A comparative study on the production of ethanol from lignocellulosic biomass by chemical and biological method

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ABSTRACT

Ethanol derived from non-edible biomass is renewable and a clean source of energy. It is independent of the food industry and it is economically feasible. The first generation biofuel or bioethanol is still not a very convenient source of energy as it prominently depends on the availability of grains. The main objective of this work is to develop an industrious efficient process to produce ethanol from lignocellulosic biomasses like wood and leaf in a lab scale. Two processes were compared. The first process involved an alkaline pre-treatment of the powdered biomass followed by dilute acid hydrolysis. The second process involved an alkaline treatment followed by direct hydrolysis of the biomass by use of a fungal species obtained from rotting wood. Following hydrolysis, fermentation was performed using *Saccharomyces cerevisiae* and ethanol produced was measured. The process methodologies performed here are liable to be scaled up easily. The final study determines factors such as temperature, strength of the reagents and retention time to maximize ethanol production.

Keywords: Cellulosic Ethanol; Biofuel; Lignocellulosic; Biomass; Hydrolysis

1. INTRODUCTION

Petroleum today is the most indispensable fuel in the world. Unfortunately, the world crude oil resources are depleting at an accelerated rate. Therefore, attention must be diverted to developing an alternative source to fossil fuel. Bioenergy holds the answer to this challenge. The most important form of bioenergy that exists in today's market is ethanol. USA and Brazil are the two frontrunners in the commercialization of fuel ethanol. In fact, USA and Brazil together produced a massive 5 billion gallons of fuel based ethanol accounting per year but it is considered less in magnitude to make an impact on the global petroleum use [1,2] . The qualities of ethanol that account for its preference are the high energy capacity, high calorific value and clean combustion . The problems associated with ethanol produced from edible plant parts are the requirement for large land area to cultivate the grains or grow the cane [pretreatment key]. In addition, pressure is placed on agriculture to meet the demands of food secuirty for a nation as well. Therefore, scientists have explored 'cellulosic ethanol', ethanol produced from the non-edible parts of the plant. However, the production of cellulosic ethanol is tedious in the biological context as there is high dependance on cellulase enzyme [1,3,4]. To circumvent this challenge, chemical treatment by acid hydrolysis has proved efficient and provided feasibility to industries to scale up easily . In this study, we explore the possibilities of both biological and chemical treatments for ethanol production.

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2. MATERIALS AND METHODS

2.1. Raw materials

Leaf litter
Red padak (*Pterocarpus*) sawdust
Plywood sawdust

2.2. Culture collection

The yeast (*S. cerevisiae*) culture used for fermentation processes was obtained from Microbial Type Culture Centre (MTCC). Micro-organism from the slant culture was inoculated in 50 ml of YPD broth (1% yeast extract, 2% peptone, 2% dextrose) and incubated at 30 °C for 4 days. This was used as seed culture.

2.3. Analytical methods

2.3.1. Dry weight estimation of lignin

The dry weight estimation of the constituents was done according to the procedures of Sun *et al.* [5] and Adsul *et al.* [6] (2005); the Sun *et al.* [5] and Badal *et al*. [7] (2005).

Lignin estimation: A considerable weight (X) of the ground, air dried substrate was suspended in 200 ml of 1% (w/v) aqueous sodium hydroxide. The sample was autoclaved in a 500 ml conical flask for 1 h at 121 °C. The residue was collected and washed with tap water till neutral pH. The residue was air dried in hot air oven at 80 °C for 48 h and weighed (x). The weight loss ($X - x$) was an approximate estimate of the lignin content.

Hemi-cellulose estimation: The delignified sample was suspended in 100 ml 1% (v/v) sulphuric acid. The suspended mixture was autoclaved at 121 °C for 1 h in a 250 ml conical flask. The residue was collected and washed with tap water till neutral pH. The residue was air dried at 80 °C for 48 h and weighed (y). The difference in weight (x) to the final weight (y) is the hemi-cellulose content.

Cellulose estimation: The final residual weight (y) after acid hydrolysis is the cellulose content.

2.4. Alkaline pretreatment

Alkaline pretreatment was performed on the biomass samples and identified the optimum temperature for lignin removal [3,4]. Conical flasks (9 × 500 ml) were taken and divided into groups of three. In the first group, to all three flasks was added 2 g of ground leaf litter. To the second group, to all three flasks was added 2 g of ground plywood sawdust. To the third group, to all three flasks was added 2 g of ground red padak sawdust. To all nine flasks was added 200 ml of 1% (w/v) aqueous sodium hydroxide (NaOH). One flask from each group was kept at room temperature for 1 h. One other flask from each group was kept in a preheated water bath at 100 °C for 1 h. The last flask from each group was autoclaved at 121 °C for 1 h. After an hour, the contents of each flask were filtered and the residue washed thoroughly until neutral pH. The residues in each flask were air dried for 48 h at 80 °C following which their dry weights were noted. Supernatant (2 ml) from each flask was stored to test for the presence of lignin.

2.5. Estimation of lignin

Folin's ciocalteu test was performed to confirm the presence of lignin semi-quantitatively . Distilled water (150 µl) was added to 50 µl of sample and mixed well. To this mixture was added 1.5 ml of 7% Na_2CO_3 followed by 1.5 ml of Folin's Ciocalteu reagent. A blank was prepared with 200 µl distilled water and the aforementioned reagents. The blank and the samples were incubated at room temperature for 90 min. The OD of these solutions was observed at 765 nm with the blank as the reference. OD values were compared to the dry weights of the corresponding residues.

2.6. Acid hydrolysis

Determined the optimum percentage of the acid for cellulose hydrolysis [1,3,4,9-11]. Each of the nine residues was split into three fractions, each fraction weighing 0.5 g. Total sample test tubes were 27. To one set of these three samples was added 10 ml 1% H_2SO_4 with reaction time of 1 h. To another set of sample was added 10 ml of 10% H_2SO_4 with reaction time of 1 h. The third set of sample was treated with 10 ml of 1% H_2SO_4 with overnight incubation. One set of nine test tubes from each group was kept at room temperature for 1 h. One other set of nine test tubes from each group was kept in a pre-heated water bath at 100 °C for 1 h. The last set of nine test tubes from each group was autoclaved at 121 °C for 1 h. The 27 acid hydrolysed samples which were subsequently neutralized using anhydrous sodium bicarbonate ($NaHCO_3$). The sample were tested for glucose by the DNS method.

2.6. Screening and isolation of cellulolytic fungi

A fungal colony was sterile picked up from a decaying piece of wood. The colony, being relatively large and rigid was ground using a mortar and pestle and diluted in 1% PBS. Serial dilution was performed to 10^{-5} times. 0.1 ml from the 10^{-4} dilution was inoculated onto a 25 ml PDA (0.4% Potato infusion, 2% dextrose, 1.5% agar) petri-dish using the spread plate technique [8]. The plate was incubated at 30 °C for 7 days. From the colonies that grew, four prominent colonies of different morphological features were isolated. The isolated colonies were grown on new PDA petri-dish for 7 days at 30 °C. The four colonies were tested for cellulose activity by growing them on CMC Agar (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$,

0.05% KCl, 0.2% CMC, 0.02% peptone, 1.7% agar). These colonies were inoculated on a single CMC plate and incubated for 3–4 days at 30 °C. On the 4th day, the plate was flooded with Gram's Iodine solution and decanted to check for zones of clearance on the CMC media . The colony that generated the largest zone of clearance was selected and stored for further studies.

2.7. Optimised physical method vs. biological method

Comparison of the optimised physical method and the biological methods was performed on basis of glucose and ethanol yield.

2.8. Optimised physical method

3.75 g of mixed waste biomass substrate (1.25 g ground leaf litter, 1.25 g ground plywood sawdust, 1.25 g ground red padak sawdust) was treated with 200 ml of 1% NaOH for 1 h at room temperature on an orbital shaker at 80 rpm [4,9-12]. The residue was collected and washed with water till neutral pH. The residue was suspended in 200 ml of the 1% H_2SO_4 with a reaction time of 1 h at room temperature. The sample was neutralized by adding anhydrous sodium bicarbonate ($NaHCO_3$) till the effervescence had ceased. Immediately after neutralization had been done, the entire volume was centrifuged at 10,000 rpm for 5 min. The supernatant was tested for glucose using the DNS method. This neutralized sample was poured into a 500 ml conical flask and inoculated with *S. cerevisiae* for fermentation to take place under at 30 °C under anaerobic conditions. Glucose and ethanol levels were tested every 24 h until ethanol levels were found to depreciate. Glucose and ethanol were estimated using the DNS and potassium dichromate assay, respectively.

2.9. Optimized biological method

The isolated cellulolytic fungi was grown in a broth containing 5 g of the mixed sample which was used as the cellulosic substrate .This is the fungal seed culture. The substituted CMC media was prepared with the similar CMC media composition but 3.75 g of mixed substrate replaced the traditional CMC in the composition. Two hundred millilitres of the substituted CMC media was prepared. The broth was autoclaved at 121 °C for 30 min with pressure at 15 psi. Ten percent of fungal seed culture was inoculated in it. Every 24 h the broth was tested for amount of glucose using the DNS method. As and when the level of glucose was found to reduce, the broth was centrifuged. The supernatant was inoculated with *S. cerevisiae*. Ethanol levels were tested every 24 h after *S. cerevisiae* inoculation.

3. RESULTS

3.1. Preliminary analysis

Initially, the dry weights of lignin, hemi-cellulose and cellulose in three different substrates were estimated. The dry weights that were estimated are depicted in the following table:

Table 1. Biomass composition in weight (%).

Sample	WPS (%)	RPS (%)	LL (%)
Lignin	39	47	59
Hemi-cellulose	23	14	12
Cellulose	26	43	22

Inference: Lignin content was found to be maximum in leaf and that of cellulose was maximum in Red Padak Sawdust.

3.2. Alkaline pretreatment

The next step carried out was the estimation of lignin after the biomass had been subjected to alkaline treatment at three different temperatures. The dry weights were calculated first followed by the Folin's Ciocalteu Test which was done to corroborate the results of the dry weight estimation.

Table 2. Weight loss (%) in biomass post alkaline pretreatment at different temperatures.

Temperature	Substrate		
	WPS (%)	RPS (%)	LL (%)
Room Temperature	33	29	65
100 °C	41	33	81
121 °C	44	38	83

Inference: Weight loss was found to be the maximum in leaf litter at 121 °C.

3.3. Acid hydrolysis

The samples, post alkaline treatment, were washed thoroughly till neutral pH was reached and treated with varying strengths of acid over varying incubation periods. The glucose in each case was estimated using the DNS assay.

Table 3. Glucose estimation for WPS glucose post acid hydrolysis at different concentration.

Sample	Temperature (°C)	Acid (%)	OD (575 nm)	Glucose (mg/ml)
		1	0.528	1.53
	RT	1 (ON)	0.072	0.20
		10	0.805	2.34

White		1	0.325	0.94
Plywood	100	1 (ON)	0.012	0.03
Sawdust		10	0.557	1.61
		1	0.322	0.93
	121	1 (ON)	0.003	0.008
		10	0.516	1.50

Inference: Acid hydrolysis was found to be most efficient at room temperature with 10% sulphuric acid.

Table 4.Glucose estimation for RPS glucose post acid hydrolysis at different concentration.

Sample	Temperature (°C)	Acid (%)	OD (575 nm)	Glucose (mg/ml)
		1	0.113	0.32
	RT	1 (ON)	0.036	0.10
		10	0.125	0.36
Red		1	0.148	0.43
Padak	100	1 (ON)	0.034	0.09
Sawdust		10	0.162	0.47
		1	0.012	0.03
	121	1 (ON)	0.003	0.008
		10	0.015	0.04

Inference: The glucose yield was found to be the maximum at 100 **°C**.

Table 5. Glucose estimation for LL glucose post acid hydrolysis at different concentration.

Sample	Temperature (°C)	Acid (%)	OD (575 nm)	Glucose (mg/ml)
		1	0.600	1.74
	RT	1 (ON)	0.045	0.13
		10	0.670	1.94
Leaf		1	0.318	0.92
Litter	100	1 (ON)	0.105	0.30
		10	0.350	1.01
		1	0.120	0.34
	121	1 (ON)	0.105	0.30
		10	0.125	0.36

Inference: Room temperature was found to be the most favourable condition to carry out acid hydrolysis.

3.6. Screening and isolation of cellulolytic fungi

A pure fungal colony was isolated from decaying wood .The fungus, tested positive for cellulase activity on CMC media using Gram's Iodine solution.

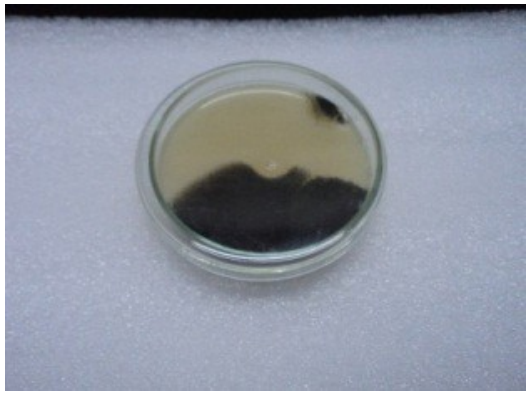

Fig. 1. Fungal colony isolate.

Fig. 2. Cellulase screening on CMC media using Gram's Iodine.

Fig. 3. 45× view of fungal isolate.

Inference: The colony that gave the largest zone of clearance produced more units of cellulase in the shortest time.

3.7. Optimised physical method vs. biological method

After the preliminary and confirmatory analysis of the best suited method was done, two optimized methods were followed one involving chemical hydrolysis by 1% sulphuric acid at room temperature and the other involving biological hydrolysis by the fungal organism isolated from a decaying piece of wood. The glucose and ethanol in these samples were tested every 24 h to give a profile that predicted the best time of incubation.

Table 6. Calculated values for glucose and ethanol.

Time (h)	Chemical hydrolysis		Biological hydrolysis	
	Glucose (mg/ml)	Ethanol (%)(v/v)	Glucose (mg/ml)	Ethanol (%) (v/v)
0	7.6011	Yeast inoculated	0.2691	–
24	7.1713	0.0899	0.3268	–
48	6.4388	0.1354	0.396	–
72	5.8791	0.098	0.4797	–
96	5.1205	0.0819	0.4422	–
120	–	–	0.3758	Yeast inoculated
144	–	–	0.3729	0.0819
168	–	–	0.3701	0.0605

Inference: Glucose concentration was found to be maximum immediately after chemical hydrolysis and ethanol percentage peaked at 48 h incubation. Glucose concentration during biological hydrolysis was found to be maximum at 72 h of incubation and ethanol percentage peaked at 24 h incubation.

4. DISCUSSION

4.1. Preliminary analysis

It can be concluded from Table 1 that if all the three biomasses were mixed and treated together, the average cellulose content present would be an approximate 30%. This is a very good percentage given that the sustrates are waste materials. Leaves, have a profoundly high starch content, but, the leaves that were used in the experiment were categorized as litter and thus had a drained starch content.

Red padak sawdust was found to have maximum cellulose content, quite greater than white plywood sawdust. The logical explanation that can be given here is that red padak sawdust is less processed when compared to white plywood sawdust, which would clearly point to the fact that lesser cellulose would have been drained during the compression process of the plywood, hence giving it a higher cellulose content. The substrate thus proved to be rich in cellulose and suitable for use as a source of cellulosic ethanol.

4.2. Alkaline pretreatment

Tables 2 and 3 suggest that the lignin removal was highest from leaf treated at 121 °C. The semi-quantitative Folin's test also suggests the same fact. One explanation for the very high OD values in the Folin's test of the leaf sample could be the presence of other phenolic compounds like isoflavones and flavonoids which could have interfered with the results. Another possible reason for the loss of weight at high incubation temperatures could be associated with the fact that 100 °C and 121 °C are temperatures at which part of the hemi-cellulose would have been hydrolysed and thus washed away with the water, hence resulting in a higher weight loss. From the results, 121 °C appears to be the ideal temperature for lignin removal but from a large scale industry point of view, 121 °C would be an impractical temperature to sustain coupled with a pressure of 15 psi.

The method that clearly promised economic feasibility as well as fairly high removal of lignin was the alkaline treatment at room temperature. The comparatively low variance in the OD values of the RT and 121 °C samples directly mean that the actual content of lignin also would not vary drastically. Again, the wide difference in the leaf samples was attributed to the presence of other phenolic compounds. Considering all of these factors, alkaline treatment at room temperature seemed to be the best method to follow.

4.3. Acid hydrolysis

4.3.1. White plywood sawdust

According to the data in Table 4, overnight incubation proved to be the least effective since excessive exposure to sulphuric acid would have led to the dehydration of the glucose and production of inhibitors like sulphur oxide.

Even though 10% acid proved to be the most effective strength of acid to carry out hydrolysis, on an industrial scale it is more difficult to maintain when compared to acid of 1% strength. The variance of 1% acid OD values from the 10% acid OD values is not very high and thus on a long term basis, carrying out acid hydrolysis by using 1% acid would thus give a higher overall yield when compared to carrying out the process with 10% acid.

4.3.2. Red padak sawdust

From the data in Table 5 the small variances in the values at room temperature and 100 °C could be ignored when large scale considerations were made. Even in this case, room temperature proved to be a better choice to carry out acid hydrolysis.

4.3.3. Leaf litter

The difference between 1% and 10% acid OD values is in fact very minute, as the Table 6 depicts.

1% of acid at room temperature was found to be the best condition to carry out acid hydrolysis.

4.4. Screening and isolation of cellulolytic fungi

Fig. 1 shows the pure colony isolated. From Fig. 2, the region with the largest zone of clearance was the area where the fungal micro-organism produced the maximum units of cellulase. The cellulase produced in the region broke down the carboxy methyl cellulose in the media to glucose which did not stain Gram's iodine solution in that region. In conclusion, it was the most favourable colony to be used in the biological hydrolysis process. The fungus of this colony was later identified to be a member of the *Aspergillus* species. This decision was made by comparing the hyphae structures of the colony in Fig. 3 to a database of fungal species (doctorfungus.org, tnenvis.nic.in, aspergillus.org.uk). Even though the decision was made based on morphological characteristics, there is striking resemblance in the hyphae structures.

4.5. Optimised physical method vs. biological method

From Tables 6 it can be noted that after chemical hydrolysis the glucose concentration consistently reduced indicating that the glucose is converted to ethanol by *Saccharomyces cerevisiae*. The ethanol was found to peak at 48 h incubation and after that was found to reduce. Via biological hydrolysis, the glucose concentration was first found to increase and then decrease after peaking at 72 h incubation. This can be attributed to the fact that the fungus breaks down the cellulose and after 3 days of incubation, when it has sufficient glucose in the media, utilizes that glucose for its own growth.

The 0^{th} h OD reading of glucose via chemical hydrolysis corresponds to a glucose concentration of 7.6011 mg/ml which would give a total of 1.5202 g of glucose in the 200 ml of acid hydrolysed sample. This would give a hydrolysis efficiency of approximately 40.53% since 3.75 g of the substrate had been hydrolysed. With respect to ethanol production, the maximum OD value was seen at 48 h of incubation which corresponds to 0.1354% ethanol in the sample. It can therefore be calculated that in the 200ml of the inoculated sample was present 0.2708 ml of ethanol that is 0.2137 g of ethanol. Substrate (1 kg) would thus yield 57 g of ethanol with an efficiency of 5.69%.

The hydrolysis efficiency of biological hydrolysis is however very low at 2.56%. The alcohol percentage peaked at 24 h incubation with 0.0819% ethanol in the sample. This would mean that 3.75 g of substrate via fungal hydrolysis and a subsequent fermentation gave 0.129 g of ethanol. Substrate (1 kg) would thus give 34.4 g of ethanol. Comparing both, chemical hydrolysis was clearly the better choice. Further research is required to produce better results with biological method of pretreatment.

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