Research Article

Conversion of Lignocellulosic Wastes to Sustainable Energy using *Saccharomyces cerevisiae* and *Zymomonas mobilis*

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ABSTRACT

Climate change impacts and growing environmental concerns over the use and depletion on non-renewable energy resources, greenhouse gas (GHG) emissions, together with the recent increase in price and instability of the oil markets have spurred researchers and industries to look at clean energy options for the sake of both the global and local environments. In this respect, the replacement of petroleum-based gasoline with biomass-based ethanol will result in a new industrial revolution and a sustainable carbohydrate economy. Lignocellulosic biomass is considered as the only foreseeable feasible and sustainable resource for renewable fuel. The structural complexity of the lignocellulosic material hinders enzymatic hydrolysis for what their conversion to bioethanol requires a pretreatment step. The substrates (wheat straw, rice straw and sugarcane bagasse) were pretreated with an acid and an alkali followed by enzymatic hydrolysis and then subjected to two methods of fermentation namely, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The study reveals that the ethanol production by SSF was higher (53.203%) with the highest initial reducing sugar (6.724 gg^{-1}), after 72 h of fermentation at different concentrations of the inoculum level from the mixed cultures after supplementation of sugarcane molasses to the fermentation media.

Keywords: Bioethanol, Saccharification Pretreatment, Lignocellulosic biomass, Fermentation.

INTRODUCTION

Biomass-based ethanol is well entrenched in a policy as a potential substitute for gasoline. Due to the ever increasing demand for energy, rapid growth in population, fast depletion of crude oil reserves and industrialization, globally there is an increased interest in alternative fuels.[1] One of the primary benefits of switching to bioethanol is because of the easy adaptability of this fuel to existing engines, it is a cleaner fuel and its biomass renewability can potentially provide a sustainable fuel supply.[2,3] The production of second generation biofuels from renewable lignocellulosic biomass will result in a new industrial up rise from fossil based economy to a sustainable carbohydrate economy.[4] The lignocellulosic materials are made of three structural polymers: cellulose, hemicelluloses and lignin.[5]

Cellulose is a homopolysaccharide, consisting of β-D glucopyranose attached with linear chains. It is crystalline in nature. Hemicellulose is a heteropolysaccharide composed of pentoses (D-xylose, D- arabinose), hexoses (D-mannose, D-glucose and galactose) and sugar acids. Lignin is largely composed of phenyl-propane units mostly linked by ether bonds.[6]

The biomass is a less expensive source of carbon and includes wheat straw,[7] rice straw,[8] sugarcane bagasse,[9] willow,[10] corncobs and waste newspapers.[11] Among the agro-residues, wheat straw, rice straw and sugarcane bagasse are substrates of high potential for biotechnological processes. Cellulose, hemicelluloses and lignin content of rice straw are in the range of 32-47, 19-27, and 5-24% respectively,[12] while wheat straw contains 33-40% of cellulose, 20-25% of hemicelluloses and 15-20% of lignin,[13] whereas sugarcane bagasse comprises of 40-44% cellulose, 24-28% hemicelluloses and 10-14% lignin.[14]

Bioconversion process is considered as the most promising available technologies for ethanol production from lignocellulose based on enzymatic hydrolysis. This process involves four steps, i.e pretreatment, enzymatic hydrolysis, microbial fermentation and separation. Enzymatic saccharification can be integrated and separated into simultaneous saccharification and fermentation and separate hydrolysis and fermentation respectively.[15] Of these four steps, pretreatment is perhaps the most important step in cellulosic ethanol production process, the goals being, minimizing the formation of inhibitors for subsequent fermentation steps, avoiding sugar degradation, disrupting hydrogen bonds in crystalline cellulose and removing the cross-linked matrix of lignin and hemicelluloses that embeds the cellulose fibers.

Several physicochemical and chemical pretreatment methods are currently employed to overcome the recalcitrance of the lignocellulosic biomass, improve the monomeric sugar yield and increase enzyme efficiency. These include dilute acid,[16] alkaline,[17] liquid hot water,[18] organosolv[19] and ammonia explosion[20] pretreatment technologies.

Among these techniques, dilute sulfuric acid and sodium hydroxide pretreatment have been considered as leading pretreatment process that are currently under commercial development. The acid medium attacks the polysaccharides, especially hemicelluloses which are easier to hydrolyse than cellulose.[21] Alkaline treatment

digests the lignin matrix and makes hemicellulose and cellulose available for enzymatic degradation.[16] Alkaline treatment was followed by enzymatic hydrolysis which involves cellulases mostly produced by soft-rot fungi such as *Trichoderma, Penicillium* and *Aspergillus*.[22] Endo- and -exoglucanase and β-glucosidases are a cocktail of cellulases required in order to break down the microfibril structure of cellulose into its carbohydrate components in an effective manner.[23]

A wide variety of microorganisms like yeast and bacteria are used to ferment the above said lignocellulosic materials to produce bioethanol.[24] The strains must contain adequately balanced cellulase and fermentation activities for a good production of ethanol. For economical viability on an industrial scale, lignocellulosic ethanol production must be above 4% (v/v) in the fermentation broth. To enhance the production, cane molasses can be added to the fermentation media.

In the present study, wheat straw, rice straw and sugarcane bagasse were chosen as the raw lignocellulosic materials. In order to obtain high ethanol concentrations, a combination of an acid and an alkali pretreatment were used to fractionate the biomass to obtain substrates with high cellulose content. An addition of cane molasses to the fermentation media helps in supplementation of macro and micro nutrients. Ethanol production was investigated by using *Saccharomyces cerevisiae* and *Zymomonas mobilis* in both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

MATERIALS AND METHODS

Microorganisms

Aspergillus flavus (KUMBF1308) was obtained from the Department of Microbiology, Karpagam University. The fungus produces cellulolytic enzymes that convert carbohydrate polymers into fermentable sugars. The yeast strain *Saccharomyces cerevisiae* was obtained from Sakthi Sugars Ltd., Bhavanisagar, Tamilnadu, India and was grown in YEP broth media (contained (w/v) yeast extract 0.3%, peptone 1.0%, dextrose 2%, pH 6.0). *Zymomonas mobilis* (MTCC 10988) was collected from the Microbial Type Culture Collection Chandigarh, India and was grown in a nutrient rich medium containing dextrose 2%, yeast extract 1.0%, KH_2PO_4 0.2% and pH 6.0. After incubation for 24 h at 120 rpm, they were used as inoculums for ethanol production.

Pretreatment Strategies

Wheat straw, rice straw and sugarcane bagasse were obtained from the local market, Coimbatore, Tamil Nadu, India. They were sliced into small pieces and spread on trays. After sieving, they were used as substrates.

The lignocellulosic materials were treated with 2% H_2SO_4 at the ratio of 1 g solid per 10 mL liquid autoclaved for 45 min. The lignocellulosic materials were then filtered and washed with tap water until neutral. Then the washed materials were treated with 2% NaOH at a ratio of 1 g original raw material to 6 mL liquid. The solids were separated by filtering, washed with tap water until neutral then dried at 105°C.[25]

Enzyme Preparation

For enzyme production, *Aspergillus flavus* (KUMBF1308) was cultured on Potato Dextrose Agar slants, incubated at 28°C for 7 days for spore suspension preparation. All the substrates were dried in a mechanical dryer at 50°C till constant moisture content was obtained. Solid state fermentation was carried out by taking 10 g of each substrate, dispensed into 500 mL Erlenmeyer conical flask and moistened with 10 mL salt solution (Glucose: 0.6, KH_2PO_4: 0.1%, $MgSO_4.7H_2O$: 0.05% and KCl: 0.05%). The flasks were autoclaved at 121°C for 25 min, cooled to room temperature and inoculated with 2 mL of the fungal conidial suspension (10^6 spore/mL). The inoculated flasks were mixed thoroughly and incubated at 30°C for 7 days in a static incubator. At the end of the fermentation, the supernatant was harvested by centrifuging at 10,000 rpm for 20 min at 4°C and was used as crude cellulase enzyme. Cellulase enzyme filter paper activity (FPA) assay was studied by.[26]

Enzyme Saccharification

The enzyme preparation was added to the pretreated substrates suspended in sodium citrate buffer 0.05 M (1% w/v), pH 5 at a concentration of 1 IU of FPA activity/ mL and incubated at 50°C. At regular time intervals, samples were withdrawn and assayed for the amount of reducing sugars released. The reducing sugar content was assayed by DNS method[27] and the percentage of saccharification was calculated by the formula proposed by Tewari.[28] The cellulose content was estimated following the method of Updegraff, (1969).[29]

$$Saccharification\ (\%) = \frac{Reducing\ sugar\ formed\ \times 0.9 \times 100}{Cellulose\ content\ of\ pretreated\ substrate}$$

Molasses Preparation

The novel strategy of using 5% (v/v) sugarcane molasses promotes complete sugar consumption and increases the ethanol yield. The molasses sugar was adjusted to a specific concentration by diluting with distilled water at a dilution of approximately 180 g/L. The solid particles were removed by decantation. The reducing sugar content was estimated by DNS method.[27] The hydrolysates obtained after saccharification process were then supplemented with 5% molasses.

Separate Hydrolysis and Fermentation

The hot air oven dried pretreated biomass by a combination of an acid and an alkali was subjected to enzymatic saccharification using crude cellulase enzyme at 5 IU of FPA activity/mL. After filtration with Whatman No. 1 filter paper, the hydrolysates were supplemented with 5% cane molasses after a specific dilution with distilled water of approximately 180 g/L and then allowed to ferment for 72 h after inoculation with the mixed

cultures of *S.cerevisiae* and *Z.mobilis* at varying concentrations (0-10% and 10-0%).

Simultaneous Saccharification and Fermentation

The acid/alkali pretreated biomass containing both the residue and the hydrolysates were supplemented with 5% of sugarcane molasses after a specific dilution with distilled water of approximately 180 g/L. At a concentration of 5 IU of FPA activity/mL, the crude enzyme was loaded along with both cultures of *S.cerevisiae* and *Z.mobilis*. Fermentation was carried out for 72 h at varying concentrations (0-10% and 10-0%) of the inoculums.

Analytical Method

Analysis of the ethanol content was done by spectrophotometric method.[30]

Ethanol Yield

The ethanol yield was calculated by the modified formula proposed by.[31]

$$Ethanol\ yield\ (\%) = \frac{Ethanol\ produced\ \times 100}{Reducing\ sugar\ utilized}$$

Statistical Analysis

Data were subjected to analysis of variance by One Way ANOVA using AGRES software and Duncan's Multiple Range Test (DMRT).[32]

RESULTS AND DISCUSSION

The lignocellulosic biomass is composed of three structural polymers: cellulose, hemicelluloses and lignin and small quantities of other compounds. Among these components, cellulose and hemicelluloses can be pretreated and saccharified and eventually fermented to obtain bioethanol. For pretreatment, a combination of an acid and an alkali aided in the final ethanol production. The purpose of the pretreatment was to disrupt the crystalline structure of cellulose, remove lignin and/or hemicelluloses and to increase the porosity of the substrates for enzymatic attack.[33] High ethanol production depends on a high cellulose concentration that is best affected by removal of non-cellulose components by pretreatment.[34] Cellulosic ethanol has recently been produced from agricultural residues (straw and bagasse) which are cheap feed stocks easily available and also does not have the ethical concern associated with the use of potential food resources. This is currently a hot spot in the bioenergy research field.[35]

The cellulose content of the pretreated agro wastes was analyzed and tabulated as shown in (Table 1). Acid/alkali pretreatment had a great influence on the reducing sugars released through enzymatic hydrolysis by the crude cellulase enzyme (5 IU FPA activity/mL) prepared from *A. flavus* (KUMBF1308). Generally cellulases penetrate into the substrate to access and hydrolyse, unlike many common enzymes which take their substrates into the active site pockets. They have specific domains for binding with their substrate so that the enzyme works on the polymer and causes a slow degradation.[36] The highest initial reducing sugar released was 6.724 gg^{-1} as shown in (Figure 1; Table 2) with a gradual decrease in the percentage of saccharification where 60.516% was the highest percentage attained at 24 h and the lowest 12.114% at 72 h as shown in (Table 2).

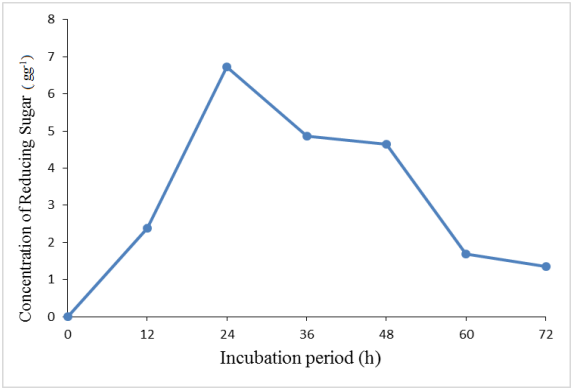

Figure 1: The Effect of Crude Cellulase Enzyme on Release of Reducing Sugars

Geeta et al., (2001)[37] reported a maximum release of reducing sugars of 313 mg g^{-1} by treating *Samanea saman* with hot water and 3% H_2SO_4. The alkaline hydrolysis of wheat straw resulted in 85% conversion of cellulose to form glucose.[38] Arthe et al., (2008)[39] reported a gradual increase of the amount of reducing sugars of 55 mg mL^{-1} and 295 mg mL^{-1} when cotton waste was treated with 0 and 0.5% acid respectively. 3% acid concentration gave 400 mg mL^{-1}.

Hydrolysis of cellulose to glucose in aqueous media catalyzed by the cellulase enzyme suffers from slow reaction rates due to highly crystalline linear structure of cellulose which makes penetration of enzymes to the active side very difficult.[40] Pretreatment necessitates the removal of lignin to expose other molecules to enzymatic action. Enzymatic hydrolysis and fermentation process can be accomplished using different strategies; separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF).[16] The fermentation process would be economically viable only if both hexose and pentose sugars present in the hydrolysates are converted to ethanol. *Saccharomyces cerevisiae* and *Zymomonas mobilis* have already been accepted as the most promising microorganisms for fermentation, as they produced considerably more ethanol yield and showed higher volumetric rate from sugar mixtures of pentose and hexoses.[41] The synergistic effect of *S. cerevisiae* and *Z. mobilis* is mentioned since the characteristics of these two organisms are mutually beneficial in fermenting sugars even under anaerobic conditions that may be created during growth phases. *Saccharomyces cerevisiae* is by far the most commonly used microbial species for bioethanol production and is well adapted to the

industrial scenario. It produces ethanol with stoichiometric yields, high specific rate of sugar intake and tolerates a wide spectrum of inhibitors. *Zymomonas mobilis* has been projected as the future ethalogen due to its high specific rate of sugar intake, can tolerate (up to 14% v/v) of ethanol, energy efficiency and high ethanol yield (up to 97% of theoretical). Yeast *Saccharomyces cerevisiae* and bacteria *Zymomonas mobilis* are the best commonly employed organisms used in alcohol fermentation.[42] The mixed cultures had a great influence in the prodution of bioethanol after their integration at different levels of the inoculum size. *Zymomonas mobilis* showed high titres at the concetration of 6 to 4 for both SSF and SHF in all the four experiments. In this research work, SHF and SSF were considered. SSF gave better results as compared to SHF as indicated in Table 5 (2.401 gg^{-1}) and Table 6 (2.541 gg^{-1}) ethanol production and a yield of 51.435% and 53.203% respectively. For SHF the results were slightly lower with a production of 2.220 gg^{-1} (Table 3) and 2.321 gg^{-1} (Table 4) and a yield of 49.191% and 50.227% respectively.

Table 1: Analysis of Purified Cellulose and Pretreated Agro Wastes

S. No.	Substrate	Original Concentration mg/mL	Cellulose Found mg/mL	% Cellulose Recovered from Substrates
1	Purified cellulose	40.0	40.2	40.2
2	Pretreated agro wastes	20.0	10.0	10.0

Table 2: Saccharification of Cellulosic Wastes by *Aspergillus flavus*

S. No.	Incubation Period (h)	Reducing Sugar (gg^{-1})	Saccharification (%)
1	12	2.373 ± 0.130	21.357
2	24	6.724 ± 0.111	60.516
3	36	4.862 ± 0.071	43.758
4	48	4.641 ± 0.143	41.769
5	60	1.685 ± 0.076	15.165
6	72	1.346 ± 0.050	12.114

Values are mean ± SD of three samples.

Table 3: Ethanol Production by Separate Hydrolysis and Fermentation by *S.cerevisiae* and *Z.mobilis*

Incubation Period (h)	Inoculum Size		RSU (gg^{-1})	EP (gg^{-1})	EY (%)
	S.cerevisiae	*Z.mobilis*			
24	10	0	4.426 ± 0.053	1.921 ± 0.130	43.403
48	8	2	4.482 ± 0.621	2.115 ± 0.069	47.189
72	6	4	4.513 ± 0.325	2.220 ± 0.164	49.191
96	4	6	4.406 ± 0.521	2.108 ± 0.148	47.844
Initial reducing sugar concentration in the hydrolysate (gg^{-1})	6.724 ± 0.111				

RSU: Reducing sugar utilized (gg^{-1}); EP: Ethanol Production (gg^{-1}); EY: Ethanol Yield (%). Values are mean ± SD of three samples

Table 4: Ethanol Production by Separate Hydrolysis and Fermentation by *S.cerevisiae* and *Z.mobilis*

Incubation Period (h)	Inoculum Size		RSU (gg^{-1})	EP (gg^{-1})	EY (%)
	S.cerevisiae	*Z.mobilis*			
24	0	10	4.327 ± 0.266	1.934 ± 0.029	44.696
48	2	8	4.475 ± 0.059	2.127 ± 0.256	47.531
72	4	6	4.621 ± 0.820	2.321 ± 0.000	50.227
96	6	4	4.416 ± 0.335	2.116 ± 0.164	47.917
Initial reducing sugar concentration in the hydrolysate (gg^{-1})	6.724 ± 0.111				

RSU: Reducing sugar utilized (gg^{-1}); EP: Ethanol Production (gg^{-1}); EY: Ethanol Yield (%). Values are mean ± SD of three samples

Table 5: Ethanol Production by Simultaneous Saccharification and Fermentation by *S. cerevisiae* and *Z. mobilis*

Incubation Period (h)	Inoculum Size		RSU (gg^{-1})	EP (gg^{-1})	EY (%)
	S.cerevisiae	*Z.mobilis*			
24	10	0	4.317 ± 0.145	1.962 ± 0.029	45.448
48	8	2	4.505 ± 0.256	2.134 ± 0.058	47.370
72	6	4	4.668 ± 0.029	2.401 ± 0.071	51.435
96	4	6	4.494 ± 0.050	2.218 ± 0.014	49.355

RSU: Reducing sugar utilized (gg^{-1}); EP: Ethanol Production (gg^{-1}); EY: Ethanol Yield (%). Values are mean ± SD of three samples

Table 6: Ethanol Production by Simultaneous Saccharification and Fermentation (SSF) by *S.cerevisiae* and *Z.mobilis*

Incubation Period (h)	Inoculum Size		RSU (gg^{-1})	EP (gg^{-1})	EY (%)
	S.cerevisiae	*Z.mobilis*			
24	0	10	4.412 ± 0.033	2.108 ± 0.111	47.779
48	2	8	4.423 ± 0.050	2.172 ± 0.256	49.107
72	4	6	4.776 ± 0.029	2.541 ± 0.000	53.203
96	6	4	4.515 ± 0.058	2.300 ± 0.148	50.941

RSU: Reducing sugar utilized (gg^{-1}); EP: Ethanol Production (gg^{-1}); EY: Ethanol Yield (%). Values are mean ± SD of three samples

Table 7: Influence of Sugarcane Molasses on Ethanol Production

Experiment	Strains		Incubation Time (h)	EP (gg^{-1})	EY (%)
Separate hydrolysis and fermentation	*S.cerevisiae* and *Z.mobilis*	Without molasses	24	0.817 ± 0.033	20.726
			48	1.048 ± 0.057	26.148
			72	1.201 ± 0.029	29.146
			96	0.960 ± 0.033	23.460
		With 5% molasses	24	1.934 ± 0.029	44.696
			48	2.127 ± 0.256	47.531
			72	2.321 ± 0.000	50.227
			96	2.116 ± 0.164	47.917
Simultaneous saccharification and fermentation	*S.cerevisiae* and *Z.mobilis*	Without molasses	24	0.872 ± 0.100	21.735
			48	1.154 ± 0.029	27.955
			72	1.428 ± 0.058	33.839
			96	1.196 ± 0.153	28.640
		With 5% molasses	24	2.108 ± 0.000	47.779
			48	2.173 ± 0.050	49.107
			72	2.541 ± 0.111	53.203
			96	2.300 ± 0.256	50.941

Values are mean ± SD of three samples

Simultaneous saccharification and fermentation (SSF) plays a crucial role to overcome enzyme inhibition. This process combines enzymatic saccharification with ethanol fermentation to keep the concentration of glucose low. The accumulation of bioethanol in the fermenter does not inhibit cellulases as much as high concentration of glucose, making SSF an efficient strategy for increasing the overall rate of cellulose to ethanol conversion.[43]

Chadha et al., (1995)[44] reported an improved bioethanol yield when rice straw underwent SSF. Saha and Cotta (2006)[45] evaluated the performance of both SSF and SHF on wheat straw and due to the reduction of glucose inhibition in the enzymatic hydrolysis, the detoxifying effect of fermentation and the positive effect of inhibitors present in the pretreated hydrolysate, excluding the aspect of time, they suggested SSF approach is more advantageous and works well than SHF.

Suresh et al., (1999)[46] used *Aspergillus niger* and *Saccharomyces cerevisiae* for simultaneous saccharification and fermentation of grains and obtained ethanol of 2.90% (v/v). Zayed and Meyer, (1996)[47] used wheat straw for the production of ethanol by SSF and reported a yield of 11.8 g/L by using *T. viride* and *P. tannophilus.*

Nutritional requirements as well as growth factors play an important role in increasing the cell mass and thereby ethanol production. Sugarcane molasses is one of the liquors resulting from crystallization step in cane sugar processing although it may vary in composition, but it usually contains 50-55% fermentable sugars and 8-14% ash, where major components are calcium (1.5%), potassium (4.0%), magnesium (1.0%), silica (0.4%) and phosphate (0.2%).[48]

Molasses had a great influence on the production of ethanol when it was supplemented in the fermentation media (Table 7). Its addition to the medium may have aided to regulate the maintenance energy requirements seen as lower growth reporter for xylose utilization when compared with glucose utilization, supported by the suggestion that production of ATP from xylose is lower than that from glucose.[49]

Due to its high osmolality, molasses has the advantage that it can be stored for extended periods of time without microbiological spoilage. Dilution of the sugarcane molasses aids to avoid inhibitory ethanol concentrations in the fermentation step.[50]

Saiga and Vishwanathan, (1984)[51] demonstrated an increase in the rate of bioethanol production by addition of vegetable oils and fatty acids as supplements. These findings clearly indicate that some additives including cane molasses can be influential in enhancing the production of ethanol.

CONCLUSION

The combination of acid and alkali pretreatment was effective in preparing the substrates for ethanol production. Pretreatment removed most of the non-cellulosic materials making cellulose more accessible to enzymes that convert it into fermentable sugars.

From the study, enzymatic saccharification with the crude cellulase enzyme from the fungi and fermentation by *S. cerevisiae* and *Z. mobilis* after the media was supplemented with cane molasses, the produce was high with a 53.203% yield.

When comparing the two methods of fermentation, SSF produced large amounts of ethanol hence making the method preferable. This study indicates a better solution for waste management through the utilization of wheat straw, rice straw and sugarcane molasses for ethanol production that could be used in various industrial applications.

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