabling the production of biofuels as well as a wide range of bio-based chemicals. However, its complex structure, composed of cellulose, hemicellulose, and lignin, strongly resists biochemical and chemical degradation [2]. Biomass conversion typically involves three main steps: pretreatment to disrupt the lignocellulosic matrix and remove lignin, enzymatic hydrolysis to convert cellulose into fermentable sugars, and fermentation to produce biofuels such as ethanol [2]. Among these, pretreatment is the most critical step, as lignin and hemicellulose hinder enzyme accessibility to cellulose. Water hyacinth contains approximately 60% cellulose, 8% hemicellulose, and 17% lignin, making it a suitable candidate for enzymatic saccharification and bioconversion into value-added products such as bioethanol, biogas, and platform chemicals [1]. Despite its relatively low lignin content, strong interactions between hemicellulose and lignin still limit enzymatic accessibility, necessitating effective pretreatment strategies.

Deep eutectic solvents (DESs) have gained attention as sustainable and eco-friendly substitutes for traditional acid and alkaline reagents in the pretreatment of lignocellulosic biomass. DESs exhibit high efficiency in selectively solubilizing lignin and hemicellulose, producing cellulose-rich substrates with improved enzymatic digestibility [3]. They offer several advantages, including low cost, biodegradability, ease of preparation, recyclability, and reduced toxicity. Nevertheless, challenges such as the potential toxicity of certain components and limited long-term environmental data remain [3]. Earlier research has confirmed the capability of the DES-based process for water hyacinth. For example, a choline chloride/urea system was reported to remove 64.32 ± 4.08% of lignin under optimized conditions determined through response surface methodology (RSM) [4]. Other pretreatment approaches, including alkaline and acid-based methods optimized by RSM, have also been reported to enhance sugar yields, biogas production, and ethanol fermentation efficiency [1,5]. However, studies focusing on choline chloride/formic acid (ChCl/FA) DES pretreatment of water hyacinth remain limited.

Accordingly, this study aims to optimize the pretreatment parameters of water hyacinth using a choline chloride/formic acid (ChCl/FA) deep eutectic solvent to improve reducing sugar production during enzymatic hydrolysis, utilizing RSM with a Box–Behnken design.

2 Materials and Methods

2.1 Materials

Water hyacinth was harvested from the Chao Phraya River in Nonthaburi, Thailand. The collected biomass was washed, separated into plant components, and cut into small pieces. The material was dried at 60 °C overnight, ground into powder, and sieved to obtain uniform particle size.

Choline chloride and cellulase enzyme (CTec2) were obtained from Sigma-Aldrich (USA), while formic acid (89.0–91.0% purity) was supplied by KEMAUS (Australia). All reagents were of analytical grade. Distilled water was provided by the laboratory facility.

2.2 Deep Eutectic Solvent (DES) Synthesis

The DES was carried out by mixing choline chloride (hydrogen bond acceptor) and formic acid (hydrogen bond donor) at a molar ratio of 1:2. The mixture was heated at 90 °C with continuous stirring until a clear and homogeneous liquid was formed. The prepared solvent was stored in airtight containers prior to use.

Table 1. Presents the experimental design used in the RSM for pretreating water hyacinth biomass with the ChCl/FA DES.

Run	S/L ratio (w/w)	Temperature (°C)	Time (min)	Sugar yield (%)
1	1/5	130	150	22.494
2	1/10	130	240	26.628
3	1/15	110	240	26.265
4	1/15	110	60	23.769
5	1/10	110	150	28.778
6	1/5	110	240	25.055
7	1/10	90	60	17.352
8	1/5	110	60	29.165
9	1/10	130	60	20.488
10	1/10	110	150	18.371
11	1/10	110	150	24.350
12	1/10	110	150	21.771
13	1/5	90	150	32.126
14	1/15	90	150	26.191
15	1/15	130	150	25.722
16	1/10	90	240	25.970
17	1/10	110	150	21.794

2.3 RSM Experiment Design

Optimization was performed using RSM with a Box–Behnken design. Three independent variables were investigated: temperature (90–130 °C), reaction time (60–240 min), and solid-to-liquid ratio (1/5–1/15 w/w). A total of 17 experimental runs were generated.

The experimental data were fitted to a quadratic polynomial model:

$$Y=\beta_0+\beta_1 A+\beta_2 B+\beta_3 C+\beta_{11} A^2+\beta_{22} B^2+\beta_{33} C^2+\beta_{12} AB+\beta_{13} AC+\beta_{23} BC \quad (1)$$

where Y represents reducing sugar yield and A, B, and C are process variables.

2.4 DES Pretreatment of Water Hyacinth

The dried and milled water hyacinth biomass was subjected to pretreatment with ChCl/FA under the conditions outlined in Table 1. After the pretreatment process, the mixture was allowed to cool to the surrounding temperature to stop the reaction. The pretreated biomass was subsequently rinsed with distilled water at a ratio of 50 mL/g of biomass to eliminate any remaining DES, continuing until the pH of the wash water reached neutrality, ensuring suitability for subsequent enzymatic hydrolysis. The samples were divided via vacuum filtration. The collected solids were dried overnight and stored in a desiccator prior to enzymatic hydrolysis and Fourier-transform infrared (FTIR) characterization.

2.5 Enzymatic Saccharification of Water Hyacinth

Enzymatic saccharification was carried out using pretreated biomass at a concentration of 2.5% (w/v), with an enzyme dosage of 30 FPU per gram of biomass. The mixture was maintained at 50 °C with continuous agitation at 150 rpm for 72 h. After hydrolysis, samples were centrifuged and the supernatant was collected for sugar analysis.

2.6 Reducing Sugar Analysis

Reducing sugars were quantified using the DNS method. Absorbance was measured at 540 nm, and concentrations were calculated based on calibration curves [6].

2.7 Fermentation and Ethanol Analysis

Fermentation was carried out using Saccharomyces cerevisiae under controlled conditions. Ethanol concentration was determined using a dichromate oxidation method and measured spectrophotometrically.

3 Results and Discussions

3.1 Optimization of DES Pretreatment by Box Behnken Design (BBD)

BBD, a response surface methodology (RSM) approach, combines two-level factorial arrangements with incomplete block designs to enable efficient optimization of response variables. In this work, analysis of variance (ANOVA) along with lack-of-fit evaluation was employed to determine the adequacy and predictive reliability of the developed model.

The integration of RSM and BBD was used to optimize the pretreatment process by examining three independent factors: temperature (A, 90–130 °C), reaction time (B, 60–240 min), and solid-to-liquid ratio (C, 1/5–1/15 w/w) via Design-Expert software (version 7.0.0) with reducing sugar yield (Y) selected as output variable. The amount of reducing sugars released after enzymatic hydrolysis was quantified using the DNS method, and the corresponding results are summarized in Table 1.

The experimental data were further analyzed using RSM to develop a mathematical model describing the relationship between the independent variables and the response. A

backward quadratic model was found to suitably represent the effects of pretreatment conditions on reducing sugar yield, yielding a high coefficient of determination (R^2 = 0.9636). The model's significance and reliability were evaluated through ANOVA, where a p-value (Prob. > F) of less than 0.05 indicated statistical significance. The ANOVA results revealed that the ChCl/FA pretreatment model was highly significant, with an F-value of 26.45 ($p < 0.0001$), confirming the robustness and adequacy of the model.

The experimental data were processed using response surface methodology (RSM) to construct a mathematical model describing the system. A backward quadratic model was found to best represent the effect of pretreatment variables on reducing sugar yield, with a high coefficient of determination ($R^2 = 0.9636$). The model's validity and significance were evaluated through analysis of variance (ANOVA), where a p-value (Prob. > F) less than 0.05 indicated statistical significance. The ANOVA results confirmed that the ChCl/FA pretreatment model was highly significant, as evidenced by an F-value of 26.45 ($p < 0.0001$), demonstrating its reliability and strong predictive performance.

Figure 1 illustrates the three-dimensional response surface plots. The plots reveal that the interactions among temperature, time, and S/L ratio exert either synergistic or antagonistic effects on sugar release during enzymatic hydrolysis. Notably, increasing all three variables beyond optimal levels resulted in a decrease in sugar yield. This trend is consistent with theoretical expectations, as excessive pretreatment severity, particularly prolonged reaction times and elevated temperatures, can promote sugar degradation and oxidative reactions, ultimately reducing total sugar production [7].

According to the RSM results, the optimum pretreatment parameters were identified as a temperature of 91.85 °C, a time of 60.70 minutes, and a solid-to-liquid ratio of 1:5.07, corresponding to a predicted reducing sugar content of 0.21 g/g. Experimental validation under these conditions produced an actual sugar yield of 0.19 g/g, corresponding to a percentage error of 7.98% compared to the predicted value. This close correlation between predicted and experimental outcomes confirms the reliability and strong predictive performance of the developed model for optimizing ChCl/FA pretreatment of water hyacinth.

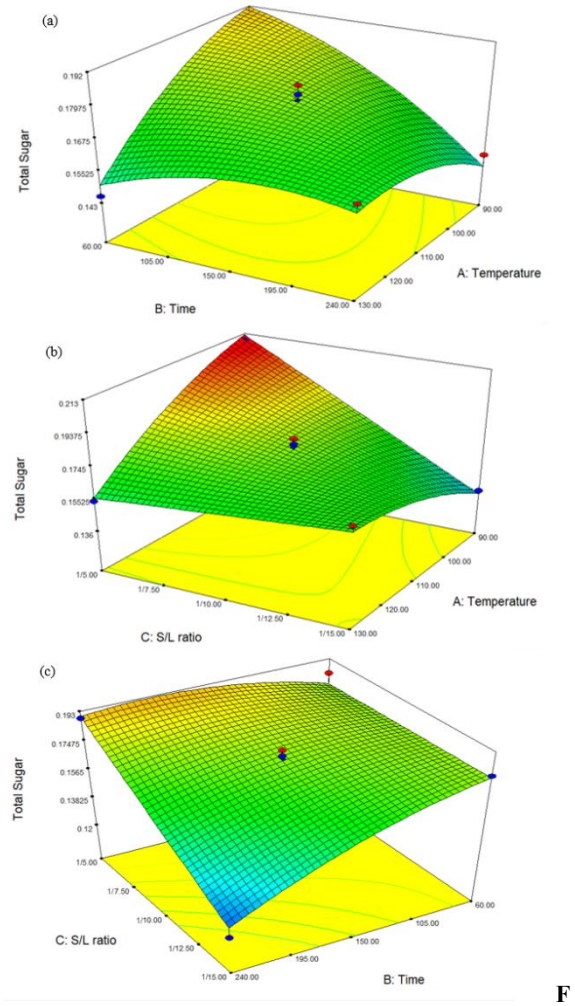

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Fig. 1. illustrates the three-dimensional response surface plots showing the combined effects of (a) temperature and reaction time, (b) solid-to-liquid ratio and temperature, and (c) solid-to-liquid ratio and time on reducing sugar yield under optimal conditions.

The regression equation presented in Equation (2):

$$Total\ Sugar = 0.35456 - 4.42971\times10^{-6}A - 3.88309\times10^{-4}B - 0.021852C + 8.1308\times10^{-6}AB + 2.17463\times10^{-4}AC - 3.49466\times10^{-5}BC - 1.68146\times10^{-5}A^2 - 8.76091\times10^{-7}B^2 \quad (2)$$

3.2 Diagnostics Plots for Model Validation

To evaluate the validity of the developed model, appropriate levels of the process variables were applied to predict and verify improvements in the response. The normal probability plot of internally studentized residuals, used to assess the assumption of residual normality, is shown in Figure 2a. The approximate linear distribution of the data points indicates that the residuals follow a normal distribution, thereby validating the applicability of regression analysis. The near-linear distribution of the data points suggests that the residuals are normally distributed, supporting the suitability of the regression model [8]. Minor deviations

from the straight line may be attributed to experimental variations, such as non-uniform heat distribution during pretreatment or minor instrumental measurement errors.

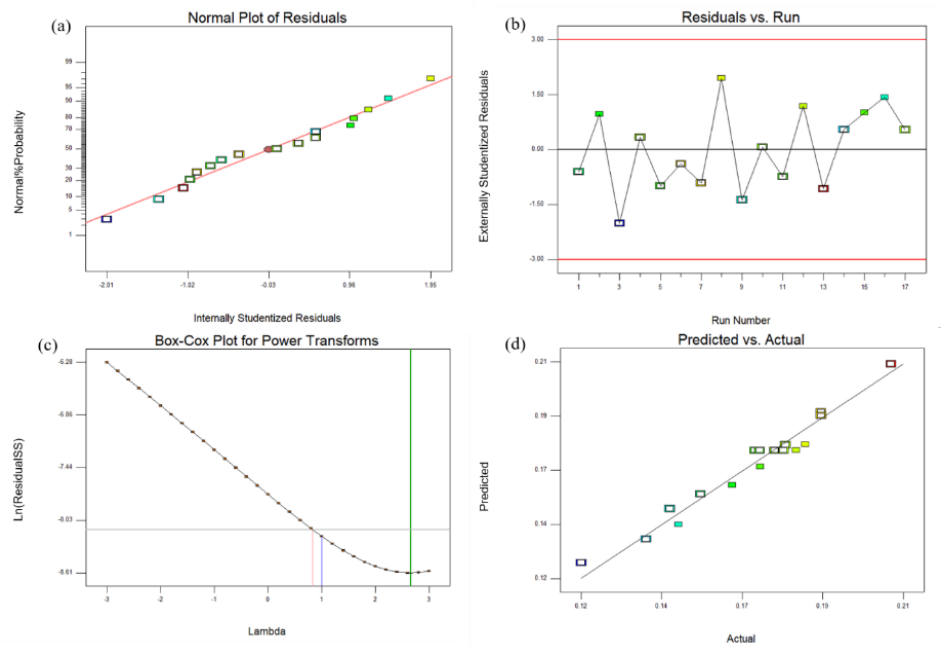

Fig. 2. (a) Normal probability plot, (b) residual vs. the experimental run number, (c) Box–Cox plot, (d) predicted data vs. actual data plot of the influential variables over the reducing sugar.

Figure 2b presents the plot of internally studentized residuals versus the experimental run number, which was employed to evaluate the randomness and independence of residuals. The residuals are randomly scattered around zero, with no data points exceeding the control limits, indicating that no hidden or uncontrolled variables significantly influenced the response. This observation suggests the absence of systematic trends or time-dependent bias. Although slight deviations are observed, likely due to unavoidable experimental uncertainties, the lack of any discernible pattern further confirms the consistency of the experimental design and the reliability of the proposed model [8].

The Box–Cox plot (Figure 2c) was employed to identify the most suitable power transformation for the response variable. Such transformations are generally applied when the residual variance varies with the predicted response magnitude. In this case, the current lambda value within the 95% confidence interval was 1, while the optimal lambda value was 2.66, which corresponded to the minimum residual sum of squares (SS). This indicates that no transformation was necessary to ensure the adequacy of the model.

Figure 2d compares the predicted and experimental reducing sugar yields (g/g). The experimental data points are closely distributed along the parity line (y = x), demonstrating strong agreement between predicted and observed values and further confirming the high predictive accuracy of the developed RSM model.

3.3 FTIR Analysis

Figure 3 presents the structure of water hyacinth biomass through the FTIR spectra. The peak observed at 1422 cm^{-1} is attributed to aromatic skeletal vibrations [8], whereas the band at 1607 cm^{-1} is indicative of lignin-related structures [9]. Another peak at 1726 cm^{-1}

corresponds to C=O stretching of carboxylic functional groups [10]. Additionally, the band at 2433 cm^{-1} can be associated with asymmetric stretching vibrations of H_2O molecules coordinated with aluminum ions [11].

Moreover, the absorption band at 2849 cm^{-1} is assigned to C–H stretching vibrations on the biomass surface [12]. A prominent peak at 2933 cm^{-1}, also related to C–H stretching, reflects contributions from both cellulose and acrylic polymer structures [13]. The band at 3319 cm^{-1} corresponds to O–H stretching of alcoholic and carboxylic groups [14], while the peak observed at 3655 cm^{-1} is attributed to free hydroxyl (–OH) groups [15].

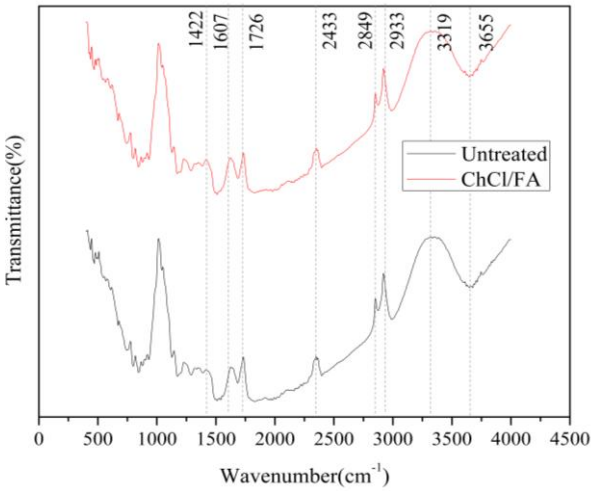

Fig. 3. Fourier-transform infrared spectroscopy (FTIR) spectra of untreated and pretreated water hyacinth biomass.

3.4 Mass Balance

Under optimal DES pretreatment conditions, 3 g of untreated water hyacinth, initially composed of 0.98 g cellulose, 0.41 g hemicellulose, and 0.36 g lignin, was converted into 1.54 g of pretreated water hyacinth containing 0.67 g cellulose and 0.22 g lignin. Enzymatic hydrolysis was conducted by using 0.3 g of the DES (ChCl/FA) pretreated water hyacinth and commercial cellulase of 30 FPU/g biomass under optimal conditions. Experimental results indicated that a total reducing sugar of 0.06 g was obtained. The DES-pretreated hydrolysate exhibited an increased reducing sugar concentration suitable for bioethanol production, resulting in an improvement in ethanol yield from 6.85% of untreated biomass to 8.50% of the pretreated sample.

The fundamental principle of comprehensive lignocellulosic biomass utilization lies in the effective separation of its major components, including cellulose, hemicellulose, and lignin, through appropriate pretreatment strategies. The mass balance of the bioconversion process, starting from 100 g of raw water hyacinth and proceeding through optimal DES pretreatment followed by enzymatic saccharification, was analyzed and is illustrated in Figure 4.

Under optimal deep eutectic solvent (DES) pretreatment conditions, 3 g of untreated water hyacinth, initially comprising 0.98 g of cellulose, 0.41 g of hemicellulose, and 0.36 g of lignin, was converted into 1.54 g of pretreated biomass containing 0.67 g of cellulose and 0.22 g of lignin. Enzymatic hydrolysis was subsequently performed using 0.3 g of the ChCl/FA-pretreated biomass with a commercial cellulase loading of 30 FPU/g biomass under

optimal reaction conditions. The experimental results indicated that a total of 0.06 g of reducing sugars was produced.

The DES-pretreated hydrolysate exhibited an increased reducing sugar concentration suitable for downstream bioethanol production, leading to an improvement in ethanol yield from 6.85% for untreated biomass to 8.50% for the DES-pretreated sample.

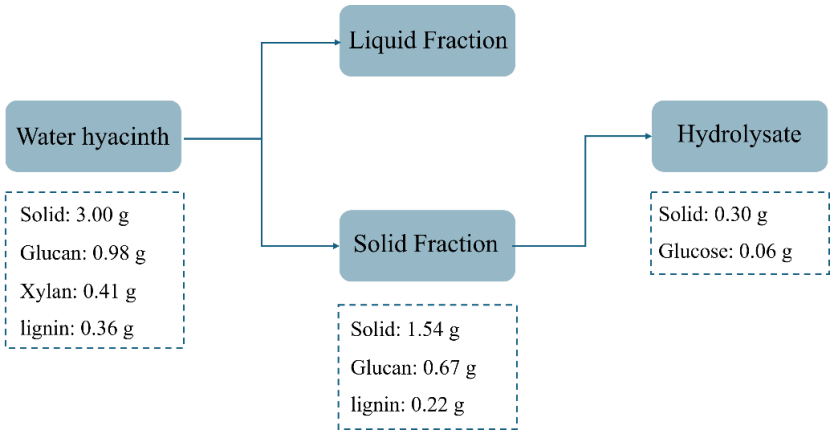

Fig. 4. The mass balance of DES pretreatment of water hyacinth at optimal conditions.

4 Conclusion

This study effectively optimized the ChCl/FA pretreatment of water hyacinth to improve enzymatic saccharification performance. The use of RSM with a BBD enabled the determination of optimal processing conditions, leading to a notable enhancement in reducing sugar yield along with strong model accuracy. FTIR analysis verified structural changes within the lignocellulosic matrix, particularly the disruption of lignin, which facilitated greater enzyme accessibility. These results demonstrate the promise of DES-based pretreatment as a sustainable and efficient approach for converting invasive aquatic biomass into value-added products. Future studies should emphasize solvent recovery, economic feasibility, and process scale-up to advance applications in biorefinery and bioenergy production.

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