Bioethanol Production From Lignoellulosic Biomass

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ABSTRACT

The various thermophilic strains were isolated from the soil samples collected from the dumping site of sugarcane bagasse and screened by using phenol red broth. Only one microorganism showed fermentation activity on all the substrates except lactose and cellulose. This strain was selected for further fermentation studies and characterized as yeast *Kluyveromyces* sp. IIPE453. The yeast strain showed the growth and fermentation on glucose, mannose, galactose, xylose, sucrose, cellobiose and lactose. The optimum conditions for growth and fermentation were found to be 50 ºC temperature and 5.0 pH.

About 92 % of the sugars present in the bagasse biomass could be recovered by acid hydrolysis in two steps, firstly with dilute acid to hydrolyze hemicelluloses and then with concentrated acid to hydrolyze cellulose. Fermentation of the bagasse hydrolysate with *Kluyveromyces* sp. IIPE453 was studied separately for hydrolysates obtained by dilute acid hydrolysis and by concentrated acid hydrolysis.

Introduction

A sustainable development of mankind has to be based on renewable energy (1). Reserves of fossil fuels such as petroleum and coal are depleting fast leading to unprecedented and unpredictable price rise and uncertainty in availability of these fuels. Energy from fossil fuels is environmentally unfriendly and is blamed for the global climate change to a large extent.

Fuels derived from biomass have the potential of providing clean, carbon neutral, and sustainable energy. The ethanol production from lignocellulosic biomass through biochemical route has attracted worldwide attention because of the potential of ethanol to be used as an alternative automotive fuel, preferably in a blend with gasoline. The use of ethanol in transport sector is very important for such countries as India as it depends heavily on import of crude oil and spends a huge sum of its annual budget for the oil import. The closed carbon cycle that results when bio-based feed-stocks are used for the production of ethanol as also the use of ethanol as a fuel has a potential of reducing carbon dioxide emission into the atmosphere (2-5). Ethanol can be easily blended with either gasoline or diesel and can be used as an automotive fuel.

In the present study, a thermophilic strain that can ferment glucose and xylose to ethanol has been screened. The strain is then used to ferment sugars obtained from acid hydrolysis of sugarcane bagasse.

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Literature Review

The traditional feed-stocks for ethanol production such as corn, food grains, sugarcane juice, and cane molasses face social and economic barriers as these materials are used substantially for human and animal consumption. Lignocellulosic biomass, on the other hand, is available abundantly and can be used as the alternative feed-stock for bioethanol production. Lignocellulosic biomass includes **forest residues** such as wood; **agricultural residues** such as sugarcane bagasse, corn cob, corn stover, wheat and rice straw; **industrial residues** such as pulp and paper processing waste, lignin from pulp and paper mills and municipal solid wastes, and **energy crops** such as switch grass. These have the potential for use as feed-stock for the production of fuel ethanol (6-10). The nature and availability of lignocellulosic feed-stocks in different parts of the world depend on climate and other environmental factors, agricultural practices, technological developments, and current usage/consumption pattern (11-12).

Composition of Biomass

Lignocellulosic biomass is a complex polymeric material that contains cellulose (20-50 %), hemicellulose (20-40 %), polyphenolic lignin (15-25 %) and other components. Cellulose, the major constituent of lignocellulose, is a linear polymer composed of thousands of glucose subunits linked by β-(14)-glycosidic bonds. Hemicellulose, the second major constituent of lignocelluloses, is a highly branched and complex heteropolymer that contains hexoses (glucose, galactose, mannose, rhamnose, fucose), pentoses (xylose and arabinose) and uronic acids (glucuronic acid and galacturonic acid). Hemicellulose is more easily hydrolyzed to its constituent monosaccharides than cellulose. Lignin is an aromatic polymer containing phenolic residues such as trans-*p*-coumaryl alcohol (*p*-hydroxy phenyl propanol), trans-*p*-coniferyl alcohol (guaiacyl propanol) and trans-*p*-sinapyl alcohol (syringyl propanol), which serve to bind the cellulose fibers. Coniferyl alcohol is the principal component of softwood lignins, whereas guaiacyl and syringyl alcohols are the main constituents of hardwood lignins (13). Approximate sugar composition in various lignocellulosic raw materials is given in Table-1.

Conversion Routes of Biomass to Biofuels

Figure 1 schematically shows various biochemical and chemical routes to produce biofuels from biomass (14). The main processes are fermentation of sugars to alcohol, gasification and

Table 1: Composition of sugars in various lignocellulosic raw materials.

Raw materials	Sugar content, %				
	Glucose	Mannose	Galactose	Xylose	Arabinose
Corn stover	39	0.3	0.8	14.8	3.2
Wheat straw	36.6	0.8	2.4	19.2	2.4
Rice straw	41	1.8	0.4	14.8	4.5
Sugarcane Bagasse	38.1	--	1.1	23.3	2.5
Rice hulls	38.1	3.0	0.1	14.0	2.6
Switch grass	31.0	0.2	0.9	0.4	2.8
Rye grass	23.9	--	--	14.7	2.8
Cotton gin	37.1	1.1	2.4	9.4	2.3
Sugar beet pulp	24.1	4.6	0.9	18.2	1.5
Hybrid poplar	44.7	2.2	0.97	14.56	0.82
Eucalyptus	49.5	1.27	0.76	10.73	0.31
Pine	44.55	11.43	2.56	6.3	1.6

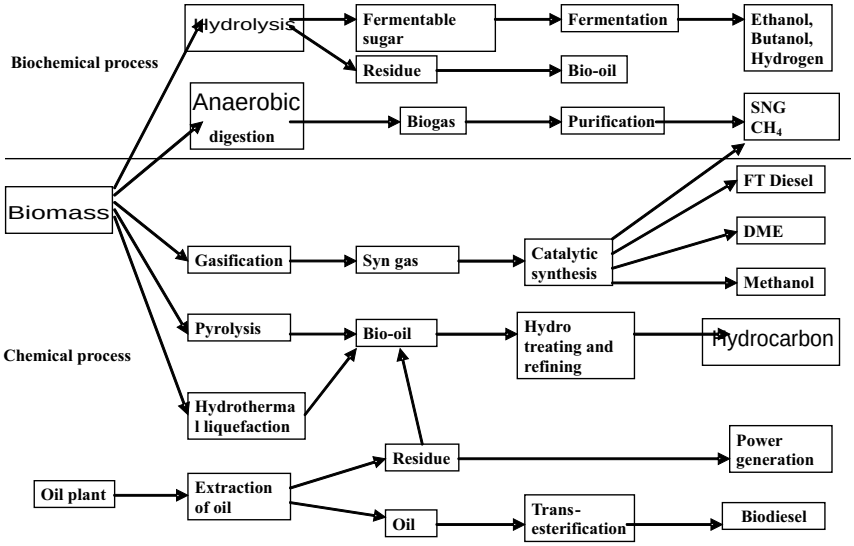


Figure 1: Overview of conversion routes of biomass to biofuels (14)

chemical synthesis, and direct liquefaction. Many different fuels such methanol, ethanol, hydrogen, synthetic diesel, biodiesel, and bio-oil can be produced from biomass.

Processes for Converting Biomass to Ethanol

Ethanol can be produced from lignocellulosic materials through biochemical route in various ways. All processes comprise the same unit processes/operations: hydrolysis of the hemicellulose and the cellulose to monomer sugars, separation of sugars, fermentation of sugars, and product recovery and concentration by distillation. These processes differ mainly in the hydrolysis step, which can either be performed by acid or by enzymes (15).

However, the fermentation of various sugars to ethanol has certain limitations. The sachharification of sugars has limitations due to metabolic inefficiency of well known ethanologens such as *Saccharomyces cerevisae* or *Zymomonas mobilis* (16-19). *Saccharomyces cerevisae* ferments glucose, mannose and fructose as well as the disaccharides (sucrose and maltose) via the Emden-Meyerhof pathway of glycolysis, while ferments galactose via combined action of the Leloir pathway and glycolysis (12).

For economical production of ethanol, both cellulose and hemicelluloses present in a typical biomass should be hydrolysed (20-22). Therefore, it is required to have microorganisms that are capable of fermenting both glucose and xylose (16, 23-25).

Various thermophilic bacteria and yeast have been used for the fermentation of hexose and pentose sugars to ethanol. Many references are available on use of *Thermoanaerobacter ethanolicus* (26-29), *Thermoanaerobacterium saccharolyticum* (30), *Clostridium thermocellum* (31-34), and yeast *Kluyveromyces* sp. (35-37). It is, however, difficult to maintain strict anaerobic conditions in large scale fermentations restricting the use of thermophilic anaerobes whereas the facultative aerobes like *Kluyveromyces* sp. have the potential for industrial applications.

By-Products in Bioethanol Production

In spite of several breakthroughs, the cost of bioethanol produced from lignocellulosic feed-stocks remains high (38). Fermentation processes that give higher ethanol recovery and possibility of recovering some value-added by-products along with ethanol may improve the economy of the lignocellulosic ethanol production processes. Table 2 lists examples of value-added by-products from ethanol production using biomass.

Table 2 By-products during ethanol production from biomass (39).

S. No.	Value-added by-product	Stage where by-product is formed	End use
1	Xylitol	Chemical or biological conversion of xylose obtained during hydrolysis of biomass to xylitol	Prevention of tooth decay and ear infection in children, sugar substitute for diabetic patients
2	Furfural	Conversion of xXylose to furfural	Valuable chemical
3	Single cell protein	Utilization of xylose solution for growing *Candida utilis*	Animal feed
4	Lignin	Residue after extraction of sugars from the biomass	Fuel additive, pellets for domestic fuel, raw material for production of adsorptive materials

Experimental Work

Isolation of Thermophilic and Thermotolerant Strains

The thermophiles were isolated from the soil samples in the media nutrient broth (NB) and yeast extract peptone and dextrose (YPD) medium as shown in Table 3. The soil samples were collected from dumping sites of crushed sugarcane bagasse in a sugar mill.Pure colonies were isolated by using 2% agar and 1% gelrite as solidifying agent at 45 °C and 60 °C, respectively.

Screening of Isolates for Ethanol Production

The thermophilic and thermotolerant ethanologens were screened using different sugars like glucose, mannose, galactose, xylose, arabinose, sucrose, cellobiose and lactose. The phenol red broth medium was inoculated with new isolates and incubated over night at temperatures ranging 45-60 °C. The change in pH due to acid production by the thermophilic and thermotolerant ethanologens was indicated by color change from red to yellow by which the potential strains could be selected. One potential yeast *Kluyveromyces* sp. IIPE453 was selected based on faster growth and color change in the phenol red medium.

Table 3: Media used for isolation of strains

	Nutrient Broth (NB)	Dextrose (YPD) Medium
Yeast Extract, (g/l)	10	10
Peptone, (g/l)	10	20
Sodium Chloride, (g/l)	5	--
Glucose, (g/l)	10	20
pH	6.5	5.0 at 45 °C and 60 °C.

Microorganisms and Culture Conditions

The medium used for growth of isolated strain *Kluyveromyces* sp. IIPE453 was salt medium (SM)(in g/l): di-sodium hydrogen ortho phosphate, 0.15; potassium di-hydrogen ortho phosphate, 0.15; ammonium sulphate, 2.0; yeast extract, 1.0; carbon source e.g. glucose, xylose, 20. The pH was adjusted 5.0 by 1N hydrochloric acid. The cells were grown in 250 ml flasks in shaker at 50 °C and 150 rpm on glucose, mannose, galactose, xylose, arabinose, sucrose, cellobiose and lactose, 10 g/l each separately. The cells were also produced in large quantity by growing in a Bioflow-110 bioreactor (ca. 5 liters) on glucose and xylose. The temperature, pH and dissolved oxygen were controlled at 50 °C, 5.0 and 40 % saturation, respectively, during the growth phase.

Hydrolysis of Sugarcane Bagasse

In another experiment of first stage hydrolysis, 2 Kg crushed bagasse was soaked in 16 L dilute acid of 2-10 % (w/w) concentration in a digester of 30 L capacity. The digester was maintained at temperature of 100 °C and 200 rpm. The samples were withdrawn at 15 min interval. After 90 min the digester was stopped and cooled to room temperature. The digested bagasse was filtered through cloth and washed residual bagasse twice with 4 l water and again filtered. Total filtrate was again filtered through Whatman filter paper using vacuum filtration unit. The solid to liquid ratio was also optimized 1:8 to 1:4.

In second stage hydrolysis, residual bagasse of first stage hydrolysis was soaked in concentrated acid of 18-40 % (w/w) concentration in a digester of 30 L capacity. The digester was maintained at temperature of 80 °C and 200 rpm. The samples were withdrawn at 15 min interval. After 90 min the digester was stopped and cooled to room temperature. The digested bagasse was filtered through cloth and washed residual bagasse twice with 4 L water and again filtered. Total filtrate was again filtered through Whatman filter paper using vacuum filtration unit.

Fermentation Conditions

The medium for fermentation containing in g/l, di-sodium hydrogen ortho phosphate, 0.15; potassium di-hydrogen ortho phosphate, 0.15; ammonium sulphate, 1.0; yeast extract, 1.0 was used. Batch fermentation was performed in Bioflow-110 bioreactor (ca. 2 liters) with hydrolysate solution obtained from sugarcane bagasse by free cells of *Kluyveromyces* sp. IIPE453. The temperature and pH dissolved oxygen were controlled at 50 °C and 5.0 respectively during the process.

Analytical Methods

Sugars (glucose, fructose, sucrose and xylose) and xylitol were analyzed by HPLC using High Performance Carbohydrate Column (Waters) at 30 °C with acetonitrile and water mixture (75:25) as mobile carrier at a flow rate of 1.4 ml min^{-1} and detected by a refractive index detector (Waters 2414). Ethanol was analyzed by gas chromatography using Ashco Neon II Gas Analyzer with a 2 m long and 1/8" dia Porapak-QS column with mesh range 80/100. Sample was injected at inlet temperature 220 °C, oven temperature 150 °C and flame ionization detector temperature 250 °C using nitrogen gas as a carrier.

Results And Discussion

The various thermophilic strains were isolated from the soil samples collected from the dumping site of sugarcane bagasse where the temperature was usually high, and screened by using phenol red broth. Only one microorganism showed fermentation activity on all the substrates except lactose and cellulose. This strain was selected for further fermentation studies and characterized as yeast *Kluyveromyces* sp. IIPE453 (deposited in 'Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India)' with deposition no. MTCC 5314). The yeast strain showed the growth and fermentation on glucose, mannose, galactose, xylose, sucrose, cellobiose and lactose. The optimum temperature and pH for growth and fermentation were found to be 50 ºC and 5.0, respectively. The sugars obtained by hydrolysis of bagasse were fermented to ethanol using the yeast *Kluyveromyces* sp. IIPE453.

Hydrolysis of Sugarcane Bagasse

The bagasse was hydrolyzed by sulfuric acid in two stages, firstly with dilute acid and then with concentrated acid. Recovery of sugars and furfural from hydrolysis at 100 ºC for 1h at different acid concentrations and solid to liquid ratio is given in Table 4.

Figure 2 shows the concentrations of sugars and furfural as functions of time during hydrolysis at 100 ºC with different concentrations of sulfuric acid. Figure 3 shows the effect of solid to liquid ratio during hydrolysis. The week acid predominantly hydrolyzes the hemicelluloses to xylose and the strong acid predominantly hydrolyzes the cellulose to glucose. Hydrolysis to furfural is low for both week acid as well as strong acid. Maximum recovery

of xylose was obtained at acid concentration of 8 % and solid to liquid ratio of 1:4. Figure 4 shows the concentrations of sugars and furfural at different acid concentrations during concentrated acid hydrolysis at 80 ºC. For concentrated acid hydrolysis, the maximum recovery of glucose was obtained at acid concentration of 40 %. 92 % of total sugars present in bagasse could be recovered in the two-stage acid hydrolysis.

Table 4 Recovery of sugars and furfural (% age of bagasse) from hydrolysis of bagasse

	Acid concentration % (w/w)	Solid to liquid ratio	Recovery as % age of bagasse		
			Xylose	Glucose	Furfural
Dilute acid hydrolysis	2	1:10	9.2	0.6	0.12
	4	1:8	13.6	0.9	0.16
	6	1:8	19.6	1.4	0.23
	8	1:8	23.1	4.4	0.31
		1:5	25	4.6	0.41
		1:4	26.14	4.6	0.63
	10	1:8	25	5.8	0.95
Concentrated acid hydrolysis	18	1:4	3.5	6	0.21
	25	1:4	2.5	9.7	0.16
	32	1:4	4.64	14.5	0.14
	40	1:4	3.34	18	0.11

Sugar Recovery from the Hydrolyzate by Ion-exchange

The sugars and the acid from mixture obtained after hydrolysis of the bagasse could be separated by ion-exchange technique. About 95 % acid free sugars were recovered with strong anion and weak anion mixture in the ratio of 5:2 and residence time 44 minutes, and about 95 % acid was recovered in the regeneration step.

Fermentation of Bagasse Hydrolysate to Ethanol

Fermentation of the bagasse hydrolysate with *Kluyveromyces* sp. IIPE453 was studied separately for hydrolysates obtained by dilute acid hydrolysis and concentrated acid hydrolysis. Table 5 shows the results of fermentation of bagasse hydrolysate

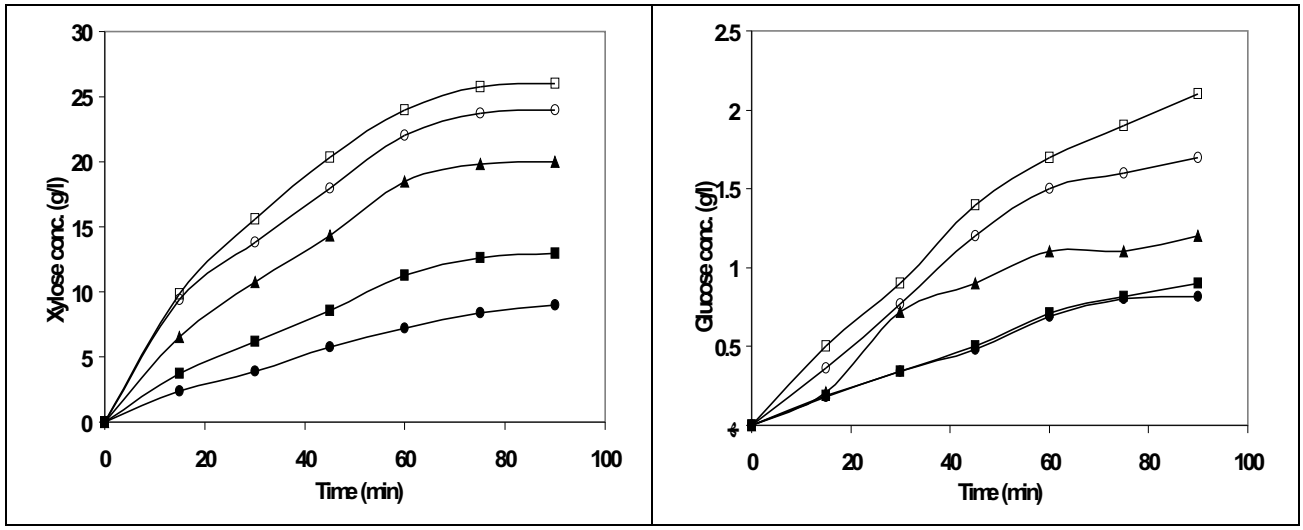
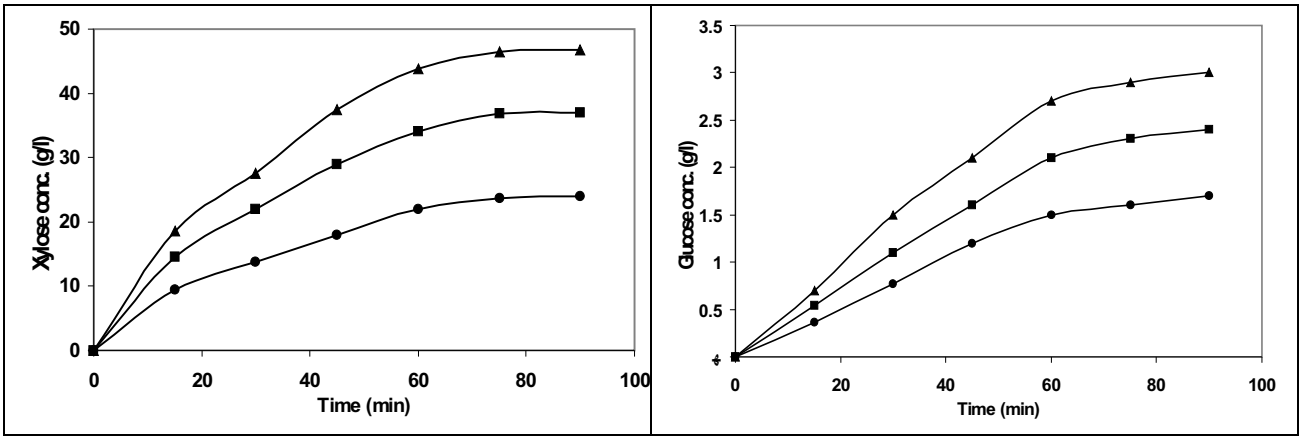


Figure 2: Concentrations of xylose, glucose, and furfural during dilute acid hydrolysis at 100 °C for different acid concentrations: (●) 2 %; (■) 4 %; (▲) 6 %; (○) 8 %; (□) 10 %



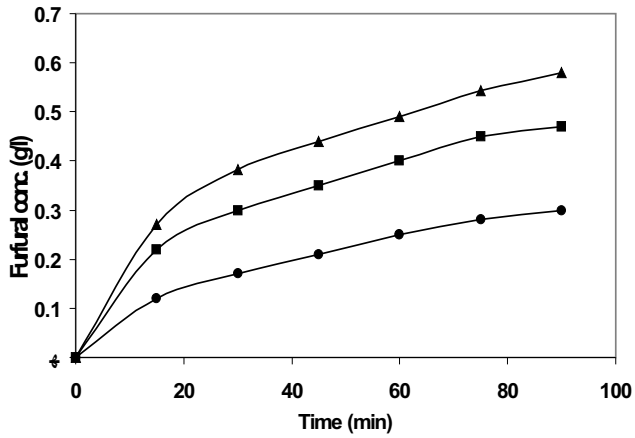
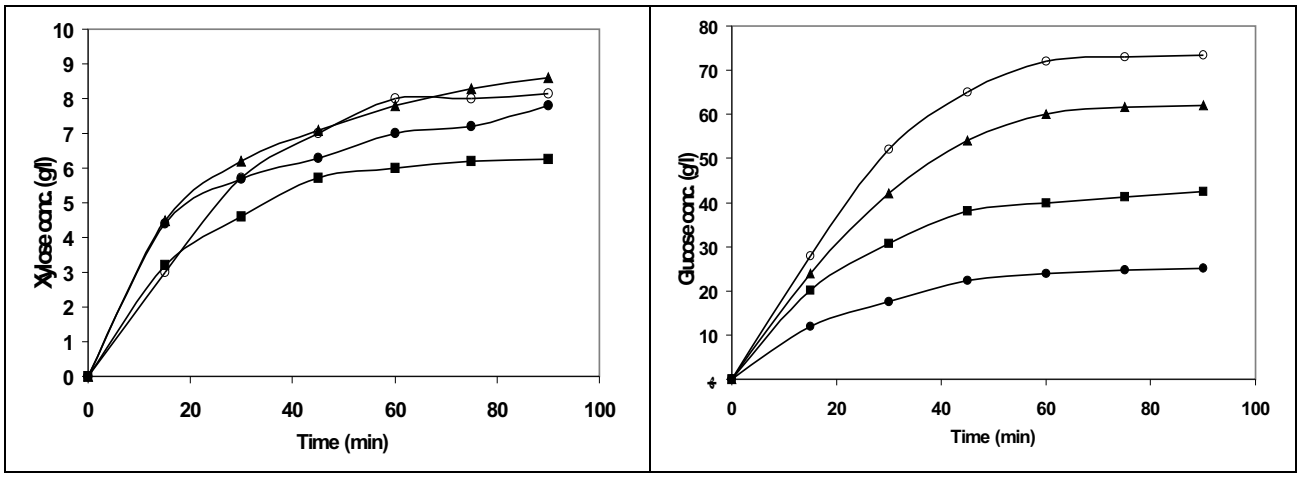


Figure 3: Concentrations of xylose, glucose, and furfural during dilute acid hydrolysis at 100 °C with acid concentration 8 % at different solid to liquid ratios: (●) 1:8; (■) 1:5; (▲) 1:4



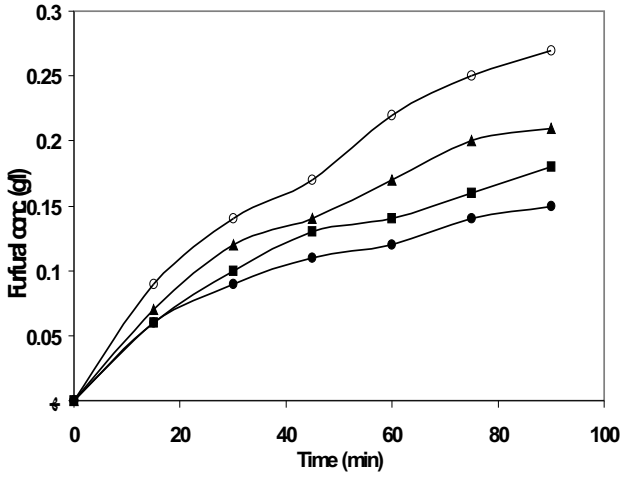


Figure 4: Concentrations of xylose, glucose, and furfural during concentrated acid hydrolysis at 80 °C for different acid concentrations: (●) 18 %; (■) 25 %; (▲) 32 %; (○) 40 %

obtained from dilute acid hydrolysis (7 g/l glucose and 18 g/l xylose) at 50 °C in a batch process. The total sugar present in hydrolysate was consumed within 8 h. Figure 5 shows the concentration of sugars and the fermentation products during the fermentation process.

The yeast strain *Kluyveromyces* sp. IIPE453 showed high ethanol productivity of 0.73 $g.l^{-1}.h^{-1}$ in sugarcane bagasse hydrolysate as compared to ethanol productivity of 0.23 $g.l^{-1}.h^{-1}$ in SSF on pretreated switchgrass by using *Kluyveromyces marxianus* IMB4 at 45 °C (39Tada et al., 2004), 0.2 $g.l^{-1}.h^{-1}$ on pretreated barley straw in SSF by using *Kluyveromyces marxianus* IMB3 (39). The strain could also produce xylitol from xylose present in hydrolysate with high yield and productivity. The ethanol yield was, however, low due to the low percentage of glucose present in hydrolysate. No inhibition was observed during the fermentation by the inhibitors present in the sugarcane bagasse hydrolysate such as furfural, hydroxy methyl furfural etc.

Figure 6 shows the concentration of sugars and the fermentation products during the fermentation of bagasse hydrolysate obtained from concentrated acid hydrolysis. Batch fermentation at 100 °C of glucose rich bagasse hydrolysate obtained by concentrated acid hydrolysis resulted in maximal 14.8 g/l ethanol concentration in broth with an ethanol yield of 83.44 % of theoretical yield on consumed sugar in 20 h. The average sugar consumption rate, volumetric productivity and specific productivity in batch fermentation were 1.74 $g.l^{-1}.h^{-1}$, 0.74 $g.l^{-1}.h^{-1}$ and 0.3 $g.g^{-1}.h^{-1}$, respectively. Ballesteros et al. (40) reported 16-19 g/l ethanol concentration in 72-82 h on different lignocellulosic biomass in SSF by *Kluyveromyces marxianus* CECT 10875 at 42 °C whereas by the same strain Tomás-Pejó et al. (24) reported a

Table 5: Concentration, yield, and productivity of ethanol and xylitol obtained by batch fermentation of dilute acid bagasse hydrolysate.

	Ethanol	Xylitol
Maximum concentration, g/l	5.4	11.7 g/l
Yield, % of theoretical yield on the basis of total sugars	42.3	46.8
Yield, % on the basis of Xylose, %		65
Productivity, $g.l^{-1}.h^{-1}$	0.73	1.46

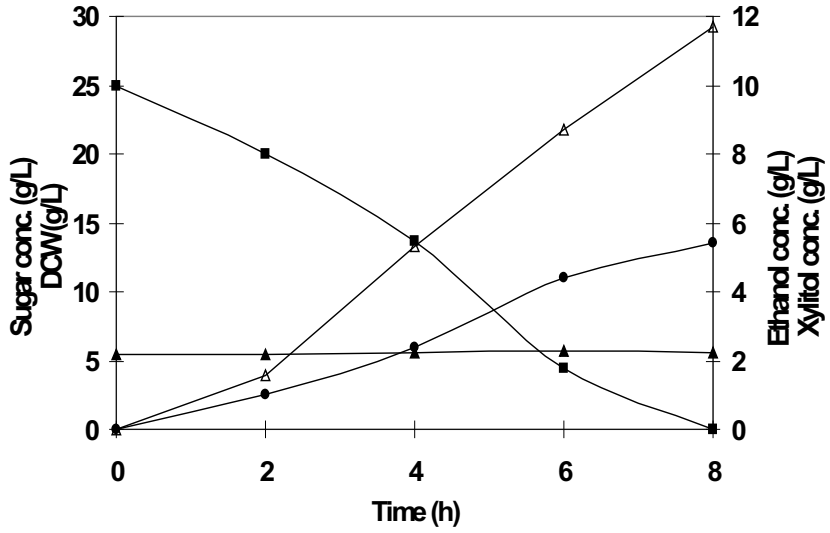


Figure 5: Fermentation with *Kluyveromyces* sp. IIPE453 at 50 ºC of bagasse hydrolysate obtained from dilute acid hydrolysis at 121 °C: (■) sugar concentration; (●) dry cell weight (DCW); (□) ethanol concentration; (Δ) xylitol concentration

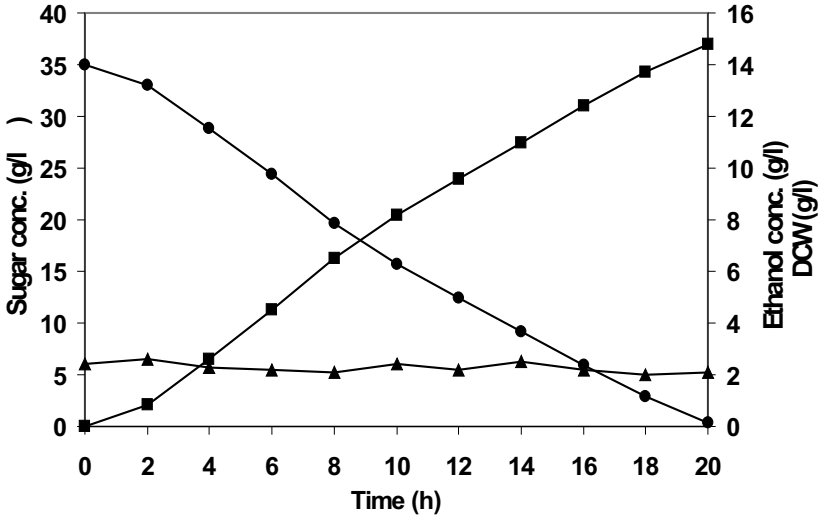


Figure 6: Batch ethanol fermentation by *Kluyveromyces* sp. IIPE453 at 50 ºC on bagasse hydrolysate obtained from concentrated acid hydrolysis at 80 ºC: (●) sugar concentration; (■) ethanol concentration; (▲) dry cell weight in broth

maximum 32 g/l ethanol with productivity of 0.44 $g.l^{-1}.h^{-1}$ on wheat straw in SSF batch process. The dry cell weight was almost constant throughout the process. The final ethanol concentration in broth was low due to low initial sugar concentration in the hydrolysate, which could be overcome either by improvement of saccharification of sugarcane bagasse or by mixing other high sugar containing feed-stocks.

Conclusions

1. A thermophilic strain isolated from the soil samples collected from the dumping site of sugarcane bagasse showed fermentation activity on all the sugars present in bagasse except lactose and cellulose. The strain was characterized as yeast *Kluyveromyces* sp. IIPE453 (deposited in 'Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India)' with deposition no. MTCC 5314).
2. The yeast strain showed the growth and fermentation on glucose, mannose, galactose, xylose, sucrose, cellobiose and lactose. The optimum temperature and pH for growth and fermentation were observed to be 50 ºC and 5.0 respectively.
3. About 92 % of the sugars present in the bagasse biomass could be recovered by acid hydrolysis in two steps, first with dilute acid to hydrolyze hemicelluloses and then with concentrated acid to hydrolyze cellulose.
4. The sugars obtained by hydrolysis of bagasse could be fermented to ethanol using the yeast *Kluyveromyces* sp. IIPE453 either in batch process or in continuous process. No inhibition was observed during the fermentation by the presence of such inhibitors as furfural, hydroxymethyl furfural in the hydrolysate.
5. The ethanol yield was, however, low due to low fermentation of xylose to ethanol and low percentage of glucose present in the bagasse hydrolysate. The yield could be increased by increasing the glucose concentration in the hydrolysate through addition of molasses, sugarcane juice or some similar material.

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