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Short Note

Development of Technology for Production of Second Generation Biofuel Ethanol from Bagasse

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Abstract: Bagasse is abundantly available agricultural crop waste obtained from renewable source, so it was used as raw material for ethanol production. Acid and heat pretreatment in combination with biological saccharificarion was evaluated for conversion of bagasse cellulose and hemicelluloses to fermentable sugars. Maximum yield of monomeric sugars (13.98 w/v) was achieved when acidic pretreatment in combination with heat was subsequently followed by enzymatic saccharificarion. The maximum ethanol production from *S. cerevisae* cells (1.6×10^9) entrapped in sodium alginate beads of diameter 2.0-2.5 mm 14.0 g/L was achieved with ethanol productivity of 10 g L^{-1} h^{-1} and sugar utilization of 85% at dilution rate of 0.25 h^{-1} by feeding 60 g/L glucose concentration in packed bed reactor at temperature 25°C and pH 5.5. This system for ethanol production was operationally stable for 136 h (cycle of 68 h).

Key words: Bagasse, saccharificarion, bioethanol producton, packed bed reactor.

Introduction

Lignocelluloses materials have gained attention as renewable sources of fermentable sugars for bioconversion into ethanol as biofuel and other value added metabolites (Adsul et al., 2011). This bioconversion generally consists pretreatment and enzymatic hydrolysis. In recent years, there has been an increasing trend towards more efficient utilization of lignocelluloses agro-industrial residues for production of second generation bioethanol and among them, sugar cane bagasse is one of the most abundant low-cost lignocelluloses material which result to reduce cost of biofuel production (Hahn-Hägerdal et al., 2007; Cardona et al., 2010; Ojeda et al., 2011; Seabra et al., 2010). Most of bagasse (75%) is used as in-house fuel for power generation or as raw material for producing low-value products such as mulch or ceiling tiles (Dawson and Boopathy, 2008; Boopathy, 2004) and remaining 25% is considered as waste that goes to landfill or is allowed to decay. Million tons of bagasse, which could be used as a raw material for cellulosic ethanol production, contain cellulose, hemicelluloses, lignin and miscellaneous components about 50, 27.5, 9.8 and 11.3% respectively (Kewalramani et al., 1988; Fengel and Wegener, 1989).

The main component is cellulose, which is biodegradable natural raw material categorized by attractive properties such as hydrophilicity, chirality and chemical modification capacity. It is the most common organic polymer and is considered an almost inexhaustible source of raw material. Second generation ethanol production from cellulose, hemicelluloses consist of pretreatment and hydrolysis of cellulosic material (Alvira et al., 2010). The pretreated solids are separated from the hydrolysed carbohydrates liquor using a filter for fermentative ethanol production which is a clean burning renewable biofuels. Demand for ethanol as an alternative fuel source has steadily increased in many parts of the world, due to declining fossil fuel

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resources and increased gasoline prices (Sheoran et al., 1998). The aim of this study was to obtain the best conditions for saccharificarion of sugarcane bagasse with acidic and alkaline treatment in combination with heat, subsequently followed by biological treatment targeting a higher production of fermentable monomer sugars and production of biofuel ethanol from immobilized *S. Cerevisiae* in continuous packed bed reactor (PBR).

Materials and Methods

Raw Material and Pretreatment

Sugarcane bagasse obtained from local sugar mill was dried, by spreading it over a shallow bed for two days and milled to pass through mesh number 20. In pretreatment, H_2SO_4 (3M) and NaOH (3M) was used in 1:4, solid to liquid in combination with heat (121°C) for different time intervals (15, 30, 45 and 60 min). After each pretreatment, lignin, cellulose, hemicellulose and reducing sugar contents were determined by standard procedures (TAPPI, 1991; Updegroff, 1969; Miller, 1959) with minor modifications.

Biological Saccharificarion of Pretreated Bagasse for Glucose Production

Bacillus sp., isolated from soil in laboratory, was cultured in a medium containing (g/l) KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.2; CMC, 10.0; Yeast extract, 1.0; Peptone, 1.0; Na_2HPO_4, 2.5; $(NH_4)_2SO_4$, 1.0; pH 7.0. Culture was incubated in rotary shaker at 150 rev. min^{-1} at 37°C and biomass was harvested after 36 h cultivation by centrifugation at 8000 RPM for 20 min for the microbial saccharificarion. Pretreated bagasse with 10% loading was inoculated with 10% (v/v) bacterial inoculums (7×10^7 cells/ml) in 1000 ml closed Erlenmeyer flasks. Total volume of the solution was 400 ml and flasks were agitated in a shaker for 60 h at 37°C and 100 rev. min^{-1}. The supernatant containing the hydrolysed bagasse content was harvested after different time interval by centrifugation at 8000 RPM for 20 min for the quantitative analysis. In second biological saccharificarion, pretreated bagasse (pH 6.5) with 10% loading was inoculated with cellulase enzyme (15 IU/ml) produced from *Bacillus* sp. in 1000 ml closed Erlenmeyer flasks in rotary shaker at 50 rev. min^{-1} (temp. 37°C) and samples were collected after different time intervals and heated at 100°C for 5 min to inactivate the enzyme. Supernatant and insoluble biomass was harvested after different time intervals by centrifugation (8000 RPM for 20 min) and quantified for the cellulose and reducing sugars contents by standard methods.

Production and Immobilisation of S. Cerevisiae Biomass for Ethanol Production

S. Cerevisiae was cultured in 500 ml of heat sterilized medium containing (g/L) glucose, 10; yeast extract, 1; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.1 and $(NH_4)_2SO4$), 0.1 in a 200 ml flask, at 30°C for 24 h using rotary shaker at 100 rev. min^{-1}. The yeast biomass was harvested by centrifugation at 10000 RPM for 20 min and suspended in the saline solution for the desired cell concentration. Sterile sodium alginate solution was added in the cell slurry to get final concentration of 2% (w/v). The beads were obtained by dropping the mixture in sterile and chilled $CaCl_2$ (0.1M) drop by drop with help of nozzle to produce spherical beads of 1.0-1.5, 2.0-2.5 and 2.5-3.0 mm diameter (Palmieri et al., 1994). The fermentation set-up for ethanol production consist of double jacketed glass column, having total working volume of 500 mL (20 cm height and 5 cm inner diameter), connected to a water supply vessel at both ends. The vessel was equipped with a stirring device, pH electrode and inlet for pumping in fresh medium.

The influent in the glass column entered from the bottom of the reactor. Recirculation of medium was achieved by a peristaltic pump to ensure an up-flow with required flow rate. Medium was recirculated into the glass column reactor at different dilution rates varying from 0.5 to 1.25 h^{-1}. The entire reactor system was autoclaved at 121°C for 20 min. Before operation, the reactor system was flushed with N_2 gas for 15 min in order to maintain anaerobic conditions. The reactor was filled with enzymatic treated bagasse supernatant and continuous operational mode was started by inoculating immobilized cell suspension of *S. Cerevisiae*. Liquid samples were collected at regular time intervals and analyzed in duplicate for quantification of ethanol and residual glucose concentration. Ethanol in the fraction was distilled at 74° and distillate collected in acidified potassium dichromate was quantified for the ethanol by Williams and Reese (1950) method with small modification. Different physical and chemical process parameters i.e dilution rate, initial sugar concentration, temperature, pH, size of beads and operational stability of beads for ethanol production was optimized.

Results and Discussion

Pretreatment of Bagasse for Saccharificarion

Pretreatment methods disrupt the ordered cellulose structure and the lignin-carbohydrate complex, and increase the surface area accessible to enzymes, promote the hydrolysis, and increase extent of hydrolysis of

cellulose in various lignocellulosic residues (Fan et al., 1982). Acidic and alkaline pretreatment in combination with heat was evaluated for hydrolysis of bagasse. Glucose and other carbohydrates were measured after each treatment and observed that acidic treatment in combination with heat was more effective as compared to alkali with heat. Chemical characterisation of the materials shows that there was a growing decline in the percentage of hemicelluloses and such behaviour was different for cellulose and lignin (Table 1). The hemicelluloses amorphous in structure was more easily degraded and reached a loss of more than 70 and 55% after 60 min by acid and alkali treatment respectively. The cellulose has a crystalline character and so, it is not so easily broken and amount can be considered satisfactory until the time of 45 min. There was no alteration in the value of lignin loss due to the specific structural specificity with acidic and alkaline treatment. Glucose concentration doubled with 50 min of reaction time by pretreatment of bagasse with acidic treatment in combination with heat, but the amount was very low (per 100 gm of bagasse) and affirm more certain reaction time, aiming a low cellulose removal concomitantly with the highest possible removal of hemicelluloses, and cellulose from the raw materials bagasse. Candido et al. (2012) found that the best time for bagasse pretreatment was 40 minutes, in which it reached a loss of hemicelluloses around 70% and lignin and cellulose, around 15%. Over this time, cellulose loss increased and there was no loss of lignin and hemicelluloses.

Biological Saccharificarion of Pretreated Bagasse

For microbial saccharificarion of lignocelluloses biomass, two enzymes ligninases to open structure and cellulase to hydrolyze the carbohydrate are important. In this case, since bagasse biomass was chemically and physically pretreated, cellulase was most important enzymes for saccharificarion. *Bacillus* sp. of 36 h was used, since the cellulase activity was maximum at this time. Cellulose content was decreased satisfactorily until the 48 h of microbial fermentation and above this cellulose utilization was not effective. Microbial treatment does not influence reduction in lignin content due to non-effectiveness *Bacillus* sp. for the ligninases enzyme production to hydrolyse the lignin content of pretreated bagasse. In saccharificarion process glucose concentration increased 0.89 to 3.67% as the fermentation time was increased up to the 60 h. Increase in the glucose production is very less effective and extract produced shows no evident increase in the saccharificarion with time. This behaviour was reproducible; the same profile in the measured saccharificarion rate was observed in a second independent experiment.

This reduction can be due to the utilization of sugar by the microorganism for the growth. To examine the enzymatic hydrolysis on acid heat pre-treated bagasse, 10% loading of biomass was saccharified by cellulase enzyme (15 IU/ml). Glucose concentration increased 0.89-13.7% (per 100 of bagasse) as enzymatic hydrolysis time was increased up to 1 h and after this time it was almost constant. Increase in the glucose production is effective and shows conspicuous increase in the saccharificarion. This behaviour was reproducible and measured saccharificarion rate was observed in a repeated independent triplicate experiment. Cellulose contents decreased by 41.5% in 1 h with crude cellulase enzyme hydrolysis bio reaction in closed Erlenmeyer flasks at rotary shaker (50 rev. min^{-1}, temp. 37°C). Cellulose, hemicelluloses lignin and glucose content were 38, 9.67, 11.7 and 13.98% respectively and quantified in 100 gm of saccharified bagasse (Table 2).

Table 1: Saccharificarion of bagasse by acidic and alkaline treatment in combination with heat

Treatment	*Time (min)*	*Cellulose (%)*	*Lignin (%)*	*Hemicelluloses (%)*	*Glucose (%)*
Acidic	15	26±2	5.2±2	20±2	0.89±0.5
	30	40±2	7.5±2	18±2	1.0±0.5
	45	51±2	10±2	16±2	1.67±0.5
	60	53±2	10±2	14±2	1.63±0.5
Alkaline	15	19±2	6±2	18±2	0.69±0.5
	30	38±2	7.6±2	12±2	0.79±0.5
	45	42±2	8.2±2	11±2	0.98±0.5
	60	43±2	9.4±2	10±2	1.35±0.5

Table 2: Biological saccharificarion of pre-treated bagasse by microbial and enzymatic methods

Saccharificarion methods	*Treatment time (h)*	*Cellulose (%)*	*Hemicelluloses (%)*	*Lignin %*	*Glucose (%)*
A. Microbial	0	51±2	14±2	11±1	0.89±0.5
	12	49 ±2	13.6±2	12±1	1.0±0.5
	24	42±2	12.4±2	11±1	2.67±0.5
	36	32 ±2	11.34±2	10.76±1	3.0±0.5
	48	28±2	11.1±2	11.0±1	3.54±0.5
	60	27±2	9.97±2	10.87±1	3.67±0.5
	0	51.0±0.5	14±2	10.87±1	0.89±0.5
B. Enzymatic	0.25	47±0.5	12.5±2	11.34±1	3.0±0.5
	0.5	28±0.5	10.2±2	11.0±1	8.7±0.5
	1.0	27.4±0.5	9.67±2	10.87±1	13.98±0.5
	1.5	32.7±0.5	8.78±2	10.76±1	13.78±0.5

Bioethanol Production from Saccharificed Bagasse

Effect of Dilution Rate

The sodium alginate entrapment method was chosen for immobilisation of *S. cerevisiae* for the production of ethanol because of its easy availability, natural source and simple procedure of immobilisation. Yeast cells (8×10^7 cells /ml) were entrapped in 200 ml with 85±5% entrapment efficiency. The average cell mass concentration in column was 1.6×10^9 in total packed volume.

The medium containing glucose concentration of 60 g/L was passed through the column at different dilution rates varying from 0.25 to 1.5 h^{-1} (Table 3). At a dilution rate of 0.25 h^{-1}, 14.2±0.7 g/L ethanol concentration was obtained in the outlet with an ethanol yield ($Y_{p/x}$) 0.56 of its theoretical yield at steady state. At a dilution rate of 0.5 h^{-1}, 13.9 g/L ethanol concentration was obtained in the outlet with an ethanol yield of 0.56. As the dilution rate was increased ethanol concentration decreased but ethanol yield on glucose consumed rate was almost constant. The ethanol productivity (q_p) and specific ethanol productivity (q_{sp}) was increased when the dilution rate was increased from 0.25 to 1.5 h^{-1}, but the ethanol concentration and sugar utilisation decreased significantly. The ethanol concentration of 14.0 with 85.8% sugar utilisation was achieved at dilution rate of 0.25 h^{-1}. In the present study, highest ethanol concentration with the highest ethanol productivity and maximum sugar utilisation at a dilution rate of 0.25 h^{-1} was achieved.

Table 3: Effect of dilution rate (I) and initial sugar concentration (II) on kinetic parameters for ethanol fermentation by immobilized *S. cerevisiae*

Dilution rate (h^{-1})	*I. Effect of dilution rate on kinetic parameters**						
	S_o *(g/L)*	S_f *(g/L)*	P_c *(g/L)*	$Y_{P/S}$ *(g/g)*	q_p *(g L^{-1} h^{-1})*	q_{sp} *(g g^{-1} h^{-1})*	μ *(%)*
0.25	60	5.1	14.20	0.56	10.0	1.35	85
0.5	60	4.9	13.9	0.57	12.0	2.45	82
1.0	60	4.6	12.2	0.56	13.5	3.6	78
1.5	60	4.4	11.9	0.56	14.8	3.98	74
	*II. Effect of initial sugar concentration on kinetic parameters**						
	S_o *(g/L)*	S_f *(g/L)*	P_f *(g/L)*	$Y_{P/S}$ *(g/g)*	q_p *(g L^{-1} h^{-1})*	q_{sp} *(g g^{-1} h^{-1})*	μ *(%)*
0.25	50	4.7	12.0	0.54	11.0	1.2	86
0.25	100	35	13.9	0.57	14.0	3.45	65
0.25	150	55	10.2	0.56	17.5	4.20	63

*S_o = Glucose concentration in feed; S_f = Residual glucose concentration in outlet; P_c = Ethanol concentration in outlet; $Y_{P/S}$ = Ethanol yield on glucose consumed; q_P = Volumetric ethanol productivity; q_{SP} = Specific ethanol productivity; μ = sugar utilization.

Effect of Initial Sugar Concentration

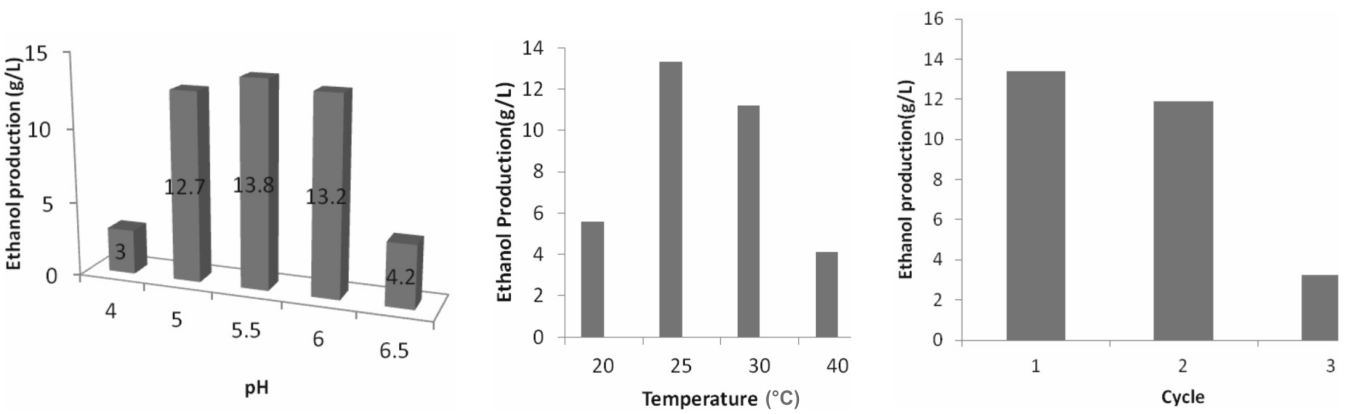
Ethanol concentration and ethanol productivity increased significantly with the increase in initial sugar concentration of 50, 100 and 150 g/L, but theoretical ethanol yield was almost constant with the increase of initial sugar concentration. Above a critical substrate concentration, decreased water activity and plasmolysis caused a decrease in the rates of fermentation. Maximum volumetric ethanol productivity (17.5 $gL^{-1}h^{-1}$) was achieved at 150 g/L initial sugar concentration. The sugar utilisation decreased on increasing glucose concentration in feed due to high ratio of glucose to cell mass concentration. Ethanol concentration increased when sugar concentration was increased in feed from 50 to 100 g/L and decreases when sugar concentration was further increased due to lower sugar utilisation. The ethanol yield on feeding glucose at any concentration was almost same. Therefore, no effect was observed of glucose concentration in feed on ethanol yield. But Ozmihci and Kargi (2008) reported that the ethanol yield was decreased by increasing feed sugar on fermenting cheese whey powder by *Kluyveromyces marxianus* in a packed column bioreactor.

Effect of pH, Temperature and Bead Size on Ethanol Production

Saccharificed bagasse containing 60 g/L total sugar at different pH values (4.5, 5.0, 5.5, 6.0 and 6.5) were fed into the PBR continuously. The dilution rate was 0.25 h^{-1} and the temperature was controlled at 30°C. Maximum ethanol concentration (13.8 g/L) was obtained at pH 5.5 from bagasse extract by Ca-alginate immobilized *S. cerevisiae*; this was due to the good yeast growth over this pH range (Figure 1a). Sharp decrease in ethanol fermentation was reported when pH of the medium was varied from the above range. The effect of temperature on ethanol production was monitored in PBR at different temperatures (20, 25, 30 and 40°C), but optimum ethanol production was at 25°C (Figure 1(b)) which is relevant to growth temperature used for the growth of *S. cerevisae.* In order to determine the effect of bead diameter on ethanol production, beads with diameters of 1.0-1.5, 2.0-2.5 and 2.5-3.0 mm were prepared by using different nozzles and used for continuous ethanol production in the packed-bed bioreactor at a dilution rate of 0.25 h^{-1}. Maximum ethanol production was obtained with 2.0-2.5 mm beads, further increases in bead diameter decreased ethanol production, because increase in size effect the diffusion coefficient of substrate from the macroenvironment to microenvironment and this leads to non-availability of substrate to the immobilised enzyme in the interior of beads. But decrease in bead size reduces entrapment efficiency of cells.

Operational Stability of Immobilised Cells

Operational stability of immobilised *S.cerevisae* cells (1.6×10^9 cells) for ethanol production was studied over an interval of 68 h, for three times. In second and third cycles, ethanol production decreased by 14 and 72% respectively with comparison to first cycle (Figure 1c). Immobilised cells were operationally stable for 136 h (cycle of 68 h) and after that leaching of cells in the outlet increases progressively due to disintegration of beads, so ethanol production was decreased.



(a) **(b)** **(c)**

Figure 1: Effect of (a) pH, (b) temperature and (c) operational stability on ethanol production in PBR from immobilized *S. Cerevisiae* biomass.

Conclusion

In this study pretreated bagasse with acidic and heat treatment illustrate maximum saccharificarion as compared to alkaline and heat treatment. Subsequently enzymatic saccharificarion was more effective with respect to the microbial treatment to hydrolyse the pretreated bagasse into fermentable sugar. The highest ethanol production and theoretical yield for ethanol was obtained when 60g/L initial sugar concentration (pH 5.5) at the dilution rate of 0.25 h^{-1} was applied in reactors packed with beads of 2.0-25 mm diameter. The packed-bed bioreactor worked efficiently and was stable for a period of 136 h without bead disintegration.

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