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Research Article

Tobacco - A Platform for Efficient Biofuel Production: Pre-Treatment to Bioethanol Production from Lignocellulosic Biomass of Tobacco.

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Abstract

Statement of the Problem: The escalating industrial and domestic demands on non-renewable energy resources have led to the rapid depletion of fossil fuels. This has resulted in the emergence of bioethanol derived from fermentation of food crops such as maize and corn which has increased the prices of food commodities. Second generation bioethanol based on raw materials rich in complex carbohydrates such as cellulose reduces the competition with the food industry. Tobacco is grown in large fields all over the world and generates multiple harvests per year, thus producing large amounts of inexpensive green biomass. The process to obtain second generation bioethanol involves four basic steps: pretreatment, enzymatic hydrolysis, sugar fermentation, and ethanol recovery

Methodology & Theoretical Orientation: The dried tobacco leaves and stalk were pretreated with water, buffer (0.1M Citrate buffer) and dilute acids (H_2SO_4, HCl, HNO_3 at 1%, 4% and 6%) at different temperatures (60◦ C, autoclave - 121◦ C and 130◦ C) and microwave treatment (700 W, 2 min). The pretreated biomass was subjected to enzymatic hydrolysis using cellulase from Trichoderma reesei (~700 U/g of substrate) and β-glucosidase (60 U/g of substrate). The total yield of glucose and ethanol produced for each pretreated biomass was assayed by standard procedures.

Findings: A considerable loss of biomass was observed after pretreatment with dilute acids compared to pretreatment with steam in water or citrate buffer. The highest glucose and ethanol yield was obtained in the pre-treated stalk with steam at 121◦ C in citrate buffer.

Conclusion & Significance: Results from the presented experimental work indicate that leaves and stalk of tobacco have a vast potential for the production of sugars that eventually can be used for producing bio-ethanol. Despite declining cigarette sales worldwide, the use of tobacco to produce bio-ethanol can be an alternative approach to save tobacco farmers. As tobacco is not a food source it will not drive up food prices.

Introduction

The fast depletion of fossil fuels has had a lot of harmful effect on the climatic change and the world economy as well as the dependence on fossil fuels has increased both industrially and domestically. This has resulted in the alternative means by which fuels can be derived, Biofuel has become the most viable means to attain this. Bioethanol are derived from food crops such as maize and sugarcane which in turn result in the high price of food commodities in the markek [1], hence there has to be another means of getting biomass for the production of bioethanol in a cheaper of less economy affecting way, these has been extensively been investigated on over the years [2-5].

Based on the stated problem we carry out a small laboratory scale experiment in other to use a commonly known plant (*Nicotiana tabacum*) which has been misused worldwide, tobacco has been a manageable source of income in many countries such as China and India and it has its disadvantages on health and climate based on the misuse of this crop [6], therefore our aim is to show that this misused crop can be a good source or a useful material for the betterment of bioethanol production research. The process for the production of bioethanol remains the same from any lignocellulosic biomass to bioethanol, every lignocellulosic biomass contains three major components of cellulose, hemicellulose and lignin, digestion of lignin is an important process owing to the fact that cellulose and or hemicellulose is the major component needed for ethanol production as they contain monomers of sugar. Conversion by enzymatic hydrolysis of lignocellulosic biomass can't be a direct method because of the high crystallinity of cellulose and the presence of other components which prevents the proper amount of cellulose to be exposed to enzyme for bioconversion. This is the importance of pretreatment which increases the porosity and surface area of the biomass thereby allowing a proper amount of cellulose to be exposed to enzyme during hydrolysis. Pretreatment methods undertaken are the physical, chemical and radiation [7], each of these pretreatments were carried out differently for both stem and leaf blade of tobacco biomass in other to determine the effect of each pretreatment and also the result of each pretreatment on the biomass to bioethanol conversion.

In this paper we use a tobacco as an alternative in other to save

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farmers who grow tobacco all over the world and also bring the awareness of research scientist to this plant which has been misused in the society. In other words, tobacco can be an essential alternative owing to the high cellulosic content and also to prevent its total restriction on farming.

Materials and methods

Pre-hydrolysis treatment

Milling

Tobacco biomass was collected from the farmland (Andhra Pradesh South India), immediately after the drying process by sunlight, both stem and leaf blades were separated so they can be treated separately, this is followed by grinding each sample separately into powdery forms, these was then followed by storage in a dark dry environment at room temperature before treatment. (The dried biomass was weighed before the further treatment was done).

The next important steps are the physical treatment, chemical treatment, physiochemical treatment and treatment by radiation. Tobacco biomasses of both stem and leaf blades were weighed at 1g each before subjection to pre-treatment.

Physical treatment: This involved the use of thermal and mechanical treatment where the weighed samples were mixed in deionized water in Erlenmeyer flask (without the presence of added chemicals), this is followed by thermal heat by steaming at 60°C for 180 minutes before the use of thermal heat by pressure. Following the steam process, thermal heat by pressure was introduced by autoclaving the steamed samples at 121°C with a mild pressure of 150 psi for 20 mins after which the samples were brought to room temperature and then the content was filtered with double layer muslin cloth, the biomass residue was dried in room temperature for 24 hours for further enzymatic hydrolysis process.

Chemical treatment: The chemical treatment was carried out with different chemicals such as Acids and buffer, this was done using varying concentrations of acids including sulphuric acid (H_2SO_4), nitric acid (HNO_3), and hydrochloric acid (HCl) in order to examine the effect of varying conditions on the biomass. Therefore, 1g of the dried biomasses of stem and leaf blade was soaked in different Erlenmeyer flasks, individually containing different concentrations of chemicals before subjecting all samples to chemical treatment.

Acid treatment: 50ml each of 4% sulphuric acid (H_2SO_4), 4% nitric acid (HNO_3), and 4% hydrochloric acid (HCl) (i.e. 4ml of each acid diluted in 100ml of deionised water) were added to a measured 1g of leaf blade biomass, the same was done for tobacco stem, followed by steaming for 180 min at 600C. Likewise, 50ml each of 6% sulphuric acid (H_2SO_4), 6% nitric acid (HNO_3), and 6% hydrochloric acid (HCl) (i.e. 6ml of acids diluted in 100ml of deionised water) followed by the protocol stated above and then steaming for 180 min at 60°C. The samples were washed using deionized water until the pH is set to be neutral while filtration is undertaken simultaneously, the biomass residue was dried in room temperature for 24 hours for further enzymatic hydrolysis process.

Physio-chemical treatment: The same acid-soaking treatment at different concentration was repeated using the same solid/liquid ratio for another set of dried leaf blades and stem and steam treatment for 180 min at 60°C followed by thermal heat with pressure using autoclave for 20 min at 121°C and 15 psi pressure. The same process was done for buffer treated sample, after steam treatment, autoclaving was done as stated above. This is to compare the effect of the selected acids to other solution such as water was used for the pre-treatment of leaf blade and stem samples separately in the first physical treatment. All samples were washed using deionized water until the pH is set to be neutral while filtration by double-layered muslin cloth was undertaken simultaneously, the biomass residue was dried in room temperature for 24 hours for further enzymatic hydrolysis process.

Buffer treatment: 0.1M Citrate buffer was prepared (by dissolving 2.9g of sodium citrate in 100ml deionised water. Also, 46.5ml of citric acid with 3.5ml of sodium citrate solution made up to 100ml with deionised water. Standardisation was done with pH meter). The same protocol as pressure treatment was followed.

Radiation treatment: The last treatment was by irradiation which was achieved using microwave oven at 700 W for 2 min, using the same procedure of acid-soaking treatment, that is 1g of each biomass of leaf blade and stem in separate open beakers each containing 50ml of 4% sulphuric acid (H_2SO_4), 4% nitric acid (HNO_3), and 4% hydrochloric acid (HCl), also, 6% sulphuric acid (H_2SO_4), 6% nitric acid (HNO_3), and 6% hydrochloric acid (HCl) and then the mixtures were kept in the microwave at 700W for 2mins each, proper precaution was taken during the irradiation process of 2 min, to prevent the samples from turning to coal, interval of 30 sec was introduced to complete the 2 min time. All samples were washed using deionized water until the pH is set to be neutral while filtration by double-layered muslin cloth was undertaken simultaneously, the filtrate was stored for analysis while the biomass residue was dried in room temperature for 24 hours for further enzymatic hydrolysis process

These four pre-treatments were done to identify the effect of pre-treatment on the biomass and to know which of the methods used has higher impact on degrading lignocellulosic components leaving enough cellulose for the hydrolysis process. Therefore, portions of the dried pre-hydrolysed samples were sent for SEM analysis (figure A-D) and the remaining samples were kept for enzymatic hydrolysis.

Enzymatic hydrolysis

Dried pre-hydrolysed samples were used as substrate in setting up the enzymatic hydrolysis. 0.5g of substrate was kept in 50ml vials together with 0.1ml of cellulase enzyme gotten from *Trichoderma viride* and *Trichoderma reesei* (~700 U/g of substrate T. viride and cellulase from T. reesei ATCC26921, respectively; Sigma,) along with this reaction mixture, 1mg β-Glucosidase (60 U/g of substrate) enzyme was added. The liquor pH was maintained at 4.8 and this was achieved by using 0.1 M citrate acid-sodium citrate buffer, the total volume of the mixture was approximated to 2ml. This was incubated in an incubator shaker (Orbitek, Scigenics Biotech) at 68rpm for 72 hours with a temperature of 50°C. On the 72nd hour, the hydrolysates were collected, part of which were sampled for glucose estimation [8] and the remaining was stored for fermentation procedure.

Fermentation of Glucose

Baker's yeast *Saccharomyces cerevisiae* was the choice of catalyst, 4.2g of yeast sample was dissolved in 21 ml of deionised water, the number of viable cells were counted using hemocytometer and a colony forming unit (CFU) of 1×10^{-7} was maintained. From the yeast sample, 0.5ml was added to 100µl of hydrolysate then followed by adding 500µl deionised water. This set-up was incubated at 37°C for 24hrs. The extracted liquor was estimated for bioethanol using (dichromate method [9]).

FESEM analysis

Both untreated and treated tobacco biomass was sent for observation under Field emission scanning electron microscope (Zeiss, Sigma) at Rahman Institute of Science. The Fig. 1 below shows the results from the FESEM taken at 300nm resolutions.

Results and discussion

The results of the experiments of both pre-hydrolytic treatment

and enzymatic hydrolysis can be seen in Table 1 and 2 below showing percentage yield of both glucose yield and ethanol yield. The tables are represented graphically in Graph A-D in all the graphs the effect of pre-treatment can be observed in both leaf blade and stem biomass differently although not much differences as it appears in the first two graphs showing the percentage of glucose yield. The percentage of ethanol yield was dropped as expected in the Table 2 as this is due to the amount of glucose lost during the pretreatment process before the enzymatic process took place. FESEM micrographs can be observed in Figure 1 (A-D) showing the pretreated biomass' components.

Effect of pretreatment

Milling

The process of milling was done using the domestic grinder, a total weight of 20g was measured each for leaf blade and stem which are to be used separately, this method does not have any effect on the lognocellulosic content but has a measurement effect on weight per cellulose yield [10].

Physical pretreatment

The effect of thermal heat and pressure with deionised water (autoclaving [11-13]) is a common method used in pretreatment of biomass, but in our experiment this method appears to have minimal effect on the percentage of glucose yield in Table 1 thus sowing that the amount of cellulose exposed to enzymatic treatment is very less, also the significan difference portrayed in our experiment is that stem response to pretreatment by autoclaving is quite high showing there is more of cellulosic material in stem than that which is present in leaf blade.

Furhermore, the result in Table 2 shows the amount of ethanol yield in percentage carried out through the experiment, this shows autoclave with water alone cannot be enough pretreatment process for the bioconversion process to bioethanol. This can also be observed in the FESEM result in Fig. 1 (B).

Table 1: Percentage yield for glucose estimation of both Stem and Leaf Blade.

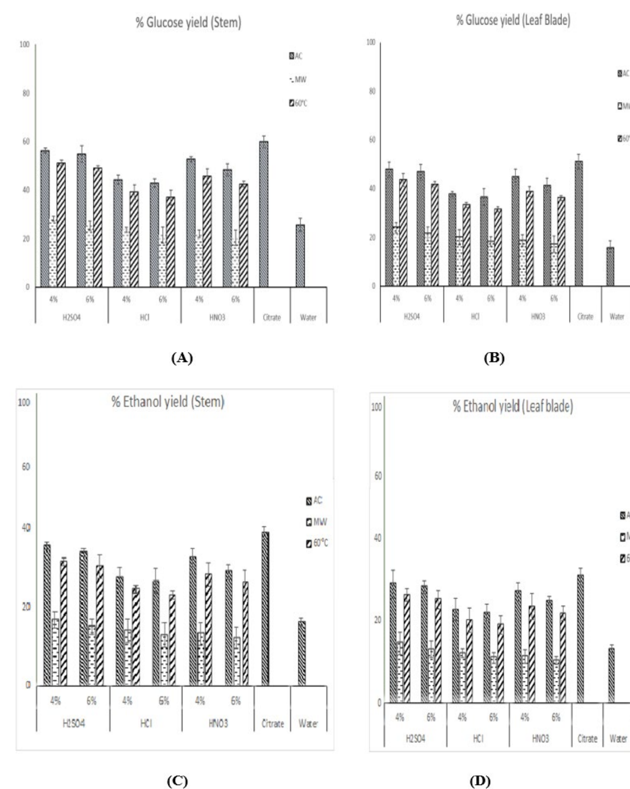
				STEM				
	H_2SO_4		HCl		HNO_3		Citrate	Water
	4%	6%	4%	6%	4%	6%		
AC (%)	56.19	54.86	44.19	42.86	52.67	48.43	59.85	25.67
MW (%)	28.12	25.24	23.45	21.45	21.95	20.34		
60°C (%)	51.14	48.94	39.14	36.94	45.56	42.44		
				LEAF BLADE				
AC (%)	47.76	46.63	37.56	36.43	44.76	41.16	50.87	15.67
MW (%)	23.9	21.45	19.93	18.23	18.65	17.2		
60°C (%)	43.46	41.59	33.26	31.39	38.75	36.07		

Table 2: Percentage yield for ethanol estimation of both Stem and Leaf Blade.

				STEM				
	H_2SO_4		HCl		HNO_3		Citrate	Water
	4%	6%	4%	6%	4%	6%		
AC (%)	35.72	33.92	27.52	26.72	32.61	29.06	38.91	16.41
MW (%)	16.88	15.15	14.07	12.87	13.17	12.21		
60°C (%)	31.69	30.37	24.49	23.17	28.34	26.47		
				LEAF BLADE				
AC (%)	28.66	27.98	22.54	21.86	26.86	24.7	30.53	13.09
MW (%)	14.34	12.87	11.96	10.94	11.19	10.32		
60°C (%)	26.08	24.96	19.96	18.84	23.25	21.65		

Stem was confirmed to have more percentage yield in both glucose and ethanol percentage yield. Compare Table 1 and 2. The FESEM micrograph Fig. 1 (C) shows sulphuric acid when compared to the untreated sample in Fig. 1 (A), how the cellulosic material has been exposed, we were only able to sample the FESEM of the autoclaved sulphuric acid dried sample.

Citrate buffer chemical shows the most effetcive of all pretreatment chemical used although we only used the pressure treatment in our experiment for citrarte buffer, we were able to



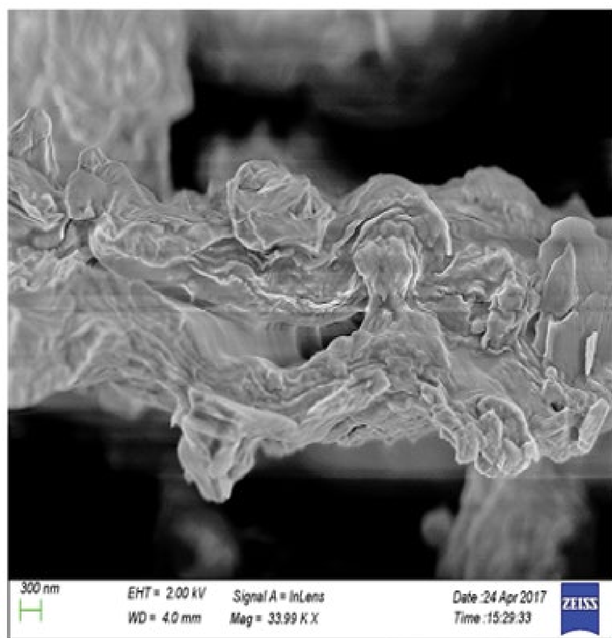
Graph A-D

Chemical pretreatment

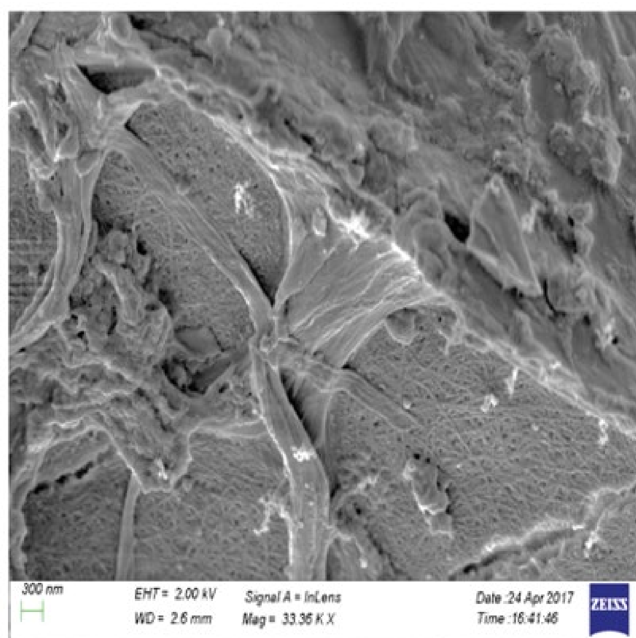
The most effective method in pretreatment for lignocellulosic biomass [14,15], dilute acid fractions of sulphuric acid (H_2SO_4), nitric acid (HNO_3), and hydrochloric acid (HCl) in the acid treatment followed by steaming for 180 min at 60°C shows how important the presence of chemical is, for the crystallization of cellulose to break down and to release the sugars in order for enzymatic process to take place. In TABLE 1, the percentage of glucose yield after treatment by each of the acids shows the capacity to release cellulose for enzymatic hydrolysis, 4% Sulphuric acid (H_2SO_4) shows a greater yield when compared to the rest, while 6% hydrochloric acid (HCl) shows the minimum effect on the pretreatment of the lignocellulosic biomass, this can be observed for both stem and leaf blade and stem where stem shows a higher chance of glucose yield compared to leaf blade.

Physio-chemical treatment

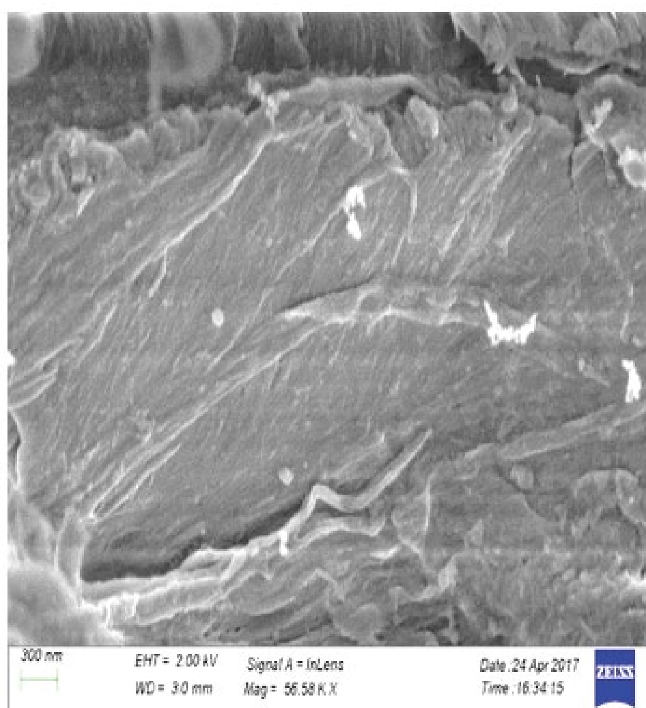
Highly effective treatment above all other treat and much more productive. This method involves the regular acid soaking treatment as stated in the protocol above followed by the steaming for 180 min at 60°C then autoclaving for 20 min at 12zd1°C and 15 psi pressure thus resulting in a higher glucose yield and after the regular process of enzymatic hydrolysis, it can be observed that this method gave the best result as seen in Table 2. As expected the percentage yield in glucose and ethanol for both stem and leaf blade varies where



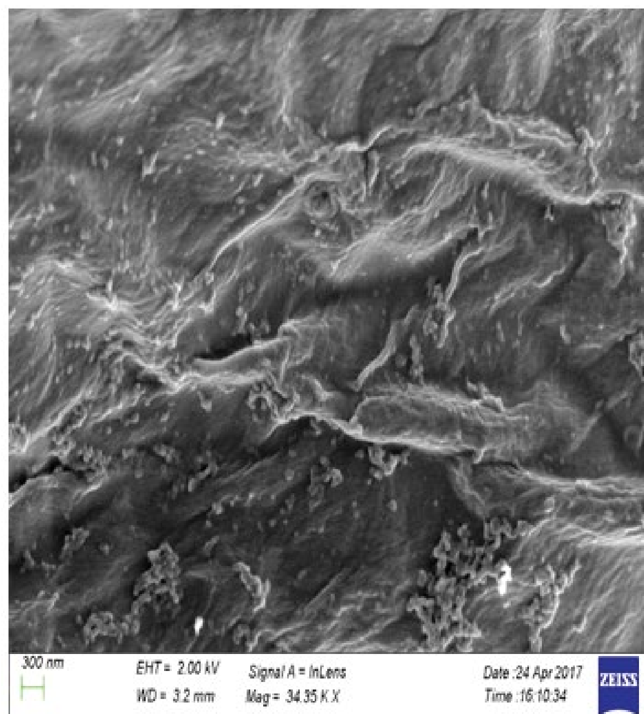
(A)



(B)



(C)



(D)

Figure 1: FESEM images of untreated and treated biomass sample **(A)** FESEM image of untreated biomass **(B)** Physical treatment by steam **(C)** Physio-chemical treatment by sulphuric acid **(D)** physio-chemical treatment by citrate buffer.

observe a igher percentage yield for both glucoe and ethanol yield. Moreso citrate buffer could be the best pretreatment chemical used in the bioconversion of lignocellulosic material.

Radiation treatment

When compared to water treatment and steam, microwave radiation even after the aid soaking mthod, radiation seems to show a very less effect yield in terms of percentage both before enzymatic hydrolysis and after hydrlysis, the amount of bioethanol produced based on radiaton exposure is variably less, this implies it would not be of a good or effective pretreatment method on tobacco biomass.

Conclusion

Nicotiana tabacum which has becom3served in our result to be more effective in a large-scale production of bioethanol. Finally, we believe the result of this experiment will be a good impact and a good potential biomass in the production of biofuel.

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