Applied Science and Engineering Progress, Vol. 20, No. 4, 2027, 8373

Research Article

Low-Power Ultrasound-Assisted Methanesulfonic Acid–Formic Acid Pretreatment Coupled with Simultaneous Saccharification and Fermentation for Bioethanol Production from Sugarcane Leaves

Apinya Kaoloun and Jiraparn Khawsurichan
The Sirindhorn International Thai-German Graduate School of Engineering, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand

Keerthi Katam
Department of Civil Engineering, Ecole Centrale School of Engineering, Mahindra University, Hyderabad, Telangana, India

Atthasit Tawai*
Biorefinery and Process Automation Engineering Center (BPAEC), The Sirindhorn International Thai-German Graduate School of Engineering, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand

* Corresponding author. E-mail: atthasit.t@tggs.kmutnb.ac.th DOI: 10.14416/j.asep.2026.11.005
Received: 19 August 2026; Revised: 30 September 2026; Accepted: 7 October 2026; Published online: 11 November 2026
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Abstract
This study investigates an energy-efficient and environmentally sustainable approach to enhancing bioethanol production from sugarcane leaves through synergistic pretreatment using low-power ultrasound and an organic acid co-solvent system. To reduce the energy demand and environmental impact associated with conventional pretreatment methods, methanesulfonic acid (MSA) and formic acid (FA) were combined under low-power ultrasonic conditions. A Box–Behnken design (BBD) based on response surface methodology (RSM) was employed to optimize the FA concentration, ultrasonication time, and ultrasound power. The optimal conditions were identified as 29.17% FA, 18.70 min of ultrasonication, and an ultrasound power of 16.61 W, with the MSA concentration maintained at 5%. These conditions promoted the disruption of the lignocellulosic structure through acoustic cavitation and acid-assisted modification, thereby enhancing cellulose accessibility while minimizing energy consumption. Subsequent simultaneous saccharification and fermentation (SSF) using Saccharomyces cerevisiae at 32 °C with an enzyme loading of 50 FPU/g achieved an ethanol yield of 73.22% of the theoretical yield (on a cellulose basis), corresponding to an ethanol concentration of 12.602 mg/mL after 48 h. Extending the fermentation time to 72 h resulted in only a marginal increase in ethanol yield of 0.8%, suggesting that a 48 h incubation period represents a potentially techno-economically optimal operating window by reducing processing time and associated production costs. Compared with separate hydrolysis and fermentation (SHF), SSF achieved a comparable ethanol concentration while shortening the overall processing time and eliminating the need for a separate hydrolysis step at 50 °C. These findings demonstrate that low-power ultrasound-assisted MSA–FA co-solvent pretreatment is a promising strategy for converting sugarcane leaf residues into bioethanol under mild and potentially cost-effective operating conditions.

Keywords: Bioethanol, Methanesulfonic acid, Response surface methodology, Simultaneous saccharification and fermentation, Sugarcane leaves, Ultrasound-assisted pretreatment

1 Introduction

The increasing global demand for energy, driven by rapid industrialization and technological advancement, has intensified the dependence on fossil fuels. However, the extensive use of fossil resources has led to severe environmental concerns, including greenhouse gas emissions and ecological degradation. Bioethanol has emerged as a promising alternative fuel due to its renewable nature and potential

to reduce carbon emissions. Among the various feedstocks, lignocellulosic biomass (LCB) such as sugarcane bagasse or leaves is particularly attractive owing to its abundance, low cost, and eco-friendly conversion potential [1]. A possible green method for improving the efficiency of biological, chemical, and physical processes, including enzymatic reactions, is ultrasound. In addition to producing chemical effects through the production of free radicals (–OH and –H) from the breakdown of water, ultrasound also produces physical effects through shock waves, shear forces, and the collapse of the microbubbles created by acoustic transmission [2]. Recent studies have demonstrated the potential of ultrasound-assisted pretreatment in biomass conversion. From the findings [3], the ultrasonic conditions (70 W at 45 kHz) have significantly increased the hydrolysis of Ramon seed flour, with optimal performance achieved at a relatively low temperature of 45 ˚C. This approach resulted in enhanced fermentation efficiency of up to 91% without inhibition of enzymatic activity, demonstrating the potential of low power ultrasound as an energy-efficient method for the production of bioethanol. Similarly, [4] demonstrated that the combination of ultrasound with alkaline pretreatment significantly enhances cellulose accessibility in LCB such as sugarcane bagasse and enset fibre (*Ensete ventricosum*). Furthermore, ultrasound-assisted pretreatments using organic acid systems have shown promise in accelerating hemicellulose solubilization and lignin degradation under mild thermal regimes [5], [6]. However, most existing studies rely on single-acid systems or high-temperature conditions.

Although there are limited studies on investigating the use of low power ultrasound in combination with environmentally benign solvent systems for biomass pretreatment. In particular, the synergistic effects of organic acids such as methanesulfonic acid (MSA) and formic acid (FA) under low-energy ultrasonic conditions have not been extensively explored. Following the pretreatment process, the exposed cellulose must be converted into fermentable sugars via enzymatic hydrolysis and subsequently fermented into bioethanol. This bio-conversion is generally operated through two main configurations: separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) [7]. SHF offers distinct operational flexibility by allowing independent optimization of temperature and pH for each process step—typically around 50 °C for cellulase activity and 30–32 °C for *Saccharomyces cerevisiae* fermentation. This flexibility makes SHF particularly advantageous when utilizing specialized pentose-fermenting microorganisms or handling pretreated feedstocks containing high concentrations of inhibitors (e.g., acetic acid or xylooligomers), where uncoupling hydrolysis from fermentation prevents severe microbial inhibition [8]. However, a major bottleneck of SHF remains end-product feedback inhibition: as glucose and cellobiose accumulate during the hydrolysis stage, they severely suppress cellulase activity, ultimately limiting total sugar yields and requiring higher enzyme dosages [7], [9]. Conversely, SSF integrates enzymatic hydrolysis and yeast fermentation into a single reactor. In SSF, released sugars are immediately consumed by the yeast as they are produced, effectively relieving feedback inhibition and maintaining higher hydrolysis rates [7].

Although SSF requires operating at a compromise temperature (typically 35–38 °C) and renders yeast recycling difficult due to unfermented solids, its practical advantages—such as reduced overall processing times, lower capital investment, reduced risk of microbial contamination due to the continuous presence of ethanol, and higher overall ethanol productivity—make it highly desirable for industrial scaling [9], [10]. In this study, the low power ultrasonic pretreatment method is employed to enhance the conversion efficiency of sugarcane leaves for bioethanol production while minimizing energy consumption. MSA is a strong, environmentally friendly, and biodegradable organic acid. In this study, MSA was incorporated into the sulfonation-based formic acid pretreatment system at a fixed concentration of 5% (w/w). The selection of 5% MSA was informed by the findings of Wu *et al.,* [11], who reported that this concentration significantly improved lignin sulfonation and enzymatic hydrolysis efficiency in biomass, with no further gains observed at higher acid concentrations. This fixed dosage provides sufficient acidity to effectively hydrolyze hemicellulose and cleave recalcitrant ether linkages in the lignin-carbohydrate complex (LCC), while preventing excessive sugar degradation into toxic fermentation inhibitors such as furfural and 5-hydroxymethylfurfural (5-HMF) [12]. Maintaining MSA at this fixed concentration allowed the response surface methodology (RSM) to focus specifically on optimizing the concentration of FA, which serves as the primary delignifying solvent for solubilizing fragmented lignin. Furthermore, to fully

evaluate the bioethanol potential of this mild and eco-friendly pretreatment, the performance of the pretreated biomass was assessed using the SSF configuration [12].

Implementing SSF in this context allows us to investigate whether the increased cellulose accessibility achieved by low-power ultrasound and organic acid synergy can seamlessly translate into enhanced bioethanol yields within a streamlined, single-vessel system, providing a cost effective, energy-efficient, and environmentally sustainable pathway for bioethanol production.

2 Materials and Methods

2.1 *Biomass and reagent preparation*

Sugarcane leaves were obtained from a local supplier in Thailand. Firstly, the leaves were washed with distilled water to remove impurities and dried at 60°C in a hot-air oven. The dried leaves were ground, screened through a 10-mesh sieve, and stored in plastic bags at room temperature. This enhances initial surface area and accessibility. Methanesulfonic acid (MSA, 99%) and formic acid (FA, 85%) were purchased from Thermo Fisher Scientific Inc. Commercial cellulase from *Trichoderma reesei* (activity: 278.51 FPU/mL) was supplied by Sigma-Aldrich Co. Ltd. For analytical purposes, potassium dichromate ($K_2Cr_2O_7$) and 3,5-dinitrosalicylic acid (DNS) reagents were prepared to quantify ethanol and reducing sugars, respectively. All other chemicals used were of analytical grade.

2.2 *Co-solvent pretreatment*

The co-solvent pretreatment was implemented. The experiments were conducted utilizing 250 mL high-pressure glass bottles to withstand the vapor pressure generated during the thermal process. For each experimental run, the solid-to-liquid ratio was strictly maintained at 1:10, combining 10 g of dry biomass with 100 mL of the prepared co-solvent mixture. The catalytic baseline of 5% (w/w) MSA was selected according to prior work by Wu *et al.,* [11], which demonstrated that 5% MSA was optimal for promoting lignin sulfonation and facilitating subsequent enzymatic digestion in poplar biomass, whereas higher concentrations yielded negligible further improvements. Adapting this framework from woody biomass to agricultural residues, 5% MSA was adopted as the fixed acid baseline for sugarcane leaves pretreatment in this study. Maintaining MSA at this baseline allowed response surface methodology (RSM) to focus specifically on optimizing formic acid (FA) concentration, ultrasonic treatment duration, and ultrasonic power rate.

The inclusion of MSA was established based on its demonstrated strong organic acidity and superior efficacy in cleaving highly stable lignin-carbohydrate complexes (LCC), thereby facilitating extensive delignification [12]. The maximization of biomass deconstruction was systematically approached via statistical optimization. A three-factor, three-level Box-Behnken Design (BBD) under RSM was employed to evaluate the interactive effects of the critical processing parameters. The three independent variables investigated were FA concentration (A: 10–30%), ultrasonic irradiation time (B: 15–30 min), and ultrasonic power rate (C: 16–33 W). Prior to thermal incubation, the sealed reactors were subjected to ultrasonic treatment according to the BBD matrix; this mechanical acoustic cavitation was introduced to induce localized shear forces, effectively disrupting the cell wall morphology and enhancing chemical penetration. Following sonication, the reactors were transferred to a temperature-controlled laboratory oven and heated to the target core temperature. Upon completion of the residence time, the reactors were cooled to room temperature. The resulting slurry was subjected to vacuum filtration to separate the pretreated solid residue from the liquid hydrolysate containing solubilized lignin and hemicellulose fractions. The recovered solid fraction was washed repeatedly with copious amounts of deionized water until the filtrate reached a neutral pH, ensuring the total elimination of residual volatile acids and inhibitory chemical by-products. The neutral solid cakes were subsequently dried at 60°C until a constant weight was achieved. The dry chemical composition of both untreated and pretreated biomass was determined for cellulose, hemicellulose, and lignin content following the standard National Renewable Energy Laboratory (NREL) analytical protocols before proceeding to subsequent enzymatic hydrolysis and fermentation stages.

2.3 *Response surface methodology*

Three crucial parameters were examined in order to optimize the pretreatment process of sugarcane leaf waste using RSM: concentration of FA (10–30%), pretreatment time (15–30 min), and power rate

(16–33 W). BBD framework of RSM was employed because it is highly effective at analyzing quadratic response surfaces with a minimized number of experimental runs. In order to enhance the subsequent lignocellulosic bioconversion, this design methodically varied the selected parameters within the predetermined ranges to evaluate their effects on the physicochemical characteristics of the sugarcane leaves waste. The relationship between the independent variables and the response was mathematically modeled using the following second-order polynomial equation, as shown in Equation (1).

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

Table 1: Independent variables along with coded values.

Factor	Units	-1 level	+1 level
A – Concentration of FA	%	10	30
B - Time	min	15	30
C - Power rate	W	16	33

Table 2: BBD matrix RSM-based ultrasonic and sulfonation pretreatment experimental setup for sugarcane leaves.

Run	X_1 Concentration of FA (%)	X_2 Time (min)	X_3 Power rate (W)
1	10	22.5	33
2	30	30	24
3	30	22.5	16
4	20	30	33
5	20	15	16
6	20	15	33
7	20	22.5	24
8	10	15	24
9	20	30	16
10	30	22.5	33
11	10	30	24
12	20	22.5	24
13	20	22.5	24
14	20	22.5	24
15	10	22.5	16
16	30	15	24
17	20	22.5	24

where Y represents the ethanol yield, X_i and X_j are the independent variables, k is the number of factors (independent variables), and β_0 , β_i, β_{ii} and β_{ij} are the regression coefficients. Table 1 and Table 2 show the experimental design for the RSM-based sulfonation pretreatment experimental setup for sugarcane leaves waste. For Equation (1), which is used to observe the influence of various variables on the ethanol response (mg/mL), please refer to the final equation in terms of coded factors (Equation (2)).

$$\begin{aligned} Ethanol = {} & 10.53 + 0.22A + 0.38B + 0.33C \\ & + 0.50AB + 0.20AC + 0.25BC \\ & + 0.23\mathrm{A}^2 + 0.59B^2 - 1.05C^2 \end{aligned} \quad (2)$$

From Equation (2), we can see the influence of the various variables that are converted into codes. Since the variable values are all adjusted to the same scale (-1 to +1), we can directly compare the influence of each factor from the size of the coefficients. We find that the variable that has the greatest influence on the response is the first factor, which has the greatest influence on the variable, C^2 (Power rate^2), with a coefficient of -1.05. This is the most influential factor on the ethanol quantity response in this experiment. A very negative value (-1.05) indicates a quadratic effect (concave down). In physical terms, this means that when the power rate is increased, the ethanol quantity increases until it reaches an optimum point. However, if the power rate is increased beyond that point, the ethanol quantity begins to decrease significantly. This may be due to excessively high energy levels destroying important substances or causing reverse reactions. Next is B^2 (Time^2), with a coefficient of 0.59, indicating that time (Time^2, B^2) has a significant and positive effect. This reflects a non-linear relationship between time and ethanol quantity, where time (Time^2, B^2) in square form has a greater impact on changes in ethanol quantity than linear changes in concentration or power. Over longer periods, the effect of time is amplified (synergistic effect), resulting in a dramatic improvement in reaction efficiency. Finally, the factor AB (Concentration X Time), with a coefficient of +0.50, represents the most significant interaction effect. The positive coefficient (+0.50) indicates that the concentration and time factors have a synergistic effect. Therefore, it can be assessed that increasing concentration (A) will yield significantly better results only when combined with increasing time (B). If only one factor is increased, the response will not be as high as when both factors are increased simultaneously.

$$Ethanol = 11.78376 - (0.27673)A - (0.65219)B + (0.61848)C + (0.00662)AB + (0.00229)AC + (0.00391)BC + (0.00235)A^2 + (0.01056)B^2 - (0.01455)C^2 \quad (3)$$

The regression equation in actual factors evaluates real-unit effects on ethanol yield, with the quadratic power rate (C^2 = -0.014550) exerting the primary influence through a parabolic trend, where yield reaches a distinct peak before declining under excessive power. Conversely, the positive quadratic time term (B^2 = +0.010561) exhibits an accelerating effect, indicating that extending duration toward the later stages yields a greater increase in ethanol production than early-stage extensions. Furthermore, the positive interaction between concentration and time (AB = +0.006617) reveals a synergistic effect, demonstrating that simultaneously increasing both variables optimizes process efficiency more effectively than adjusting either parameter independently.

2.4 *Simultaneous saccharification and fermentation (SSF) procedure*

The bioconversion of pretreated sugarcane leaves into bioethanol was performed using an integrated SSF configuration, a strategy deliberately selected to mitigate product inhibition by continuously removing fermentable sugars and to streamline the overall production process. For the enzymatic preparation phase, commercial cellulase (Cellic®CTec2), supplied by Sigma-Aldrich Co. Ltd., served as the primary biocatalyst responsible for cellulose degradation. The filter paper activity of this enzyme cocktail was determined to be 278.51 FPU/mL, established via the standard filter paper assay in conjunction with the 3,5-dinitrosalicylic acid (DNS) colorimetric method [13]. In order to systematically investigate the direct impact of enzyme dosage on the final ethanol yield, two distinct enzyme loadings, specifically 25 and 50 FPU per gram of dry biomass, were evaluated within the system. The subsequent fermentation procedure was conducted utilizing *Saccharomyces cerevisiae* Type II at a consistent initial inoculation density of 1.0 g/L. To rigorously evaluate the efficiency of the optimized pretreatment method, the SSF process was executed at a 10% (w/v) solid loading, which utilized 1.0 g of the pretreated sugarcane leaves substrate submerged in a 10 mL total working volume.

To facilitate rapid yeast acclimatization and ensure robust microbial activity throughout the cycle, the fermentation medium was supplemented with 1% (w/v) D-glucose and 1% (w/v) yeast extract to serve as vital initial carbon and nitrogen nutrient sources. Regarding process optimization and environmental controls, the initial pH of the reaction system was precisely maintained at 5.0—representing the optimal compromise for both cellulase hydrolysis and yeast viability—by employing a 0.05 M sodium acetate buffer prepared from 99.8% glacial acetic acid and 99% sodium acetate hydrate. The compiled reaction mixtures were incubated inside a shaking incubator operated at a constant speed of 150 rpm to maintain homogeneous mass transfer and substrate-enzyme contact. To identify the definitive optimal operational parameters required to achieve the maximum ethanol titer, a multi-variable range of experimental conditions was comprehensively investigated, which involved the shifting of enzyme loadings between 25 and 50 FPU/g-biomass, the variation of incubation temperatures across 32, 35, and 37 °C, and the tracking of fermentation periods at 24, 48, and 72 hours. All experimental runs and conditions in this study were performed in triplicate to ensure strict statistical reliability, and the resulting fermented broth from each vessel was subsequently collected and analyzed to quantify the final ethanol concentration.

2.5 *Analytical methods and ethanol quantification*

Upon completion of the fermentation, the fermented broth was centrifuged at 6,000 rpm for 5 minutes to remove yeast cells and solid residues. The ethanol concentration in the resulting supernatant was quantified using a modified dichromate-oxidation spectrophotometric method coupled with solvent extraction to ensure high precision [14]. Briefly, ethanol was selectively extracted from the aqueous phase using tri-n-butyl phosphate (TBP). Following phase separation via centrifugation, the ethanol-rich upper TBP layer was reacted with an acidic potassium dichromate ($K_2Cr_2O_7$) reagent. The oxidation of ethanol prompted a distinct colorimetric shift to blue-green, indicative of the formation of chromium (III) ions. The absorbance of the reaction product was measured at 595 nm using a UV/Vis spectrophotometer. All measurements were performed in triplicate to ensure statistical accuracy.

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2.6 *Fermentation configurations: SSF and two-stage SSF*

To evaluate and compare the dynamic profiles of bioethanol production, the enzymatic hydrolysis and fermentation processes were carried out utilizing two distinct operational configurations, namely conventional SSF and two-stage SSF, where both systems were executed in identical reactors with a final working volume of 10 mL. For the conventional SSF configuration, the pretreated solid residue was mixed thoroughly with the commercial cellulase cocktail and immediately inoculated with *Saccharomyces cerevisiae* at the very beginning of the process, allowing the entire integrated system to operate continuously at a fixed compromise temperature of 32 °C for 48 hours to ensure immediate sugar assimilation by the active yeast cells.

Conversely, the two-stage SSF configuration was temporally decoupled into two sequential phases, beginning with an initial pre-saccharification or liquefaction stage conducted at the enzyme's thermodynamic optimum temperature of 50 °C for 24 hours without the presence of yeast cells in order to trigger rapid biomass depolymerization and effectively reduce the physical viscosity of the high-solid slurry. Upon completion of this pre-hydrolysis period, the core temperature of the partially liquefied broth was decreased to 32°C for 48 hours, and the system was subsequently inoculated with *Saccharomyces cerevisiae* to initiate the concurrent saccharification and fermentation stage, which was sustained for the remaining duration of the total cultivation timeline. Throughout the fermentation period of both configurations, broth samples were collected at periodic intervals and subjected to centrifugation, after which the resulting supernatants were collected to investigate the sugar consumption profiles and determine the exact bioethanol concentrations according to the analytical framework and chromatographic protocol [14].

3 Results and Discussion

3.1 *Comparative study of SSF process conditions*

To maximize the bioethanol yield from the pretreated sugarcane leaves, the operational parameters of the SSF process were systematically investigated. The experimental variables evaluated included enzyme loadings (25 and 50 FPU/g-biomass), incubation periods (1 to 3 days), and fermentation temperatures (32 to 37°C). The experimental profiles (Figure 1) revealed that the highest bioethanol concentration of 12.602 mg/mL was achieved under the condition of 50 FPU/g-biomass at 32 °C after a 3-day (72 h) incubation period. Notably, a 2-day (48 h) fermentation under identical conditions (50 FPU/g-biomass at 32 °C) produced a competitive ethanol concentration of 12.496 mg/mL, representing a negligible statistical difference compared to the 3-day yield. Driven by the objective of maximizing process energy efficiency and reducing operational costs for future industrial scale-up, the 2-day incubation period was selected as the baseline condition for further optimization across the 17 RSM experimental runs. This finding strongly aligns with previous work [9], which evaluated bioethanol production from oil palm empty fruit bunches (EFB) via SSF. Their study demonstrated that higher enzyme loadings (up to 40 FPU/g-biomass) drastically accelerated sugar conversion, whereas insufficient loadings (e.g., 10 FPU/g-biomass) severely limited cellulase reaction rates, failing to provide adequate glucose for yeast metabolism. Furthermore, the optimal fermentation efficiency in [9] peaked at 48 hours, beyond which ethanol yields plateaued or marginally decreased, further corroborating the efficiency of our selected 48 h operational window.

Regarding temperature optimization, the effects of thermal variation (32 to 37°C) on SSF performance were systematically monitored (Figure 1). The empirical data identified 32°C as the optimum temperature, yielding 12.496 mg/mL of bioethanol. To further highlight the superiority of the integrated system, a comparison was made with the conventional SHF method. Under identical biomass inputs, the SSF process outpaced the SHF method (which yielded 8.664 mg/mL at 30 °C) by 30.7%. The consolidation of enzymatic saccharification and microbial fermentation into a single bioreactor vessel significantly reduces both processing time and overall utility demands. In conventional SHF configurations, enzymatic hydrolysis typically necessitates elevated temperatures of approximately 50 °C for extended periods before the slurry is cooled to 30–32 °C to sustain yeast viability during the subsequent fermentation phase [15]. For instance, a previous study [14] reported an ethanol titer of 12 mg/mL using SHF approach that required 3 days of saccharification at 50 °C followed by 2 days of fermentation at 30 °C, totaling 5 days operational cycle. This extended

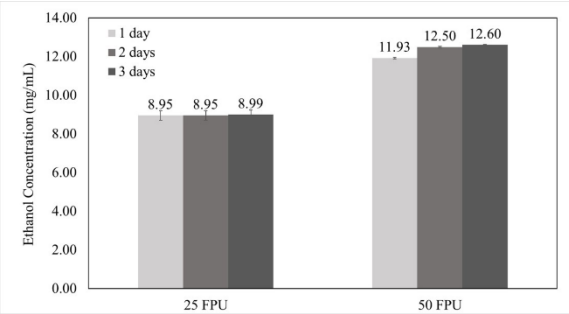


Figure 1: Ethanol production from SSF process. The following variables were selected: 1.) FPU consisting of 25 and 50 FPU, 2.) Incubation time ranging from 1 to 3 days.

timeline imposes a substantial energy burden on industrial operations. The economic and energy-saving benefits of transitioning from SHF to SSF are further supported by process simulation data. An Aspen Plus simulation framework conducted for commercial-scale bioethanol production upgrades [16] revealed that the independent hydrolysis unit operation was the most energy-intensive stage, consuming 154,008 kWh of electricity and accounting for 85.98% of the total utility costs (approximately 6,160 USD), primarily due to the energetic demand of maintaining a constant 50°C temperature for 72 hours.

Consequently, the low-temperature (32°C) integrated SSF strategy developed in this study provides an effective benchmark and guideline for heavy industry to minimize thermal maintenance costs, decrease total utility footprint, and substantially lower overall bioethanol production costs. From the experimental results to find the optimal conditions for SSF, the following variables were selected: 1.) FPU consisting of 25 and 50 FPU, 2.) Incubation time ranging from 1 to 3 days, and 3.) Temperature ranging from 32 to 37 °C. These were used to test and determine the optimal conditions for fermenting ethanol from pretreated biomass to obtain the highest ethanol yield. This test revealed that the most effective SSF condition for ethanol fermentation was 50 FPU, 3 days at 32°C, achieving the highest ethanol yield of 12.602±0.04 mg/mL. However, the 50 FPU, 2-day fermentation at 32°C condition resulted in an ethanol yield of 12.496±0.04 mg/mL. All experimental runs were performed in duplicate, which was similar to the 3-day fermentation period (no statistically significant difference). Driven by the objective of maximizing process energy efficiency and reducing operational costs for future industrial scale-up, the 48-hour incubation period and optimal enzyme loading were selected as baseline conditions for further multi-variable optimization across 17 experimental runs via RSM. This rational design aligns with previous findings on bioethanol production from oil palm EFB via SSF, which demonstrated that an elevated enzyme loading of 40 FPU/g-biomass was required to maximize ethanol yields (up to 6.05%) and that fermentation efficiency peaked at 48 hours before plateauing [14]. These findings strongly support our selection of a 50 FPU/g enzyme loading and a 48-h operational window to optimize bioethanol productivity and energy utilization.

To determine the optimal temperature for ethanol fermentation using the SSF method, temperatures were varied from 32 to 37°C, with the ethanol yield shown in the graph above. Furthermore, a comparison was made with the SHF method (Figure 2). The optimal temperature for SSF fermentation was found to be 32°C, yielding an ethanol production of 12.496 mg/mL, compared to 8.664 mg/mL for SHF at 30°C – a difference of 30.7%. Therefore, the optimal temperature for fermentation, selected from the 17 conditions tested using the RSM, is 32°C. Combining the enzyme and yeast processes into a single reactor not only saves time but also reduces operating costs and overall energy consumption. Therefore, the SSF process is conducted at a temperature of 32°C. This is the same temperature throughout the digestion and fermentation process, unlike the SHF process, which typically requires temperatures as high as 50°C for enzymatic digestion and then lowered to 32°C for yeast activity in the fermentation phase [9]. In comparison with previous studies, a bioethanol concentration of 12 mg/mL was reported using the SHF method under enzymatic saccharification conditions of 50 FPU/g at 50 °C for 3 days, followed by a 2-day fermentation phase at 30 °C [14]. This conventional configuration requires a prolonged operational cycle of 5 days, imposing a substantial thermal and energy burden. To evaluate the economic impact of such extended processing, an Aspen Plus process simulation for commercial-scale bioethanol production revealed that the independent hydrolysis unit was the most energy-intensive operation, consuming 154,008 kWh of electricity and accounting for 85.98% of the total utility costs (approximately 6,160 USD), primarily due to the energy required to maintain a constant temperature of 50 °C for 72 hours [15]. Consequently, the low-temperature integrated SSF strategy developed in this study provides a viable framework to minimize thermal maintenance demands, shorten overall processing duration, and substantially lower bioethanol production costs.

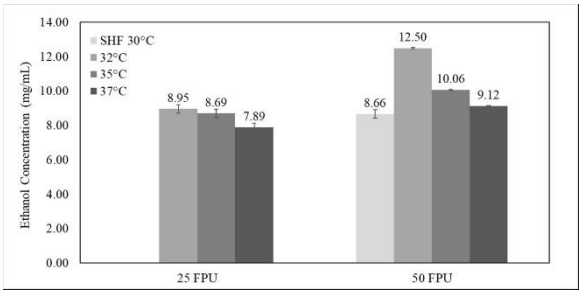


Figure 2: Ethanol production from SSF process. FPU consisting of 25 and 50 FPU, temperature ranging from 32 to 37 °C.

3.2 *Experimental findings to determine optimum SSF conditions*

Figure 3 is a 3D response surface graph showing the relationship between variable A, which is the concentration of FA (%), and variable B, which is time (min). This graph is consistent with the coefficient from the final equation in terms of coded factors (Equation 3) in front of the time variable (Time, B^2), which is equal to +0.59. The surface is curved upwards (saddle-like or concave up), indicating that over time, the effect of time exponentially accelerates the increase of ethanol. The power rate (C^2) coefficient is -1.05 (maximum influence), having a negative effect and forming a concave curve. The surface is curved upwards (saddle-like or concave up), indicating that over time, the effect of time exponentially accelerates the increase of ethanol. The power rate (C^2) coefficient is -1.05 (maximum influence), having a negative effect and forming a concave curve. This means that increasing the power to a certain point will yield the best results, but if it is too high, the ethanol content will decrease rapidly. The *AB* Interaction coefficient is +0.50, indicating a synergistic effect between concentration and time. Therefore, this graph shows the response from the variable that has the greatest influence on ethanol yield: FA concentration at 30% and a time of 30 minutes, with a constant power rate of 24.5W. This can be observed from the dark red or green areas in the corners of the graph, which indicate the highest ethanol yield. Increasing only one factor alone cannot achieve the same ethanol yield as increasing both factors simultaneously. Therefore, after studying the relationship between the variables, the predicted optimal condition can be obtained from the program. The predicted optimal conditions are an FA concentration of 29.17%, a time of 18.70 minutes, and a power rate of 16.61W (Table 3).

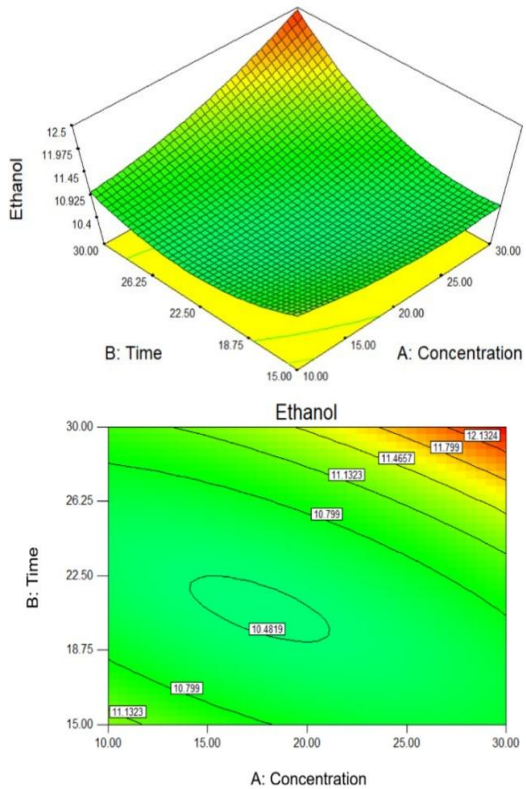


Figure 3: Response surface and contour plots for ethanol production as a function of substrate concentration (A) and fermentation time (B) at a constant power rate.

Table 3: Predicted optimal conditions.

Parameter	Optimized Value
FA Concentration (%)	29.17
Reaction Time (min)	18.7
Ultrasonic Power Rate (W)	16.61

The Figure 4 shows a 3D surface graph illustrating the relationship between the concentration of FA, A, and the power rate (W), C. This graph clearly shows the curvature of the curve, which is dome-shaped or inverted (parabolic shape). Along the power rate (C) axis, it can be seen that the peak of the dome, representing the optimum point, is in the range of approximately 24.50 - 28.75 W. Therefore, as the power is increased initially, the amount of ethanol will increase, as shown in the dark green area of the contour plot. However, if the power is increased beyond 28.75 W, the amount of ethanol will tend to decrease.

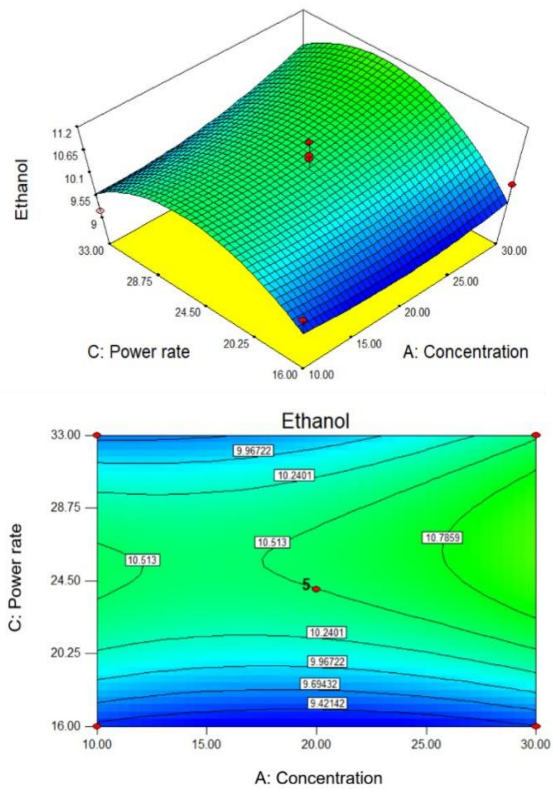


Figure 4: 3D response surface and 2D contour plots illustrating the interaction effect between FA concentration (A) and power rate (C) on ethanol yield.

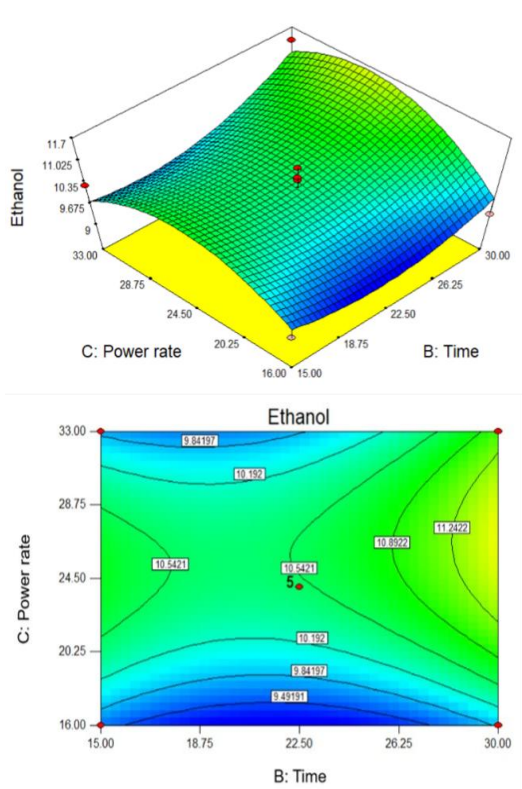


Figure 5: Response surface (3D) and contour plots showing the interaction effect between fermentation time (B) and power rate (C) on ethanol yield.

Figure 5 shows a 3D surface graph illustrating the relationship between time (B) and power rate (C). The graph has a curvature, or a peak in the middle. From the graph, it can be seen that the optimal ethanol output is in the power range of approximately 24-28 W, which is the optimal point (optimization). The contour plot shows red dots and dark yellow/red areas representing regions with the highest ethanol content (approximately greater than 11.18). A detailed analysis reveals that if the power consumption is too low (near 16) or too high (near 33), the ethanol content decreases. As time increases (from 15 to 30 minutes), the ethanol content tends to increase significantly, as evidenced by the upward trend on the right-hand side of the graph (bright green to yellow). This indicates that time is a positive factor directly affecting sugarcane leaf conditioning to promote ethanol production under these conditions.

3.3 *Bioconversion efficiency and bioethanol production from ultrasonication-sulfonation pretreated biomass*

The optimized response surface methodology conditions were 29.17% FA, 18.70 min pretreatment time, and 16.61 W ultrasonic power. Based on these conditions, this study evaluated the potential of ultrasonication-sulfonation pretreated sugarcane leaves for bioethanol production. The effects of the pretreatment on sugar yield and subsequent yeast fermentation are summarized in Figure 6. Although the response contour and surface plots showed a high ethanol-yield region at higher ultrasonic power levels (20-30 W), the numerical optimum was obtained by simultaneously considering the interactions among FA concentration, pretreatment time, and ultrasonic power. Thus, the predicted optimum represents a balanced condition rather than only the visually highest region in the response plots. Experimental validation under these conditions confirmed a high ethanol yield, supporting the suitability of the selected optimum for SSF bioethanol production.

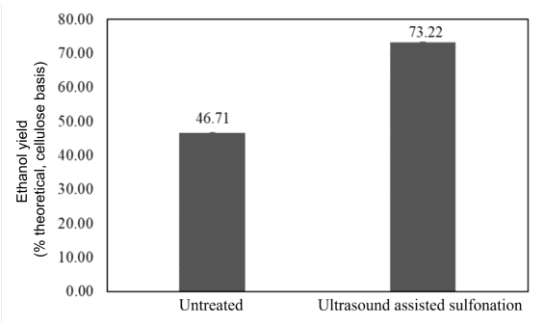


Figure 6: Ethanol yield (% theoretical, cellulose basis) obtained from untreated biomass compared with biomass pretreated via ultrasonic sulfonation.

As illustrated in Figure 6, the results for ethanol yield (% theoretical, cellulose basis) indicate a significant enhancement in the sugar release potential for the pretreated samples. Specifically, the low power rate sample, which represents the optimized conditions including ultrasonication, achieved a yield of 73.22%. This is a dramatic improvement over the untreated biomass, which yielded only 46.71%. These findings indicate a high initial reducing sugar concentration under optimized pretreated conditions, aligning with the initial target of 39.58 mg/mL, and supporting the observation that the cellular matrix was successfully broken down by subsequent cellulase hydrolysis into disaccharides (cellobiose) and monosaccharides, including hexose (C_6) and pentose (C_5) sugars, which are readily fermentable by yeast. During the SSF process, this increased sugar yield resulted in enhanced microbial activity. The yeast was able to utilize the higher carbohydrate concentration effectively, corresponding to the previously determined total reducing sugar consumption of 30.77 mg/mL. The overall fermentation efficiency was notably high, with an obtained ethanol yield of 18.7 mg/g of pretreated biomass and an ethanol yield from reducing sugar of 29.56%. The improved values in Figure 6 for the pretreated biomass demonstrate that the open structure created by ultrasonication facilitates sulfonation pretreatment, where sulfonate acid groups can readily bind with pseudo-lignin [12]. This prevents enzyme interference and enhances yeast activity, thus enabling higher bioethanol titers during the SSF process.

This substantial structural disruption, implied by the increased sugar yield in Figure 6 [17], is attributed to ultrasound waves inducing acoustic cavitation, the formation and violent collapse of microbubbles. This phenomenon generates localized high-temperature and high-pressure hot spots within the liquid matrix, physically severing the chemical bonds linking lignin, cellulose, and hemicellulose. This effect loosens the rigid biomass structure, allowing chemical agents to penetrate deeper and react more effectively. The observation [15] demonstrated the efficacy of sonication-assisted pretreatment, showing that optimized acoustic conditioning significantly enhances the release of monosaccharides, yielding up to 32.19 g/L of glucose from lignocellulosic residues. Consequently, the distinct improvement shown in Figure 6 confirms that the combination of ultrasonication with sulfonation pretreatment effectively destroys the complex structural barriers of sugarcane leaves and maximizes downstream fermentation efficiency.

To further evaluate this efficiency, the assumption of a 36% initial cellulose content for sugarcane biomass was established, which is highly supported by recent compositional analyses of various sugarcane fractions. Research evaluating the chemical composition of untreated sugarcane biomass reveals that different parts of the plant possess consistent cellulose levels clustered around this value. For instance, the analysis of sugarcane tops (SCT) demonstrates an initial cellulose content of 35.0% [18]. Similarly, another study examining different fractions of sugarcane found comparable cellulose values: 36.4% in the nodes, 37.4% in the leaves, 37.5% in the external fractions, and 37.7% in the leaves [19]. By correlating the 35.0% cellulose content found in sugarcane tops with the 36.4%–37.7% range found in the leaves, nodes, and leaves, adopting an average baseline of 36% initial cellulose content is a statistically sound and representative value for heterogeneous sugarcane residues.

Furthermore, this baseline of 36% initial cellulose content was utilized to evaluate the fermentation efficiency by calculating the theoretical ethanol yield. By converting the potential glucose content derived from this cellulose baseline—assuming complete stoichiometric conversion where 1 g of cellulose yields 1.11 g of glucose, and subsequent fermentation yields 0.51 g of ethanol per gram of glucose the maximum theoretical ethanol yield was established. The actual ethanol yield obtained from the simultaneous saccharification and fermentation

process was then compared against this theoretical maximum to precisely determine the percentage of theoretical ethanol yield achieved under the optimized ultrasonication-sulfonation pretreatment, serving as a key indicator of the process's overall economic and technical viability. While full NREL compositional tracking across every pretreated fraction fell beyond the primary scope of this ultrasonication-focused investigation, the precise chemical mechanisms and compositional fates of cellulose, hemicellulose, and lignin under this mild sulfonation system are strongly validated by our previous NREL compositional study on sugarcane leaves [16]. In that work, standard NREL protocols demonstrated that mild sulfonation effectively drives extensive hemicellulose solubilization and partial delignification, yielding a solid residue highly enriched in accessible cellulose. In the present study, combining this chemical action with acoustic cavitation further synergizes biomass deconstruction—effectively severing the recalcitrant LCC matrix, exposing glucan chains for cellulase action, and mitigating non-productive enzyme adsorption onto residual lignin. Consequently, these structural and chemical alterations directly translate into the enhanced sugar release and superior bioethanol titers observed.

The physical and morphological alterations of the sugarcane leaves induced by the optimized pretreatment configuration were systematically investigated via Scanning Electron Microscopy (SEM), as illustrated in Figure 7. The SEM micrograph of the untreated biomass (Figure 7a) exhibited a highly intact, rigid, and ordered surface structure with dense fibrils packed in a well-defined parallel alignment, which restricts enzyme accessibility to the internal cellulose fibers. Conversely, when the biomass was subjected to the chemical-mechanical co-solvent pretreatment combining sulfonation and ultrasonication (Figure 7b), the microscopic view revealed a significantly high density of micro-voids, severe perforations, and extensively fragmented particles of relatively small sizes. This structural disruption is attributed to the intense localized shear forces generated by acoustic cavitation along with the chemical action of the co-solvent system, which thoroughly shattered the cellular matrix and dramatically enhanced the surface area available for subsequent enzymatic attack.

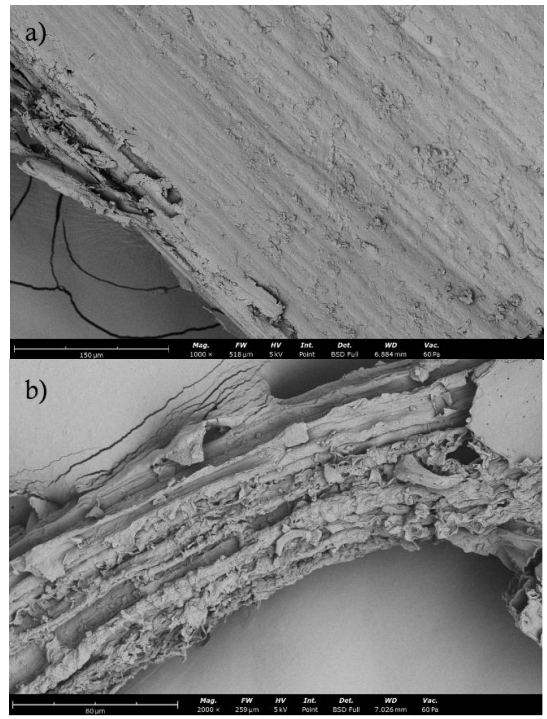


Figure 7: SEM images of sugarcane leaves: (a) untreated biomass, (b) pretreated biomass with ultrasonication and sulfonation.

3.4 *Effect of SSF and two stage SSF*

Evaluation of the overall process performance showed that conventional SSF and two-stage SSF achieved comparable final ethanol titers and overall sugar consumption at the end of fermentation. No statistically significant difference was observed in the final ethanol yields obtained from the two configurations, indicating comparable conversion of the available fermentable carbohydrates. Under the investigated solid loading and enzyme dosage, both configurations therefore exhibited similar overall fermentation performance and substrate utilization. These findings suggest that the principal advantage of the two-stage configuration may not lie in improving the final ethanol yield, but rather in providing operational flexibility for initial sugar observation.

In conventional SSF, initial enzymatic hydrolysis proceeds at 32°C, where cellulase activity is lower than its thermal optimum [20]. In contrast, the brief high-temperature pre-saccharification phase (50°C for 24 h) in the two-stage system allows for rapid initial sugar liberation prior to yeast inoculation. Crucially, since no enzyme inactivation was performed after the first stage, the active enzymes continued to synergistically

hydrolyze the remaining biomass alongside the yeast during the subsequent fermentation phase at 32°C. This approach successfully facilitated initial sugar liberation without altering the ultimate ethanol yield of the combined yeast and enzyme system.

4 Conclusion

This study demonstrates the efficacy of integrating low-power ultrasonication with an MSA–FA co-solvent system for mild pretreatment of sugarcane leaves to enhance bioethanol production. The RSM-optimized pretreatment conditions—29.17% FA, 5% MSA, 18.70 min ultrasonication time, and 16.61 W ultrasound power—effectively disrupted the recalcitrant lignocellulosic structure. Acoustic cavitation, coupled with the action of the acid system, solubilized hemicellulose, removed lignin/pseudo-lignin, and enhanced cellulose accessibility for enzymatic attack. Subsequent SSF using *Saccharomyces cerevisiae* at 32°C with 50 FPU/g enzyme loading achieved an ethanol yield of 73.22% (% theoretical, cellulose basis) within 48 h, corresponding to an ethanol concentration of 12.496 mg/mL. Extending the fermentation to 72 h yielded only an additional 0.8%, confirming that the 48 h process is techno-economically optimal by avoiding unnecessary energy consumption and processing costs. Furthermore, SSF offered performance comparable to SHF while eliminating the high-temperature hydrolysis step (50 °C) and reducing total process time. Overall, ultrasound-assisted MSA–FA pretreatment represents a viable, time-efficient strategy for converting sugarcane residues into bioethanol, with techno-economic assessments and pilot-scale validation recommended for future work.

Acknowledgements

This research budget was allocated by National Science and Innovation Fund (NSRF), and King Mongkut's University of Technology North Bangkok, (Project no. KMUTNB-FF-69-B-26.)

Author Contributions

A. K.: Investigation, methodology, data curation, formal analysis, and writing – original draft. J. K.: Investigation, data curation, and validation. K. K.: Methodology and writing – review and editing. A. T.: Conceptualization, methodology, investigation, formal analysis, supervision, funding acquisition, and writing – review and editing. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors utilized the Gemini tool to enhance the language and readability of the manuscript.

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