Research Article

Influence of Acetic Acid Pretreatment and its Residue on Bioethanol and Biogas Production from Water Hyacinth

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Abstract
Water hyacinth, an invasive species in natural water habitats, poses ecological challenges but also holds promise as a biofuel resource due to its abundant biomass. To optimize sugar yield for biofuel production, this study focuses on pretreating water hyacinth with acetic acid (AC) using Response Surface Methodology (RSM). Comparing AC, hydrochloric acid (HA), and untreated samples, AC-pretreated samples yielded the highest sugar content at 28.26 g/100 g of biomass, nearly 1.97 times higher than that of untreated samples. Additionally, AC-pretreated samples produced the maximum biogas (2573 mL) after 45 days of anaerobic digestion, while HA pretreatment yielded the highest ethanol production (9.32 g/L) within 48 h. The structural changes in the pretreated and untreated water hyacinth samples were compared using FTIR analysis, and the results showed that the pretreatment approaches exposed more cellulose to hydrolysis. Furthermore, the study investigated the impact of post-washing following acid pretreatment of water hyacinth and discovered that AC residues had no adverse effects, suggesting that the post-washing phase was unnecessary for ethanol production. These findings demonstrate that AC pretreatment can effectively enhance hydrolysis and biofuel production and that eliminating post-washing may reduce wastewater generated during the pretreatment process.

Keywords: Acetic acid, Biorefiney, Ethanol, Post-washing, Pretreatment, Water hyacinth

1 Introduction

The continuous reliance on fossil fuels as the primary energy source has resulted in global climate change, environmental degradation, and adverse effects on human health [1]. Renewable energies, including biomass, play a crucial role in addressing these issues, as biomass is considered a "carbon-neutral" fuel [2]. In many tropical places throughout the world, water hyacinth (*Eichhornia crassipes*) represents a potential

source of lignocellulose biomass [3]. This water weed belonging to the Pontederiaceae family exhibits rapid growth and can produce 2 tons of biomass per acre by doubling its population every 5–15 days [4]. Significant issues with water ecosystems have resulted from it, including limited light penetration, decreased dissolved oxygen concentration, water loss in irrigation systems due to increasing evaporation, sedimentation from trapping soil particles, and obstruction of shipping lanes and water flow during flooding [5]. Efforts to eradicate this weed have proven costly and labor-intensive, with nonpermanent removal of water hyacinths [6].

Water hyacinth possesses a substantial amount of cellulose and hemicellulose, but its lignin content is comparatively low [7], [8]. This composition makes water hyacinth an excellent candidate for enzymatic conversion, as it can be converted into fermentable sugars. This conversion process provides significant chances for the production of several valuable products such as bioethanol [9], biohydrogen [10], biogas [11], platform chemicals [12], and biochemicals [13]. Despite, the abundant hemicellulose and cellulose content and its rapid growth, the strong association between hemicellulose and lignin in water hyacinths limits the availability of hemicellulose for microorganisms.

To address this issue and optimize the production of biofuels and biochemicals, it is crucial to include a pretreatment step in the biorefinery process for lignocellulosic biomass [14]. Pretreatment plays an essential role in breaking down the complex organic structure of biomass into simpler molecules, making them more amenable to saccharification and fermentation processes [15]. Various methods are employed for pretreating lignocellulosic biomass, including physical, chemical, and biological approaches. In a specific study, water hyacinth was pretreated by using sulfuric acid at a concentration of 5% v/v. The results showed an improved biogas yield of about 107 mL/g of volatile solids (VS). Nevertheless, this pretreatment also led to the formation of compounds that degrade sugars after 45 min, potentially affecting subsequent saccharification and fermentation processes [16].

Harsh pretreatment conditions involving high acid concentration, elevated temperatures, and prolonged reaction times can result in the loss of sugar and the production of inhibitors that hinder the activities of hydrolytic enzymes during enzymatic saccharification and fermentation. Therefore, detoxification becomes crucial in removing these inhibitory compounds to maximize the process efficiency. Adjusting the pH to the optimal range (5.0–5.5) for cellulase enzyme activity during enzymatic saccharification is crucial. However, pH adjustment using NaOH or KOH to neutralize acidity introduces NaCl or KCl, respectively, which can negatively affect cellulase activity [17], [18]. Additionally, pH neutralization through washing with approximately 6 liters of deionized water for a 5 g sample results in a significant amount of wastewater generation [19]. These challenges can be reduced by utilizing organic acid in the pretreatment process. A comparative study exploring the effects of three types of organic acids (oxalic acid, acetic acid (AC), and citric acid) and inorganic acids (hydrochloric acid (HA)) on the pretreatment of palm trunk biomass for bioethanol production demonstrated that oxalic acid and citric acid yielded the highest quantities of reducing sugar (0.144 g/g-pretreated biomass) and ethanol (16.27 g/L), respectively [20]. AC (CH_3COOH) is an organic acid commonly found in vinegar and is recognized as one of the simplest carboxylic acids. Due to its cost-effectiveness and widespread availability, AC is widely used in various industries. Consequently, comparing the pretreatment effects of AC and the inorganic acid of water hyacinth represents an interesting avenue for improving the pretreatment process.

In this study, AC and HA were employed to pretreat water hyacinths to enhance ethanol and biogas production. Response Surface Methodology (RSM) was employed to optimize the pretreatment parameters to maximize the release of sugar content. Additionally, the influence of post-washing following acid pretreatment on the substrate's effectiveness in producing bioethanol and biogas was examined to minimize wastewater generation during the pretreatment process. Furthermore, the study examined the effects of acid pretreatment on the chemical bonding arrangements within the biomass to gain insights into the mechanism of acid pretreatment.

2 Materials and Methods

2.1 *Preparation and analysis of water hyacinth*

Water hyacinth was obtained from a freshwater canal

in Nonthaburi Province, located in the central region of Thailand. The biomass was then manually trimmed to decrease its size to 1 cm. Subsequently, the moisture was removed by subjecting it to drying in a hot air oven at 60 °C until a constant weight was achieved and the weight of the dried biomass was recorded. The dried biomass was further ground using a household homogenizer and screened using a 20-mesh aluminium sieve. To assess the cellulose, hemicellulose, and lignin compositions of the water hyacinth, the standard method described by Licitra *et al.* [21] was employed. The determination of the total solids (TS) and VS of the biomass was conducted using the established protocols of water and wastewater analysis [22].

2.2 *Design of experiment*

The pretreatment optimization experiments were conducted using (RSM) with a Box-Behnken experimental design (BBD), employing the Design-Expert software. The main objective of RSM was to optimize the independent variables, which represented the pretreatment factors, to achieve the maximum output for the dependent variable, which in this study was the concentration of reducing sugar. The pretreatment parameters in the experimental design were time (X_1: 30, 60, and 90 min), temperature (X_2: 100, 120, and 140 °C) and AC concentrations (X_3: 2, 7, and 12% (w/v)). The reducing sugar (YRS, mg/mL), was considered as the dependent variable in this study. The coded values were assigned to represent different levels of the independent variables, as shown in Table 1. The BBD comprised a total of 17 experimental runs, incorporating various combinations of the factors being tested, as presented in Table 2. The statistical analysis of the experimental data was carried out using the Design-Expert software, which allowed for the formulation of a second-order model as shown in Equation (1):

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{J=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where, Y is the expected reducing sugar yield, β is the regression coefficient, and X is the independent variable.

Table 1: Independent variables along with coded value levels

Factor		Units	Tested Level		
			–1	0	+1
Time	X_1	min	30	60	90
Temperature	X_2	°C	100	120	140
AC concentration	X_3	%w/v	2	7	12

Table 2: BBD matrix that was designed for optimization experiment. The impact of AC pretreatment conditions including time (X_1), temperature (X_2) and AC concentration (X_3) was tested in 17 runs on sugar yield (Y_{RS})

Run No.	Time (X_1), min	Temperature (X_2), °C	Acid Concentration (X_3), %w/v	Y_{RS} (mg/mL)
1	60	100	2	2.36
2	30	100	7	2.60
3	90	100	7	3.60
4	60	100	12	2.05
5	30	120	2	1.08
6	90	120	2	1.70
7	60	120	2	1.62
8	60	120	7	1.14
9	60	120	7	0.77
10	60	120	7	1.43
11	60	120	7	1.51
12	30	120	12	1.00
13	90	120	12	1.72
14	60	140	2	0.88
15	30	140	7	1.52
16	90	140	7	1.27
17	60	140	12	1.56

2.3 *Acetic acid and hydrochloric acid pretreatment of water hyacinth*

The pretreatment process involved utilizing AC under specific conditions, as outlined in Table 2. A benchmark for AC pretreatment was established based on HA pretreatment, which entailed a pretreatment time of 80 min, a pretreatment temperature of 104 °C, and an acid concentration of 1.41% [23]. The volume of 50 mL that contains biomass with 10% (w/v) concentration in each acid solution loaded in screw-capped bottles underwent pretreatment by heating in a hot air oven, with the temperature and time determined according to the RSM research methodology (Table 2). Afterwards, the solid fraction of the pretreated biomass was separated through filtration, employing a fritted-glass

filter, and subsequently rinsed with deionized water until a neutral pH was achieved. The washed solid fraction was then dried in a hot air oven (Model: WOF-50, make: Daihan Scientific, Korea) at 60 °C for 48 h, and the weights of the dry samples were recorded, before being subjected to enzymatic saccharification. The compositions of dried residual solids were further analyzed to evaluate the alteration in cellulose, hemicellulose, and lignin resulting from the pretreatment process. Moreover, both washed and unwashed pretreated water hyacinths were evaluated, and their saccharification yields were compared. For the unwashed sample, the solid fraction obtained after pretreatment was resuspended with 50 mL of deionized water and the pH of the mixture was brought to 7.0 by adding NaOH solution. The residual solid biomass samples were subsequently dried in a hot air oven at 60 °C for 48 h before undergoing enzymatic hydrolysis.

2.2 *Enzymatic saccharification*

The pretreatment effectiveness of each experimental run was assessed by measuring the enzymatic saccharification efficiency of the pretreated water hyacinth by using a commercially available cellulase enzyme mixture. The mixture comprised 20 FPU/g-substrate of Celluclast 1.5 L (Sigma-Aldrich, USA) and 100 CBU/g-substrate of Novozyme 188 (Sigma-Aldrich, USA) [24], [25]. The enzymatic hydrolysis of biomass was performed individually in Falcon tubes, each containing 20 mL of 50 mM sodium citrate buffer (pH 4.7), 200 µL of 2 M sodium azide and 0.5 g of pretreated biomass. The hydrolysis reactions were conducted at 50 °C for 72 h in a shaking incubator set at 200 RPM. The quantity of reducing sugars released in the hydrolysate was measured utilizing the 3,5-dinitrosalicylic acid (DNS) technique [26]. Following the saccharification process of both washed and unwashed solids, the resulting liquid hydrolysate was obtained through centrifugation at 8000 xg for 10 min. Subsequently, the hydrolysate was subjected to fermentation. To minimize variability in the experimental data, all the experiments were performed in triplicate.

2.4 *Bioethanol fermentation*

The fermentation process involved the use of *Saccharomyces cerevisiae* TISTR 5606, received from the Thailand Institute of Scientific and Technological Research (TISTR). In a tightly-sealed flask with a capacity of 50 mL, 1 mL of yeast suspension with an absorbance of 1.0 at 600 nm was inoculated in 19 mL of liquid hydrolysate. To support the initial proliferation of the yeast culture in hydrolysate, glucose (1% w/v) and yeast extract (1% w/v) were introduced as carbon and nitrogen suppliers [27], and the pH of the medium was adjusted to 5.0 by adding 1N HA. Subsequently, the flask was placed in a shaking incubator set at 32 °C and 100 RPM for 48 h. Following the incubation, the supernatant was separated by centrifugation at 8000 xg for 10 min, and the ethanol concentration was analyzed using Gas Chromatography with Flame Ionization Detection (GC-FID) with a model GC-2010 (Shimadzu, Japan). The GC system was equipped with an FID detector and a Dura Bond (DB) wax column (30 m x 0.25 mm, 0.25 µm) (Agilent Technologies, Inc., USA). The ethanol analysis was conducted using an oven program with a temperature setting of 40 °C for 4 min, followed by a ramp to 100 °C (5 °C/min), and then a further ramp to 200 °C (10 °C/min). For the ethanol sample analysis, the injector volume was maintained at 1 µL in split mode (1:20) [28]. The yeast pellet obtained after centrifugation was dried in a hot air oven at 60 °C for 48 h to measure yeast growth based on its dried weight. The ethanol concentration was determined after subtracting the ethanol yield from the negative control, which consisted of a buffer without glucose. The fermentation process was conducted in triplicate to minimize variability in the experimental data and the ethanol yields obtained from each treatment were analyzed statistically (p-value < 0.05) based on Analysis of Variance (ANOVA) by using Statistical Package for the Social Sciences (SPSS) software version 22.0.

2.6 *Biogas production*

The impact of both AC and HA pretreatment on biogas production from water hyacinth was evaluated through an anaerobic digestion experiment. The TS and VS of the seed inoculum were determined following the method described in [22], resulting in values of 65.8 ± 0.7 g/L and 38.6 ± 0.5 g/L, respectively. Before initiating the assays, the inoculum was subjected to a 48 h starvation period at 35 °C with 100 RPM agitation, without the addition of any substrate. The batch biogas

production experiments were conducted using tightly-capped Erlenmeyer flasks with a final working volume of 500 mL. Each flask contained 3 g-TS wastewater sludge of anaerobic digester, 3% wt of pretreated biomass, 10 mL of Nutrient broth media (Himedia, India), and bicarbonate buffer (15 mL) with pH 7.0. The final pH of each flask was to be 7 before closing the cap. To maintain anaerobic conditions, each flask was purged with nitrogen gas to remove any remaining air. The reactors were then incubated at 35 °C in a static water bath for 40 days. The biogas production was determined by monitoring the displacement of water volume caused by the release of biogas. Each experimental test was performed in triplicate.

2.7 *Fourier transform infrared spectroscopy (FTIR) analysis*

The structural alterations in water hyacinth caused by the pretreatment were examined using an FTIR spectrometer (Spectrum 2000, Perkin Elmer, USA). Spectra of both the untreated and pretreated water hyacinth samples were recorded, with a resolution of 4 cm^{-1} and 16 scans per spectrum, encompassing the range from 4000 to 400 cm^{-1}.

3 Results and Discussion

3.1 *Optimization of organic acid pretreatment*

Using the optimization design of this study, RSM was employed to predict and assess the influence of individual AC pretreatment conditions on the sugar yields released during enzymatic saccharification from water hyacinth. The impacts of pretreatment parameters (time, temperature and acid concentration) on released reducing sugar, as well as the interactions between two pretreatment parameters, were statistically analyzed by ANOVA, with results expressed as F-value (Table 3). A regression model was developed based on the experimental data from 17 runs, with a significance level of the model at a p-value < 0.05. The effect of each pretreatment parameter and the interaction between the two parameters, i.e. term models, were found to be significant based on p-value < 0.1. According to these criteria, the model terms, including Temperature (Temp) and TempxConcentration (Conc), were identified to be the significant terms in AC pretreatment.

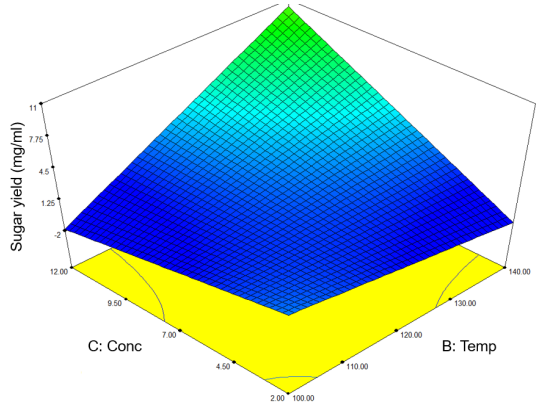

Figure 1: Response surface plots expressing the influence of AC concentration and pretreatment temperature on sugar yields.

Equation (2) represents the second-order models generated for AC pretreatment of water hyacinth.

$$Y_{RS} = +20.96 - 0.17 \text{ X Temp} - 4.43 \text{ X Conc} + 0.3982 \text{ X Temp X Conc} \quad (2)$$

The generated response surface plot is a tool to visualize the impact of two parameters on sugar yield at a time (Figure 1). In this study, AC pretreatment exhibited a high sugar yield when both factors (temperature and acid concentration) increased. To validate the mathematical models obtained from RSM, the predicted optimal condition (Time = 48.10 min, Temp = 140 °C and Conc = 12% w/v) was experimentally conducted again and the sugar yield obtained from the experiment was compared with the predicted yield. The findings indicated that the predicted sugar yield at 0.41 g/g-biomass was similar to the yield obtained from the validated experiment at 0.44 g/g-biomass, suggesting that the optimization of RSM is robust and accurate.

Table 3: ANOVA analysis to determine the significance of AC pretreatment parameters on sugar yield

Source	Sum of Squares	df	Mean Square	F-Value	p-value (Prob > F)
Model	122.61	3	40.87	5.05	0.0155
Temp	35.32	1	35.32	4.37	0.0569
Conc	23.86	1	23.86	2.95	0.1096
Temp X Conc	63.43	1	63.43	7.84	0.0150
Residual	105.17	13	8.09	-	-

3.2 *Enzymatic saccharification of pretreated water hyacinth*

Reducing sugar yields obtained from washed and unwashed solids of water hyacinth after AC pretreatment at optimum conditions by enzymatic saccharification were measured. The washing process is essential to eliminate salt traces, phenolic compounds, and sugar degradation compounds that could inhibit cellulase activity [19]. Therefore, the treatment of wastewater generated from the washing step for the second generation of bioethanol production becomes an operational and economical burden of lignocellulose biorefinery. Understanding the influences of pretreatment chemical residues on downstream steps, especially enzymatic saccharification and fermentation was necessary to skip the washing process to save process cost and operational time.

In the current study, the effect of pretreatment on both washed and unwashed samples was analyzed using the reducing sugars derived after saccharification. The enzymatic hydrolysis of washed solids (AC and HA) by cellulase cocktail led to increased output of reducing sugars than unwashed solids. The reducing sugars yield elevated by 1.94 and 1.62-fold for the washed AC and HA pretreated solids, respectively, then the unwashed AC (0.23 g/g-biomass) and HA pretreated solids (0.32 g/g-biomass). The high yield of reducing sugar was released from washed HA-pretreated samples (0.52 g/g-biomass) that were explained by hydronium ions originating from the acid catalyst. Hydronium ions cause the breakdown of the long cellulose and acetyl groups of hemicellulose chains, which increases the accessibility of the enzyme to cellulose into sugar monomers [29]. On the other hand, AC-pretreated solid exhibited a lower yield of reducing sugar (0.44 g/g-biomass) than HA-pretreated solid. For unwashed samples, the reducing sugar yields obtained in AC and HA pretreated samples increased by 1.09 and 1.52-folds, respectively, compared to untreated water hyacinth (at 0.21 g/g-biomass). This result indicated that inorganic salt (from pH neutralization of HA and NaOH) remains in hydrolysis reaction inhibited enzyme hydrolysis and decreased sugar yields in both AC and HA pretreated samples. This observation agrees accordingly with previously reported studies that show a decrease in the sugar yield by 23% of Celluclast 1.5 L when the hydrolysis

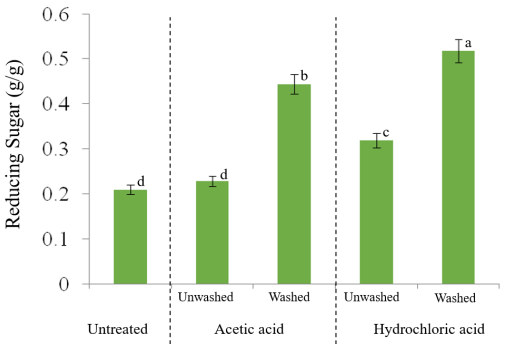

Figure 2: Reducing sugar yield obtained from enzymatic saccharifications of untreated and treated water hyacinth (washed and unwashed) by AC and HA. Each alphabet represents the statistical difference in sugar yield.

reaction of Carboxymethylcellulose (CMC) contains 0.5 M concentration of inorganic salt [30]. In addition, Celluclast 1.5 L and Accellerase 1500 exhibited a reducing sugar yield from Avicel substrate to less than 70% when containing residue of ionic liquid salt, 0.5 M EMIM-Ac, in saccharification [18]. Comparing the reducing sugars obtained from washed and unwashed samples (Figure 2), it could be concluded that post-washing after pretreatment should be conducted to maximize the efficiency of enzymatic saccharification.

To understand how acid pretreatment promotes the enzymatic saccharification of biomass lignocellulose, the composition analysis of cellulose, hemicellulose and lignin of untreated and pretreated samples was performed to monitor the changes in these compositions caused by pretreatment (Table 4). Compared to untreated samples (23.99%), cellulose contents in AC- and HA-pretreated samples were enriched to 25.51% and 33.34%, respectively. Oppositely, hemicellulose contents in both pretreated samples decreased from 32.03% in untreated samples to 28.48% and 28.62% in AC- and HA-pretreated samples, respectively. Removal of hemicellulose by acid pretreatment helps to expose cellulose fibrils to cellulase which subsequently increases the efficiency of enzymes for sugar production [31]. The AC pretreatment recovered less reducing sugar content in the hydrolysate, which may be because AC is moderately effective in removing xylan in hemicellulose than other acids [32]. In the case of lignin, the percentages of lignins were increased in AC- and HA-pretreated samples for 2.20 and 1.69 fold-time when compared to untreated water hyacinth.

According to these results, it could be explained that the acid pretreatment leads to the hydrolysis of hemicellulose into its oligomers and monomers, however, it is not effective to remove lignin from lignocellulose [15]. Organic acids were reported to be milder pretreatment and assisted the solubilization of hemicellulose with minimal sugar breakdown and lower production of inhibitory compounds [33]. The increase of lignin content caused by AC and HA in this work is in agreement with the previous studies. In most severe pretreatment conditions, i.e. acid concentration of more than 1% [34], after hemicellulose removal, lignin is liberated from biomass fibrils. After that, lignin could re-aggregate, condense and re-deposit on the surface of pretreated biomass, so-called pseudo-lignin, which subsequently reduces the accessibility of cellulase to biomass and sugar yield from biomass [35].

Table 4: Composition analysis of HA- and AC-pretreated water hyacinth at optimal pretreatment conditions

Samples	Cellulose (%wt)	Hemicellulose (%wt)	Lignin (%wt)
Untreated	23.99 ± 0.032	32.03 ± 0.007	15.57 ± 0.078
HA-pretreated	33.34 ± 0.025	28.62 ± 0.020	26.37 ± 0.038
AC-pretreated	25.51 ± 0.040	28.48 ± 0.021	34.31 ± 0.035

3.3 *Fourier-transform infrared spectroscopy analysis*

FTIR analysis was conducted to study the chemical structure and chemical bonding arrangement of water hyacinth before and after different pretreatment methods. The peaks in the FTIR spectra represent different functional groups present in the biomass, and changes in peak intensity can be used to infer changes in the chemical structure of the biomass. Figure (3) shows the FTIR spectra of untreated and pretreated water hyacinth for understanding the change in the peak height that is representative of different functional groups. The peak at 1049 cm^{-1}, representing hemicellulose, was found to have decreased in intensity after pretreatment with both AC and HA compared to the untreated biomass [36]. The peak at 1319 cm^{-1}, ascribed to cellulose, was slightly higher for both pretreated biomass contrasted to the untreated water hyacinth, indicating that more cellulose was retained during pretreatment. Previous reports have noted comparable observations regarding changes in

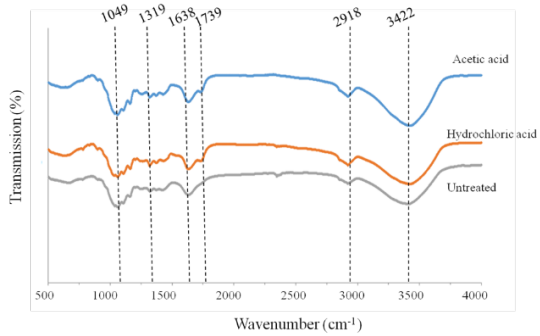

Figure 3: FTIR spectra spanning the untreated and the pretreated water hyacinth using AC and HA.

cellulose in sugarcane bagasse caused by pretreatment using different acids [37]. The peak at 1638 cm^{-1}, ascribed to lignin, showed insignificant changes in intensity after pretreatment suggesting that lignin was less affected during pretreatment.

A decrease in height of the peak at 1739 cm^{-1} was observed for both AC and HA-pretreated water hyacinth but was absent in the untreated, indicating the removal of ferric and p-coumaric acids of lignin during pretreatment [38]. The peak at 2918 cm^{-1}, allocated to the CH bond in cellulose, was found to be almost the same for both AC and HA pretreated biomasses compared to the untreated water hyacinth, indicating that more cellulose was retained during pretreatment [39]. The broad peak at 3300–3500 cm^{-1} represents the hydroxyl stretching vibration in carbohydrates. The intensity of the peak at 3422 cm^{-1}, corresponding to the stretching vibration of the OH bond in AC, was found to be stronger than that in HA and the untreated biomass, indicating more hydroxyl groups were released during AC pretreatment [40]. These results agree with the compositional studies shown in Table 4. Overall, the FTIR analysis confirms that the pretreatment methods have led to cellulose fractionation and removal of hemicellulose, which exposes more cellulose to hydrolysis.

3.4 *Effect of pretreatment chemical residues on bioethanol production from pretreated water hyacinth*

The fermentation after saccharification was conducted to estimate and compare the bioethanol production using the washed and unwashed pretreated hydrolysates. The results showed the unwashed sample was found to

produce bioethanol at the same level when compared to the washed sample in HA-pretreated samples (Figure 4(a)). The highest bioethanol production was obtained is 9.32 g/L for unwashed HA sample followed by HA washed, AC washed, AC unwashed and untreated (4.84 g/L), respectively. The reason for this is that washing can remove some of the sugars and other soluble components that were generated during pretreatment. These components can be used as carbon sources [41] and promote the microbe's tolerance to inhibitors during fermentation [42]. Suresh *et al.* [41] studied HA/H_2SO_4 hydrolysis optimization of agar waste and bioethanol production efficiency was investigated. The results exhibited that galactose (Gal), 5-hydroxymethylfurfural (HMF) and levulinic acid (LA) were generated as hydrolysis products. The result shows that low concentrations of HMF can act as cellulase inhibitors but yeast also can utilize it as carbon sources during fermentation. Furthermore, Greetham *et al.* [42] reported that the presence of low concentrations of AC (20 mM) promotes yeast tolerance to HMF and furfural.

During biomass pretreatment, some soluble components that are raw materials for biofuel production are removed leading to lower yields of biofuels during fermentation. In the study performed by Zeng *et al.* [43] HA-pretreated corn stover was washed with water or ethanol after pretreatment. They found that washing of biomass led to a significant reduction in the concentrations of sugars and other soluble components, and as a result, the ethanol yields during fermentation were lower in the washed samples compared to the unwashed samples [43]. Another study on switchgrass for the production of biofuels compared different washing methods, including water washing, acid washing, and ethanol washing. The researchers found that all of the washing methods led to a reduction in the concentration of soluble components, which in turn reduced the yields of biofuels during fermentation. They concluded that minimizing the washing steps or avoiding them altogether can lead to higher yields of biofuels [44]. Therefore, the present study proves that retaining the components during pretreatment by avoiding washing steps can help to improve the efficiency of biofuel production from lignocellulosic biomass.

Additionally, the effects of the post-washing step on the growth of *S. cerevisiae* TISTR 5606, were also assessed (Figure 4(b)). The cell densities of yeast cultures in the form of dried weights were examined on the pretreated, (HA and AC) washed and unwashed samples. It was found that the yeast cell density in the washed HA-pretreated sample (2.34 g/L) dropped compared to other samples (2.78–2.98 g/L). This may be due to the removal of some of the soluble components such as sugars and other nutrients, during the washing step, which might have resulted in a less favorable environment for the growth of yeast cells, leading to lower cell density. Yu *et al.* [45] investigated the effect of washing on the growth of S. cerevisiae during lignocellulosic hydrolysate fermentation. They found that washing the hydrolysate led to a decrease in the concentration of nutrients, such as amino acids and vitamins, which resulted in a lower cell density of yeast. The yeast cell density was higher for untreated biomass than pretreated biomass (2.98 g/L). The lower

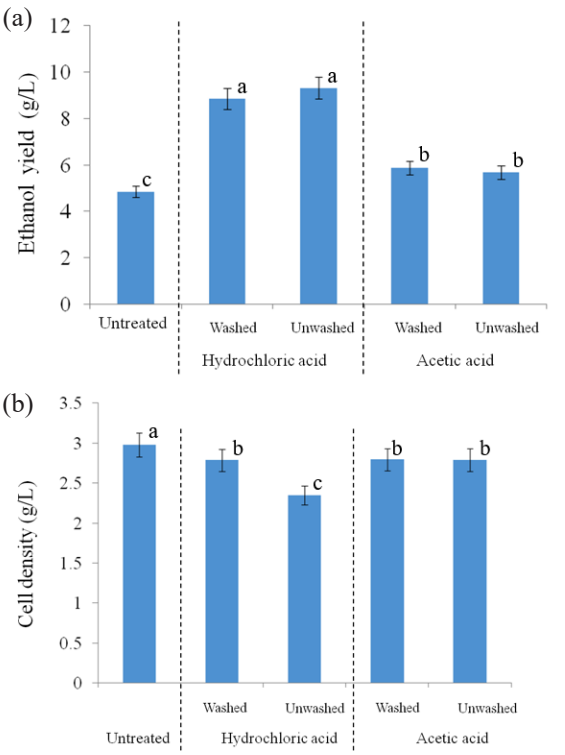

Figure 4: (a) Ethanol yields obtained from yeast fermentation of water hyacinth hydrolysates (b) yeast cell density in the form of dried weight obtained after ethanol fermentation. Each alphabet represents statistical difference in ethanol yield and cell density.

cell density of yeast observed in the pretreated biomass samples may be due to the formation of inhibitory compounds during the pretreatment process, such as furans and phenolics, which can affect the growth of yeast cells [46]. Additionally, the pretreatment process can cause changes in the physical and chemical properties of the biomass, such as changes in pH, particle size, and composition, which can also impact the growth of yeast cells [47]. A study by Liu *et al.* [47] investigated the effect of different pretreatment methods on the growth of *S. cerevisiae* during bioethanol fermentation of corn stover. They found that the highest yeast cell density was in the untreated biomass, while the lowest cell density was noticed in the biomass pretreated with acid. The authors attributed the lower cell density in the pretreated biomass to the presence of inhibitory compounds formed during acid pretreatment. However, additional research is necessary to comprehend the impact of inhibitory compounds on the growth of yeast cells.

3.5 *Biogas production*

The effect of pretreatment using HA and AC in pretreatment was investigated through the production of biogas from water hyacinth using an anaerobic digester for 45 days. As the pH is one of the significant factors that cause biogas production, it is maintained to 7.0 unless there is a failure of the process. Therefore, the analysis of the post-washing step is not conducted further for biogas production to maintain the pH concentration. The cumulative biogas production from day 1 to day 45 for untreated, HA and AC were 1948.5 mL (125.95 mL/g-TS), 2116.5 mL (136.81 mL/g-TS) and 2573 mL (166.32 mL/g-TS), respectively (Figure 5). The maximum yield of biogas for AC-pretreated water hyacinth that was higher than the untreated sample for 1.32 fold-time was due to several reasons. One of the reasons is that AC pretreatment may lead to the generation of more fermentable sugars by breaking down the hemicellulose and cellulose components of the biomass [48]. Additionally, AC is less harsh than HA and may not cause as much degradation of the biomass components, which can result in higher yields of biogas [49]. A study by Nimje *et al.* [49] investigated the effect of different pretreatment methods on biogas production from water hyacinth biomass. They found that AC pretreatment resulted in the highest biogas

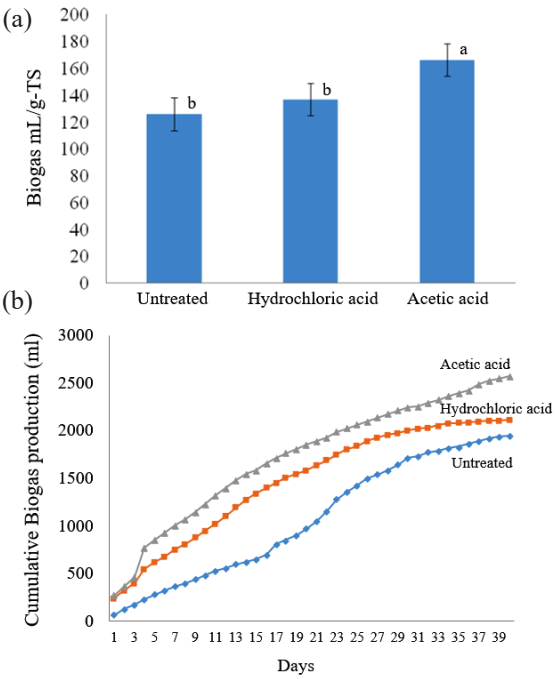

Figure 5: (a) Total biogas production during 40 days obtained from batch anaerobic digester of each condition and (b) cumulative biogas production for untreated and pretreated water hyacinth. Each alphabet represents the statistical difference in biogas yield.

yield, with a methane content of 68.5%. This was attributed to the generation of more soluble sugars and less degradation of the biomass components compared to other pretreatment methods, such as HA and steam explosion. Another study by Pandey *et al.* [48] also reported similar results, where they compared the effectiveness of different pretreatment methods on the biogas production from water hyacinth. They found that AC pretreatment resulted in the highest biogas yield, with a methane content of 57.6%. This can be ascribed to the efficient elimination of hemicellulose and the production of more fermentable sugars, which resulted in higher biogas yields.

The mass balance of the overall experiment from untreated and pretreated water hyacinths to reducing sugar, ethanol, and biogas was estimated based on the experimental results and summarized in Table 5. Per 100 g of raw biomass, the AC pretreated biomass provided the highest conversion yields of reducing sugar (28.26 g), ethanol (14.99 g) and biogas (1.06 L)

compared to HA pretreatment and untreated biomass. This is in contrast to the findings of Gunduppalli *et al.* (2022), whose study on water hyacinth subjected to oxalic acid and citric acid pretreatments yielded lower amounts of reducing sugar (23.04 g and 27.27 g, respectively) and ethanol (17.41 g and 15.31 g, respectively) [23]. Interestingly, the post-washing step was found to be unnecessary for ethanol fermentation of the AC-pretreated sample. Further studies are needed to evaluate whether the cost of the washing process, separation unit, and wastewater treatment facility is justified by the higher yields of reducing sugar and biogas obtained from the AC pretreatment compared to the HA pretreatment.

Table 5: Mass balance calculation to represent the conversion of 100 g of raw biomass to reducing sugar, ethanol and biogas

Samples	Reducing Sugar Yield (g)	Ethanol Yield (g)	Biogas Yield (L)
Untreated	14.32	13.23	0.86
HA-pretreated	1.29	11.85	0.46
AC-pretreated	28.26	14.99	1.06

4 Conclusions

The present study highlights the potential of water hyacinth as a valuable resource for biofuel production and underscores the significance of optimizing the pretreatment process to maximize sugar yields. The use of AC as a pretreatment agent, guided by RSM, proved highly effective, resulting in a substantial increase in sugar content compared to untreated samples. Additionally, AC-pretreated biomass exhibited excellent performance in biogas production through anaerobic digestion. Notably, the study also revealed the feasibility of eliminating the post-washing phase after AC pretreatment, reducing the environmental impact of the process. These findings collectively support the viability of AC pretreatment for enhancing hydrolysis and biofuel production while promoting sustainability by potentially minimizing wastewater generation. This approach represents a novel application of AC pretreatment specifically tailored to water hyacinths, highlighting the importance of customizing pretreatment methods for different feedstocks. Future research endeavors should focus on optimizing the AC pretreatment process further and explore the integration of water hyacinth-based biofuel production within the broader context of sustainable biorefineries. Additionally, comprehensive environmental and economic assessments will be vital to ascertain the viability of large-scale implementation and the potential benefits for both the bioenergy sector and the environment.

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Author Contributions

D.J. and P.T.: conceptualization, investigation, reviewing and editing; D.J., A.T. and D.D.: investigation, methodology, writing an original draft; V.P. and Y.C.: research design, data analysis; M.S. and P.T.: conceptualization, data curation, writing—reviewing and editing, funding acquisition, project administration. 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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