The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
20(3): 750-760, 2025 www.thebioscan.com

Optimization of Lignocellulosic Waste Pretreatment for Bioethanol Production Using Response Surface Methodology

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DOI: 10.63001/tbs.2025.v20.i03.pp750-760

Received on:

04-08-2025

Accepted on:

08-09-2025

Published on:

09-10-2025

ABSTRACT

The increasing energy demands and depletion of fossil fuel reserves necessitate advancing sustainable alternatives like second-generation bioethanol derived from lignocellulosic biomass. This study explores the optimization of various pre-treatment methods and fermentation parameters to enhance bioethanol yield from agricultural, vegetable, fruit, and mixed lignocellulosic wastes. Five chemical pre-treatment methods were evaluated for their effectiveness in cellulose and hemicellulose breakdown, including sulfuric acid, kraft lignin, alkaline peroxide, organosolv, and soda lignin. Sulfuric acid and organosolv treatments showed superior performance in reducing sugar release. Enzymatic hydrolysis using *Trichoderma reesei* and *Aspergillus niger* further enhanced saccharification, with *A. niger* demonstrating significantly higher cellulase activities. Co-fermentation with *Saccharomyces cerevisiae* was optimized using Response Surface Methodology (RSM), considering inoculum size, temperature, pH, and time. The highest ethanol yield—8.18% and 64.57 g/L—was obtained from organosolv pretreated mixed biomass under optimized conditions. These findings underscore the potential of integrating statistical optimization and advanced pre-treatment for efficient and scalable bioethanol production from waste biomass.

INTRODUCTION

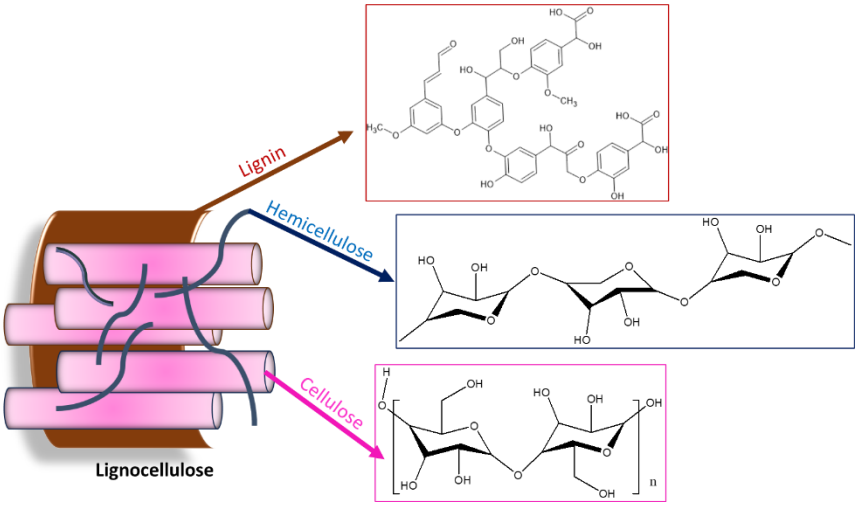
The substantial dependence on fossil fuels, which constitutes approximately 80% of global energy consumption, presents a formidable challenge. Traditionally, fossil fuels have been viewed as the primary energy source. However, their availability is projected to decline with rising energy demands. Sustainable biofuels are recognized as carbon neutral (Singh *et al.*, 2022; Gude & Martinez-Guerra, 2018). Addressing the energy crisis and emissions concurrently necessitates the development of a cost-effective and environmentally sustainable energy production framework. Recent studies indicate that lignocellulosic biomass is a viable renewable energy alternative (Chakraborty *et al.*, 2019; Jambo *et al.*, 2016; Karimi *et al.*, 2019). Converting biomass into energy or fuels reduces waste and offers a renewable, cost-efficient solution (Sampath *et al.*, 2020). About 84 million barrels of crude oil are utilised daily in the energy and transportation sector, which is projected to increase to 116 million barrels by 2030 (Ahorsu *et al.*, 2018).

Bioethanol is classified into four generations based on feedstock: first-generation (food crops), second-generation (lignocellulosic biomass like forest waste, agricultural waste, industrial waste, municipal waste, kitchen waste, APMC yard waste) (Anwar *et al.*, 2014)), third-generation (algae), and fourth-generation (genetically modified algae or crops) (Dandasena & Shahi, 2023) (Devi *et al.*, 2023) (Jain & Kumar, 2024) (Khairati, 2024). The second generation of biofuels from lignocellulosic biomass is deemed superior due to its abundance and sustainability (Moshood *et al.*, 2021). Nonetheless, lignocellulosic biomass poses significant challenges during deconstruction to produce intermediates like glucose and xylose for bioethanol conversion (Kavitha *et al.*, 2020). Lignocellulosic biomass comprises cellulose, hemicellulose, lignin, and minor components such as pectin, protein, and minerals. Cellulose and hemicellulose account for 70–80% of dry weight, while lignin represents 10–25%. Lignin's recalcitrance necessitates specific pre-treatment to hydrolyse cellulose and hemicellulose into sugars (Liguori & Faraco, 2016).

The pre-treatment of lignocellulosic waste is essential for augmenting the efficiency and sustainability of bioethanol production (Ferreira *et al.*, 2009). Implementing effective pre-treatment strategies, including acid and alkali treatments, as well as advanced methodologies such as organosolv, kraft lignin, and alkaline peroxidase, can markedly enhance the accessibility of cellulose and hemicellulose for enzymatic hydrolysis to improve the enzymatic saccharification, fermentation, and anaerobic digestion process, thereby influencing the structural integrity of the biomass and significantly improving bioethanol production through the enhanced degradation of lignocellulosic biomass (Pereira *et al.*, 2021).

Optimizing these pre-treatment conditions, it is possible to maximize bioethanol yields and improve the overall efficiency of the production process. In particular, Response Surface Methodology (RSM) allows for systematic optimization of multiple variables. Additionally, RSM can help identify the optimal conditions for pre-treatment, such as temperature, time, and chemical concentration, enhance the efficiency of bioethanol production, and contribute to the sustainable utilisation of agricultural waste resources. This systematic approach is vital for addressing the challenges of lignocellulosic waste management and biofuel production.

Figure 1:Representation of lignocellulosic biomass structure



This study intended to adopt statistical frameworks to boost total reducing sugar concentration during agricultural, mixed, vegetable and fruit waste biomass pretreatment and enzymatic hydrolysis to support increased bioethanol production. Central composite design within response surface methodology was utilized to investigate the optimization of pretreatment and hydrolysis processes, followed by co-fermentation with S. cerevisiae. Compositional variations between untreated and pretreated lignocellulosic biomass were analysed using various chemical techniques. The characterization of pretreated biomass for lignin, hemicelluloses, and cellulose degradation through analytical methods including FTIR and SEM. Findings indicate that lignocellulosic biomass holds potential as a viable substrate for bioethanol production.

1. Methodology:

1.1. Collection of lignocellulosic biomasses:

Agricultural waste (AW), including rice straw, wheat straw, rice husk, corn cobs, and maize stems, was procured from the Agricultural Produce Market Committee (APMC) located in Bangalore. In contrast, fruit waste (FW) encompassing banana peels, Amla, avocado peels, and various discarded fruits such as papaya, mosambi, watermelon peels, pineapple peels, orange peels, apple peels, and black grapes were sourced from multiple fruit juice centres throughout Bangalore, alongside vegetable waste (VW) comprising peppermint, outer cabbage leaves, cucumber peels, ridge gourd, snake gourd, tomato peels, aubergine, cauliflower, onion, beetroot, peppers, sweet potato, apple gourd, tora, yam, and elephant foot yam collected from multiple locations within Bangalore. The collected organic wastes were placed in transparent plastic bags, cleaned, dried, and milled to a 2–3 mm particle size before being homogenized in a 1:1 ratio and subsequently stored in hermetically sealed containers for subsequent experimental applications.

1.2. Compositional analysis of untreated lignocellulosic biomass:

The gathered raw lignocellulosic residues were analysed to quantify lignin, cellulose, and hemicellulose content. The composition of NREL corn stover was assessed by our laboratory in conjunction with the National Renewable Energy Laboratory, following the standardized protocols established by NREL (NREL, 2005a, 2005b, 2008a, 2008c). The biomass was dried in an oven at 60 ℃ for 6 hours to eliminate any residual moisture. All experimental procedures were conducted in triplicate, and the resultant values were reported as means.

1.3. Various pretreatment methodologies for biomass:

1.3.1. Chemical pretreatment and enzymatic hydrolysis:

The pretreatment of lignocellulosic biomass encompassed chemical pretreatments utilizing agents such as H_2SO_4, as well as enzymatic hydrolysis employing *Trichoderma reesei*, in addition to Kraft lignin, alkaline peroxide, organosolv, and soda lignin methods with minor modifications to the established conditions. the pretreatment involved varying concentrations of H_2SO_4 (1 - 5%, v/v) with a 10% (w/v) solid biomass. Hydrolysis was performed in an autoclave at 121 ˚C for intervals of 15 to 45 minutes. The hydrolysate was obtained by filtering through double-layered muslin cloth. The pH was measured with a pH meter, followed by washing the residue with distilled water until neutral, and drying at 60 ˚C overnight. The 3,5-dinitrosalicylic acid (DNSA) method assessed the changes in reducing sugar yield of the pretreated samples (Reese *et al.*, *1963*).

1.3.2. Kraft lignin method

This method involved a 10 g biomass pulping with white liquor (1:10) at 100 ℃ for 4 h, then cooling and filtering to separate holocellulose from lignin (Demuner *et al.*, 2019). The holocellulose was washed to neutral pH and dried at 50 to 60 ℃ for 3–5 days. Post-drying, the holocellulose weight was recorded for documentation.

1.3.3. Alkaline Peroxide method

A 100 ml solution of 2% NaOH and H_2O_2 (9:1) was combined with 10 g of ground biomass at a 1:10 ratio. The mixture was boiled for 3 h, cooled, and pH adjusted to 7.0 using concentrated H_2SO_4 (Tandon & Sharma, 2018). Filtration was performed to isolate holocellulose, which was air-dried and then dried at 60˚C to achieve a consistent weight.

1.3.4. Organosolv method

This study involved delignifying 10 g of biomass using peroxy-formic and peroxy-acetic acids, pulped with an 85% organic acid mix (FA/AA 70:30 v/v) for 4 h, followed by H_2O_2 bleaching and holocellulose isolation (Sharma & Sharma, 2023; Watkins *et al.*, 2015). The process was repeated for complete lignin removal from the biomass, followed by rinsing and drying at 60˚C until a stable weight was reached.

1.3.5. Soda lignin method

10 g of biomass was treated with 100 ml of 15% NaOH at 100 ℃ for 2 hours as per Kumar *et al.*, (2018b). The black liquor was filtered to isolate fibrous components, and holocellulose was washed to neutral pH and dried at 60℃ overnight. The Organosolv method, yielding the highest lignocellulosic biomass, was selected for subsequent experiments.

1.4. Enzymatic saccharification of lignocellulosic biomass

This investigation examined fungal cellulase-producing isolates. Cultivation occurred in a nutrient broth at a controlled temperature for 24 hours. Optical density was standardized to 1.0 using sterile water. A 50 ml inoculum was added to 450 ml of cellulose-containing Riviere's broth in 1000 ml Erlenmeyer flasks, with incubation conditions maintained for 72 hours. Following incubation, centrifugation was performed to isolate the supernatant for enzyme activity assays. Various qualitative and

quantitative analyses were executed, including cellulase and reducing sugars assessments.

1.5. Enzymatic hydrolysis of lignocellulosic biomass residue

For autoclaving, one gram of pretreated lignocellulosic biomass was combined with sodium citrate buffer in Erlenmeyer flasks. Under sterile conditions, crude enzyme samples were added to the flasks. Incubation occurred at an elevated temperature with continuous agitation for 72 hours. Post-incubation, the saccharified biomass was filtered and centrifuged to retrieve the hydrolysate for analysis. Reducing sugars were quantified using the Miller (1959) method, complemented by protein assays per Lowry et al. (1951).

1.6. Bioethanol fermentation

Lignocellulosic hydrolysate was utilized for fermentation with *Saccharomyces cerevisiae* sourced from MTCC Chandigarh, India. An inoculum of *S. cerevisiae* was prepared in a growth medium in a rotary shaker incubator for 24 hours to achieve a culture with 1 O.D. Untreated and organosolv pretreated agricultural and forestry supernatant received 0.5% yeast extract and 0.5% peptone, followed by autoclaving in a bioreactor. A 10% (1 O.D.) inoculum of *S. cerevisiae*-I and *P. stipitis* was introduced into the fermentation media and maintained at 25 °C for 72 hours. The fermentation occurred under anaerobic conditions, with the pH held at 6. The batch cultivation in the bioreactor was conducted at an agitation speed of 200 × g and an agitation rate of 0.05 vvm. Ethanol estimation was performed using the method by Caputi and Wright (1969), with O.D. measured at 600 nm against a blank, and bioethanol was quantified in g/L of fermented liquor.

1.7. Optimization of fermentation parameters by using the response surface methodology (RSM) approach

Bioethanol production conditions were optimised under SHF mode using the RSM method. Independent variables included A as inoculum size (i.e. 1, 2, 3, 4 and 5 %), B as fermentation duration (i.e. 24, 36, 48, 60, 72, 84 and 96 h), C as temperature (i.e. 20, 25, 30, 35, 40 and 45 ◦C), and D as pH (i.e. 1, 2, 3, 4 and 5 %), while dependent variables were ethanol yield in percentage and concentration in gram per liter—Central Composite Design with five factors at six levels assessed interactions among factors. The design consisted of thirty iterations, with variable ranges presented in **Table 5**. Mathematical dependencies were tested for ethanol production using enzymatically hydrolysed sugary syrup and *S. cerevisiae* co-culture, modelled with a quadratic equation and represented as means of triplicate experiments.

Statistical analysis was conducted using Design Expert® version 7.0 for regression analysis, generating working parameters, polynomials, and contour plots. A second-order polynomial was derived via analysis of variance, identifying optimal medium ratios through the software's optimization toolbox. Standard deviation and R_2 values were also evaluated during the experiment. Model validation was conducted through checkpoint studies, comparing experimental data with predictions to determine prediction error.

2. Results and discussion:

2.1. Compositional analysis of lignocellulosic biomass

Onokwai *et al.*, 2022 has explained that the average structural composition analysis showed that the cellulose, hemicellulose, lignin, ether extract, and corrosive values of the biomass species are25.32 ≤ 45.80%, 20.12 ≤ 30.94%, 19.44 ≤ 45.41%, 0.20 ≤ 0.46%, and 0.0001 ≤ 0.002% respectively. Similarly in the study by Nimmanterdwong *et al.*, 2021 had characterized for 149-biomass structural component, ultimate/proximate and conducted using the ASTM standard of D317, BS EN 15148, BS EN 14775 for ultimate/proximate analyses, and the NREL standard of TP51042618 for structural compositions. Whereas ultimate analysis data, cellulose (case 77, 115, 118, and 125) and hemicellulose (case 78, 114, 117, and 126) have C, H, and O contents of 40%, 6%, and 50%, respectively. In comparison, lignin (cases 79, 93, 119, and 127) has C, H, and O contents that account for approximately 50-58%, 4-6%, and 26-45%. But in this study concurred the results of compositional analysis of untreated agricultural waste (AW), vegetable waste (VW), fruit waste (FW) and mixed waste (MW) is shown in **Table 1** where cellulose shows 28 -49%, hemicellulose shows 19-31%, lignin shows 22-28% and ash content were 3-8%.

Table 1: Compositional analysis of untreated lignocellulosic biomass (wt.%).

Biomass	Cellulose	Hemicellulose	Lignin	Ash content
Agricultural waste Biomass	49.06±0.07	22.54±0.75	31.74±1.02	8.86
Vegetable waste Biomass	28.11±0.36	19.0±0.54	22.87±0.23	5.78
Fruit waste Biomass	32.98±0.42	31.21±0.65	28.65±0.47	4.56
Mixed waste Biomass	34.06±0.34	28.28±0.15	27.97±0.45	3.87

2.2. Pretreatment methodologies for different biomass:

In the current research, chemical pretreatments to analyse the reducing sugar from the pretreated agricultural, vegetable, fruit and mixed waste biomass, the lignocellulosic components shown in Figure 2 and Table 2 represent that sulfuric acid pretreatment consistently yields the highest reducing sugar content across all waste types, which is approximately 80-85%. Sodium hydroxide pretreatment is ineffective for vegetable waste (VW), 30- 35%, but performs moderately (~75-80%) for other wastes, and sodium chloride pretreatment is relatively consistent (~68-79%), showing comparable results to sulfuric acid in WM and FW. Similarly, Kumar *et al.*, 2009a and Peinemann & Pleissner, 2020 explain that the advantage is high solubility of hemicellulose and lignin in acids, which enhances glucose yield without needing subsequent enzymatic hydrolysis. Yu *et al.*, 2015 explain an alkaline liquid derived from the Kraft process, which enhances lignin removal and increases glucan and Xylan content. Raina *et al.*, (2024) elucidated that an energy-efficient alkaline hydrogen peroxide pretreatment of sugarcane bagasse, utilizing NaOH and H2O2 at a concentration of 5% (w/w), at a temperature of 25 °C for 24 h, followed by simultaneous saccharification and fermentation (SSF), resulted in a bioethanol yield increase of 0.101 kg bioethanol/kg biomass.

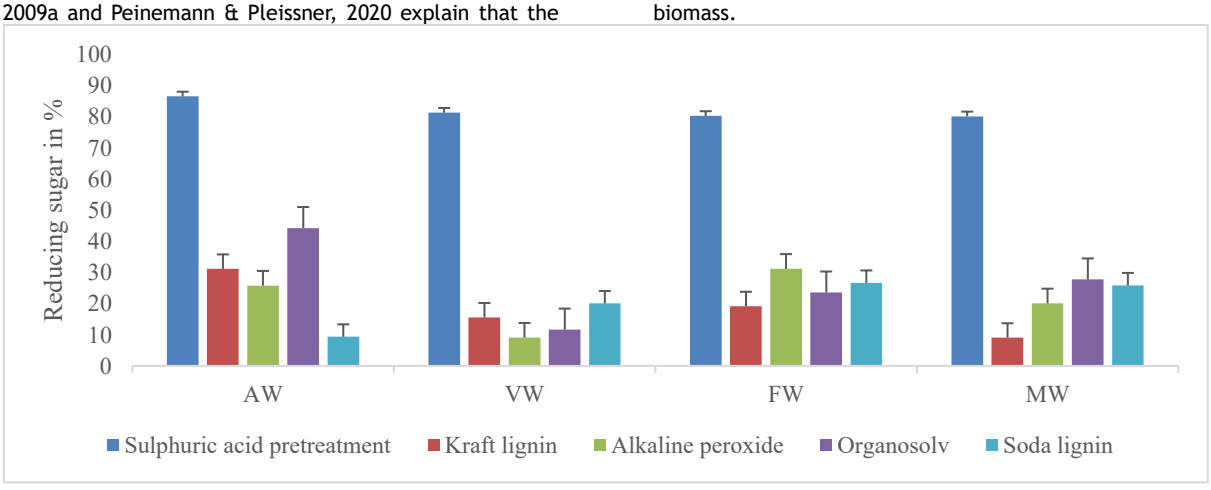


Figure 2: Pretreatment for lignocellulosic biomass

Table 2: Pretreatment of lignocellulosic biomass in %.

Lignocellulosic Biomass	H_2SO_4	Kraft lignin	Alkaline peroxidase	organosolv	Soda lignin
Agricultural waste Biomass	86.493	31.15	25.75	44.25	9.35
Vegetable waste Biomass	81.262	15.55	9.28	11.65	20.05
Fruit waste Biomass	80.226	19.15	31.15	23.55	26.63
Mixed waste Biomass	80.102	9.05	20.05	27.75	25.85

2.3. Enzymatic hydrolysis for the pretreated lignocellulosic biomasses:

Winarsih & Siskawardani, (2020) elucidate that the *T. reesei* and *A. niger* in the hydrolysis process of corncobs for producing bioethanol gives two enzymes in a ratio of 2:1 which had exoglucanase activity of 0.96 IU mL−1, endoglucanase of 1.99 IU mL−1 and ß-glucosidase activity of 0.14 IU mL−1. Pimentel *et al.*, (2021) report enzymatic activities after 72 h of submerged fermentation. *T. reesei* exhibited specific enzyme activities of 2.76 U/mg (FPase), 36.89 U/mg (CMCase), 45.03 U/mg (xylanase), and 5.18 U/mg (ß-glucosidase). *P. citrinum* LMI01 showed specific activities of 0.99 U/mg (FPase), 135.6 U/mg (CMCase), 759.6 U/mg (xylanase), and 112.9 U/mg (ß-glucosidase). *Aspergillus sp.* LMI03 revealed specific activities of 0.54 U/mg (FPase), 141.7 U/mg (CMCase), 1282.7 U/mg (xylanase), and 102.1 U/mg (ß-glucosidase). The present research co eval the enzyme activity of crude fungal strain of the strain *T. Reesei* and *A. niger* shows clear zones result from cellulase enzymes breaking down cellulose in the agar, demonstrating each isolate's ability to produce cellulase as shown in **Figure 3**. The results indicate that *A. niger* showed markedly elevated cellulolytic enzyme activities compared to *T. Reesei*. Filter Paperase (Fpase) activity was 8.059 ± 0.06 IU/mL in *A. niger*, 4.66 ± 0.03 IU/mL in *T. Reesei*, indicating enhanced cellulase efficiency. Carboxymethyl Cellulase (CMCase) activity in *A. niger* (12.37 ± 0.09 IU/mL) surpassed *T. Reesei* (7.54 ± 0.05 IU/mL) by 64%, signifying improved endoglucanase performance. The most notable difference was in ß-glucosidase activity, with *A. niger* at 4.6 ± 0.03 IU/mL, over double the 2.09 ± 0.01 IU/mL of *T. Reesei* as represented in **Table 3.**

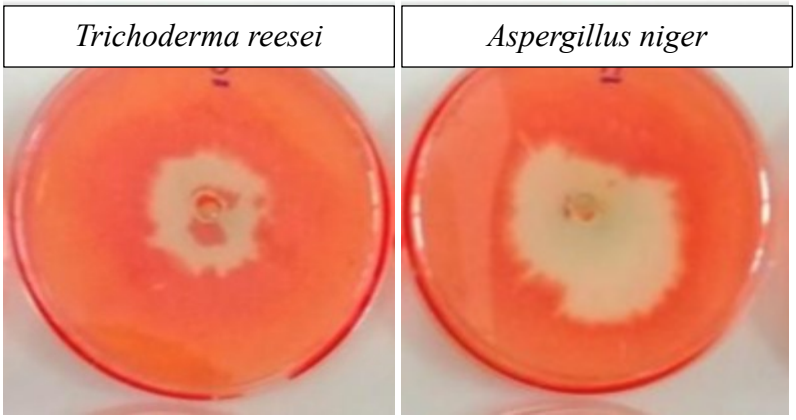


Figure 3: Isolates showing cellulase activity (zone of clearance) *for Trichoderma reesei and Aspergillus niger*

Table3: Quantitative assay observations for cellulase producing strain (*T. reesei* and *A. niger*).

Isolates	Fpase activity* (IU)	CMCase activity* (IU)	B-glucosidase activity* (IU)
Trichoderma reesei	4.66 ± 0.03	7.54 ± 0.05	2.09 ± 0.01
Aspergillus niger	8.059 ± 0.06	12.37 ± 0.09	4.6 ± 0.03

* Enzyme activity (IU): µmoles of reducing sugar released/min/ml of enzyme.

2.4. Bioethanol production from lignocellulosic biomass hydrolysate:

Hawrot & Stańczuk (2022) examined bioethanol production via separate hydrolysis and fermentation utilizing T. viride. Fungal enzymatic activity ranged from 1.25 to 1.31 irrespective of temperature. Hydrolysis and fermentation of barley straw produced 94 mL of distillate with 65% (v/v) bioethanol concentration. Furthermore, Hosny & El-Sheshtawy (2022) demonstrated that batch fermentation of municipal waste hydrolysate yielded 60.27 mL/L of bioethanol after 72 hours, as determined by GC analysis. This study found that the highest reducing sugar concentrations (86.493% and 44.25%) resulted from sulphuric acid and organosolv pretreatment of agricultural waste, compared to 20.67% from untreated waste. According to Separate Hydrolysis and Fermentation (SHF), the highest ethanol production was identified in organosolv pretreated mixed waste biomass (58.7 g/L) and organosolv pretreated agricultural waste biomass (38.44 g/L). Since sulphuric acid and organosolv pretreatment yielded significant enhancements in reducing sugars (30-36.83%) and ethanol production (40-66.6%) as shown in the **Table 4**, the biomass treated via this method was subsequently employed for optimizing fermentation parameters.

Table 4: Ethanol fermentation of different untreated and pretreated lignocellulosic biomass

Lignocellulosic biomass	Untreated Lignocellulosic biomass		H_2SO_4 pretreatment		Organosolv lignin pretreatment	
	Ethanol conc. (%)	Ethanol (g/L)	Ethanol conc. (%)	Ethanol (g/L)	Ethanol conc. (%)	Ethanol (g/L)
Agricultural waste Biomass	1.3	10.32	7.39	58.30	6.23	49.20
Vegetable waste Biomass	1.4	11.28	2.89	22.93	1.98	15.62
Fruit waste Biomass	1.12	8.83	3.01	23.74	2.32	18.30
Mixed waste Biomass	2.1	16.56	5.68	44.89	7.78	61.38
CD	0.03	0.02	0.05	0.06	0.26	0.08

SE	0.05	0.04	0.12	0.16	0.02	0.19

*Ethanol(g/L) = ethanol (%) × absolute density of ethanol

Optimization of fermentation parameters using OFAT

Table 5: Experimental design for RSM methodology

Independent variable	Units	Low	High
Inoculum size	%	1	5
Temperature	℃	20	30
Time	h	60	84
pH	-	5.0	6.0

2.5. Regression model of response

The extremum values of the experimental variables in the Central Composite Design for pretreated agricultural waste biomass are detailed in **Table 5**. Optimization of the independent variables for Response Surface Methodology was conducted through 30 experimental runs with varying combinations of four elements. Inoculum size, pH, incubation duration, and temperature were independent variables in the factorial analysis. Ethanol concentration and yield were designated as dependent variables. The experimental design, including actual factors and response values, is systematically presented for pretreated mixed waste biomass. This demonstrates significant variation in ethanol production influenced by interactions among the independent variables relevant to bioethanol fermentation. A quadratic model was developed using multiple regression analysis on experimental data from ethanol fermentation of untreated forestry waste biomass. The significant model terms were subjected to evaluation through analysis of variance (ANOVA) (**Table 6**) within the context of the optimization study ($p < 0.05$) and were identified as A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD, and CD.

Ethanol concentration (%) (Y_1) =

$$+ 1.962 + 0.1983 \times A - 0.1921 \times B + 0.1190 \times C - 0.0061 \times D + 0.1018 \times A \times B + 0.4368 \times A \times C + 0.0784 \times A \times D - 0.1743 \times B \times C + 0.0352 \times B \times D - 0.1129 \times C \times D - 0.1621 \times A^2 - 0.1574 \times B^2 - 0.2097 \times C^2 + 0.0954 \times D^2 \quad \text{Eq-1}$$

Ethanol g/L (Y_2) =

$$+ 15.464 + 1.564 \times A - 1.526 \times B + 0.9389 \times C - 0.0481 \times D + 0.0803 \times A \times B + 3.446 \times A \times C + 0.618 \times A \times D - 1.375 \times B \times C + 0.2777 \times B \times D - 0.890 \times C \times D - 1.278 \times A^2 - 1.241 \times B^2 - 1.6545 \times C^2 + 0.7527 \times D^2 \quad \text{Eq-2}$$

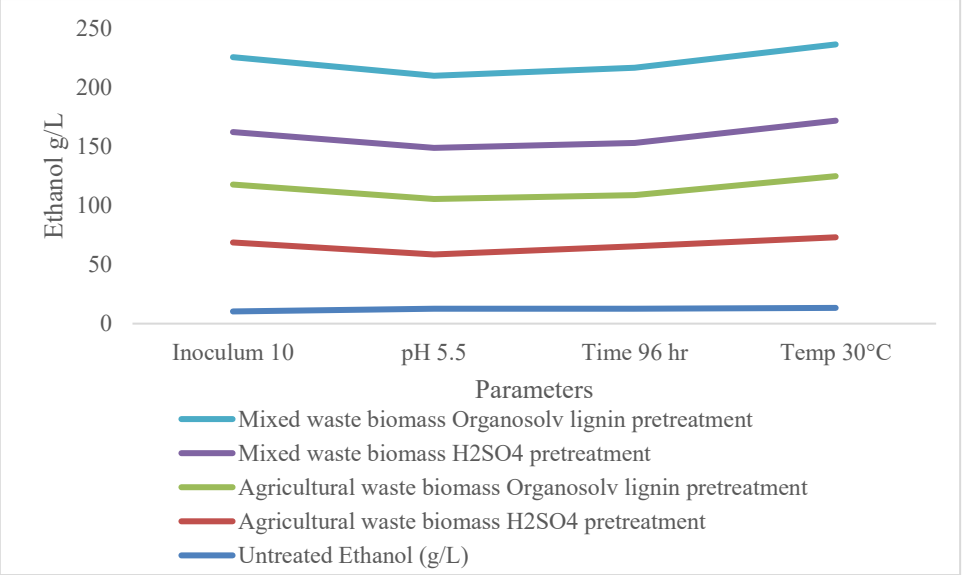


Figure 3: an increase in bioethanol yield after optimization of each fermentation parameter

Where ethanol concentration (Y1) and g/L (Y2) are response variables, as shown in **equations 1** and **2.** A is inoculum size, B is pH, C is incubation time and D is incubation temperature, 10 % inoculum size, 30 ℃ incubation temperature, 96 h incubation period and pH 5.5 for ethanol fermentation for untreated and pretreated agricultural waste biomass and mixed waste biomass for H_2SO_4 and organosolv pretreatment as shown in **Figure 3**, by optimizing the above parameters by using RSM, maximum ethanol yield obtained for mixed waste biomass, 8.18 % ethanol and 64.57 g/L. Similarly, by applying multiple regression analysis on the experimental data obtained for ethanol fermentation of pretreated mixed waste biomass and agricultural waste biomass, a quadratic model was generated for different responses of ethanol fermentation. The significant model terms were evaluated by ANOVA representing **Table 6** in the optimization study ($p < 0.05$) and were identified as A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD.

Table 6: Optimization of ethanol fermentation from pretreated Agricultural waste biomass by complete experimental design with coded independent and response variables by applying CCD of RSM

Std	Run	A: Inoculum size	B: Fermentation temperature	C: Fermentation time	D: Fermentation pH	Ethanol	Ethanol
		%	0C	H	-	%	g/L
26	1	10	25	72	5.5	2.39	9.76

17	2	0	25	72	5.5	5.72	32.32
24	3	10	25	72	6.5	1.78	5.43
15	4	5	30	84	6	3.56	20.38
8	5	15	30	84	5	5.87	36.48
30	6	10	25	72	5.5	2.23	3.76
23	7	10	25	72	4.5	4.76	37.32
29	8	10	25	72	5.5	0.45	2.15
28	9	10	25	72	5.5	3.56	29.98
12	10	15	30	60	6	4.73	4.76
5	11	5	20	84	5	0.22	15.98
19	12	10	15	72	5.5	5.12	4.98
13	13	5	20	84	6	2.94	7.98
25	14	10	25	72	5.5	1.78	37.68
20	15	10	35	72	5.5	0.67	0.98
18	16	20	25	72	5.5	4.13	4.68
7	17	5	30	84	5	5.15	3.93
14	18	15	20	84	6	3.98	4.69
9	19	5	20	60	6	4.54	0.48
22	20	10	25	96	5.5	0.11	3.95
2	21	15	20	60	5	2.23	3.76
3	22	5	30	60	5	4.31	12.37
4	23	15	30	60	5	0.75	6.05
1	24	5	20	60	5	3.22	2.2
21	25	10	25	48	5.5	0.67	0.78
27	26	10	25	72	5.5	3.57	2.97
6	27	15	20	84	5	4.39	8.65
11	28	5	30	60	6	2.78	0.76
16	29	15	30	84	6	3.11	31.48
10	30	15	20	60	6	0.45	4.88

Table 7A: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental design for pretreated lignocellulosic biomass residue ethanol percentage.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	8.93	14	0.6376	5.05	0.0018	significant
A-Inoculum size	0.9441	1	0.9441	7.48	0.0154	
B-Fermentation temperature	0.8853	1	0.8853	7.01	0.0183	
C-Fermentation time	0.34	1	0.34	2.69	0.1216	
D-Fermentation pH	0.0009	1	0.0009	0.0072	0.9336	
AB	0.1657	1	0.1657	1.31	0.27	
AC	3.05	1	3.05	24.17	0.0002	
AD	0.0983	1	0.0983	0.7781	0.3916	
BC	0.4863	1	0.4863	3.85	0.0686	
BD	0.0198	1	0.0198	0.1571	0.6974	
CD	0.2041	1	0.2041	1.62	0.2231	
A^2	0.7203	1	0.7203	5.7	0.0305	
B^2	0.6799	1	0.6799	5.38	0.0348	
C^2	1.21	1	1.21	9.55	0.0075	
D^2	0.2495	1	0.2495	1.98	0.1802	
Residual	1.89	15	0.1263			
Lack of Fit	1.58	10	0.1577	2.48	0.1636	not significant
Pure Error	0.3175	5	0.0635			
Cor Total	10.82	29				

Table 7B: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental design for pretreated lignocellulosic biomass residue ethanol concentration.

	Sum of Squares	df	Mean Square	F-value	p-value	
Model	62.87	14	4.49	3	0.0214	significant
A-Inoculum size	0.0787	1	0.0787	0.0525	0.8218	
B-Fermentation temperature	22.28	1	22.28	14.88	0.0015	
C-Fermentation time	0.0078	1	0.0078	0.0052	0.9433	
D-Fermentation pH	0.0543	1	0.0543	0.0363	0.8515	
AB	0.4192	1	0.4192	0.28	0.6044	
AC	0.7521	1	0.7521	0.5025	0.4893	
AD	0.403	1	0.403	0.2692	0.6114	
BC	0.0315	1	0.0315	0.0211	0.8866	
BD	0.4111	1	0.4111	0.2747	0.6079	
CD	11.54	1	11.54	7.71	0.0141	
A^2	2.85	1	2.85	1.9	0.1878	
B^2	1.04	1	1.04	0.6951	0.4175	
C^2	1	1	1	0.6706	0.4257	
D^2	22.3	1	22.3	14.9	0.0015	
Residual	22.45	15	1.5			
Lack of Fit	19.74	10	1.97	3.64	0.0834	not significant
Pure Error	2.71	5	0.5428			
Cor Total	85.33	29				

By optimizing the above parameters by using RSM, maximum ethanol yield obtained was 3.72 % ethanol and 29.388 g/L ethanol at 10 % inoculum size, 30 ℃ incubation temperature, 96 h incubation period and pH 5.5 for ethanol fermentation for pretreated forestry waste biomass. The statistical significance of different equations, Eq 1 and 2, was checked by F-test and ANOVA of the response surface quadratic model for untreated and pretreated Agricultural waste biomass for bioethanol fermentation has been shown. It is evident that both models are significant, as suggested by the model F-values and low probability values (Pmodel > F = 0.0001). The coefficient determination (R_2) was calculated as 0.8249 for ethanol concentration, 0.7368 for ethanol g/L for pretreated Agricultural waste biomass. Usually, a regression model having an R_2 value > 0.9 is considered to have a very high correlation. The closer R_2 (correlation coefficient) is to 1.0, the stronger the model and the better it predicts the response (Sharma & Sharma, 2023). An adequate precision value for different responses for ethanol fermentation from pretreated forestry waste was 8.9183 and 5.6055 for ethanol concentration and ethanol g/L, respectively. Adequate Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 8.9 indicates an acceptable signal. This model can be used to navigate the design space.

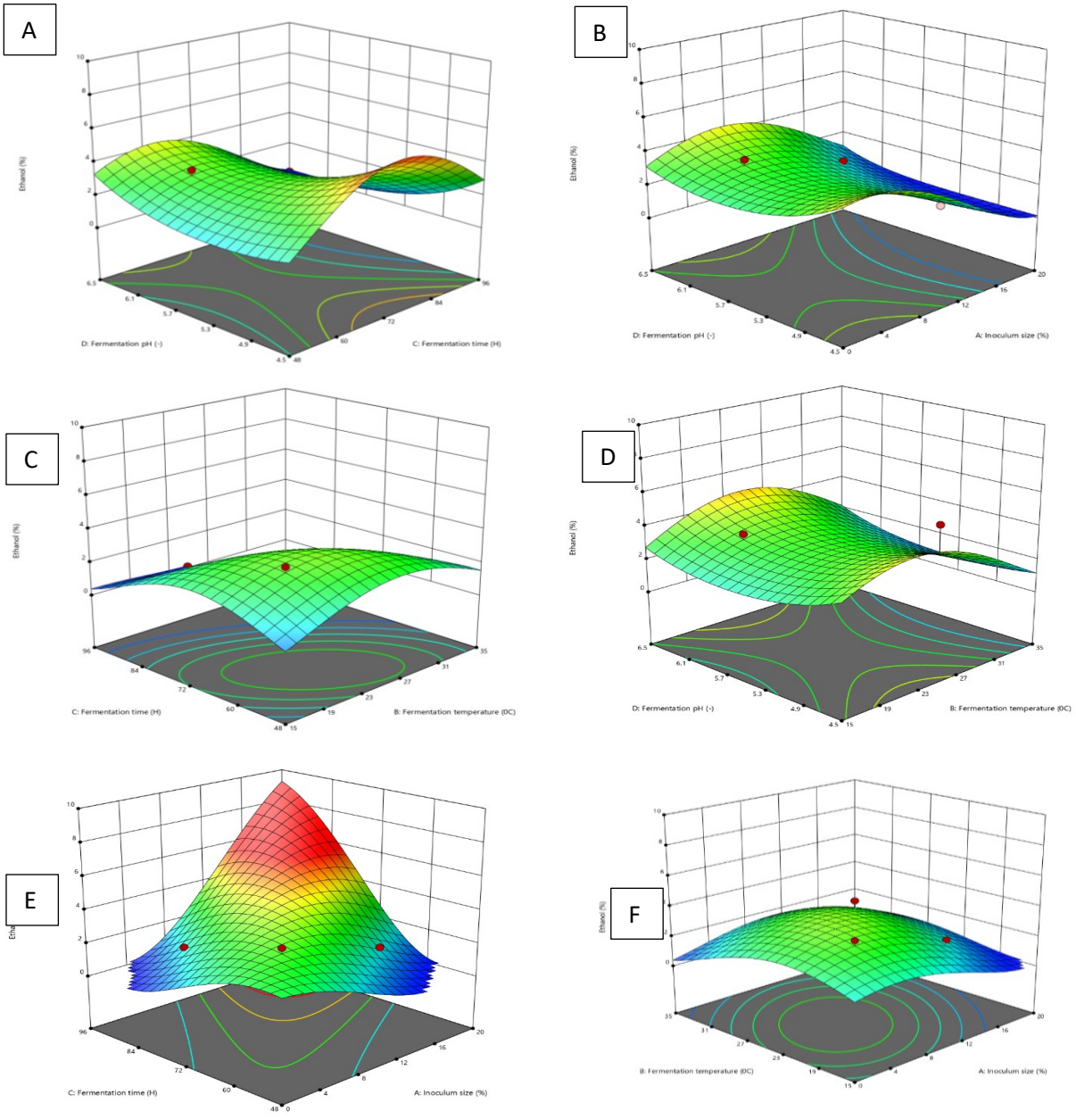


Figure 5. Response surface curves for ethanol fermentation of untreated (A-F) lignocellulosic biomass residue showing interactions between a) pH and time, b) pH and inoculum size, c) time and temperature, d) pH and temperature, e) time and inoculum size, and f) temperature and inoculum size for ethanol yield in percentage.

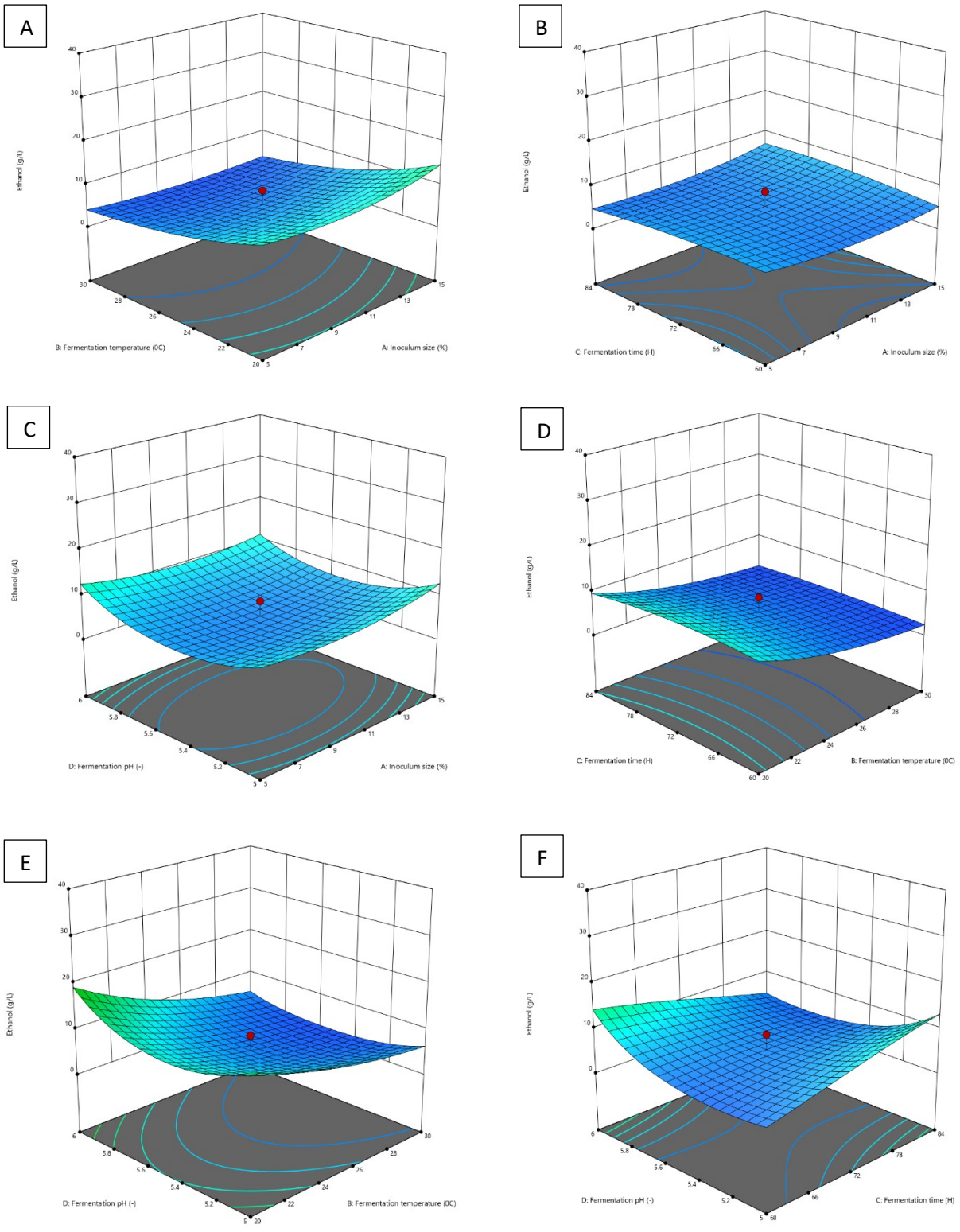


Figure 6. Response surface curves for ethanol fermentation of pretreated (A-F) lignocellulosic biomass residue showing interactions between a) pH and time, b) pH and inoculum size, c) time and temperature, d) pH and temperature, e) time and inoculum size, and f) temperature and inoculum size for ethanol yield (g/L).

The experimental design using Central Composite Design (CCD) revealed significant variability in ethanol yield based on fermentation parameters such as inoculum size, temperature,

time, and pH. Maximum ethanol concentration (36.48 g/L) and percentage (5.87%) were achieved under conditions of 15% inoculum size, 30 °C, 84 hours, and pH 5, demonstrating the synergistic effect of optimal temperature and duration as shown in **Table 6** and **Figure 5**. In contrast, extended fermentation time (e.g., 96 h) under suboptimal conditions resulted in sharply reduced yields, likely due to substrate depletion or microbial stress. ANOVA for ethanol percentage indicated a statistically significant model ($p = 0.0018$), with fermentation temperature and inoculum size emerging as critical factors as represented **Table 7A** and **Figure 6**. The interaction between inoculum size and fermentation time (AC) showed the highest significance ($p = 0.0002$), highlighting its pivotal role in optimizing bioethanol output. While fermentation pH was not significant individually, its quadratic and interaction terms affected ethanol yield, emphasizing the need for precise pH control. Similarly, the model for ethanol concentration (g/L) was significant ($p = 0.0214$), with fermentation temperature and the pH-time interaction (CD) being dominant contributors. The strong model performance is further validated by R^2 values of 0.8249 (ethanol %) and 0.7368 (g/L), along with adequate precision ratios exceeding the acceptable threshold of 4 as shown in **Table 7B**. These results confirm the effectiveness of RSM-CCD in navigating complex bioconversion systems and identify optimal operational parameters that significantly enhance ethanol yield from lignocellulosic agricultural waste.

CONCLUSION

This study confirms that lignocellulosic agricultural and food waste can serve as effective substrates for bioethanol production when optimized pre-treatment and fermentation strategies are employed. Among the tested methods, sulfuric acid and organosolv pre-treatment significantly enhanced the release of fermentable sugars. Enzymatic hydrolysis with *A. niger* exhibited higher cellulolytic efficiency than *T. reesei*. Fermentation optimization via RSM revealed critical interactions among fermentation variables, achieving a maximum ethanol yield of 64.57 g/L. The integration of chemical pre-treatment, enzymatic hydrolysis, and statistical optimization provides a scalable and sustainable model for waste-to-biofuel conversion, promoting circular bioeconomy and energy security.

REFERENCES

- Hosny, M., & El-Sheshtawy, H. S. (2022). Effect of biosurfactant on hydrolysis of municipal waste by cellulases producing bacteria for bioethanol production. *Current Research in Green and Sustainable Chemistry*, *5*, 100294.
- Hawrot-Paw, M., & Stańczuk, A. (2022). From waste biomass to cellulosic ethanol by separate hydrolysis and fermentation (SHF) with Trichoderma viride. *Sustainability*, *15*(1), 168.
- Pimentel, P. S. S. R., de Oliveira, J. B., Astolfi-Filho, S., & Pereira Jr, N. (2021). Enzymatic hydrolysis of lignocellulosic biomass using an optimized enzymatic cocktail prepared from secretomes of filamentous fungi isolated from Amazonian biodiversity. *Applied Biochemistry and Biotechnology*, *193*(12), 3915-3935.
- Winarsih, S., & Siskawardani, D. D. (2020). Hydrolysis of corncobs using a mixture of crude enzymes from Trichoderma reesei and Aspergillus niger for bioethanol production. *Energy Reports*, *6*, 256-262.
- Raina, N., Boonmee, R., Kirdponpattara, S., Narasingha, M., Sriariyanun, M., Phitsuwan, P., & Chuetor, S. (2024). Process performance evaluation of different chemical pretreatments of lignocellulosic biomass for bioethanol production. *Industrial Crops and Products*, *211*, 118207.
- Peinemann, J. C., & Pleissner, D. (2020). Continuous pretreatment, hydrolysis, and fermentation of organic residues for the production of biochemicals. *Bioresource technology*, *295*, 122256.
- Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009a). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & engineering chemistry research*, *48*(8), 3713-3729.
- Nimmanterdwong, P., Chalermsinsuwan, B., & Piumsomboon, P. (2021). Prediction of lignocellulosic biomass structural components from ultimate/proximate analysis. *Energy*, *222*, 119945.
- Onokwai, A. O., Ajisegiri, E. S. A., Okokpujie, I. P., Ibikunle, R. A., Oki, M., & Dirisu, J. O. (2022). Characterization of lignocellulose biomass based on proximate, ultimate, structural composition, and thermal analysis. *Materials Today: Proceedings*, *65*, 2156-2162.
- Liguori, R., & Faraco, V. (2016). Biological processes for advancing lignocellulosic waste biorefinery by advocating circular economy. *Bioresource technology*, *215*, 13-20.
- Kavitha, S., Kannah, R. Y., Kasthuri, S., Gunasekaran, M., Pugazhendi, A., Rene, E. R., ... & Banu, J. R. (2020). Profitable biomethane production from delignified rice straw biomass: the effect of lignin, energy and economic analysis. *Green Chemistry*, *22*(22), 8024-8035.
- Anwar, Z., Gulfraz, M., & Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of radiation research and applied sciences*, *7*(2), 163-173.
- Ahorsu, R., Medina, F., & Constantí, M. (2018). Significance and challenges of biomass as a suitable feedstock for bioenergy and biochemical production: A review. *Energies*, *11*(12), 3366.
- Pereira, L. M. S., Milan, T. M., & Tapia-Blácido, D. R. (2021). Using Response Surface Methodology (RSM) to optimize 2G bioethanol production: A review. *Biomass and Bioenergy*, *151*, 106166.
- Ferreira, S., Duarte, A. P., Ribeiro, M. H., Queiroz, J. A., & Domingues, F. C. (2009). Response surface optimization of enzymatic hydrolysis of Cistus ladanifer and Cytisus striatus for bioethanol production. *Biochemical Engineering Journal*, *45*(3), 192-200.
- Chakraborty, D., Shelvapulle, S., Reddy, K. R., Kulkarni, R. V., Puttaiahgowda, Y. M., Naveen, S., & Raghu, A. V. (2019). Integration of biological pre-treatment methods for increased energy recovery from paper and pulp biosludge. *Journal of microbiological methods*, *160*, 93-100.
- Jambo, S. A., Abdulla, R., Azhar, S. H. M., Marbawi, H., Gansau, J. A., & Ravindra, P. (2016). A review on third generation bioethanol feedstock. *Renewable and sustainable energy reviews*, *65*, 756-769.
- Gude, V. G., & Martinez-Guerra, E. (2018). Green chemistry with process intensification for sustainable biodiesel production. *Environmental chemistry letters*, *16*(2), 327-341.
- Karimi, S., Yaraki, M. T., & Karri, R. R. (2019). A comprehensive review of the adsorption mechanisms and factors influencing the adsorption process from the perspective of bioethanol dehydration. *Renewable and Sustainable Energy Reviews*, *107*, 535-553.
- Sampath, P., Brijesh, Reddy, K. R., Reddy, C. V., Shetti, N. P., Kulkarni, R. V., & Raghu, A. V. (2020). Biohydrogen production from organic waste–a review. *Chemical Engineering & Technology*, *43*(7), 1240-1248.
- Moshood, T. D., Nawanir, G., & Mahmud, F. (2021). Microalgae biofuels production: A systematic review on socioeconomic prospects of microalgae biofuels and policy implications. *Environmental Challenges*, *5*, 100207.
- Demuner, I. F., Colodette, J. L., Demuner, A. J., & Jardim, C. M. (2019). Biorefinery review: Wide-reaching products through kraft lignin. *BioResources*, *14*(3), 7543-7581.
- Tandon, D., & Sharma, N. (2018). Evaluation of different pretreatments for enhanced saccharification of Pinus roxburghii biomass by using mixture of polymerizing

enzymes and bioreactor studies for its bioconversion into ethanol. *J. Pharmacogn. Phytochem*, *7*, 3423-3432.
- Sharma, P., & Sharma, N. (2023). Lignin derived from forestry biomass as capping reagent in the biosynthesis and characterization of zinc oxide nanoparticles and there In vitro efficacy as a strong antifungal biocontrolling agent for commercial crops. *BioNanoScience*, *13*(1), 36-48.
- Watkins, D., Nuruddin, M., Hosur, M., Tcherbi-Narteh, A., & Jeelani, S. (2015). Extraction and characterization of lignin from different biomass resources. *Journal of materials research and technology*, *4*(1), 26-32.
- Kumar, N., Vijayshankar, S., Pasupathi, P., Nirmal Kumar, S., Elangovan, P., Rajesh, M., & Tamilarasan, K. (2018b). Optimal extraction, sequential fractionation and structural characterization of soda lignin. *Research on Chemical Intermediates*, *44*, 5403-5417.
- Reese, E. T., & Mandels, M. (1963). Enzymic hydrolysis of cellulose and its derivatives. In *Cellulose* (pp. 139-143).
- Miller, G.L., 1959. Use of dinitro salicylic acid reagent for determination of reducing sugars. Anal. Chem. 31, 426–428.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin-phenol reagent. Journal of Biology and Chemistry 193, 265–275.
- Dandasena, T. K., & Shahi, S. (2023). A Renewable Biofuel-Bioethanol: A Review. *Journal of Advanced Zoology*, *44*(S3), 1698-1706.
- Devi, A., Bajar, S., Sihag, P., Sheikh, Z. U. D., Singh, A., Kaur, J., ... & Pant, D. (2023). A panoramic view of technological landscape for bioethanol production from various generations of feedstocks. *Bioengineered*, *14*(1), 81-112.
- Jain, S., & Kumar, S. (2024). A comprehensive review of bioethanol production from diverse feedstocks: Current advancements and economic perspectives. *Energy*, 131130.
- Singh, T. A., Sharma, M., Sharma, M., Sharma, G. D., Passari, A. K., & Bhasin, S. (2022). Valorisation of agro-industrial residues for production of commercial biorefinery products. *Fuel*, *322*, 124284.