Research Article

Enhancing Enzymatic Hydrolysis of Sugarcane Leaves through Sulfonation-Based Pretreatment with a Reusable Organic Solvent under Mild Conditions for Bioethanol Production

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DOI: 10.14416/j.asep.2025.06.009
Received: 10 February 2025; Revised: 13 April 2025; Accepted: 19 May 2025; Published online: 26 June 2025

Abstract

Efficient pretreatment of lignocellulosic biomass is vital for enhancing bioconversion efficiency and reducing production costs sustainably. This study evaluates a sulfonation-based pretreatment strategy employing a reusable organic co-solvent system consisting of formic acid and methanesulfonic acid (MSA) for the pretreatment of sugarcane leaves. Comparative experiments were conducted with and without MSA under fixed conditions of 20% formic acid, 90 °C, and 90 min. Results indicated that the inclusion of MSA significantly enhanced sugar concentration by 1.73-fold and increased sugar yield by 70%. Optimization of pretreatment conditions was performed using response surface methodology (RSM) and a genetic algorithm (GA), with the MSA concentration maintained at 5%. Formic acid concentration, temperature, and pretreatment time were varied to determine optimal conditions. RSM identified optimal conditions at 27.5% formic acid, 81 °C, and 102 min, whereas GA optimization yielded 20% formic acid, 89 °C, and 177 minutes. The corresponding sugar concentrations were 29.4 mg/mL for RSM and 30.49 mg/mL for GA. Subsequent enzymatic hydrolysis and ethanol fermentation produced ethanol concentrations of 12.6 mg/mL under RSM conditions and 12.0 mg/mL under GA conditions. Despite GA optimization utilizing 7.5% less formic acid, ethanol yields were not significantly different compared to RSM results; however, GA required a longer processing time and slightly higher temperature. These findings demonstrate the potential of sulfonation-based pretreatment for cost-effective and environmentally sustainable bioethanol production. However, the optimization was limited to mild pretreatment conditions, and the results were validated only at the laboratory scale.

Keywords: Bioethanol, Biorefinery, Genetic algorithm, Methanesulfonic acid, Sugarcane leaves, Sulfonation-based pretreatment

1 Introduction

The rising global demand for energy and the depletion of fossil fuel resources have made the development of renewable energy essential for achieving sustainable progress. Lignocellulosic biomass, as the most abundant renewable resource, presents significant potential for the production of biofuels and high-value bioproducts. However, the complex structure of lignocellulosic biomass—composed of cellulose, hemicellulose, and lignin—poses substantial challenges to efficient bioconversion [1]–[3]. Pretreatment plays a vital role in disrupting the rigid structure of biomass by removing hemicellulose and lignin and exposing cellulose for enzymatic hydrolysis [4]–[6]. Despite notable advancements in pretreatment technologies, the inherent resistance of lignin to degradation and the structural alterations it undergoes during processing continue to hinder bioconversion efficiency. Acid hydrolysis is regarded as one of the most effective methods for the deconstruction of lignocellulosic biomass. However, this process generates degradation products such as acetic acid, hydroxymethylfurfural (HMF), and furfural, which inhibit bioethanol fermentation [7]. Furthermore, lignin degradation under acidic conditions induces condensation reactions that form stable carbon–carbon (C–C) bonds, thereby reducing biomass digestibility [8]. The degradation of hemicellulose can also produce pseudo-lignin, a by-product that mimics lignin and obstructs enzymatic access to cellulose, further diminishing the efficiency of enzymatic hydrolysis [9].

One of the major challenges in lignocellulosic biomass conversion is the non-productive binding of cellulase enzymes to lignin and pseudo-lignin. Hydrophobic, electrostatic, and hydrogen bonding interactions significantly impede enzymatic hydrolysis by limiting enzyme accessibility to cellulose [10]. To overcome these challenges, several biomass conditioning methods have been developed, including physical, chemical, physico-chemical, and biological pretreatments. Among these, chemical methods—particularly acid and alkaline hydrolysis—are effective in breaking down hemicellulose and lignin structures, although they can generate inhibitory compounds that negatively affect subsequent fermentation. In terms of sugar yield, chemical pretreatments, especially acid hydrolysis, generally achieve the highest recovery rates, often exceeding 80%, whereas physical and biological methods tend to result in lower yields. Due to its high efficiency in deconstructing biomass and enhancing enzymatic hydrolysis, despite the associated risk of inhibitor formation, acid hydrolysis remains one of the most widely used approaches. Based on these advantages, the present study focuses on a sulfonation-based pretreatment incorporating organic acids under mild conditions to improve biomass deconstruction while minimizing inhibitor generation [11]. In this context, mild conditions primarily refer to operating at lower temperatures (70–100 °C), which help to reduce the degradation of sugars and limit the formation of inhibitory by-products.

Sulfonation pretreatment effectively mitigates the inhibitory effects of lignin by introducing sulfonic acid groups, enhancing lignin hydrophilicity, and improving enzymatic digestibility. However, conventional sulfonation methods—such as acid, neutral, alkaline, and ammonium sulfite treatments—require high temperatures (140–180 °C), which limits scalability for industrial applications [8], [12]. Therefore, the development of innovative sulfonation techniques that operate under milder conditions is essential to improve commercial feasibility. Although organic acids such as methanesulfonic acid (MSA) offer advantages for sulfonation pretreatment due to their low toxicity and recyclability, research on the use of reusable organic solvents in this context remains limited. Nevertheless, several studies have explored the potential of sulfonation-based systems for lignocellulosic biomass pretreatment and related catalytic processes. Mennani *et al.*, [13] demonstrated that direct sulfonation lignin (DSL) achieved a 93.97% esterification yield, surpassing carbonized-sulfonated lignin (CSL) due to its higher density of active sites. Building on this finding, a bagasse lignin-based catalyst sulfonated with MSA achieved a 91.1% methyl stearate yield and retained 82.8% efficiency after five cycles, highlighting the catalyst's stability and reusability [14]. A sulfonated carbon catalyst derived from crambe meal initially converted 75% of oleic acid; however, its efficiency dropped sharply to 29% after four cycles due to the gradual loss of sulfonic groups [15]. Further supporting the effectiveness of sulfonation, lignin-based catalysts achieved a 96.58% biodiesel conversion from waste vegetable oil, a result attributed to enhanced porosity and increased acid site density [16]. Extending beyond catalytic applications, sulfonation-based pretreatment combined with organic solvent systems operating at mild temperatures below 100 °C has shown considerable promise for biomass processing. This method not only improved glucose conversion by 89.3% but also significantly enhanced enzymatic hydrolysis efficiency, demonstrating its potential for scalable and sustainable biofuel production [17]. Organic acid systems, recognized for their recyclability and reusability, have been widely applied in the pretreatment of poplar and other lignocellulosic biomasses [18], [19]. MSA, an important sulfonic acid derivative, is regarded as a green solvent owing to its low oxidation potential, low toxicity, and biodegradability [20]. In addition to serving as a solvent, MSA facilitates sulfonation modification of lignin, thereby improving enzymatic hydrolysis efficiency by reducing non-productive binding between lignin and cellulolytic enzymes [14]. Recent studies have explored sulfonation-based co-solvent systems, such as formic acid–MSA, for the selective fractionation of poplar biomass at mild temperatures [21]. These systems have demonstrated partial solvent recoverability and reusability, contributing to more sustainable biomass processing. However, most previous work has focused on woody biomass and solvent systems without optimization for agricultural residues like sugarcane leaves. In contrast, the present study systematically optimizes the formic acid–MSA pretreatment under mild temperatures (below 100 °C) specifically for sugarcane leaves, targeting enhanced sugar recovery and ethanol production while maintaining solvent reusability.

The present study systematically evaluates a sulfonation-based pretreatment strategy that employs a reusable organic co-solvent system composed of formic acid and MSA for the pretreatment of sugarcane leaves under mild conditions. Although sulfonation-based co-solvent systems have been previously applied to woody biomass, their application to agricultural residues such as sugarcane leaves remains limited. In addition, few studies have systematically optimized the pretreatment parameters using advanced optimization techniques. To address this gap, this study investigates the influence of MSA on sugar recovery and applies response surface methodology (RSM) and a genetic algorithm (GA) to optimize pretreatment conditions. The novelty of this work lies in the combination of using a reusable formic acid–MSA system under mild temperatures specifically for sugarcane leaves and employing dual optimization strategies to enhance biomass deconstruction efficiency while maintaining solvent reusability. This approach aims to support the development of cost-effective and environmentally sustainable bioethanol production.

2 Materials and Methods

2.1 *Materials*

Dried sugarcane leaves were obtained from Chachoengsao, Thailand. They were washed thoroughly with deionized water to remove dirt and debris, then air-dried at room temperature. The leaves were further dried at 60 °C in a hot air oven to a constant weight before being ground and sieved to a 10-mesh particle size to ensure uniformity for the pretreatment process. Methanesulfonic acid (99%) and formic acid (85%) were obtained from Thermo Fisher Scientific Inc. and were used as received without further purification. Cellulase from *Aspergillus niger* (original activity: 171 FPU/mL) was supplied by Tokyo Chemical Industry Co. (Tokyo, Japan) and was stored under recommended conditions to maintain activity. For enzymatic hydrolysis experiments, cellulase was applied at a loading of 50 FPU per gram of dry biomass. The 3,5-dinitrosalicylic acid (DNS) reagent was prepared to quantify reducing sugars during enzymatic hydrolysis. All other chemicals and reagents were of analytical grade and were obtained from reputable suppliers.

2.2 *Co-solvent pretreatment and composition analysis*

Dried sugarcane leaves were ground and sieved to a 10-mesh particle size, resulting in a coarse material rather than a fine powder. The co-solvent pretreatment experiments were conducted in a 250 mL pressure bottle. A mixture of 10 g of ground sugarcane leaves and 100 mL of the co-solvent system was thoroughly mixed in the reactor and heated using an incubator. MSA (5% w/w) was included in the solvent system based on findings from a previous study by Wu *et. al.*, [22], which reported that 5% MSA improved lignin sulfonation and enzymatic hydrolysis efficiency in poplar biomass, with no significant further improvements observed at higher MSA concentrations. Although that study focused on woody biomass, 5% MSA was selected as a starting point for the pretreatment of sugarcane leaves in the present study. The formic acid dosage, reaction temperature, and pretreatment time were optimized using response surface methodology (RSM). The reactor was sealed and heated to the target temperature, and the reaction proceeded for 3 h under controlled conditions. After pretreatment, the mixture was cooled to room temperature, and the solid and liquid phases were separated by filtration. The residual solids were washed thoroughly with deionized water to remove remaining solvents and impurities, then dried at 60 °C. The treated solids were collected and stored for subsequent enzymatic hydrolysis and fermentation experiments.

2.3 *Enzymatic hydrolysis of substrates*

The pretreated substrates were subjected to enzymatic hydrolysis. A sample of 0.3 g of residual solids was accurately weighed and transferred into a 15 mL centrifuge tube. A 0.05 M acetic acid–sodium acetate buffer solution (pH 4.8) was added to achieve a solid consistency of 5.0 wt%. Cellulase was dosed at 50 FPU/g of substrate to catalyze the hydrolysis reaction. The enzymatic hydrolysis was carried out in a constant-temperature shaking reactor at 50 °C for 72 h to ensure sufficient enzymatic activity. This duration was chosen based on results in [22], which showed that extending the time beyond 48 h did not significantly enhance yields. Following hydrolysis, the mixture was centrifuged, and the glucose concentration in the supernatant was determined using the DNS method. The glucose conversion yield was calculated using the formula from reference [1], which considers the total theoretical glucose content based on the initial cellulose composition of the substrate.

2.4 *RSM-based optimization*

Response surface methodology (RSM) was applied to optimize the sulfonation-based pretreatment process by examining the effects of three critical parameters: formic acid concentration (10–30%), pretreatment temperature (70–90 °C), and pretreatment duration (90–180 min). These parameters and their ranges were selected to maintain mild pretreatment conditions, with the temperature restricted to below 100 °C to minimize energy consumption and reduce process severity. The reaction time was controlled to limit inhibitor formation, and the concentration range was chosen based on economic feasibility. Within these constraints, the selected factors significantly influenced the disruption of the lignocellulosic matrix and the enhancement of enzymatic hydrolysis efficiency. A Box-Behnken design (BBD) within the RSM framework was employed due to its efficiency in evaluating quadratic response surfaces with a reduced number of experimental runs. This design systematically varied the selected parameters within defined ranges to assess their impact on the physicochemical properties of sugarcane leaf waste, aiming to improve lignocellulosic bioconversion. The yield of reducing sugars from enzymatic hydrolysis was used as the primary response variable, quantified using the DNS method due to its specificity and sensitivity in detecting reducing sugars. The relationship between independent variables and the response was modeled using a second-order polynomial equation, as shown in Equation (1).

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{i=1}^{k}\beta_{ii} X_i^2 + \sum_{i=1}^{k}\sum_{j=i+1}^{k}\beta_{ij} X_i X_j \qquad (1)$$

where Y represents the reducing sugar (RS) yield, X_i and X_j are the independent variables, k is number of factors (independent variables), and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients. Table 1 detailed the independent variables and their coded levels used to optimize the pretreatment conditions for sugarcane leaf waste. Table 2 outlined the experimental design for the sulfonation-based pretreatment of sugarcane leaves within the RSM framework. Response surface plots were presented to illustrate the interaction effects between formic acid concentration, temperature, and time on sugar yield.

Table 1: Independent variables for the RSM-based pretreatment conditions of sugarcane leaf waste.

Independent Variable	Coded Symbols	Levels		
		–1	0	1
Formic acid Concentration (%)	X_1	10	20	30
Temperature (°C)	X_2	70	80	90
Time (min)	X_3	90	135	180

2.5 *Genetic algorithm -based optimization*

The genetic algorithm (GA) was employed to optimize the response variable by identifying the optimal combination of concentration (X_1), temperature (X_2), and time (X_3) using the obtained experimental data. A second-order polynomial model was developed to describe the relationship between the yield of RS and the independent variables, incorporating linear, quadratic, and interaction terms, as expressed in Equation (2).

$$RS = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_1^2 + b_5 X_2^2 + b_6 X_3^2 + b_7 X_1 X_2 + b_8 X_1 X_3 + b_9 X_2 X_3 \quad (2)$$

where $b_0, b_1, ..., b_9$ are regression coefficients obtained by fitting the experimental data. The optimization was performed by minimizing the negative response function, which was defined to enable maximization of the reducing sugar yield. This objective function is expressed in Equation (3):

$$f\left(X_1, X_2, X_3\right) = -\Big(b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_1^2 + b_5 X_2^2 + b_6 X_3^2 b_7 X_1 X_2 + b_8 X_1 X_3 + b_9 X_2 X_3\Big) \quad (3)$$

Table 2: Experimental setup for RSM-based sulfonation pretreatment of sugarcane leaf waste.

Run	Pretreatment Condition		
	X_1: Formic Acid Concentration (%)	**X_2: Temperature (°C)**	**X_3: Time (min)**
1	20	80	135
2	30	90	135
3	30	80	180
4	20	70	180
5	10	70	135
6	10	90	135
7	20	90	90
8	20	80	135
9	10	80	90
10	20	70	90
11	20	90	180
12	20	80	135
13	30	80	90
14	20	80	135
15	30	70	135
16	10	80	180
17	20	80	135

This approach ensured that the GA sought to maximize the RS while exploring the design space defined by the experimental bounds: X_1 = [10,30]%, X_2 = [70,90] °C, X_3 = [90,180] minutes. The optimization process involved initializing a population of candidate solutions, evaluating their fitness based on the objective function, and iteratively applying selection, crossover, and mutation to refine the population [23], [24]. Convergence was achieved when the maximum number of generations was reached or when further improvements in fitness became negligible. The optimal conditions obtained through GA were validated against the regression model.

2.6 *Bioethanol fermentation*

Batch fermentation for bioethanol production was conducted using hydrolysate derived from sugarcane leaf lignocellulosic biomass. *Saccharomyces cerevisiae* was employed as the fermenting microorganism. The fermentation was carried out with a culture comprising 19 mL of liquid hydrolysate and 1 mL of yeast inoculum. Cell concentration was monitored spectrophotometrically at 660 nm, with an optical density (OD) of 1 selected to ensure consistent inoculation. To facilitate yeast acclimatization, glucose (1% w/v) and yeast extract (1% w/v) were added as carbon and nitrogen sources, respectively [25]. The pH was adjusted to 5.0 using a 0.05 M acetic acid–sodium acetate buffer solution prepared from glacial acetic acid (99.8%) and sodium acetate hydrate (99%). Fermentation was incubated at 30 °C for 48 h in a shaking incubator set at 150 rpm. Following fermentation, yeast cells were separated by centrifugation at 6,000 rpm for 5 minutes, and ethanol concentration in the supernatant was quantified using a spectrophotometric method [11]. Briefly, ethanol was extracted from the liquid sample using tri-n-butyl phosphate (TBP) and separated into two phases via centrifugation. The upper TBP layer was mixed with a dichromate reagent for oxidation, producing a blue-green color. The oxidized product was diluted, and its optical density was measured at 595 nm using a UV/Vis spectrophotometer. Ethanol content was determined from a standard calibration curve prepared with absolute ethanol (99.8%), with triplicate measurements performed for accuracy. Ethanol yield, sugar yield, and ethanol conversion were calculated following standard methods [26]. Ethanol conversion is defined as the ratio of ethanol produced to the initial RS content in the hydrolysate, providing a quantitative measure of fermentation efficiency.

2.7 *Statistical analysis*

All experiments were conducted in triplicate, and the results are presented as mean ± standard deviation (SD). The optimum pretreatment conditions predicted by response surface methodology (RSM) and the genetic algorithm (GA) were experimentally validated by performing the pretreatment experiments in triplicate. Subsequent enzymatic hydrolysis and ethanol fermentation experiments were also conducted in triplicate. The experimental results were compared with the predicted values to assess the accuracy of the optimization models and to evaluate the reproducibility and variance across all stages of the process.

2.8 *Scanning electron microscopy (SEM) analysis*

The morphological characteristics of untreated and pretreated sugarcane leaf biomass were examined using a Thermo Scientific Phenom Pharos G2 FEG-SEM. Samples were mounted onto aluminum stubs with double-sided carbon tape. Imaging was performed in low vacuum mode (60 Pa) at an accelerating voltage of 5 kV using a backscattered electron (BSD) detector. No conductive coating was applied to the samples prior to analysis. Representative images were selected to illustrate surface structural changes following different pretreatment conditions.

3 Results and Discussion

3.1 *Enhancement of sugar recovery by formic acid–MSA pretreatment*

The effect of applying formic acid pretreatment in the presence and absence of MSA was systematically investigated to evaluate the efficiency of the sulfonation-based pretreatment strategy. In each experiment, 10 g of dried sugarcane leaves were used as the initial biomass. Under the designed condition of 20% formic acid at 90 °C for 90 min, the RS concentration significantly increased from 7.4 mg/mL without MSA to 20.2 mg/mL with MSA, as illustrated in Figure 1. Correspondingly, the sugar yield improved from 11.97% to 20.26% based on the initial raw biomass weight. This substantial enhancement in sugar recovery highlights the critical role of MSA in promoting sulfonation during pretreatment. The introduction of sulfonic acid groups likely improves lignin hydrophilicity and disrupts the lignin structure, thus enhancing cellulose accessibility for enzymatic hydrolysis. Similar observations have been reported by Wu *et al.* [22], where in-situ lignin sulfonation using an organic solvent system resulted in a twofold increase in sugar release efficiency. Factors contributing to the improved sugar yield in the present study include enhanced delignification, reduced non-productive binding between lignin and enzymes, and greater exposure of cellulose microfibrils, consistent with the findings of Wang *et al.* [17]. Overall, the incorporation of MSA under mild pretreatment conditions effectively facilitates lignocellulosic biomass deconstruction, thereby significantly boosting enzymatic hydrolysis performance.

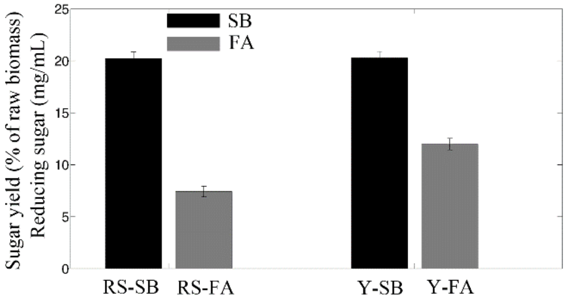

Figure 1: Comparative sugar yield and concentration from biomass: sulfonation-based (SB) and formic acid (FA) pretreatments.

The bar chart in Figure 1 compares the RS concentration and sugar yield obtained from sugarcane leaves pretreated with the sulfonation-based (SB) formic acid–MSA system and conventional formic acid (FA) pretreatment under identical conditions. RS-SB and RS-FA denote the RS concentrations resulting from sulfonation-based and formic acid pretreatments, respectively, while Y-SB and Y-FA denote the corresponding sugar yields. Error bars represent the standard deviation of triplicate measurements.

Figure 2 illustrates the effect of sulfonation-based pretreatment duration on sugar yield using 20% formic acid at 70 °C. A significant increase in sugar yield was observed after 135 min, compared to the gradual increase between 90 and 135 min. This trend suggests that extended pretreatment duration substantially improves sugar release efficiency.

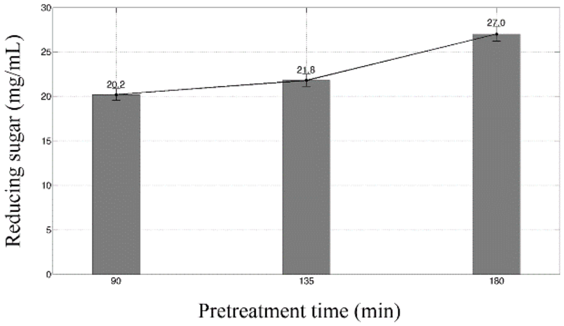

Figure 2: Effect of sulfonation-based pretreatment duration on reducing sugar yield.

3.2 *Response surface model: Pretreatment optimization for sugar yield*

The influence of experimental variables on RS yield was evaluated using three-dimensional and two-dimensional response surface plots (Figure 3). Analysis of variance (ANOVA) indicated that the interaction between pretreatment temperature and time (BC) showed a marginal effect (p-value = 0.0523), while variations in formic acid concentration (C) did not have a significant impact (p-value = 0.0896). Among all terms, the quadratic effect of temperature (B^2) was statistically significant (p-value = 0.0234), indicating that temperature plays a key role in influencing sugar yield under the conditions studied. This trend is similar to that reported by Panakkal *et al.*, [21], where higher pretreatment temperatures improved fermentable sugar release from sugarcane bagasse. However, their study was conducted at higher temperatures (up to 140 °C), whereas the present study focuses on mild conditions below 100 °C. These results emphasize the importance of temperature optimization to achieve efficient biomass deconstruction while minimizing the formation of inhibitory compounds.

Experimental data were further applied to optimize conditions using a genetic algorithm. The GA-derived conditions were validated and compared with those obtained from RSM to evaluate their effectiveness in maximizing sugar yield.

3.3 *Optimal pretreatment conditions identified by RSM and GA*

The optimal pretreatment conditions for maximizing RS concentration were determined through simulation using response surface methodology (RSM) and a genetic algorithm (GA), with the predicted results summarized in Table 3. Using a 5% loading of pretreated biomass and maintaining 5% MSA, RSM predicted the optimal conditions at 27.5% formic acid, 81 °C, and 102 min, whereas GA predicted optimal conditions at 20% formic acid, 89 °C, and 173 min. Both optimized conditions obtained from RSM and GA simulations were experimentally validated through triplicate shake flask experiments. The resulting reducing sugar (RS) concentrations were 29.40 ± 1.87 mg/mL for the RSM model and 30.49 ± 2.53 mg/mL for the GA model. These values closely align with the predicted outcomes (30.20 mg/mL for RSM and 32.24 mg/mL for GA), confirming the reliability and predictive accuracy of the optimization models. The sugar yields obtained in this study are considered acceptable for sulfonation-assisted acid pretreatment under mild conditions. Wu *et al.*, [22] reported that poplar pretreated with a formic acid–methanesulfonic acid co-solvent system under similar mild conditions achieved glucose concentrations of 10.9–13.2 g/L following enzymatic hydrolysis.

Table 3: Optimal pretreatment conditions and reducing sugar yields predicted by RSM and GA simulations, along with experimental validation results.

Method	**Optimal Pretreatment Parameter**			**Reducing Sugar Yield (mg/mL)**	
	X_1 (%)	**X_2 (°C)**	**X_3 (min)**	**Predicted**	**Experimental**
RSM	27.5	81	102	30.20	29.40 ±1.87
GA	20	89	177	32.24	30.49 ±2.53

Although direct comparison between enzymatic hydrolysates and acid-pretreated biomass must be undertaken cautiously, the higher sugar concentrations observed in the present study suggest that the pretreatment strategy effectively enhances sugar release. While GA utilized a lower formic acid concentration, it required a longer pretreatment time and higher temperature. Nevertheless, the sugar yields from both methods were comparable, demonstrating that both RSM and GA represent effective strategies for optimizing pretreatment conditions to enhance sugar recovery. Although solvent recovery was not evaluated in this study, previous research has demonstrated the potential partial recoverability and reusability of the formic acid–MSA system [20], [22], and further investigation of solvent recycling is planned as part of future work. Furthermore, scale-up simulations and experimental validation are also planned to further compare the performance of RSM- and GA-optimized pretreatment conditions under practical operating conditions. In future evaluations, factors such as total processing time, chemical consumption, and overall economic feasibility will be considered to determine the most suitable strategy for industrial application.

To provide additional insight, the influence of formic acid concentration on pretreatment efficiency warrants consideration. Although lignin solubility was not directly analyzed in this study, formic acid concentration is known to affect biomass deconstruction. Moderate concentrations facilitate hemicellulose removal and increase cellulose accessibility, whereas excessively high concentrations may induce sugar degradation, leading to the formation of inhibitory compounds such as furfural and HMF.

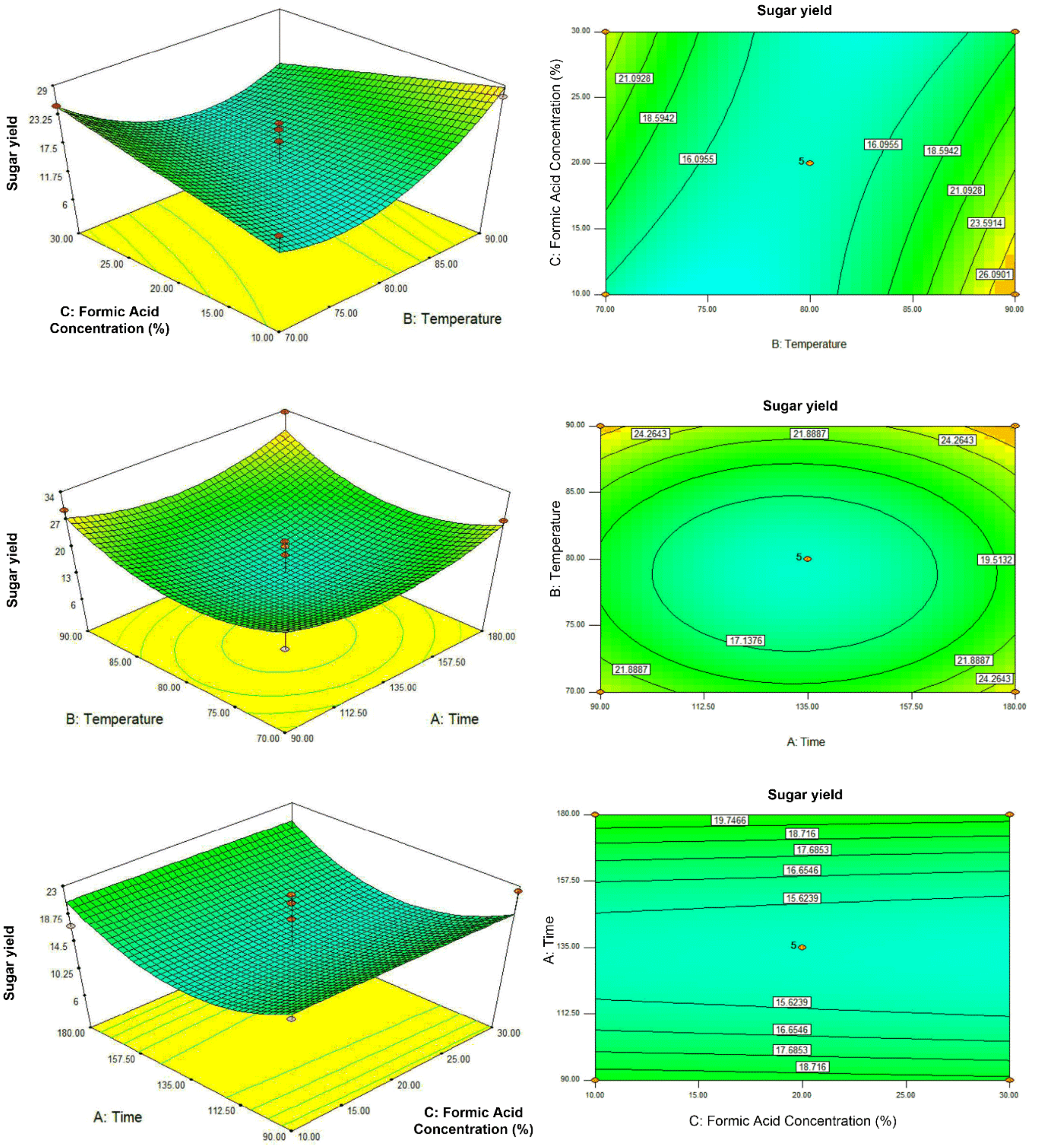

Figure 3: Three-dimensional and two-dimensional response surface analysis of factors affecting reducing sugar yield.

Furthermore, pseudo-lignin formation and lignin condensation reactions may occur under harsher conditions, which can inhibit enzymatic hydrolysis efficiency. These effects likely account for the observation that higher formic acid concentrations did not result in further improvements in sugar yield, thereby underscoring the importance of optimizing pretreatment parameters to balance effective deconstruction and inhibitor minimization. Although the formation of inhibitory compounds is discussed conceptually, detailed chemical analysis, such as HPLC quantification of HMF, furfural, and acetic acid, was not conducted in this study but should be conducted in future research to further elucidate the effects of pretreatment conditions on fermentation performance. To validate this observation, further structural analysis—specifically the scanning electron microscopy (SEM) images in Figure 4—was conducted to compare the morphology of untreated and pretreated biomass, confirming lignin disruption and cellulose exposure. Figure 4a shows untreated biomass with smooth and intact surfaces, indicating minimal structural damage under mild temperature conditions. In contrast, Figure 4(b) and (c), representing biomass pretreated with formic acid and the sulfonation-based formic acid–MSA system, show visible cracks and surface disruptions in the lignocellulosic cell walls. These structural changes likely assist fibrillation and improve enzymatic accessibility during hydrolysis.

The biomass pretreated with the formic acid–MSA system (Figure 4(c)) exhibits more severe structural disruption than the biomass treated with formic acid alone (Figure 4(b)), while the untreated sample (Figure 4(a)) retains a more compact and rigid structure. The increased porosity and fiber separation observed in the sulfonation-based system suggest more effective lignin removal and greater cellulose exposure. These structural changes correlate with the higher sugar concentrations obtained after pretreatment. This result aligns with the findings of Wu *et al.*, [27], who report that greater structural disruption improves enzymatic hydrolysis and reduces condensed lignin by introducing sulfonic acid groups into lignin molecules. The sulfonic acid groups minimize hydrophobic interactions between lignin and cellulase, contributing to the enhanced enzymatic digestibility observed in the sulfonation-based formic acid–MSA system [20], [28], [29]. Although the morphological changes were confirmed by SEM observations in this study, further chemical characterization, such as FTIR or XRD analysis, would be valuable to more directly verify the structural modifications of lignin and will be considered in future work.

3.4 *Ethanol production under optimized conditions: RSM and GA comparison*

The ethanol fermentation experiments using *S. cerevisiae* were conducted with hydrolysates obtained from biomass pretreated under optimal conditions determined by RSM and GA. As presented in Figure 5, the ethanol concentration achieved with the RSM-optimized hydrolysate was 12.6 mg/mL, while that obtained from the GA-optimized hydrolysate was 12.0 mg/mL. Statistical analysis indicates that the difference between these concentrations is not significant (p-value > 0.05), suggesting comparable fermentation performance under laboratory-scale conditions. Similarly, the ethanol yields, calculated based on the initial RS content, were 43% and 39% for the RSM and GA conditions, respectively, with no statistically significant difference observed. Although these differences are minor at the laboratory scale, it is anticipated that scaling up to bench or pilot scale may reveal more pronounced variations in fermentation efficiency.

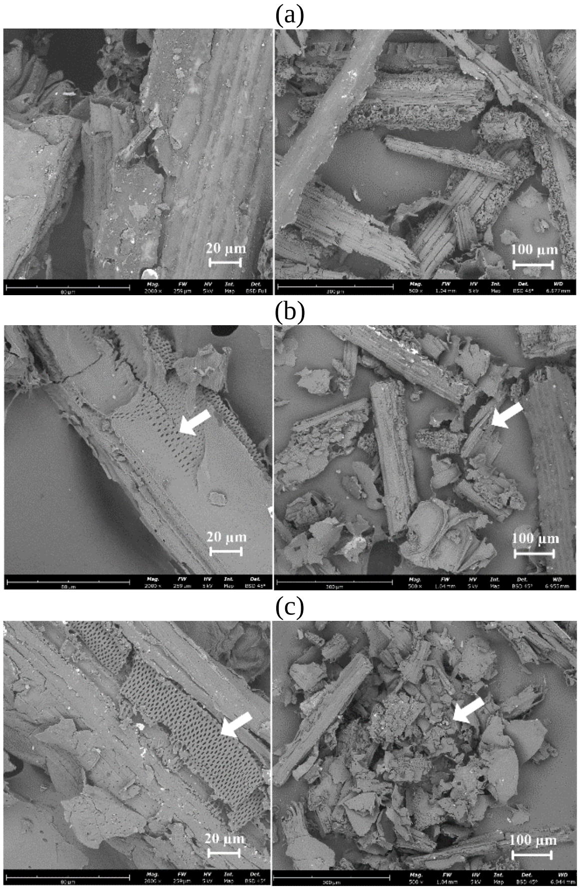

Figure 4: SEM images of sugarcane leaf biomass under different pretreatment conditions: (a) untreated biomass, (b) biomass pretreated with formic acid, and (c) biomass pretreated with the sulfonation-based co-solvent system of formic acid and MSA.

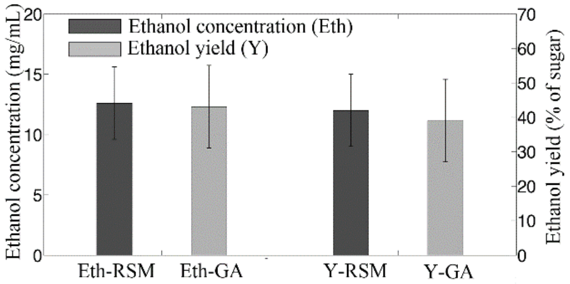

Figure 5: Comparative ethanol yield and concentration from biomass pretreated under optimal conditions identified by RSM and GA.

The marginally higher ethanol concentration and yield under RSM conditions can be attributed to differences in hydrolysate composition. Although the GA condition produced slightly more reducing sugar (30.49 mg/mL) than RSM (29.4 mg/mL), its longer pretreatment time and higher temperature may lead to the formation of inhibitory compounds (e.g., furfural, hydroxymethylfurfural), which can suppress yeast activity and reduce fermentation efficiency [30]. In contrast, the shorter processing time and moderate temperature associated with the RSM-optimized conditions likely minimize inhibitor formation, thereby enhancing yeast performance and overall ethanol productivity. Similar findings are reported by Panakkal *et al*., [21], who demonstrate that optimizing pretreatment conditions through RSM improves sugar yield while reducing the formation of fermentation inhibitors in sugarcane bagasse hydrolysates. These results suggest that, while both optimization methods are effective, the RSM-derived conditions offer a better balance between sugar recovery and fermentation efficiency. Furthermore, increasing biomass loading beyond the 5% used in this study could further improve ethanol yield by providing greater sugar availability, making this an important direction for future research. Additional strategies, such as integrating the sulfonation-based pretreatment with mild physical methods to further enhance cellulose accessibility without significantly increasing energy consumption, could also be explored. Nevertheless, it should be noted that variations in biomass composition and structure may lead to differences in hydrolysis efficiency and inhibitor formation [31]. Therefore, further experimental validation across different lignocellulosic feedstocks is essential to confirm the broader applicability and effectiveness of these strategies.

4 Conclusions

This study demonstrates the effectiveness of a sulfonation-based formic acid–MSA pretreatment strategy for enhancing sugar recovery from sugarcane leaves under mild conditions. Using RSM and GA optimization, high RS concentrations of 29.4 mg/mL and 30.49 mg/mL were achieved, respectively, leading to ethanol concentrations of 12.6 mg/mL for RSM and 12.0 mg/mL for GA. Although the GA conditions produced slightly more RS, the RSM-derived conditions offer advantages, including shorter processing time (102 min), lower temperature (81 °C), and reduced inhibitor formation, resulting in better fermentation performance. The unique contribution of this work lies in combining a mild sulfonation-assisted pretreatment with statistical optimization, applied to sugarcane leaves—a low-cost agricultural residue—to achieve high sugar yield without complex multi-step processing. These findings provide a practical basis for future scale-up to bench or pilot scale, highlighting the potential of this approach for sustainable and energy-efficient bioethanol production. While the results are promising, further investigation is needed to address the longer processing time under GA-optimized conditions, the absence of experimental solvent recovery validation, and potential scaling challenges. These aspects define directions for future work without diminishing the demonstrated effectiveness of the pretreatment strategy. Future studies should also include techno-economic evaluation and inhibitor analysis to fully assess industrial applicability.

Acknowledgments

This research budget was allocated by National Science, Research and Innovation Fund (NSRF), and King Mongkut’s University of Technology North Bangkok (Project no. KMUTNB-FF-68-B-30).

Author Contributions

A.K.: investigation, methodology; A.T.: investigation, methodology, writing an original draft, reviewing and editing; P.T.: data curation, funding acquisition; L.T.: data curation, data analysis; N.K.: investigation, writing—reviewing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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