Research Article

Dewaxing and Post-Pretreatment Washing: Impact on Sugar and Ethanol Yields from Tobacco Residue

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Abstract
Waste generated from tobacco cultivation has negatively impacted the environment due to its inappropriate disposal methods. This negative impact can be mitigated by valorizing tobacco residue. In this study, tobacco residue was pretreated and the effect of dewaxing and washing on sugar and ethanol yields was studied. Tobacco residue was pretreated with alkali (2.17 M NaOH, 94 °C, 4.5 h) or acid (2.95 wt% H_2SO_4, 133 °C, 0.92 h). The effect of dewaxing was studied by incorporating the dewaxing step prior to pretreatment. Similarly, the effect of washing was analyzed by omitting post-pretreatment washing. Compositional analysis revealed that dewaxing prior to alkaline pretreatment improved cellulose content by 80% compared to the standard pretreated sample. Enzymatic hydrolysis of the samples showed that pretreatment had improved sugar yield by up to 6.1 times. Moreover, the sugar yield further improved when dewaxing and post-pretreatment washing steps were incorporated into the process. The unwashed biomass showed a 3-fold decrease in sugar compared to untreated biomass. Furthermore, fermentation studies showed that the dewaxed alkaline pretreated tobacco residue

enhanced ethanol yield by 34% compared to standard pretreated biomass. Thus, this study reveals the potential of tobacco residue valorization and emphasizes the importance of dewaxing and post-pretreatment washing in a biorefinery.

Keywords: Biorefinery, Dewaxing, Lignocellulose, Tobacco residue, Washing

1 Introduction

Tobacco is one of the widely grown cash crops [1] and is a source of lignocellulose [2]. Globally, around 5.8 million tons per year of tobacco is produced [3] using 3.4 million hectares of arable land [1]. Tobacco production generates a waste of 4.4 to 13.4 billion tons per annum depending on the type of tobacco being cultivated [4]. There is no proper waste disposal system for the generated waste; it is either burnt or decomposed in landfills [5]. Without proper handling, tobacco residue poses significant environmental risks, as it contains harmful chemicals and toxins that can leach into soil and water systems, threatening ecosystems, and public health [6]. Furthermore, landfills result in the release of methane, which has a greater global warming potential than carbon dioxide [7]. However, effective management of tobacco residue offers opportunities for resource utilization, aligning with sustainability goals [8]. By repurposing this waste stream into value-added products like biofuels or bioproducts, the industrial sector can reduce its environmental impact and meet consumer demands for eco-friendly practices [9]. Therefore, managing tobacco residue is crucial for mitigating risks and seizing opportunities to advance environmental stewardship and sustainability in the tobacco industry.

One method for disposing of tobacco residue is composting. However, the effect of tobacco-based compost on crop yield is not definitive. Some studies suggest it can improve yield by increasing beneficial microbes in the soil around plant roots (rhizosphere) [10], [11], whereas others suggest a decline in crop yield [12]. Compositional analysis of tobacco reveals that tobacco has a similar non-structural sugar profile to that of energy crops, with the added benefit of lower lignin content [13]. Tobacco residue can be valorized into cellulose, hydrolyzed sugars, ethanol, or other platform chemicals to deal with the improper waste disposal problem [14]. Tobacco residue's compositional profile makes this valorization attractive, both technically and economically [13]. Lignocellulose, the most abundant natural biopolymer, is composed of hemicellulose, lignin, and cellulose [15]. By recognizing lignocellulose as a sustainable raw material for the biorefining process, the transition from fossil to biogenic products becomes feasible. Moreover, the extracted cellulose from lignocellulose can be hydrolyzed into fermentable sugars, which in turn can serve as a feedstock for platform chemicals [16].

Despite its advantageous properties, lignocellulose's inherent resistance to pests, and biological and chemical attack, resulting from a robust network of covalent and hydrogen bonds, makes cellulose difficult to access by hydrolytic enzymes [17]. Various pretreatment methods aim to break down the intricate structure of lignocellulose, thereby increasing cellulose accessibility [18]. Acid pretreatment has been widely used to pretreat lignocellulosic biomass due to the ability of acid to solubilize hemicellulose, rendering cellulose accessible [19]. Several studies have indicated an improvement in saccharification efficiency and ethanol yield after pretreatment of lignocellulose biomass with dilute sulfuric acid; Zheng *et al*., pretreated wheat straw, lowering the hemicellulose content from 19% to 4%, achieving 86.6% cellulose hydrolysis [20]. Sahoo *et al*., pretreated wild rice grass and obtained 163 mg of sugar per g of biomass [21]. Zhou *et al*., pretreated alfalfa stems and obtained a 51.8% ethanol yield [22]. Besides dilute acid pretreatment, alkali pretreatment is yet another popular and effective pretreatment method that makes cellulose accessible by lignin removal [23]. Research indicates enhanced performance in lignocellulosic biorefinery parameters with the utilization of NaOH pretreatment; Ningthoujam *et al*., improved the cellulose content from 29% to 45% and lowered the lignin content from 17% to 4% in rice straw [24]. Sharma *et al*., pretreated jute and obtained a maximum saccharification of 76.48% at optimized pretreatment conditions [25]. Similarly, Kooprasertying *et al*., obtained a maximum ethanol yield of 33.15 g/L by pretreating oil palm fronds at optimized conditions [26].

Acid and alkali pretreatment methods target the lignocellulosic structure. However, unlike woody biomass, non-woody biomass (like tobacco residue), has an extracellular cuticle above the plant cell wall [27] (Figure 1). The cuticular layer transpiration rate serves as a barrier for foliar compounds and

pathogenic attack [28]. The extracellular cuticle, rich in cuticular wax, provides an additional layer of resistance to cellulose accessibility [29], [30]. These waxes are composed of long-chain fatty acids and their derivatives such as aldehydes, ketones, alkanoic acids, and alkanes [31], [32]. The composition, however, varies from plant to plant [33].

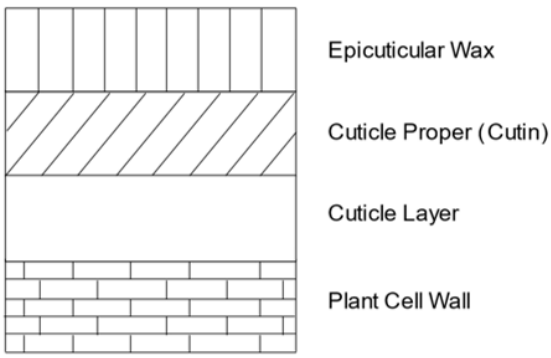


Figure 1: Visualization of epicuticular wax in relation to the cell wall.

While pretreatment studies typically focus on primary and secondary cell wall degradation, removal of cuticular wax remains underexplored in the context of improving cellulose accessibility. As the wax components are organic solvent soluble, dewaxing can be performed by organic solvent extraction [33]. Dewaxing prior to pretreatment has shown promise in improving sugar and ethanol yields from lignocellulosic biomass. Attard *et al*., assessed the effect of dewaxing *Miscanthus* on enzymatic saccharification and observed an approximately 20% increase in sugar yield through dewaxing [34]. Qi *et al*., conducted a similar study on sugarcane bagasse and reported a 42% increase in cellulose digestibility [35]. These studies, however, did not perform pretreatment after dewaxing. Other studies coupled dewaxing with pretreatment and reported increased saccharification efficiency and ethanol yield with dewaxed samples [36], [37].

An important factor that affects the economics and sustainability of lignocellulosic biorefinery is water. Water is an essential component for lignocellulosic biorefinery and is used at every step; according to Gu *et al*., second-generation biorefineries use 2.9 times more water than first-generation biorefineries [38]. Several studies have been conducted to reduce water consumption for improved economics of lignocellulosic biorefineries; Scapini *et al*., have summarized the research on switching fresh water with seawater [39], Tobin *et al*., have suggested a better integration of wastewater systems into biorefineries [40]. Although water is required at every step of second-generation bioethanol production, Zhao *et al*., has recognized post-pretreatment washing as an intensive water-consumption step [41]. The post pretreatment washing step is required to remove pretreating agent residuals and water-soluble inhibitors [41], [42]. Literature on the omission of post pretreatment washing is almost absent, therefore, further insights into this topic are required.

The current study attempts to fill the aforementioned research gap; it utilizes an underutilized and abundant biomass: tobacco residue to reduce the environmental impacts over traditional management (Table 1). Furthermore, the effect of dewaxing on enzymatic saccharification and ethanol yield is tested. Here two different pretreatment methods are employed i.e., dilute acid and alkali pretreatment, and their coupling with dewaxing is analyzed. Finally, the understudied topic of the effect of post pretreatment washing on enzymatic hydrolysis and fermentation is explored. This research is unique in that it simultaneously examines dewaxing, pretreatment, and washing, providing a unique perspective on the understudied crop waste, and tobacco residue.

Table 1: Comparison of environmental impact of traditional disposal method and current work.

Traditional Disposal Methods	This Work
Underground water contamination and soil quality degradation due to landfills [43]	Conversion of negative value waste into valuable product
Deterioration of air quality due to suspended particles from landfills [44]	The product (ethanol) can be converted into a wide range of chemicals
Suspension of particulate matter in the air due to combustion of residue [45]	Lowering the burden on landfill sites
Release of methane due to decomposition of organic matter [46]	Self-sufficient in terms of energy (utilizing lignin as an energy source)
Leaching of minerals into ground-level freshwater bodies can result in algae blooms, which can also be fatal to aquatic life [47]	Improving crop yield by diverting tobacco residue from composting to ethanol production

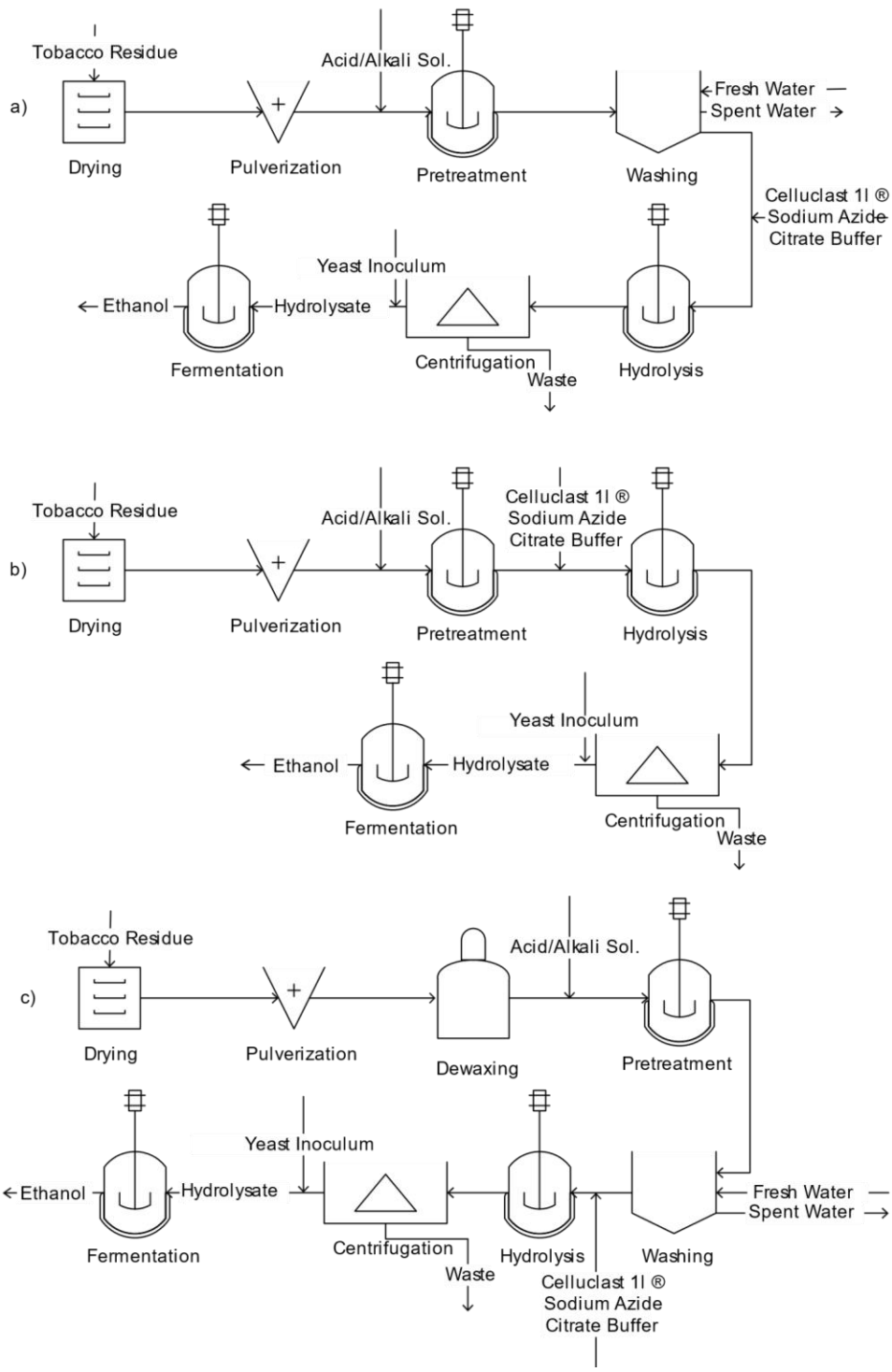


Figure 2: Block flow diagram of experimentation a) conventional pretreatment (acid or alkali), b) pretreatment without washing, c) dewaxing prior to pretreatment.

2 Materials and Methods

2.1 *Materials*

Tobacco residue was collected from Nakhonpathom, Thailand. Hexane (purity ≥ 99.5%) was purchased from Fischer Chemicals, USA. Sulfuric acid (98%), sodium hydroxide (98%), and sodium azide (98%) were purchased from Ajax Finechem Australia. CelluClast 1.5L® was purchased from Sigma Aldrich, Singapore. *Saccharomyces cerevisiae* TISTR 5606 was sourced from the Thailand Institute of Science & Technology Research.

2.2 *Biomass drying and pulverization*

The collected tobacco residue was dried at 80 °C in the convection oven (WOF-50, Daihan Scientific, Gangwon-do, Korea) until no change in mass was observed. Once dried, the tobacco residue samples were subjected to size reduction using a typical household blender. To filter large particles from the dried, crushed sample, it was passed through a 20-mesh aluminum sieve. The prepared samples were stored in air-tight containers for further use. Figure 2 shows a block flow of the experimental procedure.

2.3 *Dewaxing*

The dried ground and sieved tobacco residue was dewaxed using the Soxhlet apparatus following the procedure done by Athukorala *et al*. [48]. A round bottom flask was filled with 250 mL hexane as a solvent. The round bottom flask was kept in the water bath. The Soxhlet apparatus was fixed atop the round bottom flask. 5 g of the prepared tobacco residue was weighed into the thimble. The thimble was carefully placed in the Soxhlet apparatus. A condenser was fixed atop of the Soxhlet apparatus and was plugged at the top to avoid solvent vapor escaping. The condenser was connected to a cooling water supply. The water bath was set at 70 °C, and extraction was carried out for 6 h. The dewaxed tobacco residue was dried at 60 °C in the convection oven (WOF-50, Daihan Scientific, Gangwon-do, Korea) until no change in mass was observed. The prepared samples were stored in air-tight containers until further use.

2.4 *Pretreatment*

The specimens underwent pretreatment, encompassing both those with wax and those subjected to dewaxing procedures. Both alkali and acid pretreatment were carried out for each sample.

2.4.1 Alkali pretreatment

The samples were pretreated with NaOH aqueous solution. Optimized operating conditions were taken from a previous study [49]. A solid loading of 100 g/L was employed: 5 g of sample was added to 50 mL of 2.17 M NaOH solution. Pretreatment was carried out at 94 °C for 4.5 h in a hot air oven (WOF-50, Daihan Scientific, Gangwon-do, Korea) [50]. The pretreated biomass was separated from the liquid portion using vacuum filtration employing Whatman No.1 filter paper.

2.4.2 Acid pretreatment

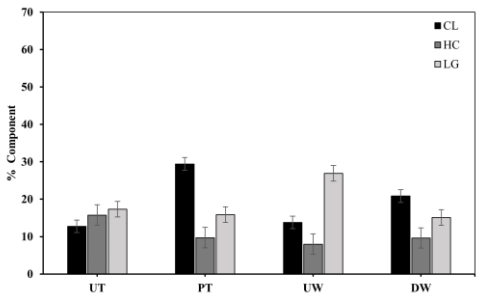
The samples were pretreated with H_2SO_4 aqueous solution. Optimized operating conditions were taken from a previous study [50]. A solid loading of 100 g/L was employed: 5 g of sample was added to 50 mL of 2.95 wt% H_2SO_4 solution. The pretreatment was carried out at 133 °C for 0.92 h in a hot air oven (WOF-50, Daihan Scientific, Gangwon-do, Korea) [50]. The pretreated biomass was separated from the liquid portion using vacuum filtration employing Whatman No. 1 filter paper.

2.5 *Washing*

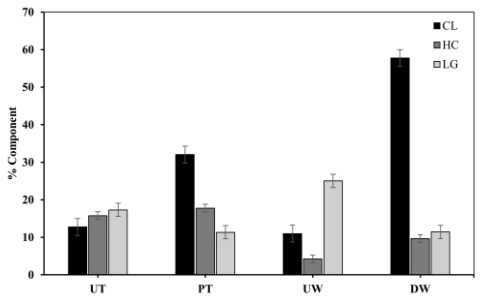
All the dewaxed-pretreated samples and half of the pretreated samples were washed with distilled water until a neutral pH was observed. The washed samples were cleared of residual water using vacuum filtration. Half of the pretreated samples were left unwashed to check the effect of washing on saccharification and fermentation.

2.6 *Enzymatic Saccharification*

Saccharification of all the samples was carried out; untreated (UT), pretreated (PT), dewaxed pretreated (DW), and unwashed pretreated (UW). This procedure was conducted with a solid concentration of 2.5% (w/v) within a 50 mM citrate buffer solution, maintaining a pH of 4.8. To safeguard against microbial contamination, 40 µL of sodium azide was incorporated into the hydrolysate. The enzymatic reaction was carried out by CelluClast 1.5L®, with enzyme loading (20 FPU/g biomass).



(a)



(b)

Figure 3: Compositional Analysis of a) acid pretreated and b) alkali pretreated Tobacco Residue. (UT: Untreated, PT: Pretreated, UW: Unwashed Pretreated, DW: Dewaxed Pretreated), (CL: Cellulose, HC: Hemicellulose, LG: Lignin).

This biocatalyzed reaction mixture was subsequently incubated at 50 ℃, 150 rpm for 72 h. After 72 h, the reaction was terminated by subjecting the mixture to a temperature of 100 ℃ for 10 min, denaturing the enzymes in the process. Following this, the hydrolysates were subjected to centrifugation at 10,000 xg by centrifuge for 5 min, after which the supernatant was extracted for the quantitative analysis of reducing sugars. The quantification was performed using the 3,5-dinitro salicylic acid (DNS) assay [51].

2.7 *FTIR*

To assess the changes in functional groups of all the samples, FTIR was carried out. The analysis was done by an FT-IR spectrometer (Invenio, Bruker, United Kingdom) at a resolution of 4 cm^{-1} and a scanning range of 400 to 4000 cm^{-1}.

2.8 *Ethanol production & quantification*

To test the impact of each procedure on ethanol production from tobacco residue, the hydrolyzed biomass was fermented with *Saccharomyces cerevisiae*. Fermentation was carried out in a 50 mL conical flask. 19 mL of hydrolysate was added into the conical flask along with 5% w/v glucose and 1% w/v yeast extract. The fermentation process continued for 72h at 30 °C in an orbital shaker (Model: JSSI-100C, JS Research Korea) at 150 rpm in a temperature-controlled environment. The post fermentation liquid was centrifuged by centrifuge for 10 min at 8000 RPM. Ethanol concentration was measured by GC-MS Analysis [52].

2.9 *Statistical analysis*

Statistical analysis was done to assess the impact of hypothesized factors on the response variables. Specifically, two-sample t-tests were conducted to check the statistical significance of each factor in relation to two key response variables: reducing sugar and ethanol yield. This analytical approach aimed to check whether the observed changes in the factors under investigation indeed wielded a significant influence on the outcomes. To assess the statistical significance of pretreatment, sugar and ethanol yields for pretreated and untreated samples were compared. Furthermore, to assess the statistical significance of dewaxing and washing, sugar and ethanol yields for standard pretreated samples were compared with sugar and ethanol yields from dewaxed pretreated and unwashed pretreated samples. The analyses were carried out by the software JMP® Pro 17.1.0.

3 Results and Discussions

3.1 *Compositional changes*

The effect of pretreatment type, washing, and dewaxing on pretreatment efficiency can be assessed by analyzing the composition profile of the different samples (Figure 3). Pretreatment had a profound impact on lignocellulosic composition, resulting in a 2.3- and 2.5-fold increase in cellulose content for acid and alkali pretreatment respectively when compared to the untreated sample. The relatively lower cellulosic content for acid pretreatment can be explained by partial hydrolysis of cellulose during acid pretreatment, whereby the hydrolyzed cellulose is lost as glucose [53]. For acid pretreatment, there was no significant change in lignin content, however, a 38% decline in hemicellulose content was observed. This is because the leading fractionation mechanism for acid pretreatment is the hydrolysis of glycosidic bonds present in hemicellulose [54]. Conversely, for

alkali pretreatment, there was a 34% decrease in lignin content but an almost similar hemicellulose content as of the untreated sample. This trend for alkali pretreatment is because alkali pretreatment fractionates lignocellulose via delignification [55].

The results for unwashed acid and alkali pretreated samples were similar. Compared to the pretreated washed sample, cellulose content decreased by 53% and 66% for unwashed acid and alkali pretreated samples respectively. For acid pretreatment, no significant change in hemicellulose content was observed, however, a 1.69-fold increase in lignin content was noted. For alkali pretreated sample, a 76% decrease in cellulose and a 2.2-fold increase in lignin was observed. This abnormal result could be attributed to the interaction of the residual pretreating agent with the compositional analysis technique (indicated in the interference section in the NREL procedure) [56]. A similar interference of residual components with the compositional analysis technique was observed by Gundupalli *et al*., where the cellulose, hemicellulose, and lignin content increased after dewaxing [36].

The results for dewaxing were mixed: on one hand, it had a pronounced effect on cellulose and hemicellulose content for the alkali pretreated samples i.e., cellulose content increased by 80% and hemicellulose content fell by 46% compared to the normal pretreated samples. On the other hand, for acid pretreated samples, cellulose content fell by 29% while the other two components remained almost unchanged. The decline of cellulose content for acid pretreatment can be attributed to an increased rate of partial hydrolysis [57]. Compared to the acid pretreatment of the sample with wax, cellulose is more exposed in the dewaxed sample, resulting in a higher amount of cellulose being hydrolyzed. This hypothesis is reinforced by the sugar yield results for the dewaxed acid pretreated sample in section 3.2.

Summarizing, the compositional analyses show that pretreatment results in a cellulose enriched pulp; this enrichment is higher for alkali pretreated samples. The enrichment is further improved by dewaxing. However, for acid pretreatment, dewaxing lowers the cellulose content compared to the standard pretreated sample. Unfortunately, due to the interference of residual pretreating agents with the compositional analysis technique for the unwashed samples, we could not reach any conclusive results about the effect of washing on lignocellulosic composition.

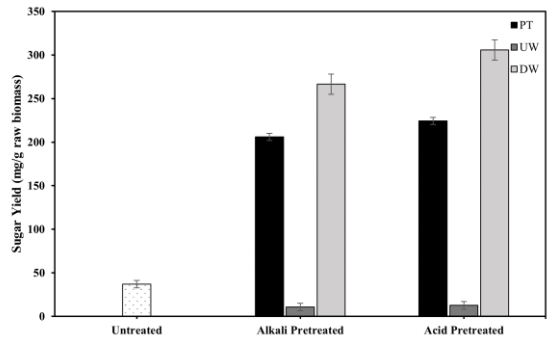


Figure 4: Reducing sugar yield calculated using DNS (PT: Pretreated, UW: Unwashed Pretreated, DW: Dewaxed Pretreated).

3.2 *Sugar Yield Analysis*

Sugar yield is one of the parameters to assess the efficiency of the lignocellulosic ethanol process. Figure 4 gives the summary of the sugar yield of all the samples subjected to enzymatic saccharification. Pretreatment, acid or alkali, had significantly improved the sugar yield; 206 mg sugar/g raw biomass and 224 mg sugar/g raw biomass were observed for alkali and acid pretreated samples respectively i.e., a respective 5.5- and 6.1-fold increase. Though alkali pretreated biomass had a higher cellulose content than acid pretreated biomass, acid pretreated biomass showed higher sugar yield. This can be explained by partial hydrolysis of cellulose via acid hydrolysis, which results in lower cellulose content, and the hydrolyzed portion of cellulose is manifested in the sugar yield [58]. Another possible reason could be the presence of free lignin liberated during alkali pretreatment, which might deposit over the cellulose rich biomass and hinder the enzymatic activity [59], [60].

The washed pretreated samples had an improved sugar yield; however, the unwashed pretreated samples had an extremely low sugar yield; even lower than the untreated sample (approximately 3 times lower) [50]. This is due to the inhibitors produced during pretreatment which inhibits cellulase activity [61]–[63]. Furthermore, free lignin has also been identified as an inhibitor that binds to cellulase, rendering the enzyme (Cellulase) inactive [60].

Wax removal has been proven to be beneficial as wax offers an additional layer of resistance to cellulose accessibility [34]. It improved the sugar yield for acid and alkali pretreated samples, the effect being more profound for acid pretreated samples due to partial hydrolysis of cellulose, a phenomenon

absent in alkali pretreatment [64]. Compared to the normal pretreated biomass, a 27% and 23% increase in sugar yield was observed for dewaxed acid and alkali pretreated samples respectively. If compared to the untreated sample, an average 85% improvement in sugar yield was observed for the dewaxed pretreated samples. This is in accordance with other studies; Gundupalli *et al*., observed a 1.17, 1.04, and 1.35-times increase in sugar yield for rice straw, Napier grass, and sugarcane bagasse respectively [37]. Similarly, Kádár *et al*., reported 67% improvement in the conversion of the carbohydrate content of wheat straw by dewaxing prior to plasma assisted pretreatment [65].

The two sample t-tests were conducted to test the statistical significance of pretreatment, dewaxing, and washing. Table 2 shows that each step was statistically significant at the 95% significance level.

3.3 *Ethanol yield analysis*

The samples subjected to enzymatic hydrolysis were further subjected to fermentation using *Saccharomyces cerevisiae*. The alkali and acid pretreated sample had a 2.8- and 4.3-fold higher ethanol yield respectively as compared to the untreated sample (Figure 5). This is in accordance with the sugar yield; acid pretreated sample had a higher sugar yield than alkali pretreated sample. However, the difference in sugar yield was not as pronounced as the difference in ethanol yield. This could be due to the generation of fermentation inhibitors during alkali pretreatment, lowering ethanol yield. A similar trend was observed by Rattanaporn *et al*., where they observed a higher sugar yield from oil palm trunk while using oxalic acid as a pretreating agent, but reported higher ethanol yield with acetic acid even though it had a lower sugar yield than oxalic acid [66]. The authors explained this anomaly by the generation of fermentation inhibitors during the oxalic acid pretreated oil palm trunk.

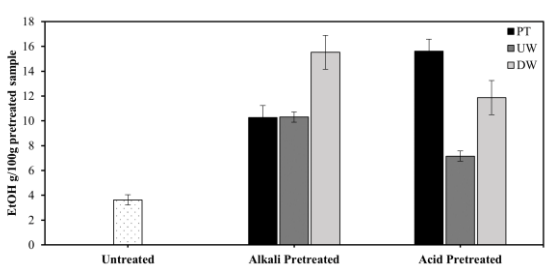


Figure 5: Ethanol yield (PT: Pretreated, UW: Unwashed Pretreated, DW: Dewaxed Pretreated)

Table 2: Two sample t-tests to determine the statistical significance of pretreatment, dewaxing, and washing on sugar yield. (SY: Sugar Yield, PT-A: Acid Pretreated, UW-A: Unwashed Acid Pretreated, DW-A: Dewaxed Acid Pretreated, PT-Al: Alkali Pretreated, UW-Al: Unwashed Alkali Pretreated, DW-Al: Dewaxed Alkali Pretreated).

S. No	μ_1 (SY)	μ_2 (SY)	*p*-value
1	UT	PT-A	<0.0001
2	PT-A	DW-A	0.0006
3	PT-A	UW-A	<0.0001
4	UT	PT-Al	<0.0001
5	PT-Al	DW-Al	<0.0001
6	PT-Al	UW-Al	<0.0001

Table 3: Two sample t-tests to determine the statistical significance of pretreatment, dewaxing, and washing on ethanol yield. (EY: Sugar Yield, PT-A: Acid Pretreated, UW-A: Unwashed Acid Pretreated, DW-A: Dewaxed Acid Pretreated, PT-Al: Alkali Pretreated, UW-Al: Unwashed Alkali Pretreated, DW-Al: Dewaxed Alkali Pretreated).

S. No	μ_1 (EY)	μ_2 (EY)	*p*-value
1	UT	PT-A	<0.0001
2	PT-A	DW-A	0.0343
3	PT-A	UW-A	0.0003
4	UT	PT-Al	<0.0001
5	PT-Al	DW-Al	<0.0001
6	PT-Al	UW-Al	0.882

As dewaxed alkali pretreated tobacco residue had a higher sugar yield compared to the standard alkali pretreated tobacco residue, therefore the dewaxed sample had a 34% higher ethanol yield [67]. Surprisingly, the ethanol yield from the unwashed samples was significantly higher than the untreated samples, although the unwashed samples had a very low sugar yield. It is possible that the sugar yield readings for the unwashed samples are not accurate. The reducing sugar yield was calculated using the DNS method. The DNS method works by reacting DNS with reducing sugar to produce 3-amino-nitrosalicylic acid, which absorbs light [68]. In the presence of residual pretreating agents such as sulfuric acid or sodium hydroxide, this reaction may be disturbed, hence producing inaccurate results. Although the unwashed samples had higher ethanol yields, the yields were lower than the washed pretreated samples, implying that post pretreatment washing improves the overall ethanol yield. These findings were further backed by the statistical analysis conducted at the 95% significance level (Table 3).

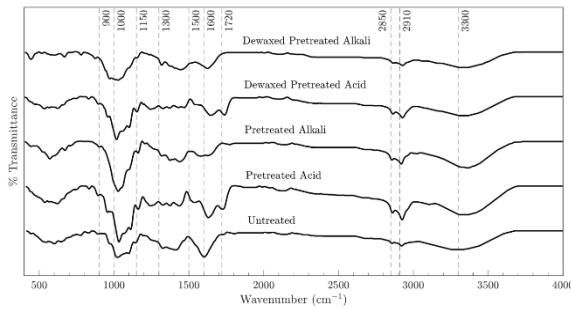


Figure 6: FTIR Spectra of pretreated, dewaxed, and untreated samples conducted at a resolution of 4 cm^{-1} and a scanning range of 400 to 4000 cm^{-1}.

The ethanol yields resulting from dewaxed alkali-pretreated and acid-pretreated biomass were comparable. However, the optimal pretreatment method is dependent upon several factors, including the specific type of lignocellulosic feedstock, reagent availability and cost, and the desired end product [69], [70]. Both acid and alkali pretreatment methods present distinct advantages and disadvantages, necessitating careful consideration based on the individual user's requirements.

Alkali pretreatment generally demonstrates superior delignification, reduced formation of inhibitory compounds, and relatively higher preservation of carbohydrates [42], [70]. Moreover, it enhances the porosity and surface area of the biomass, facilitating subsequent processing steps [71]. However, alkali pretreatment can also modify lignin structure, making it less suitable for certain applications [72]. Additionally, the high cost of alkali reagents in scaled-up systems and prolonged reaction times may pose challenges [50].

Conversely, acid pretreatment is more effective in removing hemicellulose, resulting in higher sugar yields [42]. Nevertheless, it is associated with increased inhibitor formation, higher corrosivity, and greater sugar degradation [73]. These factors can negatively impact subsequent fermentation processes and overall ethanol yield.

Therefore, the selection of an appropriate pretreatment method requires a comprehensive evaluation of the specific feedstock characteristics, process economics, and desired product profile. While alkali pretreatment offers certain advantages in terms of delignification and carbohydrate preservation, acid pretreatment may be favored for its enhanced hemicellulose removal and sugar yield. Ultimately, the optimal choice is context-dependent and should be guided by a careful assessment of the trade-offs involved.

3.4 *FTIR* Analysis

We found that all the pretreated samples had similar FTIR spectra. However, all of them were different from the untreated sample FTIR spectrum (Figure 6). This is an indicator of changes in chemical structure due to pretreatment. A fluctuation in peak intensity at approximately 1000 cm^{-1} signifies alterations in cellulose content, as this peak corresponds to the stretching of C-O bonds within cellulose molecules [74]. Compared to the untreated sample, an increased peak intensity was observed for the acid and alkali pretreated sample indicating an increase in cellulose content [75]. Comparing the peaks for acid and alkali pretreated samples, alkali pretreated sample had a more intense peak, as alkali pretreated sample had higher cellulose enrichment (Figure 3). Similarly, the standard acid pretreated sample had a more intense peak compared to the dewaxed acid pretreated sample because the rate of partial hydrolysis was higher in the dewaxed sample resulting in comparatively lower cellulose content. Similar changes are observed for the peak at around 900 cm^{-1} which is responsible for β-D-glucose linkages [76] indicating an increased cellulose content for the pretreated samples

A peak at around 1150 cm^{-1} is due to β-1,4-glycosidic linkages present in cellulose and hemicellulose [77]. Compared to the untreated sample, an increased peak intensity was observed for the pretreated samples. Although hemicellulose removal took place, the peak intensity should have been reduced in the pretreated samples, but the effect of hemicellulose removal is masked by cellulose enrichment. This can be supported by numbers from Figure 3 where 57% increase in cellulose content and 38% decrease in hemicellulose content was observed, clearly indicating cellulose enrichment dominating hemicellulose removal. Furthermore, breakage of the lignin carbohydrate complex was also detected in the FTIR spectra; the peak at around 1300 cm^{-1} indicates the presence of (O=C-O-C), the intensity of which reduced in the pretreated samples compared to the untreated sample [78]. A decrease in lignin content is observed by the peak at around 1500 cm^{-1} which is an indicator of aromatic ring in lignin [79]. The effect is more pronounced in alkali pretreated, and dewaxed alkali pretreated samples, as alkali pretreatment results in better delignification.

Asymmetric carboxylate stretching is observed at around 1600 cm^{-1} indicating the presence of wax and pectins [74]. There is a clear reduction in peak intensity in the dewaxed samples indicating wax removal. This idea is further reinforced by the peak at around 3300 cm^{-1}; a characteristic peak for OH, the intensity of which is reduced for the dewaxed samples compared to the samples with wax [80]. Furthermore, the peak at around 1720 cm^{-1} is an indicator of phenyl ester linkages [75]. This peak is almost absent in the alkali pretreated samples but present in the acid pretreated samples suggesting that alkali pretreatment breaks the lignin carbohydrate complex whereas acid pretreatment fails to do so. Lastly, the peak around 2850 cm^{-1} is representative of C-H bond stretching [81], whereas the peak at around 2910 cm^{-1} indicates methoxy C-H bond stretching [82]. The methoxy C-H peak intensity weakened in the alkali samples indicating lignin removal, the effect was not quite evident in the acid pretreated samples.

4 Conclusions

The study explored the effect of tobacco residue pretreatment, dewaxing, and post-pretreatment washing on sugar and ethanol yields. The untreated sample had an extremely low sugar and ethanol yield; 36.99 mg sugar/ g raw biomass and 3.63 g EtOH/ 100g pretreated sample. These results necessitated preprocessing of the biomass for improved sugar and ethanol yield. Therefore, pretreatment of the tobacco residue was carried out. On one hand, alkali pretreatment was observed to increase cellulose content more effectively; a 2.5-fold increase compared to untreated sample. On the other hand, acid pretreatment resulted in higher sugar and ethanol yields; a respective 6.1- and 4.3-fold improvement. The enhanced yields for acid pretreatment relative to alkali pretreatment had been attributed to partial hydrolysis of cellulose during acid pretreatment.

To enhance the sugar and ethanol yields even further, dewaxing was performed. Dewaxing enhanced cellulose enrichment and ethanol yield for alkali pretreated samples and improved sugar yield for both pretreatment methods. Dewaxing not only improves pretreatment results, but it also results in valuable products such as wax. However, further insights into the economic impact of dewaxing on the biorefinery process are required. It was observed that post pretreatment washing increases the water footprint of the process, therefore, the study also analyzed the effect of washing on sugar and ethanol yields. It was concluded that post pretreatment washing improves sugar and ethanol yields. Though it increases waste-water generation, it is a good trade-off between ethanol yield and the amount of wastewater generated.

The present study yields significant findings for the valorization of tobacco residues, a waste material that could pose substantial environmental and health risks if managed through conventional waste disposal methods. Furthermore, it highlights the importance of washing and dewaxing and the interaction of dewaxing and pretreatment methods. These results provide a robust foundation for the potential scale-up of biorefineries, offering compelling evidence for the technical feasibility of the process at a laboratory scale. In conclusion, this study provides valuable insights into the enhancement of 2G bioethanol.

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Author Contributions

E.J.P., K.C and M.S.: conceptualization; M.A.K. and M.P.: methodology; E.J.P. and M.S.: validation; E.J.P., K.C. and M.S.: formal analysis; E.J.P. and M.A.K.: investigation; S.R. and M.A.K.: data curation; M.A.K.: writing—original draft preparation; K.K., S.R., J.J. and M.S.: writing—review and editing; E.J.P., K.C. and M.A.K.: visualization; J.J. and M.S.: supervision; M.S.: project administration; M.S.: funding acquisition, 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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