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Research Article

Tobacco – A Potential Source for Sustainable Production of Bioethanol

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Abstract

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 fermented food crops such as maize and corn. Second generation bioethanol from raw materials rich in complex carbohydrates such as cellulose alleviates the use of food crops. Tobacco is grown in multiple harvests annually all over the world, thus producing large amounts of inexpensive green biomass as alternative feedstock for bioethanol production. The process to obtain second generation bioethanol involves four basic steps: pretreatment, enzymatic hydrolysis, sugar fermentation, and ethanol recovery. The dried tobacco leaves and stalk were subjected to different physio-chemical pretreatment methods. A combination of enzymes, cellulase and β-glucosidase was used to convert the pretreated biomass to glucose. The enzyme hydrolysate was then subjected to fermentation using yeast. The total yield of glucose and ethanol, produced for different pretreatments methods, was assayed by standard procedures. The effect of different pretreatment methods on biomass structure and complexity was investigated by (FESEM) studies. The results from the presented experimental work indicate that leaves are a potential source producing bioethanol. As a result, despite declining cigarette sales worldwide, the use of tobacco to produce bioethanol can be an alternative approach to save tobacco farmers.

Keywords: Pretreatment; Enzymatic Hydrolysis; Fermentation; Bioethanol

Introduction

Fossil fuels are essential drivers of the progress of technological, social, economic and development globally. Therefore, the fast depletion of fossil fuels have had a lot of harmful effect on the climatic change as well as the world's economy [1]. This has resulted in the search of alternative means by which fuels can be derived and bioethanol has become the most viable means to attain this. First-generation bioethanol has been mainly produced from sugar (sugar cane and sugar beet) and starch (maize and wheat) crops and, thereby have been responsible for food commodity price increases [2]. Consequently, a future increase of bioethanol production will need to depend on exploiting alternative food/feed associated biomass products such as lignocellulosic feedstock [3,4], food wastes [5] and alternative plant species. Tobacco (*Nicotiana tabacum* L.) is grown in more than 125 countries, have a well-developed infrastructure for crop management and harvest processing, yielding up to 160 tonnes per hectare through multiple harvests. Tobacco has been a manageable source of income in many countries such as China and India. In recent times, reduced sales of tobacco products due to their harmful effects on health and climate has affected the economy of tobacco farmers [6]. Therefore, in this paper the potential of exploiting lignocellulosic tobacco biomass (leaves and stalk) for bioethanol production was studied.

In general, all lignocellulosic biomass contains three major components namely cellulose, hemicellulose and lignin of which cellulose fraction is the major component essential for ethanol production by cellulase enzymatic conversion. Presence of lignin and the high crystallinity of cellulose hinder the enzymatic conversion of cellulose to glucose. Therefore, the lignocellulosic biomass has to be subjected to a process called pretreatment that facilitates removal of lignin and increase the porosity and surface area of the biomass to ensure proper exposure of cellulose to enzymatic hydrolysis. Tobacco stalk and leaf biomass was subjected to different

combinations of physical (milling), chemical (dilute acid, citrate buffer and water), thermal (60^0C, autoclave) and radiation (microwave, 700W) pretreatment methods in order to determine the best method that gives the highest glucose and ethanol yield. The pretreated biomass was subjected to enzymatic hydrolysis using cellulase from *Trichoderma reesei* and β-glucosidase and fermentation using baker's yeast.

Materials and Methods

Pretreatment methods

Milling

Tobacco biomass was procured from Andhra Pradesh South India and dried in an air-dry oven to prevent any biological reactions and possible contaminations following which, the stem and leaf blades were separated. The dried biomass was ground using mixer grinder (Kenstar, India).

Physio-chemical pretreatment methods

Various pretreatment methods were investigated including different chemical treatments: dilute-acid [sulphuric acid (H_2SO_4), nitric acid (HNO_3), and hydrochloric acid (HCl)] at 4% and 6%, 0.1 M citrate buffer and water; thermal: 60^0C, and autoclave; and radiation: microwave (700 W). Some of these treatments were used in combinations of two treatments applied consecutively (Table 1). Briefly, the tobacco (leaf and stem separately) biomass and dilute acid (4% and 6%) were mixed at a solid/liquid ratio of 0.5 g in 50 ml and subjected to different types of pretreatment: i) 60^0C for 180 min, ii) 60^0C for 180 min followed by autoclave at 121^0C with a pressure of 15 psi for 20 min, iii) microwave at 700 W for 2 mins.

The tobacco biomass was also subjected to 0.1 M citrate buffer and water at solid/liquid ratio of 0.5 g in 50 ml and treated in autoclave at 121^0C with a pressure of 15 psi for 20 min. The samples after pretreatment were brought to room temperature and then the content was filtered with double layer muslin cloth, repeatedly washed with water till neutral pH and biomass residue was dried in room temperature for 24 hours for further enzymatic hydrolysis process. The amount of biomass recovered was determined by analytical weighing balance.

FESEM analyses

The untreated and pretreated biomass was observed under field emission scanning electronmicroscope (Zeiss, Sigma) at Raman Research Institute, Bangalore, India. The samples were prepared by drying the biomass at 60^0C for 24 h and dried samples were spread on the carbon tape placed over the surface of FESEM stub and were sputtered with 10 nm gold in a sputter coater (SCH 620, Leo).The pictures were taken at different magnifications.

Enzymatic hydrolysis

Dried pretreated samples were used as substrate in setting up the enzymatic hydrolysis. 0.5 g of pretreated biomass, was taken in 50 ml falcon tubes, together with 0.1 ml of cellulase from *Trichoderma reesei* (700 units/g, C2730, Sigma) and 1 mg β-Glucosidase (60 U/g, 49290, Sigma). The liquor pH was maintained at 4.8 by adding 0.1 M citric acid-sodium citrate buffer. The total volume of the mixture was approximated to 2 ml and incubated in an orbital shaker at 50^0C, 68 rpm for 72 hours. The hydrolysates were then sampled for glucose estimation by DNS method [7] and the remaining was stored for fermentation.

Fermentation of Glucose

Baker's yeast *Saccharomyces cerevisiae* was the choice of catalyst, 4.2 g of yeast sample was dissolved in 21 ml of deionised water, the number of viable cells were counted using hemocytometer and diluted appropriately to 1×10^7 cells/ml. 0.5 ml was added to 100 µl of hydrolysate and then made up 1 ml with deionised water. This set-up was incubated at 37°C for 24 hrs. The extracted liquor was estimated for bioethanol using dichromate method [8].

Results and Discussion

Pretreatment methods using Tobacco biomass

All lignocellulosic biomasses are basically made up of three main constituents: lignin, cellulose and hemicellulose. The sugar moieties, which make up the cellulose and hemicelluloses are the important components for ethanol production. The physiochemical nature of any lignocellulosic biomass makes it resistant to the direct action of enzymatic hydrolysis. The presence of lignin and the high crystallinity of cellulose hinder the exposure of cellulose fraction of biomass to enzyme hydrolytic reaction. This necessitates pretreatment of raw biomass prior to ethanol production. The main objectives of pretreatment are: increasing the porosity and surface area of biomass, removal of lignin and reducing the crystallinity of cellulose [9]. All of these effects lead to higher and faster hydrolysis of cellulose, thus maximizing the extent of saccharification and yield of fermentable sugars. Several pretreatment methods have been practiced for various lignocellulosic biomasses prior to enzymatic hydrolysis. These methods are of physical, chemical or physicochemical nature.

In order to investigate the suitability of various pretreatment methods for maximum solubilization of hemicelluloses and lignin

removal, the tobacco biomass was subjected to multiple physio-chemical treatment methods. First, the air dried tobacco biomass was segregated into leaf blade and stem fractions manually and subjected to mechanical grinding. Mechanical comminution reduces the particle size and increases the surface area of biomass for pretreatment and enzymatic hydrolysis. This treatment does not affect lignin and hemicellulose or induce any microscopic effects such as disruption of cellulose crystallinity or reduction in degree of polymerization from the biomass. Thus, the obstruction for the enzyme action on cellulose is not reduced much by this treatment. The milled tobacco biomass was then subjected to different physio-chemical and thermal treatment.Various pre-hydrolysis treatments were investigated, including dilute-acid (H_2SO_4, HNO_3 and HCl (4 and 6 %)), water and citrate buffer followed by thermal, steam and microwave treatment or combinations of two of them applied consecutively (Table 1).

Physical treatment	Chemical Treatment	Thermal treatment		Steam treatment		Microwave	
		T (°C)	Time (min)	T (°C), pressure	Time (min)	Power (W)	Time (min)
Milling to approx. 1 mm particles using a mixture grinder	4 % (v/v) H_2SO_4	60	180				
	6 % (v/v) H_2SO_4	60	180				
	4 % (v/v) H_2SO_4	60	180	121, 15 psi	15		
	6 % (v/v) H_2SO_4	60	180	121, 15 psi	15		
	4 % (v/v) H_2SO_4					700	2
	6 % (v/v) H_2SO_4					700	2
	4 % (v/v) HNO_3	60	180				
	6 % (v/v) HNO_3	60	180				
	4 % (v/v) HNO_3	60	180	121, 15 psi	15		
	6 % (v/v) HNO_3	60	180	121, 15 psi	15		
	4 % (v/v) HNO_3					700	2
	6 % (v/v) HNO_3					700	2
	4 % (v/v) HCl	60	180				
	6 % (v/v) HCl	60	180				
	4 % (v/v) HCl	60	180	121, 15 psi	15		
	6 % (v/v) HCl	60	180	121, 15 psi	15		
	4 % (v/v) HCl					700	2
	6 % (v/v) HCl					700	2
	H_2O			121, 15 psi	15		
	0.1 M Citrate Buffer			121, 15 psi	15		

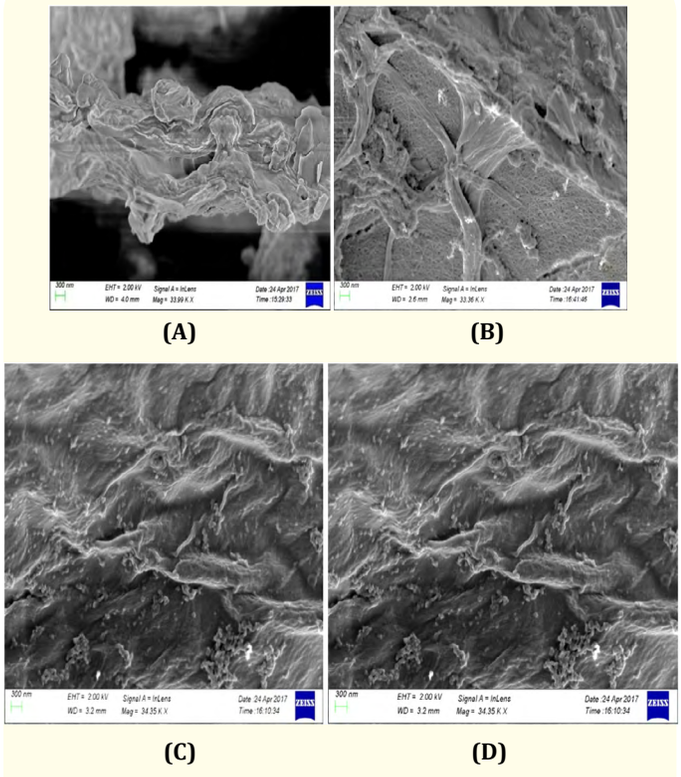
Table 1: Experimental conditions in pre-treatment of Tobacco biomass selected in this study.

Effect of Pretreatment on the structure and recovery of Tobacco lignocellulosic biomass

The pretreatment of lignocellulosic biomass is widely available in literature. In this experiment significant loss of biomass was observed during the pretreatment. FESEM images (Figure 1) below shows the effect of pretreatment on the biomass. As can be seen from the figures, citrate buffer pretreatment (Figure 1D) has produced the best result when observed under the microscope showing the maximum erosion of lignin component and exposure of the cellulose moiety.

Enzymatic hydrolysis and percentage glucose yield

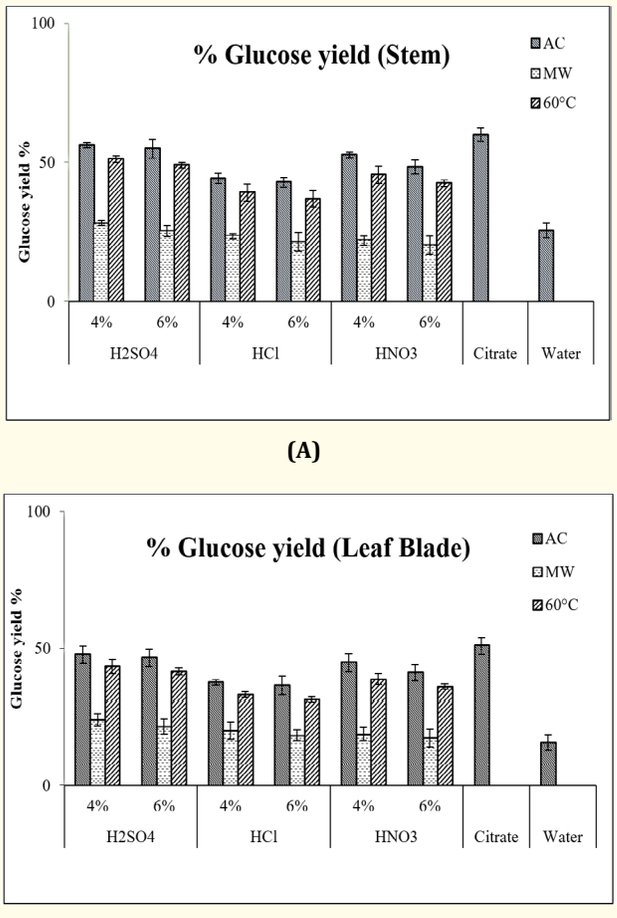
The major purpose of enzymatic hydrolysis of pretreated biomass is to release of monosacharides from their polysacharides [10]. Cellulase has a tremendous effect in the bioconversion by acting on cellulose bonds by the breakdown of the bonds to form cellobiose. The catalyst β-Glucosidase hydrolyses cellobiose, cleaving the bonds and thereby converting the cellobiose to glucose. 0.5 g of tobacco biomass was treated with cellulase from *Trichoderma reesei* (700 units/g, C2730, Sigma) and β-Glucosidase (60 U/g, 49290, Sigma) at 50°C for 72 hours while maintaining the pH at



(A) **(B)**

(C) **(D)**

Figure 1: FESEM images of untreated and pretreated biomass. (A) FESEM images of untreated biomass shows the presence of lignin in the lignocelllosic biomass. (B) FESEM images of tobacco biomass pretreated with steam in water. (C) FESEM images of tobacco biomass pretreated with steam in dilute H2SO4. (D) FESEM images of tobacco biomass pretreated with steam in 0.1 M citrate buffer.



(A)

(B)

Figure 2: Percentage yield of glucose from tobacco stem and leaf blade after pretreatment under different conditions and on enzymatic hyrdolysis using enzymes Cellulase- Trichoderma reesei (700 units/g, C2730, Sigma) and β-Glucosidase (60 U/g, 49290, Sigma). (A) Percentage glucose yield of tobacco stem pretreated at different conditions. (B) Percentage glucose yield of tobacco leaf blade pretreated at different conditions.

4.8. The glucose yield percentage was estimated using DNS method [7]. The percentage glucose yield for tobacco stem and leaf blade pretreated under different conditions is represented in table 2 and figure 2. The maximum glucose yield was observed for tobacco stem pretreated with steam in citrate buffer.

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

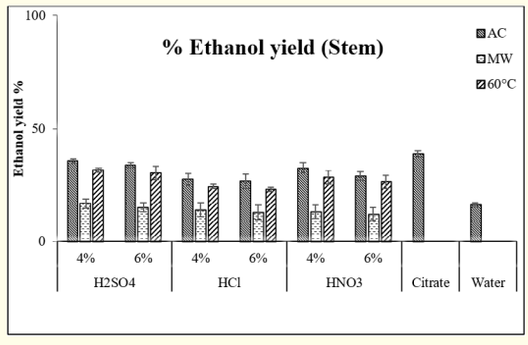
Table 2: Percentage yield of glucose from tobacco stem and leaf blade after pretreatment under different conditions and on enzymatic hyrdolysis using enzymes Cellulase- Trichoderma reesei (700 units/g, C2730, Sigma) and β-Glucosidase (60 U/g, 49290, Sigma).

Fermentation and percentage ethanol yield

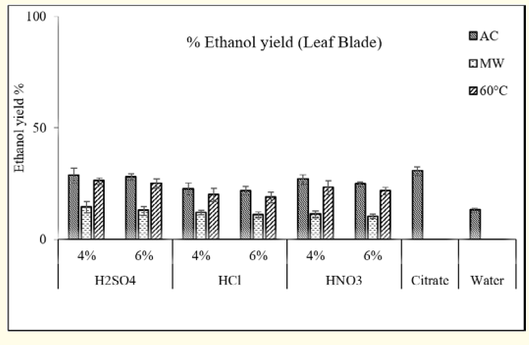
Fermentation process is the bioconversion of the sugars to bioethanol by the use of yeast *Saccharomyces cerevisiae*. The effect of the bioconversion by yeast depends on the factors of temperature, pH, aeration and nutrient supplements [11]. 0.1 ml of the enzyme hydrolysate incubated with bakers's yeast at 37°C for 24 hrs. Following the fermentation process, the extracted liquor was estimated for bioethanol using dichromate method. The percentage ethanol yield for tobacco stem and leaf blade pretreated under different conditions is represented in table 3 and figure 3. As expected, the tobacco stem pretreated with steam in citrate buffer gave the maximum yield of 38.9 %.

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

Table 3: Percentage yield of ethanol from tobacco stem and leaf blade after pretreatment under different conditions and on fermentation using baker's yeast.



(A)



(B)

Figure 3: Percentage yield of ethanol from tobacco stem and leaf blade after fermentation with baker's yeast (1×107 cells/ml) at 37°C for 24 hours. (A) Percentage ethanol yield of tobacco stem pretreated at different conditions. (B) Percentage ethanol yield of tobacco leaf blade pretreated at different conditions.

Conclusion

Even though bioethanol is primarily produced from food crops, such as sugar cane, maize and other grain cereals, alternative starchy and lignocellulosic species are under investigation. However, well-adapted plants like tobacco could foster the geographical expansion of bioethanol production. Tobacco has been formerly proposed as a production platform for biodiesel by genetically engineering the leaves to produce increasing amount of lipids in leaves [12]. In this paper, we suggest that tobacco could be developed into an unconventional energy crop for production of bioethanol, particularly in countries with committed infrastructure despite the continuous reduction of cultivated area due to harmful effects of tobacco. We have been able to, in laboratory scale harness the potential ability of tobacco biomass for bioethanol production which might be considered in a large-scale production in the nearest future.

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