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Saccharification of alkali treated biomass of Kans grass contributes higher sugar in contrast to acid treated biomass

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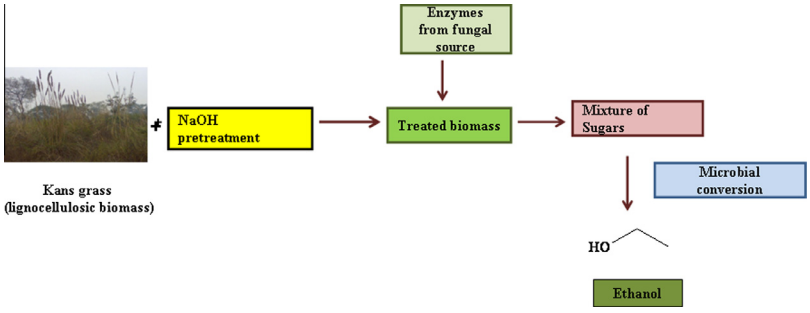
HIGHLIGHTS

- In present study as Kans grass biomass was used as a potential substrate for bioethanol production.
- Alkali pretreatment of Kans grass was followed by the saccharification by using crude cellulase enzyme.
- Resulted Sugar after enzymatic hydrolysis was used for ethanol production by *Saccharomyces cerevisiae* and *Pichia stipitis*.
- A high yield of ethanol was obtained.
- On site produced crude enzyme may be more cost effective for bioethanol production process.

GRAPHICAL ABSTRACT



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ABSTRACT

Economical production of biofuel is prerequisite to depletion of fossil fuel. In recent years, biomass of numerous food crops was used as a feedstock for bioethanol production. Unfortunately, due to limited availability as well as confliction with food, these sources may hold back for continuous production of bioethanol. Therefore, in the present study a waste land crop "Kans grass" was utilized as feedstock for microbial production of bio-ethanol. The Kans grass biomass obtained after NaOH pretreatment at optimum conditions (in term of lignin removal) was subjected to enzymatic saccharification by using crude enzyme (obtained from *Trichoderma reesei*) to total reducing sugars (TRSs), which was further fermented for bioethanol production using yeast strains. Different time (30, 60, 90 and 120 min), concentrations of NaOH (0.5%, 1%, 1.5% and 2%) as well as temperatures (100, 110 and 120 °C) were used for pretreatment study. At 120 °C, approximately more than 50% of delignification was observed. Moreover, subsequent enzymatic saccharification contributed 350 mg g^{-1} dry biomass of total reducing sugar (TRS) production. Interestingly, TRS was approx. fivefold higher than enzymatic saccharification of acid pretreated biomass (69.08 mg g^{-1}) as reported previously (Kataria et al., 2011) and fermentation of enzymatic hydrolysate using microbes resulted in the 0.44–0.46 g g^{-1} ethanol yield which is a high yield when compared to the other existing literature. Another advantage of alkali pre-treatment was without production of toxic compounds in comparison to acid pre-treatment method. In conclusion, Kans grass was shown as potential feedstock for biofuel production via alkali and enzymatic saccharification in contrast to acid pre-treatment.

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1. Introduction

The overall energy consumption is increasing with the growing world population and rapid industrial growth, as a result resources of non-renewable energy are depleting very fast and that results in increase the price. Bio ethanol is one of the alternative clean liquid fuels that can be produced by fermentation of sugars or simple starch such as sugarcane, maize etc. However, those sources may have adverse effect on farmland or forest diversity and as well as on soil, water and food resources. Among potential alternative bio-energy resources, lignocellulosics have been accepted for production of next generation fuel [1]. Lignocellulosic substances contain cellulose, hemicellulose and lignin. The different Sources of lignocellulosics including wood, agricultural residues, water plants, grasses, and other plant substances are well known for the starting material for bioethanol. Kans grass (*Saccharum spontaneum*) is one of the novel and potential non food source of lignocellulosic material that can be cultivated on waste lands without conflicting the food crop. This plant biomass is available throughout the year without much cultivation efforts and with low supply of water. Addition to this, it is composed of sufficient amount of holocellulose content (cellulose and hemicellulose, 64.67%) and can be utilized for bioethanol production [2]. For conversion of lignocellulosic biomass to ethanol, there are majorly three steps: pretreatment, hydrolysis (sachharification) and fermentation. Pretreatment is one of the important steps where the three dimensional cell wall is disrupted for sufficient availability of cellulose by removing lignin, which is the main obstacle for cellulase action (for monomer sugar production).Cellulose (a polymer of glucose sugar) and hemicellulose (polymer of Xylose, mannose and arabonise sugar) portion of plant biomass first depolymerised in to monomer sugars and further these monomer sugar are utilized by microorganisms (yeast and bacteria).

Two approaches have been developed in parallel for conversion of lignocelluloses to fermentable sugars that may be acid based and enzyme based. The enzyme based technology is advantageous over acid based treatment (conc. H_2SO_4) due to higher conversion efficiency, absence of substrate loss due to chemical modification, lack of inhibitory compounds production, low cost, no need of recycling of acid and the use of more moderate and non-corrosive conditions like low temperatures, neutral pH [3]. Use of biodegradable and non toxic reagents is more economical than any other method. The substrate usually requires a pretreatment process before being subjected for enzymatic breakdown which is aimed at increasing the susceptibility of cellulose to enzyme. The overall performance of cellulase enzyme is highly dependant on the residual lignin present along with cellulose.

Pretreatment by sodium hydroxide is one of the conventional methods among the all pretreatment methods that received high interest over the years. It is a low energy demanding and relatively inexpensive technique which has beet studied with various lignocellulosic materials. Sodium hydroxide disrupt the structural linkages, affect the lignin barriers, reduce the cellulose crystallinity, and increase the cellulose accessibility by exposing up the structure and make it more accessible to the cellulase enzyme, which results in a sharp increase in sugar yield.

As cellulase enzyme production accounts for 40% of total cost in overall production of bioethanol [4], hence to reduce cost of production, on site production of crude enzyme is more viable than commercial cellulase due to their reasonable cost (exclusion of enzyme purification step), high enzyme production capacity, etc. The reduction in cost paves cost effective way for ethanol production [5]. *Trichoderma reesei* has been used for industrial cellulase production since 1960s [6]. But it was mainly used in food, pulp and paper and textile industry. Due to increase cost of fermentation process for bioethanol, cellulase production is one of the key steps for hydrolysis of the lignocellulosic materials. Several different strains have been developed since then to enhance the production of cellulase from the fungal strain *QM6a* [7] that is the first industrially used strain and nearly all strains have been obtained by mutating this strain in some way [8].

In the present investigation, dilute NaOH pretreatment was optimized for lignin removal for a novel lignocellulosic material Kans grass and delignified biomass was saccharified by using crude cellulase enzyme [2] to obtain reducing sugars, which further utilized for bioethanol production by using yeast strains *Pichia stipitis* and *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Kans grass biomass

Kans grass biomass was obtained from various parts of Uttrakhand, India was chopped into small pieces (0.5–1.0 cm), washed and dried at 60 °C overnight and finally stored at room temperature. As plant biomass is composed of carbohydrate (cellulose and hemicelluloses), lignin and other components hence composition estimation for cellulose, hemicelluloses, lignin, ash as well as moisture content was done on dry weight basis [2].

2.2. Dilute NaOH pretreatment

Dilute alkali (NaOH) pretreatment of Kans grass biomass (5% w/v) was done by different sodium hydroxide concentrations (0.5, 1, 1.5 and 2% w/v) for variable residence time (30, 60, 90 and 120 min) at different temperatures (100, 110 and 120 °C). After pretreatment, solid residue obtained was separated from the liquid portion. The liquid fraction (hydrolysate) was kept at −20 °C for the analysis of total reducing sugars, xylose. However, the collected solids were washed with distilled water to obtained neutral pH, dried at 60 °C. These portions of the solid residues were used for determination of total residual solid, holocellulose and lignin content. The reduction in lignin following pretreatment was calculated based on the initial dry-weight of lignin in the untreated sample and the dry-weight of lignin in the remaining solids after pretreatment. All experiment were carried out in triplicates.

2.3. Cellulase enzyme production

The crude enzyme used in this hydrolysis study was obtained from *T. reesei*, (procured from NCIM) was cultivated in enzyme production media by inoculating the fungal spores at 28 °C at pH 5 for 8 days with cellulose as carbon source (optimized condition, [2]). The fungal biomass was separated from media by centrifugation (20 min at 3220 g), the clear supernatant was analyzed for the CMCase, xylanase and total cellulase activity (filter paper activity) and stored at −80 for further use in saccharification step.

2.4. Enzymatic saccharification/hydrolysis of (NaOH) pretreated Kans grass biomass

Dilute NaOH pre-treatment as carried out with different NaOH concentrations, time and temperatures and the conditions were optimized for maximum removal of lignin. At 120 °C the maximum removal of lignin (14–79.30%) was observed with solubilization of sugars. As more the lignin was removed the solubilization of total reducing sugars was also observed thereby the reduction of holocellulose content of the biomass. Hence the solid residue remained (all NaOH concentration and pretreatment duration at 120 °C) after

pretreatment were washed with distilled water and dried at 60 °C over night and used for enzymatic saccharification.

The 250 mL conical flasks containing citrate buffer (0.05 M, pH 4.8) with different biomass loading of NaOH pretreated Kans grass (2, 4, 5 and 6% (w/v)) were autoclaved and cooled and then 20 FPU g dry biomass^{-1} (gdb) of filtered sterilized crude *T. reesei* cellulases was added aseptically. The flasks were incubated at 50 °C for 96 h, at 200 rpm. Samples collected at 12 h interval and analyzed for total reducing sugars (TRSs) liberated. The results shown are the mean of three independent experiments.

2.5. Production of ethanol

TRS concentration of hydrolysate (obtained after optimized conditions of saccharification) was maintained to 10 g L^{-1} and was then supplemented with other essential medium components for both *P. stipitis* and *S. cerevisiae* respectively.

2.6. Lignin, holocellulose, cellulose and hemicellulose estimations

The lignin [9,10] and ash content [11] were determined as per NERL protocol whereas holocellulose and hemicellulose were determined by according to Han and Rowell [12]. Cellulose was determined as the difference between holocellulose and hemicellulose contents.

2.7. Sugars estimation

Total reducing sugars were estimated by DNS method [13] however, xylose estimation was done by phloroglucinol assay [14]. All the calculation were done from corresponding standards.

2.8. Estimation of cellulases (Endoglucanase or CMCase, FPA and xylanase) activity

The supernatant of *T. reesei* production media obtained after regular intervals of time was analyzed for endoglucanase, cellulase and xylanase activity. Endoglucanase activity (CMCase) activity was estimated using the method described by Xiao et al. [15], whereas Xylanase activity was measured using oat spelt xylan as substrate and method described by Bailey et al. [16]. Cellulase activity (measured as filter paper hydrolyzing activity, using a 1 × 6 cm strip of Whatman No. 1 filter paper) was assayed according to the method recommended by Ghosh [17]. One unit of enzyme (CMC, FPU and xylanase) is defined as one micro mol production of glucose/xylose per mL per minute. All colorimetric observations were recorded using UV–Vis spectrophotometer. The total reducing sugars were analyzed by DNS method [13].

2.9. Maintenance and cultivation of S. cerevisiae and P. stipitis

The yeast strain *S. cerevisiae* (MTCC 170), procured from MTCC, *IMTECH*, *Chandigarh* India, was grown at 30 °C and maintained at 4 °C on MGYP agar plates containing (g L^{-1}) glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; agar 20 (pH 5.0). Fermentation was carried out with sugar hydrolysate (10 g L^{-1}) by supplementation of other essential medium components [18] (g L^{-1}) yeast extract, 6.0; $CaCl_2{\cdot}2H_2O$, 0.3; $(NH_4)2SO_4$, 4.0; $MgSO_4.7H_2O$, 1.0 and KH_2PO_4, 1.5 (pH 5.5) in conical flasks with 100 mL working media on a shaker (150 rpm) with addition of 5% (v/v) of mid exponential phase (12 h) inoculum at a temperature of 30 °C, the initial pH was adjusted to 5.0.*P. stipitis* strain (NCIM 3497), obtained from NCIM, Pune, India, was grown at 30 °C and maintain at 4 °C on MGYP agar plates containing (g L^{-1}): glucose, 10; yeast extract, 30; peptone, 5; agar 20 (pH 5.0).The fermentation media consists of the following composition: (g L^{-1}): yeast extract, 1; $(NH_4)HPO_4$, 2; $(NH_4)SO_4$, 1; $MgSO_4{\cdot}7H_2O$, 0.25; and trace element solution 1 mL per L. The trace element solution contained (g L^{-1}): $CuSO_4{\cdot}H_2O$, 2.5; $FeCl_3{\cdot}6H_2O$, 2.7; $MnSO_4{\cdot}H_2O$, 1.7; $Na_2Mo_2O_4{\cdot}2H_2O$, 2.42; $ZnSO_4{\cdot}7H_2O$, 2.87; $CaCl_2{\cdot}6H_2O$, 2.4 and concentrated sulphuric acid (18 N), 0.5 mL. Medium pH was adjusted to 5.0 [19]. Fermentation was carried out in conical flasks with 100 mL working media (10 g L^{-1} sugar hydrolysate with fermentation media components) on a orbital shaker (150 rpm) with addition of 5% (v/v) of mid exponential phase (16 h) inoculum at a temperature of 30 °C, the initial pH was adjusted to 5.0.

A set of parallel control experiment was also carried out for both yeast strains (*P. stipitis* and *S. cerevisiae*) by using additive synthetic sugars (glucose in place of sugar hydrolysate) with all other media components. All experiments were performed in triplicates. Aliquots of 5 mL were withdrawn periodically for the estimation of cell mass, ethanol and residual sugars in fermentation broth. All the experiments were performed in triplicates.

2.10. Biomass estimation for P. stipitis and S. cerevisiae

To estimate dry cell biomass of the yeasts, a calibration curve between optical density and biomass concentration was prepared. The optical density of the samples was measured at 600 nm and dry cell biomass was further calculated from calibration curve.

2.11. Ethanol determination

Analysis of ethanol was carried out by using a capillary gas chromatograph (DANI GC) coupled with a flame ionization detector on a solgel wax column. The column temperature was initially adjusted at 100 °C for 2 min; it was increased to 180 °C for 1 min at a rate of 10 °C/min and was further increased to 220 °C with a rate of 5 °C min^{-1} for 5 min. The injection and detector temperatures were set at 210 °C. The carrier gas (N_2) flow-rate was kept at 1 mL min^{-1} whereas auxiliary and hydrogen gas flow rates were maintained at 350 and 30 mL/min respectively.

2.12. SEM analysis

Untreated as well as pretreated Kans grass samples were scanned under SEM (model No. LEO 435VF, England) for the morphological structure analysis.

3. Results and discussion

3.1. Composition analysis

Kans grass biomass which is known as one of the cultivar of switchgrass. The holocellulose content was estimated to be 64.7% of dry wt. which composed of cellulose (43.7%) and hemicellulose (21.1%). The compositions of the other switch grass verities given by NREL's biomass feedstock composition and properties database [20] were compared with Kans grass. In all other varieties holocellulose content is found to be in the range of 56.7–63.3% and thus it is evident that the total carbohydrate content (holocellulose) in Kans grass is found to be highest among all other varieties of switch grass studied by different researcher. The moisture and ash content of Kans grass biomass were calculated to be 4.7 ± 0.06 and 2.1 ± 0.2% while lignin content was estimated to be 25.2 ± 0.6% [2].

3.2. Study of NaOH pretreatment

NaOH pretreatment of Kans grass (5% w/v) was carried out with different concentrations of NaOH (0.5%, 1%, 1.5% and 2%) for different

Table 1
Total lignin removal as well as total residual solid and holocellulose remained after dilute NaOH pretreatment with different NaOH concentration (0.5%, 1%, 1.5% and 2%) and time (30, 60, 90 and 120 min) at 100 °C.

Pretreatment conditions		Total solid (g/100 g dry wt.)	Lignin removal (%)	Holocellulose remained (g/100 g dry wt.)
Time (min)	NaOH conc. (%)			
30	0.5	87.23 ± 0.71	4.019 ± 0.59	62.19 ± 0.27
	1.0	85.23 ± 0.89	12.97 ± 1.24	61.18 ± 0.36
	1.5	82.12 ± 1.06	18.18 ± 2.65	60.58 ± 0.19
	2.0	79.71 ± 0.62	23.55 ± 2.34	59.29 ± 0.21
60	0.5	85.45 ± 0.63	11.92 ± 1.97	62.15 ± 0.12
	1.0	81.34 ± 1.07	17.39 ± 2.18	61.27 ± 0.24
	1.5	78.34 ± 0.88	24.91 ± 2.98	60.11 ± 0.17
	2.0	77.10 ± 1.24	27.65 ± 1.62	59.87 ± 0.13
90	0.5	81.34 ± 1.87	13.17 ± 1.76	59.78 ± 0.12
	1.0	79.34 ± 1.65	21.28 ± 2.10	58.28 ± 0.17
	1.5	77.4 ± 2.13	28.25 ± 2.12	57.78 ± 0.21
	2.0	77.12 ± 1.43	28.81 ± 1.98	56.14 ± 0.26
120	0.5	79.2 ± 1.19	17.31 ± 1.34	58.21 ± 0.19
	1.0	78.34 ± 1.37	26.14 ± 1.22	57.23 ± 0.12
	1.5	76.9 ± 0.94	30.56 ± 1.63	56.32 ± 0.13
	2.0	75.4 ± 1.45	31.19 ± 1.92	56.27 ± 0.15

Table 2
Total lignin removal as well as total residual solid and holocellulose remained after dilute NaOH pretreatment with different NaOH concentration (0.5%, 1%, 1.5% and 2%) and time (30, 60, 90 and 120 min) at 110 °C.

Pretreatment conditions		Total solid (g/100 g dry wt.)	Lignin removal (%)	Holocellulose remained (g/100 g dry wt.)
Time (min)	NaOH conc. (%)			
30	0.5	85.53 ± 0.65	6.24 ± 1.51	62.13 ± 0.78
	1.0	76.86 ± 1.35	30.13 ± 0.43	61.76 ± 0.83
	1.5	71.06 ± 0.87	39.39 ± 1.67	60.56 ± 0.59
	2.0	68.12 ± 1.44	43.81 ± 0.31	59.21 ± 0.67
60	0.5	80.97 ± 1.39	15.08 ± 1.43	61.56 ± 0.56
	1.0	75.84 ± 0.76	22.16 ± 0.54	59.81 ± 0.74
	1.5	69.54 ± 0.87	39.39 ± 1.29	58.23 ± 0.83
	2.0	65.32 ± 1.32	46.99 ± 0.63	57.23 ± 0.57
90	0.5	76.32 ± 0.47	26.58 ± 0.54	57.12 ± 0.53
	1.0	73.57 ± 1.39	42.57 ± 0.87	56.39 ± 0.83
	1.5	65.89 ± 0.65	48.38 ± 1.73	56.13 ± 0.63
	2.0	64.32 ± 1.54	45.92 ± 0.39	54.19 ± 0.52
120	0.5	73.42 ± 0.62	38.95 ± 0.87	56.13 ± 0.45
	1.0	71.23 ± 1.32	46.20 ± 0.87	54.13 ± 1.08
	1.5	63.42 ± 0.27	45.60 ± 0.76	53.67 ± 0.74
	2.0	60.21 ± 0.37	50.13 ± 0.66	53.13 ± 0.82

duration of time (30, 60, 90, 120 °C) and at variable temperatures (100, 110 and 120 °C). After NaOH pretreatment the solid residue was separated from the liquid fraction, washed with distilled water and stored at room temperature after drying at 60 °C overnight in an incubator and was used for further estimations of remaining solid residue, lignin content and holocellulose (Tables 1–3). The liquid fraction was stored at −80 °C for the estimations of liberated total reducing sugars (TRSs) and xylose (Figs. 1–3).

3.2.1. Lignin and sugars liberation

NaOH mediated pretreatment is more susceptible than other chemical pretreatment due to no or low toxic compounds production during pretreatment reaction. NaOH pretreatment is estimated to be one of the most effective chemical for pretreatment of different lignocellulosic feedstocks including sunflower hull [21], corn stover [22], cotton stalk [23], sunflower stalk [24] and sunflower Hull [25]. The total solid recovery, lignin removal as well as holocellulose content are shown in Tables 1–3. The total solid residue recovery after pretreatment decreased as the residence time increased which may be due to solubilization of lignin and sugars (that mainly composed of xylose). During pretreatment at 100 °C nearly 12.8–24.6% (Table 1) of solid residue loss was observed while with increase in temperature to 110 and 120 °C (Table 2 and 3) the range of solid residue lost was found to be in the range of 14.5–39.8 and 15.7–47.5% respectively. So it was evident from the Tables 1–3 that as the temperature was increased there was more loss in solid residue.

Higher concentration of NaOH may be favourable to achieve the maximum removal of lignin but solubilization of the carbohydrates was also carried out which cause the less yield of holocellulose (carbohydrates). At 100 °C (Table 1) the lignin solubilization (%) was found to be in the range of 4–31.2 while at 110 °C (Table 2) this range was enhanced to 6.2–50.1 with all NaOH concentration and duration of pretreatment. The maximum lignin removal of 79.3% was observed in 90 min of residence time with 2% NaOH concentrations at 120 °C (Table 3), on the other hand under the same conditions a loss of 6.6% of xylan with respect to holocellulose was also observed. The higher concentrations of NaOH (1.5% and 2%) were significant during pretreatment reaction at 120 °C with no major difference in lignin removal and the maximum delignification was found to be in the range of 72.8–79.3%.

However at 120 °C with residence time of 120 min and for all concentrations (0.5%, 1%, 1.5% and 2%), more than 70% (70.1–73.2%) delignification was observed, but at the same time loss of

Table 3
Total lignin removal as well as total residual solid and holocellulose remained after dilute NaOH pretreatment with different NaOH concentration (0.5, 1, 1.5 and 2%) and time (30, 60, 90 and 120 min) at 120 °C.

Pretreatment conditions		Total solid (g/100 g dry wt.)	Lignin removal (%)	Holocellulose remained (g/100 g dry wt.)
Time (min)	NaOH conc. (%)			
30	0.5	84.33 ± 1.21	14.25 ± 0.56	61.59 ± 0.78
	1.0	77.57 ± 0.76	46.84 ± 0.63	60.79 ± 0.34
	1.5	69.25 ± 1.67	52.01 ± 0.84	55.85 ± 0.45
	2.0	67.77 ± 1.43	75.57 ± 0.69	56.44 ± 0.63
60	0.5	74.93 ± 0.51	19.14 ± 1.45	57.79 ± 0.12
	1.0	67.77 ± 0.82	59.29 ± 0.75	55.80 ± 0.76
	1.5	65.7 ± 1.54	71.83 ± 0.48	54.76 ± 0.96
	2.0	67.53 ± 0.63	76.80 ± 0.52	51.35 ± 0.43
90	0.5	74.01 ± 1.48	34.10 ± 1.49	55.97 ± 0.43
	1.0	65.40 ± 1.39	61.64 ± 0.98	54.22 ± 0.85
	1.5	63.22 ± 1.45	74.33 ± 1.21	49.84 ± 0.35
	2.0	60.66 ± 0.73	79.30 ± 0.78	51.90 ± 0.42
120	0.5	69.95 ± 1.45	70.43 ± 1.87	55.69 ± 0.52
	1.0	58.56 ± 1.54	72.94 ± 0.61	47.84 ± 0.82
	1.5	52.47 ± 1.42	71.91 ± 0.58	43.66 ± 0.32
	2.0	57.36 ± 0.73	72.86 ± 0.50	44.93 ± 0.44

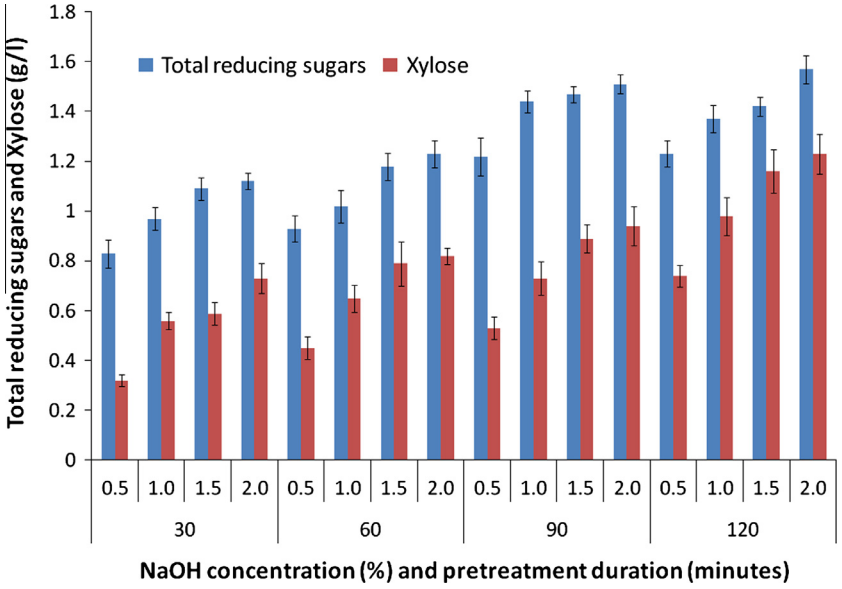

Fig. 1. Concentration of total reducing sugars and xylose liberated during NaOH pretreatment with different NaOH concentrations (0.5%, 1%, 1.5% and 2%) and residence time durations (30, 60, 90 and 120 min) at 100 °C.

holocellulose content was also observed. In a similar investigation 65.6% of delignification was found with 2% NaOH concentration in 90 min of duration.

The liquid fraction obtained after NaOH pretreatment was investigated for the total reducing sugars and xylose estimation. Xylose is one of the major pentose sugars of hemicellulose that released during alkali pretreatment with all other sugars. At 100 °C the total reducing sugars and xylose were found to be in range of 0.83–1.5 and 0.3–1.2 g L^{-1} (Fig. 1) respectively at all pretreatment conditions. At 110 °C these ranges enhanced to 1.2–2.4 g L^{-1} for total reducing sugars and 0.7–1.5 g L^{-1} for xylose (Fig. 2). However the maximum removal of total reducing sugars was estimated to be in range of 2.2–3.3 g L^{-1} with all conditions (Different NaOH concentration and residence time) at 120 °C (Fig. 3), here NaOH concentrations of 1.5% and 2% are important as both of these concentrations resulted approximately the same amount of total reducing sugars and xylose for all residence time. This released sugar may be utilized with fermentation by using pentose utilizing yeast or bacterial strain.

As NaOH pretreatment is more favoured towards lignin removal and this was observed that high temperature (120 °C) and high residence time in which lignin removal was found to be in the range of 70.4–72.9% of total lignin. The maximum lignin removal (79.3%) was observed at 90 min of duration at 120 °C with 2% NaOH. However, in another study Silverstein et al. (2007) under the same pretreatment conditions (2% NaOH, 90 min, 120 °C) reported 65% lignin removal in cotton stalk. As lignin inhibits the function of cellulase enzymes so the results from sugarcane bagasses and soft woods by various authors [26,27] suggest that lignin removal is needed to maximize the yield of fermentable sugars during the enzymatic hydrolysis. Hence removal of the lignin depends upon the lignocellulosic material and pretreatment conditions including temperature as well as residence time.

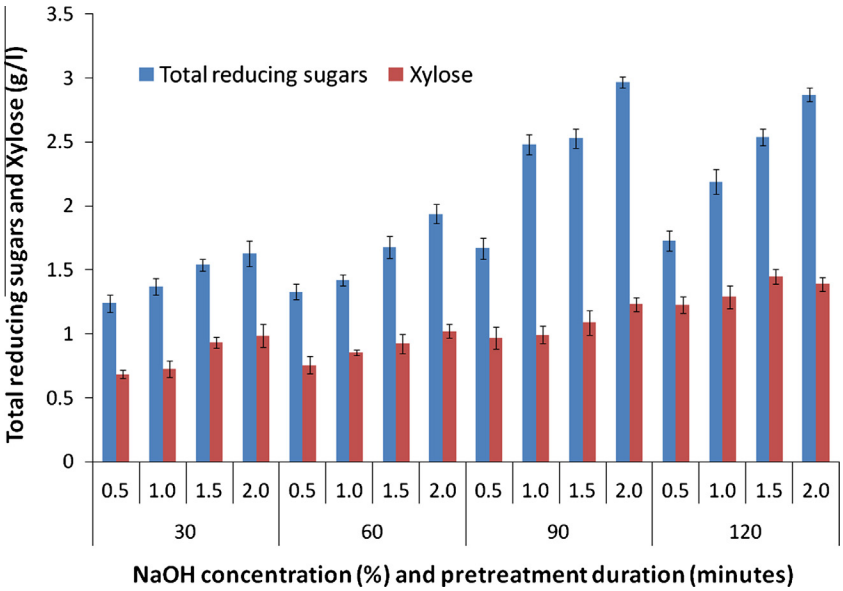

Fig. 2. Concentration of total reducing sugars and xylose liberated during NaOH pretreatment with different NaOH concentrations (0.5%, 1%, 1.5% and 2%) and residence time durations (30, 60, 90 and 120 min) at 110 °C.

3.2.2. Compositional and configuration change in Kans grass biomass after NaOH pretreatment

After optimization of pretreatment conditions, NaOH concentration of 2% and residence time of 90 min at 120 °C was found to be best in terms of lignin removal (79.3%).

The estimation of composition of Kans grass biomass before and after pretreatment was done and summarized in Fig 4. The holocellulose content of NaOH treated biomass was deceased from 64.7 g to 51.9 g per 100 g of biomass. This is due to the solubilization of the carbohydrate content during pretreatment. However the lignin content in original biomass was found to be 25.2 g/100 g biomass which was decreased to 5.3 g/100 g biomass after pretreatment. SEM was used to study the morphological features and surface characteristics of materials with untreated (Supplementary Fig. 1a) as well as pretreated Kans grass biomass (Supplementary Fig. 1b). As pretreatment resulted in significant structural changes in biomass (Supplementary Fig. 1). Sodium hydroxide pretreatment disrupted the structure of the fibers (Supplementary Fig. 1b). Furthermore, the structure of the lignocellulosic biomass was exposed up and provides higher surface area for subsequent enzymatic reactions. The similar observations of SEM analyzed were also observes on pine wood by Hui et al. [28].

3.3. Saccharification of NaOH pretreated Kans grass biomass

NaOH pretreatment was found to be more effective for the lignin removal; more than 50% of lignin was removed at pretreatment temperature of 120 °C. Hence the solid residue remained after NaOH pretreatment at 120 °C treated with different concentration of NaOH (0.5%, 1%, 1.5% and 2%) and duration (30, 60, 90 and

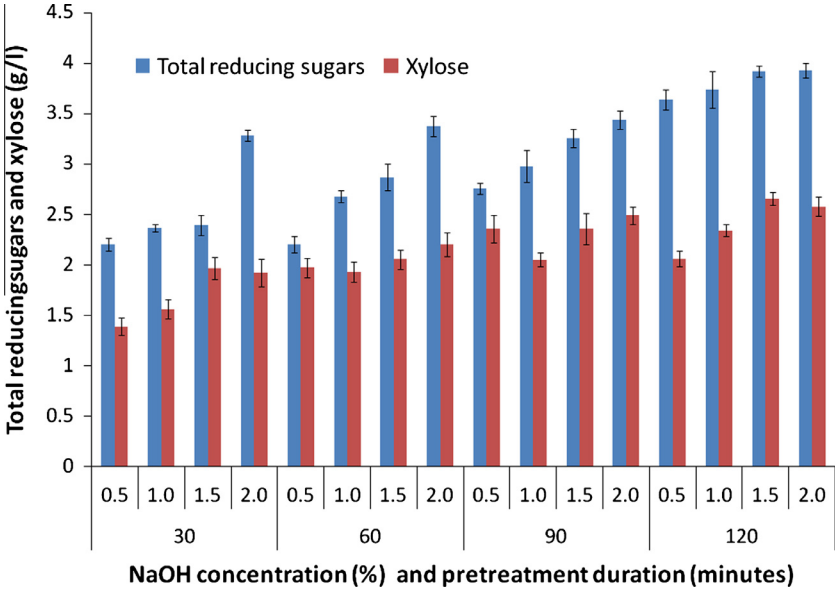

Fig. 3. Concentration of total reducing sugars and xylose liberated during NaOH pretreatment with different NaOH concentrations (0.5%, 1%, 1.5% and 2%) and residence time durations (30, 60, 90 and 120 min) at 120 °C.

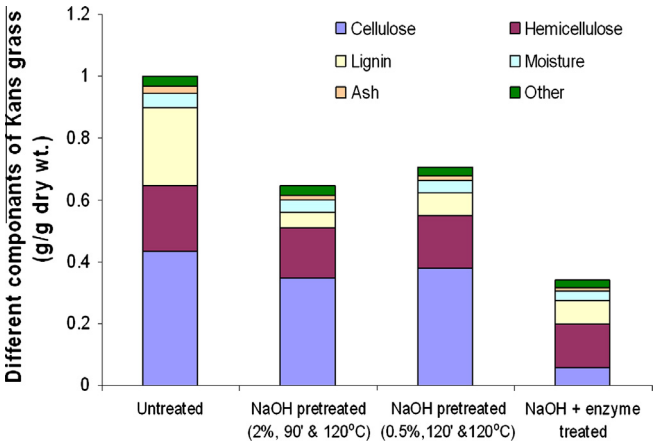

Fig. 4. Compositional change in Kans grass biomass before pretreatment, after dilute NaOH pretreatment at conditions (2%, 90′ and 1220 °C) and (0.5%, 120′ and 120 °C) and enzymatic saccharification with crude cellulase.

120 min) was further used for enzymatic saccharification with crude cellulase enzyme (20 FPU/gdb) with different biomass loading (2, 4, 5 and 6% w/v).

To investigate the optimum conditions for the enzymatic hydrolysis of pretreated Kans grass biomass (pretreated with NaOH at 120 °C), was supplied with 20 FPU/gdb of crude mixture of enzyme (derived from *T.reesei* with 1.14 FPU total cellulase activity, 1.5 U/mL of CMCase and 6.6 U/mL of xylanase activity) in citrate buffer for 96 h for different biomass loading (2, 4, 5 and 6% w/v) and then sugar liberated was estimated.

The overall reducing sugars production pattern after crude enzymatic saccharification can be clearly observed in Figs. 5a–d. A decreased in TRS after hydrolysis was observed with the increased concentrations of NaOH during pretreatment. As with maximum NaOH concentration (2%) the TRS was found to be in the range of 0.48–101 mg/gdb (Fig. 5d), the same was found to be in the range of 20.8–110.7 and 49.32–182.3 mg/gdb for 1.5 (Fig. 5c) and 1% NaOH (Fig. 5b) respectively while with 0.5% NaOH (Fig. 5a)pretreated biomass yielded 80–350 mg/gdb TRS by enzymatic saccharification with crude enzyme.

The biomass obtained after pretreatment condition of 0.5% NaOH for 120 min was found to be the most suitable for the maximum recovery of TRS after enzymatic hydrolysis with different biomass loadings (2%, 4%, 5% and 6%). With 2% Biomass loading 169 mg/gdb TRS was obtained after saccharification with crude cellulase. When biomass loading was increased to 4% and 5% the TRS concentration was also increased to 298 and 350 mg/gdb respectively. However no further enhancement was observed with 6% biomass loading, rather a drop in TRS yield was observed (328 mg/gdb).It was a general observation in all experiments that with increase in biomass loading there was decrease in TRS. This may be due to stirring difficulties and low aqueous movable phase. A similar observations also recorded by [29,30]. As higher concentration of NaOH and long residence time found to be more suitable for maximum removal of the lignin and low solid residue recovery may cause low total reducing sugars formation. The 120 min of pretreatment duration lead to maximum recovery of sugars during enzymatic saccharification (Fig. 4). At 0.5% NaOH concentration with 120 min of pretreatment duration maximum sugars recovery of 62.9% was observed on the other hand a higher concentrations (2%) and longer duration (90 min) (which is the best condition of maximum lignin removal of 79.3%) resulted in lesser sugar release (15.6%) The reason may be due to presence of low holocellulose content after removal of 79.3% lignin. The NaOH concentration of 0.5% w/v also showed significant results with supply of different biomass loading and residence time by different researchers [25,31]. In another study dilute acid pretreated sugarcane bagasse was supplied for organosolv pretreatment by using NaOH-ethanol mixture and 291 mg/gdb glucose liberation was observed [32].

As per the experiments, best pretreatment condition was selected as 0.5% NaOH pretreatment for 120 min duration with 70.4% of lignin removal and 63% of the holocellulose was released as total reducing sugars after sachharification. However the maximum removal of lignin was obtained (79.3%) at 2% NaOH and 90 min residence time, but at this condition only 15.58% of

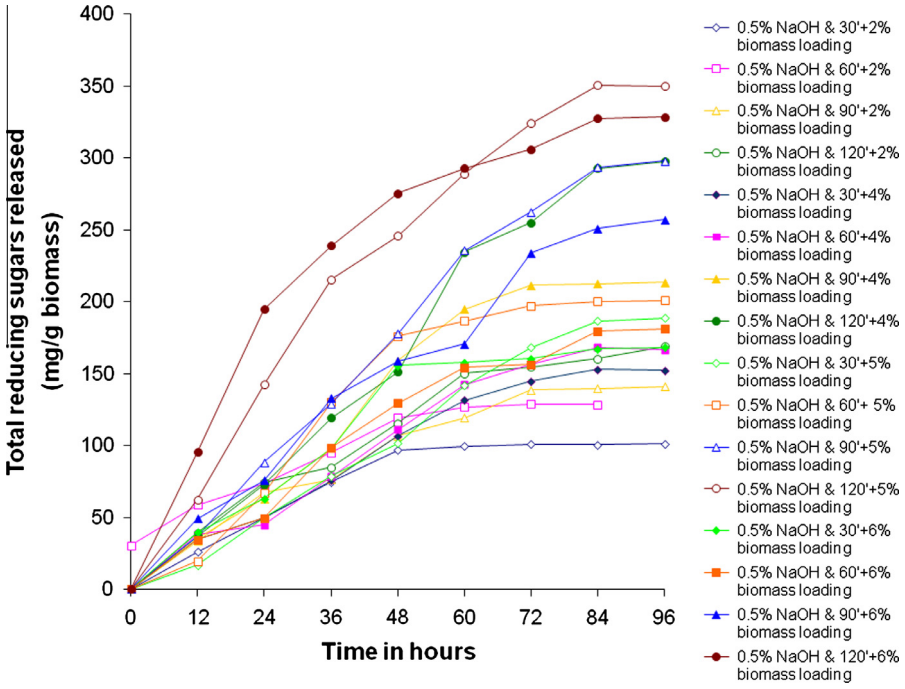

Fig. 5a. Enzymatic saccharification of 0.5% (w/v) NaOH pretreated Kans grass biomass (pretreated at 120 °C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v).

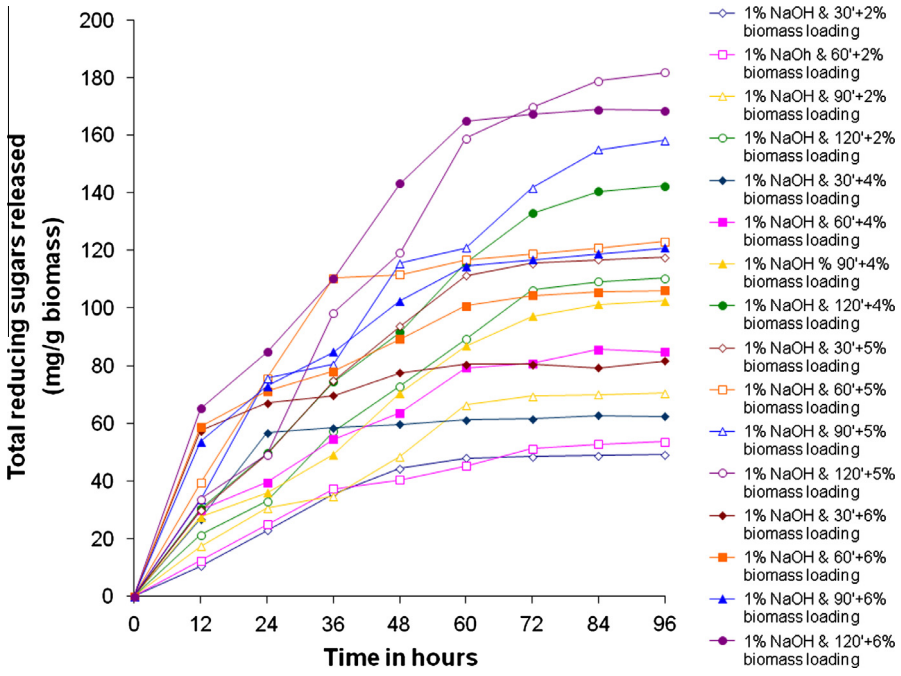

Fig. 5b. Enzymatic saccharification of 1% (w/v) NaOH pretreated Kans grass biomass (pretreated at 120 °C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v).

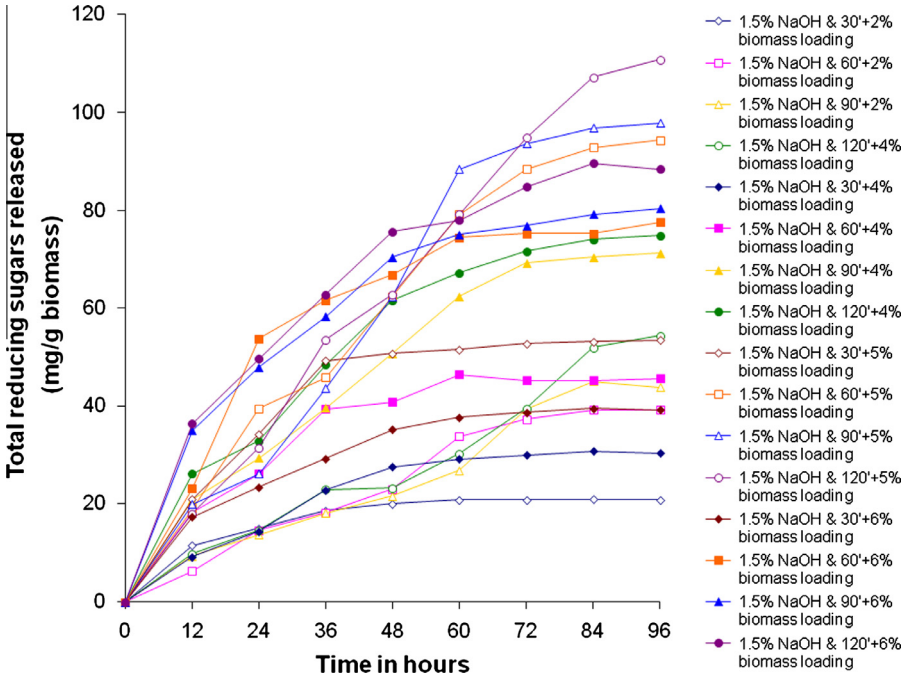

Fig. 5c. Enzymatic saccharification of 1.5% (w/v) NaOH pretreated Kans grass biomass (pretreated at 120 °C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v).

holocellulose release as soluble sugar was reported (Fig. 4). Since, lignin removal should not be the only criteria for an effective pretreatment as large amount of loss of carbohydrate at more severs conditions was found that may affect the enzymatic hydrolysis step. Hence, low concentrations of chemical as well as loading of crude enzyme mixture to obtain total reducing sugars were more cost effective process during this study.

3.3.1. Structural and compositional changes

The configuration changes were observed after SEM analysis of Kans grass biomass. A smooth structure of Kans grass biomass was observed before pretreatment (Supplementary Fig. 1a). However due to NaOH pretreatment (0.5% NaOH with 120 min of residence time) the patches formed that may be due to disruption of lignin (Supplementary Fig. 1c). Again, when this NaOH pretreated

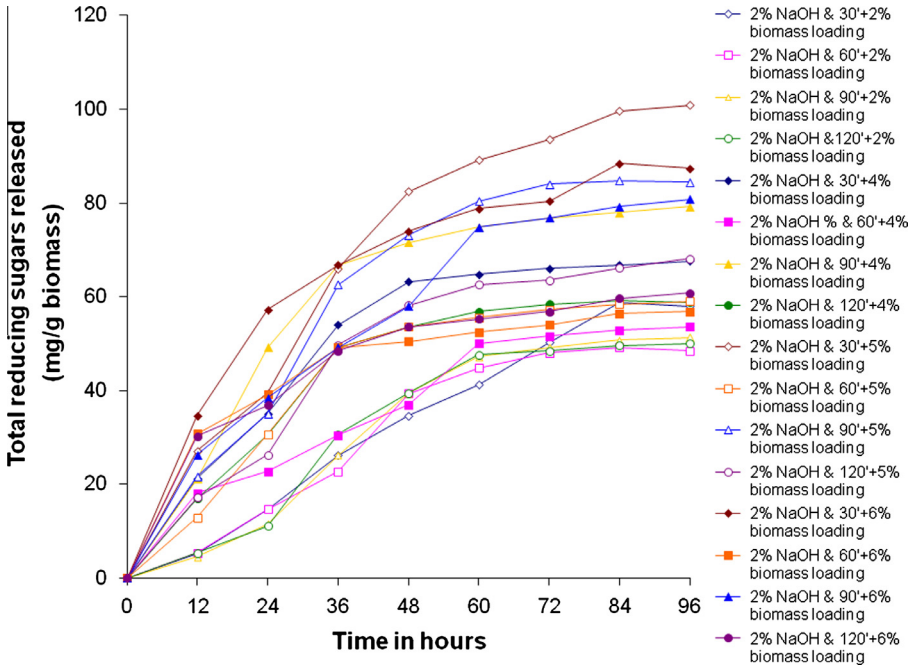

Fig. 5d. Enzymatic saccharification of 2% (w/v) NaOH pretreated Kans grass biomass (pretreated at 120 °C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v).

biomass was further treated with 20 FPU of crude cellulase, the disruption of the biomass structure was observed (Supplementary Fig. d) due to solubilization of holocellulosic portion due to enzyme action. The changes in composition also confirmed that lignin component was maximum removed by NaOH pretreatment, whereas the holocellulosic portion was solubalize in further treatment with crude cellulase enzyme.

3.4. Ethanol production using NaOH pretreated Kans grass hydrolysate obtained after enzymatic hydrolysis

The sugar hydrolysate obtained after enzymatic hydrolysis (using crude enzyme mixture obtained from *T. reesei*) of NaOH pretreated Kans grass biomass was utilized for bioethanol production by using yeast strains of *S. cerevisiae* and *P. stipitis* shown in Figs. 6a and b respectively. When the fermentation was carried out by using *S. cerevisiae* adjusting initial TRS concentration at 10 g L^{-1}, the ethanol concentration was found to be 2.89 g L^{-1} with ethanol yield (Yp) 0.38 g g^{-1} after 32 h (Fig. 6a). The specific growth rate was estimated to be 0.22 h^{-1} (Table 4).

Similarly when, fermentation was carried out using *P. stipitis* in hydrolysate media with 10 g L^{-1} of initial TRS concentration. The ethanol concentration was estimated to be 3.2 g L^{-1} after 24 h. Complete sugar consumption (g L^{-1}), ethanol production (g L^{-1}) as well as cell biomass formation (g L^{-1}) profile is shown in Fig. 6b. The bioethanol yield was found to be 0.44 g g^{-1} with

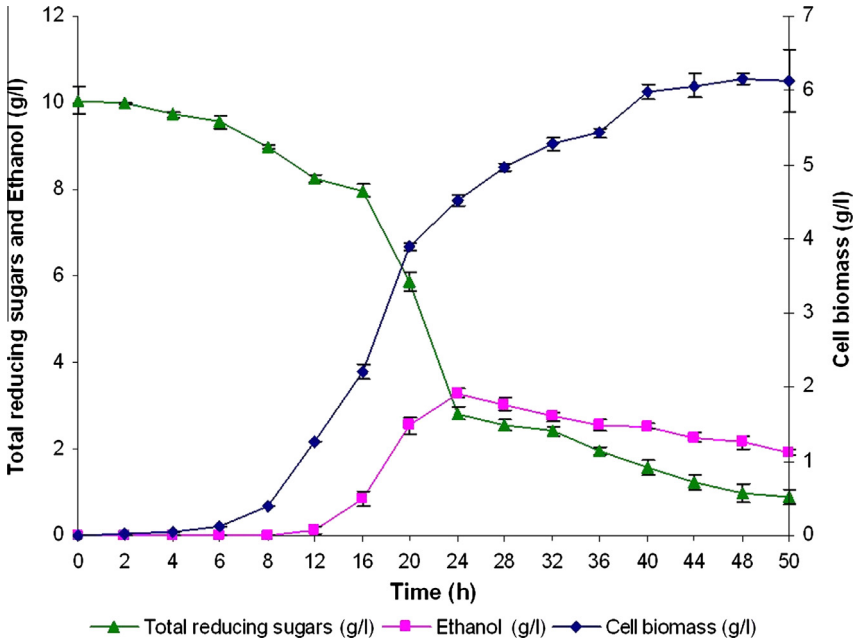

Fig. 6a. Fermentation profiles of *P. stipitis* in hydrolysate obtained after enzymatic hydrolysis.

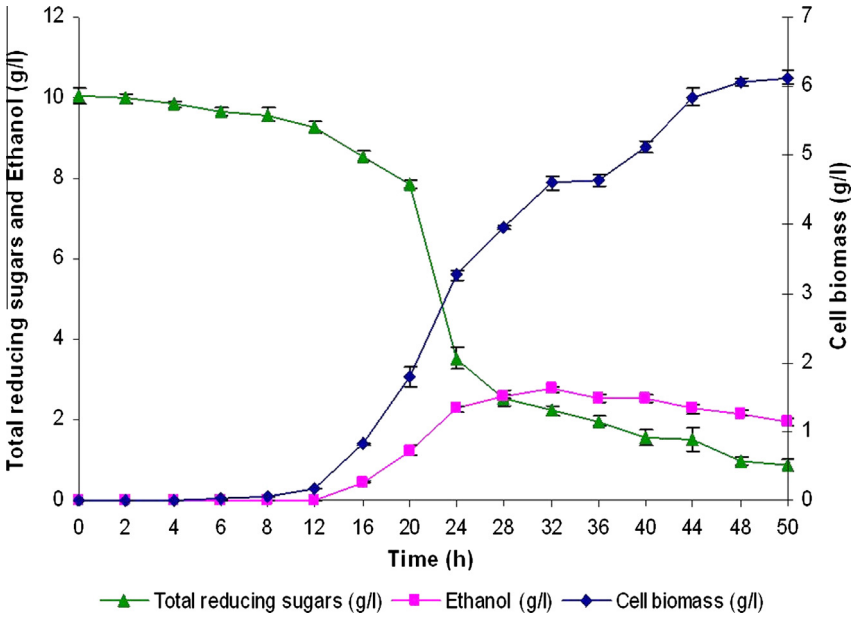

Fig. 6b. Fermentation profiles of *S. cerevisiae* in hydrolysate obtained after enzymatic hydrolysis.

Table 4
Fermentation of hydrolysate (obtained after NaOH and crude enzyme pretreatment of Kans grass biomass) by using *S. cerevisiae* and *P. stipitis*.

Fermentation parameters	Hydrolysate media		Synthetic media	
	S. cerevisiae	*P. stipitis*	*S. cerevisiae*	*P. stipitis*
Initial total reducing sugars, (g L^{-1})	10.21 ± 0.32	10.13 ± 0.27	10.33 ± 0.45	10.33 ± 0.36
Maximum ethanol concentration (g L^{-1})	2.89 ± 0.23	3.29 ± 0.31	4.64 ± 0.17	3.64 ± 0.03
Time (h)	32	24	20	24
Sugar consumed (%)	78.19 ± 1.87	74.60 ± 2.31	95.16 ± 1.31	77.81 ± 0.91
Specific growth rate, μ (h^{-1})	0.22 ± 0.04	0.220.03	0.17 ± 0.03	0.26 ± 0.004
Ethanol yield coefficient, Yp/s (g g^{-1})	0.38 ± 0.05	0.44 ± 0.07	0.47 ± 0.03	0.46 ± 0.01
Biomass yield coefficient, Yx/s (g g^{-1})	0.58 ± 0.08	0.64 ± 0.08	0.34 ± 0.03	0.68 ± 0.004
Ethanol productivity, Qp (g L^{-1} h^{-1})	0.090 ± 0.01	0.13 ± 0.06	0.23 ± 0.06	0.15 ± 0.02
Growth rate, Qx (g cells L^{-1} h^{-1})	0.14 ± 0.02	0.20 ± 0.03	0.23 ± 0.06	0.20 ± 0.03
Max. sugars consumption rate, Qs (g L^{-1} h^{-1})	0.24 ± 0.02	0.31 ± 0.02	0.16 ± 0.04	1.94 ± 0.04
Theoretical yield, η (%)	75.16 ± 1.22	86.47 ± 2.31	92.55 ± 1.21	91.17 ± 1.21

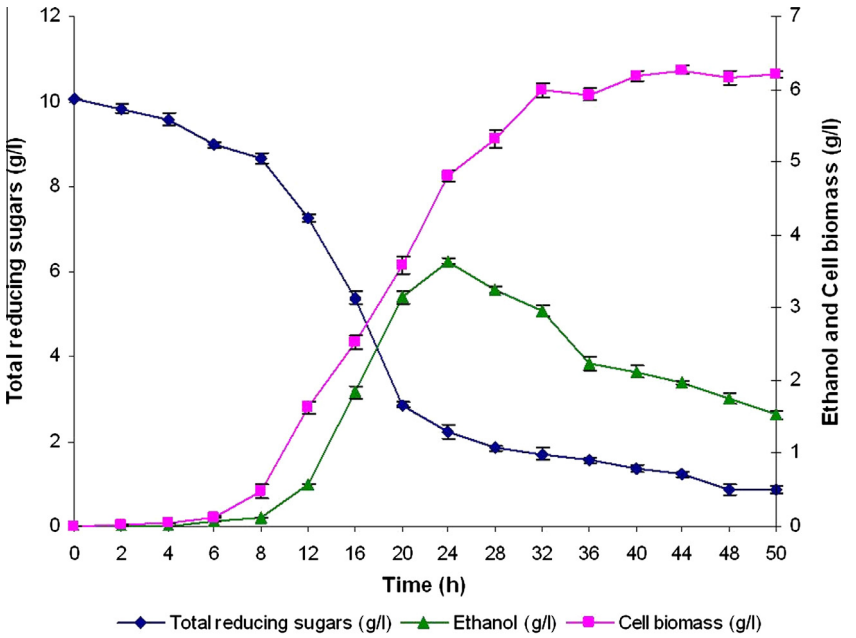

Fig. 6c. Fermentation profiles of *P. stipitis* in synthetic media.

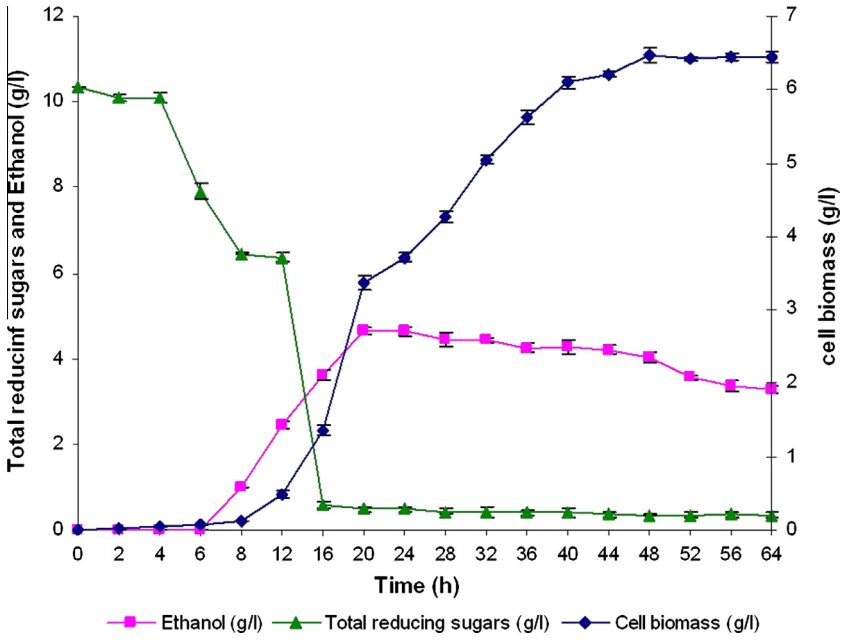

Fig. 6d. Fermentation profiles of *S. cerevisiae* in synthetic media.

Table 5
Comparison of separate hydrolysis and fermentation results from various lignocellulosic materials pretreated.

Substrates	Pretreatment	Enzyme source for Saccharification	Microorganisms for ethanol production	Ethanol yield ($g\,g^{-1}$)	Refs.
Wheat starch pre fermentation effluent	Dilute H_2SO_4	Commercial enzyme	Recombinant *Sacchromyces cerevisiae* strain REF	0.47	[33]
Wheat starch post fermentation effluent	Dilute H_2SO_4	Commercial enzyme	Recombinant *Sacchromyces cerevisiae* strain REF	0.46	[33]
Prosopis juliflora	Dilute H_2SO_4	Commercial enzyme	*Sacchromyces cerevisiae*	0.49	[34]
Sugarcane baggase	Lime alkaline	Crude cellulase	*S. cerevisiae*	0.081	[35]
Chlorococcum infusionum	NaOH	–	*S. cerevisiae*	0.26	[36]
Kans grass	***NaOH***	***Crude from T. reesei***	***S. cerevisiae***	**0.38**	***Present study***
Kans grass	***NaOH***	***Crude from T. reesei***	***P. stipitis***	***0.44***	***Present study***
Kans grass	*Acid*	*Crude from T. reesei*	*P. stipitis*	*0.46*	[2]

specific growth rate of $0.22\,h^{-1}$ (Table 4).The growth profile of *P.stipitis* and *S.cerevisiae* in synthetic media are shown in Figs. 6c and d respectively. Since in synthetic media ethanol yield (Yp/s) for both yeasts *P. stipitis* ($0.46\,g\,g^{-1}$) and *S. cerevisiae* ($0.47\,g\,g^{-1}$) was observed to be better than the hydrolysate media which may be due to the fact that glucose as sole carbon source is known to be better sugars for fermentation to produce ethanol in comparison to all other sugars.

Hence a better ethanol yield ($0.44\,g\,g^{-1}$) was observed when *P. stipitis* was used for fermentation. As *P. stipitis* is one of the yeast strains that can utilized wide range of hexose and pentose sugar including glucose, mannose, galactose, xylose and cellobiose. As the NaOH pretreated biomass is mainly composed of cellulose and hemicellulosic portion after removal of lignin content during pretreatment. The enzyme mixture that was used for enzymatic hydrolysis was composed of xylanase and cellulase activity. Hence during hydrolysis both type of sugars (pentose and hexose, mainly glucose) were solubalize and were present in this sugar mixture. The *P. stipitis* which can utilize both types of sugars for ethanol fermentation was found to be more efficient in term of ethanol yield ($0.44\,g\,g^{-1}$) to *S. cerevisiae* ($0.38\,g\,g^{-1}$) which mainly can utilize glucose sugar for ethanol fermentation. Hence the ethanol yield from both yeasts is comparable to yield obtained by all other researcher after different pretreatment and enzymatic strategies (Table 5).

4. Conclusions

Kans grass is one of the novel non food resources of lignocellulosic biomass which may be cultivated and utilized for the bioethanol production without extra pressure on food crop land. NaOH pretreatment is a conventional strategy for delignification of biomass; hence by using dilute NaOH pretreatment more than 79% of lignin removal was observed therefore for the next step of hydrolysis more biomass surface area may be available for enzyme action. As Saccharification/hydrolysis for lignocellulosic material, cellulase enzyme production accounts of 40% of the total cost of bioethanol production. So in the present study effort were done for the Saccharification of NaOH pretreated Kans grass with crude enzyme (fungal source) instead commercial enzyme to make overall process to be more economical. Further upon fermentation of sugars obtained after hydrolysis with *P.stipitis* high yield of ethanol was obtained which was found to be close to theoretical yield of ethanol and comparable to all other existing literature. In conclusion, saccharification of alkali pretreated biomass of Kans grass is

more advantageous in comparison to acid pretreated biomass since it results in higher sugar (about 5-fold).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2013.06.045.

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