DEVELOPMENT OF ALKALINE/ORGANOSOLV PRETREATMENT OF RICE STRAW

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A THESIS SUBMITTED AS A PART OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN ENERGY TECHNOLOGY AND MANAGEMENT

THE JOINT GRADUATE SCHOOL OF ENERGY AND ENVIRONMENT
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ABSTRACT

Lignocellulose represents a promising starting material for conversion into fuels and chemicals in biorefineries; however, its efficient conversion to sugar requires a pretreatment step. In the present research, the pretreatment of rice straw by alkaline/organosolv process was studied aiming to separate high quality lignin and enhance enzymatic digestibility of the cellulose-enriched solid. Effects of alkaline (NaOH, NH_3 solution, and tri-ethyl amine) on organosolv pretreatment using acetone, ethyl acetate, and ethanol at varying temperature, reaction time, acetone content, alkaline concentration, percent solid loading including solvent regeneration after pretreatment were studied. The NaOH in an acetone system was shown to be the best condition in terms of cellulose selectivity and enzymatic digestibility. The optimal alkaline-catalyzed pretreatment reaction contained 4 % (w/w) of rice straw with 20 ml of 50% (w/v) NaOH solution in 180 ml of acetone and treated at 80°C for 30 min with the initial pressure at 20 bars and mixing at 250 rpm. This led to the highest glucose yield of 869 mg/g pretreated biomass which was equivalent to the maximal glucose recovery of 78.97% based on available glucan in the native biomass. The work shows potential of alkaline pretreatment in organic solvent system for increasing digestibility of lignocelluloses. The high efficiency and selectivity of the developed pretreatment process could provide a high glucose yield with purity from lignocellulosic biomass for further conversion to value-added biorefinery products.

Keywords: Biorefinery, Lignocellulose, Alkaline/Organosolv, Pretreatment

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LIST OF ABBREVIATIONS

HPLC = High-performance liquid chromatography

DNS = 3, 5 Dinitrosalicylic acid

RS = Rice straw

NaOH = Sodium hydroxide

NH_3 = Ammonia

TEA = Triethylamine

DI = Deionized water

EtOH = Ethanol

EA = Ethyl acetate

FPU = Filter paper unit

CHAPTER 1
INTRODUCTION

1.1 Rationale and Problem Statement

Nowadays, the demand for fossil fuels is rapidly increasing, but the supply is decreasing, which leads to the increasing prices of fossil fuels. Alternative energies are key solution to solve this problem. One of most widely-used alternative fuels is gasohol which is composed of gasoline and ethanol. Besides production of bioethanol from the conventional sugar and starch feedstocks, it can be alternatively produced from lignocellulosic biomass.

Lignocellulosic biomass is a source for "second generation biofuels" and is a renewable feedstock in contrast to fossil fuels, which are non-renewable sources. This leads to an increasing research interest on conversion of lignocellulosic biomass to biofuels and biochemicals. Another problem is that fossil fuel generates lots of greenhouse gas, resulting in global climate change. Thailand is an agricultural country and has a large amount of agricultural residues. It becomes a low cost feedstock to produce variety of value added chemicals or fuels. Therefore, utilization of lignocellulosic biomass is considered an interesting approach to increase the value of waste while reduce the oil crisis problem. Lignocellulosic biomass comprises three major components which are lignin, hemicelluloses and cellulose. Lignin is a barrier in biomass utilization. It is a large heteropolymer network and acts as a glue to seal hemicelluloses and cellulose together. So the removal of lignin is necessary for producing a feasible yield of sugar for further conversion step in biorefineries. Pretreatment by delignification to disrupt lignin structure can increase the enzyme accessibility to the cellulose fraction and increase the glucose yield obtained from enzymatic hydrolysis. Glucose is an important intermediate for conversion to a variety of fuels and chemicals such as ethanol, organic acids and organic solvents by fermentation or catalytic routes. In order to develop an alternative pretreatment method, this work was focused on the study of alkaline-catalyzed organosolv process for increasing pretreatment efficiency and selectivity under low-temperature conditions. The reaction parameters i.e. types of solvents, alkaline loading, temperature, operational time, and solid loading were studied. The work provides an efficient alternative method for increasing biomass digestibility for maximized sugar yield in biorefinery

1.2 Literature reviews

The scope of this research is the modification of organosolv pretreatment by using alkaline catalyst. The review covers the pretreatment of biomass, with the focus on alkaline and organosolv pretreatment and enzymatic hydrolysis of biomass.

1.2.1 Pretreatment

The pretreatment of lignocellulosic materials is a prerequisite step to increase the digestibility of the recalcitrant biomass. This can be achieved by removal of lignin and hemicellulose, reduction in cellulose crystallinity, and increasing porosity of the raw materials resulting in increasing cellulose accessibility to hydrolytic enzymes [6]. Recent studies focused on evaluating and optimizing pretreatment techniques for the pretreatment of softwood, hardwood, and agricultural residues [5].

Ingram et al. (2011) [25] studied the conversion of lignocelluloses into valuable products. The present work compared liquid hot water (LHW) pretreatment to the soda pulping process and to the ethanol organosolv pretreatment using rye straw as a single lignocellulosic material. The organosolv pretreated rye straw was shown to require the lowest enzyme loading in order to achieve a complete saccharification of cellulose to glucose. At biomass loading up to 15% (w/w) cellulose conversion of LHW and organosolv pretreated lignocelluloses was found to be almost equal. The soda pulping process showed lower carbohydrate and lignin recoveries compared to the other two processes.

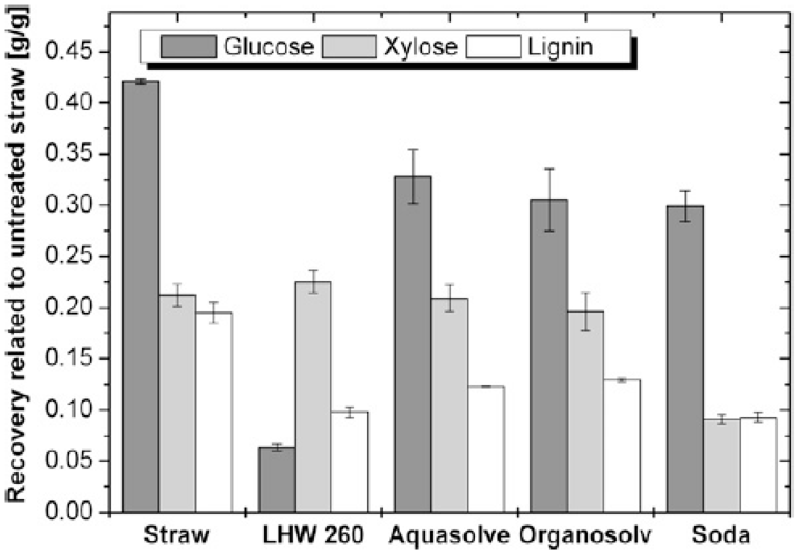


Fig. 1 Overall recovery of glucose, xylose and lignin from different processes related to untreated dried rye straw (determined by an acid hydrolysis). Enzymatic hydrolysis was performed usisng 10% (w/v) biomass with an enzyme loading of 13 FPU/g cellulose and incubated for 48 h at 323 K.

1.2.1.1 Alkaline pretreatment

Alkaline pretreatment involves the application of alkaline solutions, such as NaOH or KOH, to remove lignin and a part of the hemicelluloses, and efficiently increases the accessibility of the enzyme to the cellulose. The alkaline pretreatment can result in a sharp increase in saccharification yields. Pretreatment can be performed at low temperature but with a relatively long time and high concentration of the base. Compared with acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicelluloses and cellulose, and avoids fragmentation of the hemicelluloses polymers [8].

Wang et al. (2010) [9] studied the pretreatment of coastal Bermuda grass with NaOH at concentrations from 0.5% to 3% (w/v) with a residence time from 15 to 90 min at 121^{o}C. The pretreatments were evaluated based on total lignin removal and production of total reducing sugars, glucose and xylose from enzymatic hydrolysis of the pretreated biomass.

The optimal NaOH pretreatment condition at 121^{o}C was at 15 min with 0.75% NaOH. Under these optimal pretreatment conditions, the total reducing sugars yield was 71% of the maximum theoretical yield, and the overall conversion efficiencies for glucan and xylan were 90.43% and 65.11%, respectively [9].

Table 1 The overall glucan and xylan conversion efficiencies[a] after 72h enzymatic hydrolysis of NaOH pretreated coastal Bermuda grass at 121°C [9].

Time (min)	NaOH conc. (%)	Glucan conversion (%)	Xylan conversion (%)
15	0.5	64.56	35.79
	0.75	90.43	65.11
	1	91.56	60.98
	2	89.51	46.45
	3	91.68	37.61
30	0.5	70.19	43.18
	0.75	91.61	62.66
	1	90.73	59.27
	2	87.72	45.41
	3	87.02	34.85
60	1	88.43	57.24
	2	87.37	44.47
	3	88.36	35.06
90	1	90.27	46.25
	2	84.91	45.84
	3	86.12	35.16
Control[b]		31.22	6.73

[a] Overall glucan/xylan conversion efficiencies = (g glucose/xylose produced in the hydrolyzate) x (0.9/0.88)/(g glucan/ xylan in raw biomass).

[b] Untreated Bermuda grass sample hydrolyzed with excessive loadings of cellulase and cellobiase.

Xu and Cheng (2010) [10] studied the used of sodium hydroxide (NaOH) and lime ($Ca(OH)_2$) together to improve the cost-effectiveness of alkaline pretreatment of switch grass at ambient temperature.

Based on the sugar production from enzymatic hydrolysis, the best pretreatment conditions were determined as: solid loading of 2%, at ambient temperature (no heating) with a residence time of 6 h, NaOH loading of 0.10 g/g raw biomass and $Ca(OH)_2$ loading of 0.02 g/g raw biomass. The glucose and xylose yields were 59.4% and 57.3% of the theoretical yield, respectively. The sugar yield of the biomass that was pretreated using combination of 0.10 g NaOH/g raw biomass and 0.02 g $Ca(OH)_2$/g raw biomass was found comparable with that of the biomass that was pretreated using 0.20 g NaOH/g raw biomass under the same conditions. This led to a remarkable reduction in chemical expense due to the low cost of lime and the reduced loading of NaOH [10].

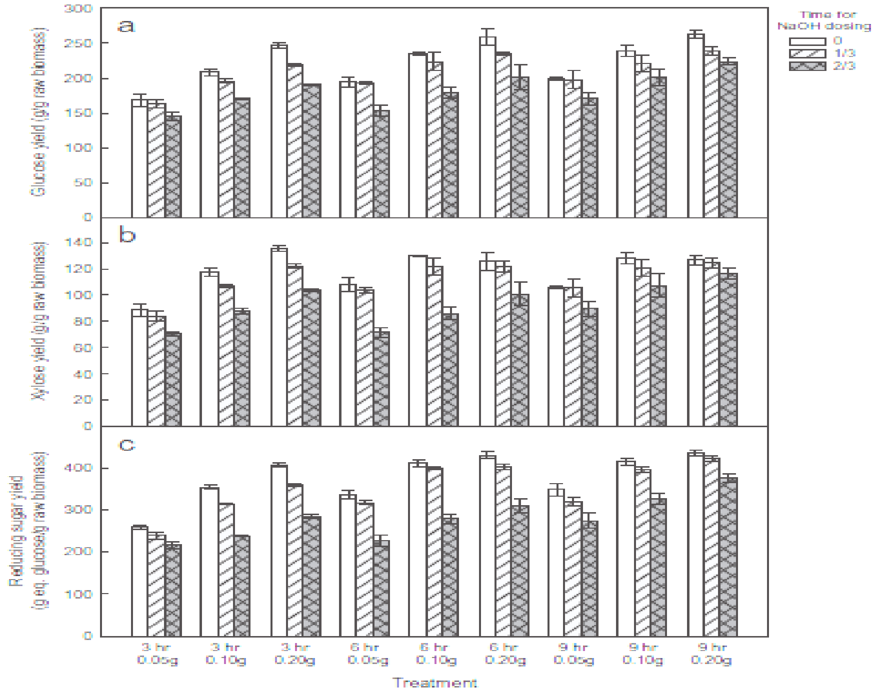


Fig. 2 Yields of (a) glucose, (b) xylose, and (c) total reducing sugars in enzymatic hydrolysis of switchgrass biomass pretreated at different combinations of residence time, NaOH loading, and time point for NaOH addition [10].

Table 2 Comparisons of carbohydrate conversions, lignin reductions, and pH changes between NaOH pretreatment and pretreatment use a combination of NaOH and lime [10].

Chemical loading (g raw/biomass)	Glucan conversion (%)	Xylan conversion (%)	Total carbohydrate conversion (%)	Lignin reduction (%)	Initial pH	Final pH	
						6 h	24 h
0.2 g NaOH	69.5 (1.09)	54.8 (0.87)	63.5 (1.01)	48.6 (1.03)	12.92 (0.02)	12.81 (0.02)	12.66 (0.04)
0.1 g NaOH	48.1 (2.12)	38.9 (2.90)	44.1 (1.73)	34.6 (1.51)	12.84 (0.02)	12.59 (0.02)	12.32 (0.03)
0.1 g NaOH+ 0.02 g $Ca(OH)_2$	59.4 (0.79)	57.3 (0.79)	59.3 (1.04)	37.8 (0.55)	12.86 (0.01)	12.77 (0.03	12.65 (0.04)

Gupta and Lee (2010) [11] studied the pretreatment of switchgrass using aqueous ammonia or NaOH to enhance the enzymatic digestibility. To increase the effectiveness of pretreatment, H_2O_2 was supplemented with alkaline reagents. Since H_2O_2 is unstable at high temperature, stepwise change of temperature was applied. This process was found to be effective in the case of NH_3/H_2O_2 treatment. The composition of pretreatment liquid indicated that hemicelluloses solubilized during alkaline treatment and existed either in the form of oligomers or as lignin-carbohydrate complex (LCC). LCC formation was prominent in ammonia treatment and in $NaOH/H_2O_2$ treatment.

Table 3 Distribution of carbohydrates after the pretreatment [11].

	15%NH_3, 120°C				5%NaOH, 85°C			
	without H_2O_2				without H_2O_2			
	solid (%)	Oligomer (%)	LCC (%)	Degradation (%)	solid (%)	Oligomer (%)	LCC (%)	Degradation (%)
Glucan	98.9	2.4	0.0	0.0	98.5	3.7	0.0	0.0
Xylan	61.7	21.6	13.0	3.7	43.4	24.2	0.0	33.5
Galactan	46.2	42.4	10.1	1.3	50.2	52.3	0.0	19.3
Arabinan	56.6	45.2	12.5	0.0	43.2	48.0	0.0	18.5
Mannan	0.0	0.0	1.0	99.0	0.0	0.0	0.0	100.0
	with H_2O_2				with H_2O_2			
	solid (%)	Oligomer (%)	LCC (%)	Degradation (%)	solid (%)	Oligomer (%)	LCC (%)	Degradation (%)
Glucan	97.6	1.8	0.4	0.2	95.2	3.3	1.2	0.3
Xylan	47.9	29.3	0.5	22.3	40.2	28.0	22.9	8.9
Galactan	39.3	38.4	1.0	21.2	46.7	54.4	21.3	0.0
Arabinan	34.4	48.6	0.9	16.0	32.0	59.7	19.8	0.0
Mannan	0.0	0.0	0.0	100.0	0.0	0.0	19.0	81.0

MacIntosh and Vancov (2010) [13] studied the physico-chemical pretreatment of wheat straw by alkaline pretreatment using NaOH. The alkaline pretreatment successfully delignified the biomass by disrupting the ester bonds cross-linking lignin and xylan, resulting in cellulose-and hemicellulose-enriched fractions. The impacts of varying pretreatment parameters (temperature, time and alkalinity) on enzymatic digestion of residual solid material were assessed.

Following pretreatment, the recoverable solids and lignin contents were found to be inversely proportional to the severity of the pretreatment process. Elevating temperature and alkaline strengths maximized hemicelluloses and lignin solubilisation, and enhanced enzymatic saccharification. Pretreating wheat straw with 2% NaOH for 30 min at 121°C improved enzyme saccharification 6.3-fold when compared to control samples. Similarly, there was a 4.9-fold increase in total sugar yields from samples treated with 2% NaOH at 60°C for 90 min [13].

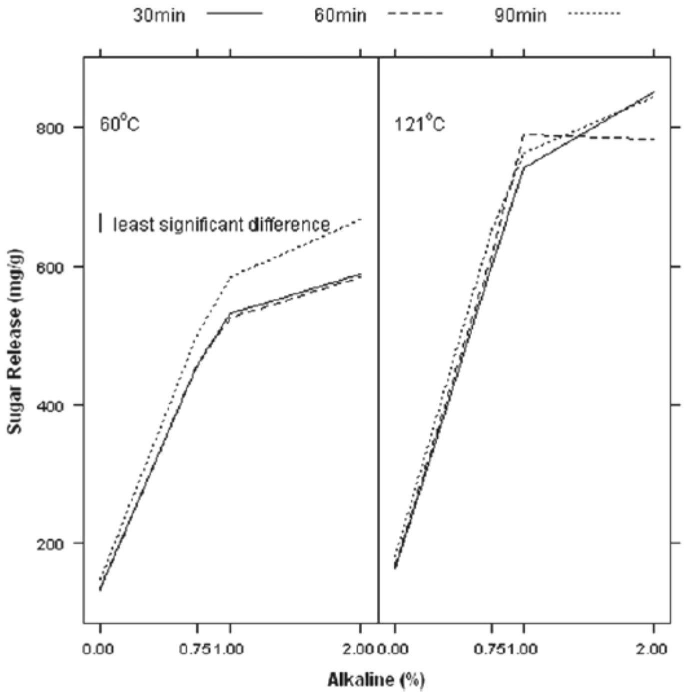


Fig. 3 Total sugar released from alkali pretreated (10%, w/v) and enzyme saccharified wheat straw. Sugar yields are expressed as mg/g pretreated material. Displayed data represents averages of three independent experiments [13].

1.2.1.2 Ammonia pretreatment

Li et al. (2010) [23] studied an integrated bioconversion process to convert corn stover-derived pentose and hexose to ethanol effectively. In this study, corn stover was pretreated by soaking in aqueous ammonia (SAA), which retained glucan (~100%) and xylan (>80%) in the solids. The pretreated carbohydrates rich corn stover was converted to ethanol via two phase simultaneous saccharification and fermentation (TPSSF). The TPSSF using 12h SAA-treated corn stover resulted in the highest ethanol concentration (22.3 g/l), which was equivalent to 84% of the theoretical ethanol yield based on the total carbohydrates (glucan + xylan) in the untreated corn stover.

The compositions of untreated and SAA-pretreated corn stover are summarized in Table 4, while Fig. 4 summarizes the sugar retentions of SAA-pretreated corn stover. The 8 h, 12 h, and 24 h SAA pretreatment resulted in 99%, 98%, and 98% of glucan retention, respectively. In the case of xylan, the retention were 90%, 86%, and 80% of xylan, respectively. These results confirmed previous observation that SAA pretreatment is an efficient method for glucan and xylan retention. The SAA retains the hemicelluloses in the solids by minimizing the interaction of ammonia with hemicelluloses during treatment at moderate temperatures and without agitation [23].

Table 4 Composition of untreated and SAA-pretreated corn stover [23].

Pretreatment conditions	Glucan (%)	Xylan (%)	Arabinan (%)	Galactan (%)	Lignin (%)
Untreated	38.3	21	2.7	2.1	17.4
8h SAA-treated	37.9	18.9	2.3	1.7	9.6
12h SAA-treated	37.6	18.1	2.2	1.7	7.3
24h SAA-treated	37.5	16.8	2.2	1.3	6.8

*Pretreatment: 15wt% aqueous ammonia, 1:11 solid: liquid ratio, 60°C for 8 h, 12 h, and 24 h

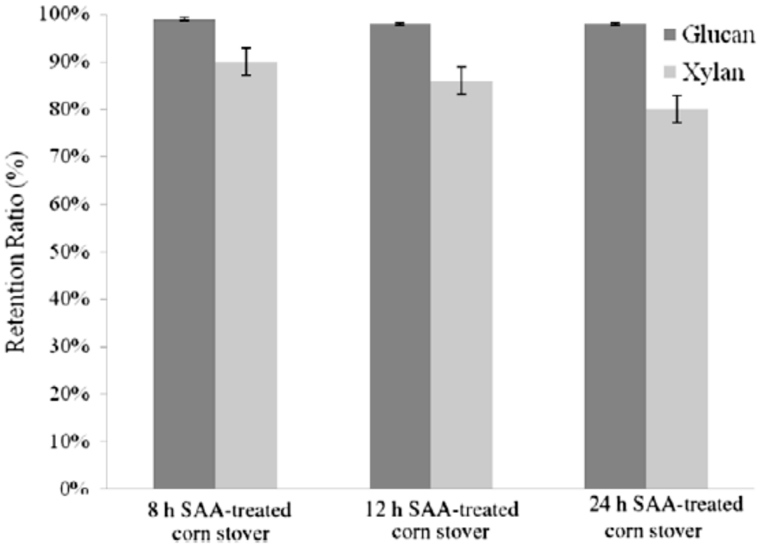


Fig. 4 Glucan and xylan retentions after SAA pretreatment. Pretreatment condition: 15 wt% aqueous ammonia, 1:11 solid: liquid ratio, 60°C for 8 h, 12 h, and 24 h [23].

1.2.1.3 Organosolv pretreatment

The organosolv process uses organic solvents such as methanol, ethanol, and ethylene glycol to fractionate biomass components. The change of cellulose crystallinity during organosolv pretreatment is not clear yet, but it has been found that the swelling of cellulose in organic solvent strongly depends on the species of organic solvent, solvent concentration and temperature.

Organosolvent pretreatment is more expensive at present than the leading pretreatment processes, but the separation and recycling of the applied solvent could reduce the operational costs of the process. It also requires strictly controlled conditions due to the volatility of organic solvents. Removal of solvents from the pretreated cellulose is usually necessary because the solvents might inhibit enzymatic hydrolysis and fermentation or digestion of hydrolysate [12].

Sannigrahi et al. (2010) [5] studied ethanol organosolv pretreatment of Loblolly pine to enhance the efficiency of enzymatic hydrolysis of cellulose to glucose. The results indicated reduced crystallinity of the cellulose following the organosolv pretreatment, which renders the substrate easily hydrolysable by cellulase. The degree of crystallinity increased and the relative proportion of *para*-crystalline and amorphous cellulose decreased after enzymatic hydrolysis, indicating preferential hydrolysis of these regions. The NMR spectra of the solid materials before and after the treatments show that hemicelluloses and lignin were degraded during the organosolv pretreatment [5].

Moradi et al. (2013) [15] studied the pretreatment of rice straw using concentrated phosphoric acid and sodium hydroxide prior to enzymatic hydrolysis that used commercial cellulase and β-glucosidase. Hydrolysis of the alkaline pretreated rice straw resulted in production of 163.5 g glucose from each kg of untreated rice straw. Additionally, concentrated phosphoric acid pretreatment and subsequent hydrolysis resulted in production of 192.3g glucose from each kg straw [15].

Table 5 Glucose yield after enzymatic hydrolysis of pretreated and untreated rice straw [15].

	Yield of enzymatic hydrolysis		
	Theoretical glucose yield (%)[a]	(g glucose/ kg RS*)	(g glucose/ kg pretreated RS)
Untreated RS	31.1	101.8	-
Alkaline pretreated	60.4	163.5	407.9
Phosphoric acid pretreated	69.8	192.3	354.1

[a] Theoretical glucose yield (%) = produced glucose (g/l) x 100/ (1.111x substrate concentration (g/l) x biomass glucan fraction)

*RS: rice straw

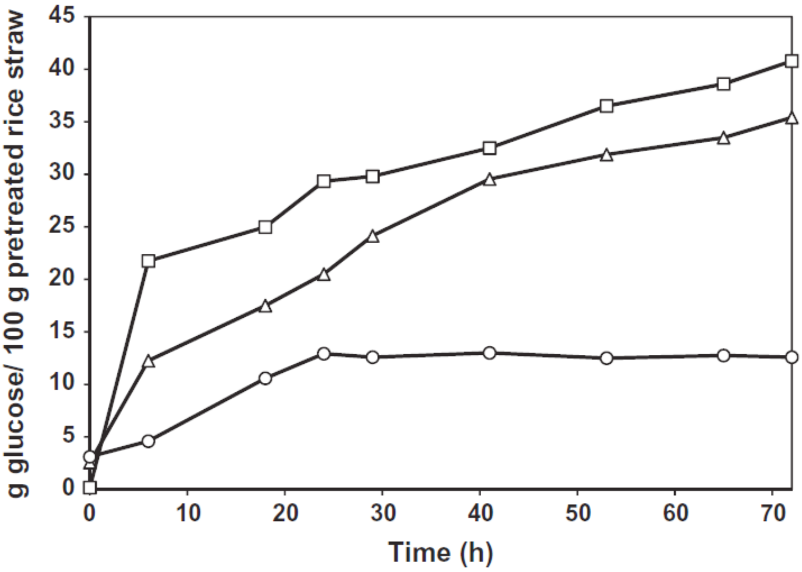


Fig. 5 Production of glucose in the enzymatic hydrolysis of untreated (○), alkaline pretreated (□), and H_3PO_4 pretreated (△) rice straw [15].

Cybulska et al. (2012) [14] studied the fractionation of switchgrass using the “Clean Fractionation” with the use of ternary solvent mixture (water/ethyl acetate/ethanol) in the presence of sulfuric acid as the catalyst. Optimization of temperature, catalyst concentration and solvent composition was performed using Response Surface Methodology and 59.03±7.01% lignin recovery, 84.85±1.34% glucose, and 44.11±3.44% aqueous fraction xylose yields were obtained at 140°C, 0.46 % w/w catalyst concentration, 36.71 % w/w ethyl acetate concentration, and 25.00 % w/w ethanol concentration. The level of inhibitory by-products in the cellulose fraction did not inhibit the fermentation performance of *Saccharomyces cerevisiae* and resulted in an ethanol yield of 89.60±2.1%.

Table 6 Experimental design for clean fractionation optimization [14].

Experiment no.	Temperature (^oC)	Sulfuric acid conc. (%w/w)	Ethyl acetate conc. (%w/w)	Ethanol conc. (%w/w)
1	140.00	0.46	50.00	10.00
2	140.00	0.46	15.00	10.00
3	140.00	0.15	50.00	35.00
4	110.00	0.46	15.00	35.00
5	140.00	0.15	15.00	35.00
6	110.00	0.15	50.00	10.00
7	110.00	0.46	50.00	35.00
8	110.00	0.15	15.00	10.00
9	99.77	0.31	32.50	22.50
10	150.23	0.31	32.50	22.50
11	125.00	0.04	32.50	22.50
12	125.00	0.57	32.50	22.50
13	125.00	0.31	3.07	22.50
14	125.00	0.31	61.93	22.50
15	125.00	0.31	32.50	1.48
16	125.00	0.31	32.50	43.52
17	125.00	0.31	32.50	22.50
18	125.00	0.31	32.50	22.50
19	125.00	0.31	32.50	22.50
20	125.00	0.31	32.50	22.50

Table 7 Results obtained from clean fractionation process [14].

Exp.	lignin recovery (%)	Hydrolysis glucose yield (%)	Total glucose yield (%)	Xylose aqueous fraction yield (%)	Hydrolysis xylose yield (%)	Acetic acid conc. in hydrolyzate (g/L)	Acetic acid conc. in aqueous fraction (g/L)
1	72.71±2.08	73.38±3.00	70.61±2.80	49.45±1.51	15.49±0.55	0.21±0.00	75.92±5.83
2	15.22±1.41	73.85±0.51	70.99±0.49	67.92±2.68	16.16±0.58	0.18±0.03	43.90±5.33
3	37.37±0.70	33.60±1.41	33.26±1.37	5.22±0.63	28.41±1.24	0.27±0.02	12.65±1.00
4	17.70±0.00	28.88±2.79	28.70±2.74	2.09±1.39	18.31±1.99	0.13±0.02	22.42±0.34
5	14.73±4.88	35.37±3.18	35.01±3.18	3.45±0.43	22.49±0.40	0.22±0.01	9.54±0.11
6	18.16±0.00	20.08±2.35	19.99±2.33	0.93±1.32	9.34±3.24	0.07±0.02	9.98±2.61
7	16.46±2.43	41.49±0.50	40.93±0.45	8.06±0.88	33.46±0.30	0.28±0.02	34.62±0.08
8	1.96±0.00	15.58±0.63	16.51±0.63	0.00±0.00	5.86±0.16	0.06±0.02	11.08±1.01
9	11.31±0.70	19.18±0.86	19.10±0.85	0.29±0.41	9.51±1.09	0.12±0.03	30.65±1.94
10	58.74±0.31	96.33±0.10	92.32±0.05	55.23±0.18	21.25±1.04	0.25±0.00	51.50±0.80
11	10.80±0.70	12.42±0.59	12.37±0.59	0.00±0.00	3.18±0.56	0.00±0.00	0.94±0.52
12	62.34±2.08	66.21±0.36	64.81±0.45	31.24±2.23	31.08±0.69	0.31±0.02	62.16±5.08
13	6.63±1.75	32.38±1.17	32.12±1.15	3.93±0.81	17.54±0.56	0.15±0.00	10.56±0.17
14	63.84±3.43	46.78±2.18	45.64±1.89	22.92±4.70	27.17±3.48	0.28±0.01	51.18±1.57
15	15.48±3.82	35.42±0.82	35.08±0.81	9.12±1.65	18.12±0.56	0.21±0.00	44.22±4.29
16	14.50±1.05	36.17±1.62	35.78±1.56	4.98±0.78	31.10±1.28	0.28±0.03	21.27±0.32
17	49.13±0.00	45.22±1.50	44.73±1.36	9.67±3.66	28.44±0.37	0.29±0.01	47.86±3.36
18	40.78±0.00	47.39±1.70	46.78±1.72	12.72±1.00	28.46±1.84	0.33±0.05	51.95±3.22
19	39.72±0.00	43.26±0.10	42.71±0.10	11.56±0.20	28.07±0.97	0.31±0.01	54.26±0.96
20	45.96±3.85	44.78±1.39	44.33±1.40	8.80±0.16	29.78±0.76	0.32±0.02	49.14±0.84

Amiri et al. (2013) [17] studied the pretreatment of rice straw with 75% (v/v) aqueous ethanol containing 1% w/w sulfuric acid at 150°C for 60 min. The pretreatment resulted in the highest total sugar concentration of 31 g/l in the enzymatic hydrolysis. Thus, the organosolv pretreatment can be applied for efficient production of the solvents from rice straw.

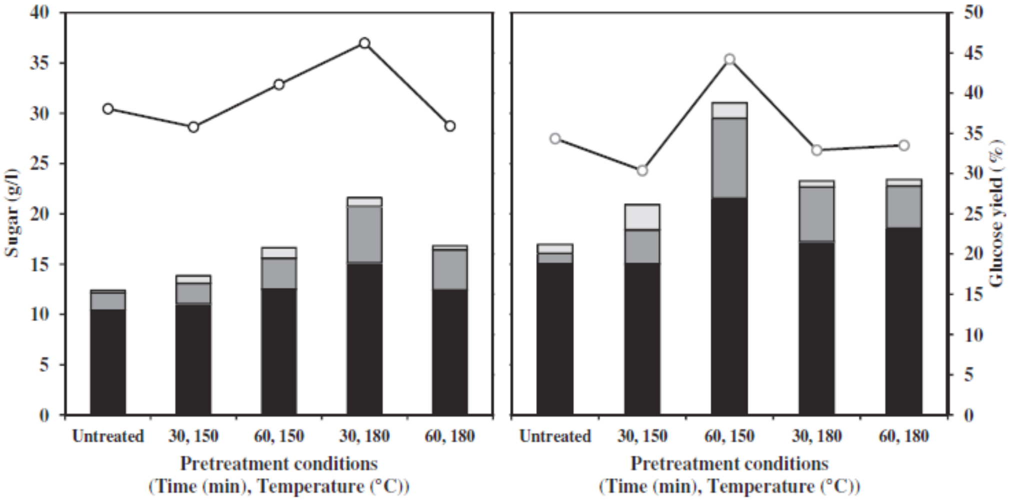


Fig. 6 Glucose (black), xylose (dark gray), and arabinose (light gray) concentrations, and theoretical glucose yield (○) after 72 h enzymatic hydrolysis of organosolv pretreated rice straw at 5% (a) and 8% (b) solid loading. Theoretical glucose yield (%) = produced glucose (g/l) x100/ (1.111xsubstrate concentration (g/l) x biomass glucan [17].

Mesa et al. (2011) [18] studied sugarcane bagasse pretreated with dilute-acid and submitted to an organosolv ethanol process with NaOH under different operational conditions (pretreatment time, temperature, and ethanol concentration). The aim was to maximize the glucose yield in the subsequent enzymatic hydrolysis stage. The optimum organosolv pretreatment conditions consisted in using 30% (v/v) ethanol at 195°C, during 60 min. Enzymatic hydrolysis of the residue then obtained produced 18.1g/l glucose correspondent to a yield of 29.1g glucose/ 100 g sugarcane bagasse.

1.2.1.4 Sequential pretreatment

Gao et a.l (2013) [7] studied different pretreatment processes including liquid hot water (LHW), sodium hydroxide (NaOH) pretreatment, and their sequential pretreatment to improve the enzymatic digestibility and sugar recovery from sugarcane bagasse (SCB). LHW pretreatment solubilized over 82% of xylan and 42% of lignin. NaOH pretreatment could remove 78% of lignin and retained most of the glucan.

For sequential pretreatment, LHW-NaOH pretreatment was able to solubilize over 92% of xylan and remove 76% of lignin. The highest total sugar recovery of 73% was achieved, while the NaOH-LHW pretreatment removed nearly 84% of lignin and solubilized 71% of xylan, showing the highest enzymatic digestibility. The pretreatment efficiency was in the order of NaOH-LHW>NaOH>LHW-NaOH>LHW [7].

Table 8 Composition of untreated and treated SCB [7].

Sample	Solid remain (%)	Pretreatment					
		Glucan (%)		Xylan (%)		Klason lignin (%)	
		Content	Recovery	Content	Recovery	Content	Recovery
Raw SCB	100	41.95	-	21.7	-	23.61	-
LHW pretreated SCB	59.05	63.12	88.84	6.49	82.35	23.07	42.31
NaOH pretreated SCB	61.2	63.19	92.18	26.63	24.88	8.55	77.85
LHW+NaOH pretreated SCB	42.5	82.45	83.53	3.97	92.22	13.07	76.47
NaOH+LHW pretreated SCB	45.55	76.91	83.5	13.75	71.15	8.13	84.33

Kuo et al. (2008) [26] studied the attempts to enhance cellulose saccharification by cellulase using cellulose dissolution as a pretreatment step. Four cellulose dissolution agents, NaOH/Urea solution, *N*-methylmorpholine-*N*-oxide (NMMO), ionic liquid (1-butyl-3-methylimidazolium chloride; [BMIM]Cl) and 85% phosphoric acid, were employed to dissolve cotton cellulose. In comparison with conventional cellulose pretreatment processes, the dissolution pretreatments were operated under milder condition with temperature <130°C. The dissolved cellulose was easily regenerated in water. The regenerated celluloses exhibited a significant improvement (about 2.7 to 4.6 fold enhancement) on saccharification rate during 1sth reaction. After 72h, the saccharification yield ranged from 87% to 96% for the regenerated celluloses while only around 23% could be achieved for the untreated cellulose. Even with high crystallinity, cellulose regenerated from phosphoric acid dissolution achieved the highest saccharification rates and yield probably due to its highest specific surface area and lowest degree of polymerization (DP). The effectiveness of cellulose dissolution pretreatments on enhancing regenerated cellulose saccharification by cellulase followed the order of phosphoric acid> [BMIM]Cl> NMMO> NaOH/urea.

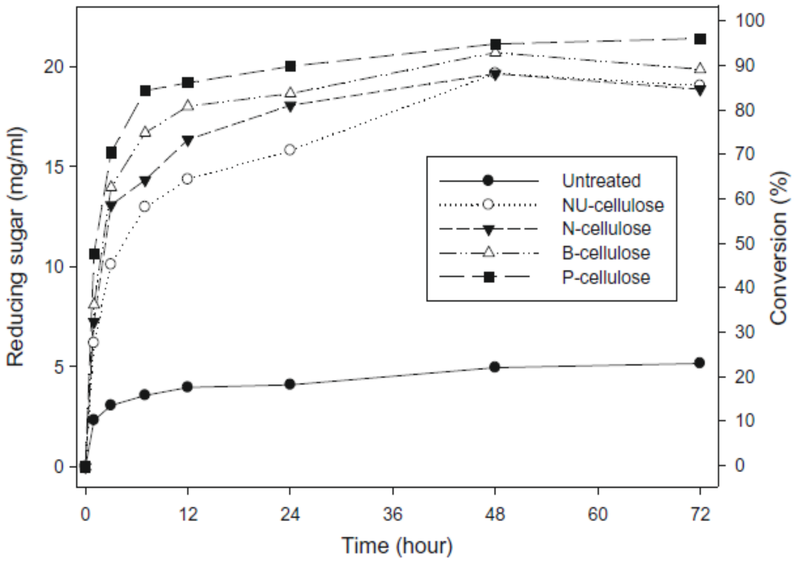


Fig. 7 Reducing sugars released during enzymatic hydrolysis of untreated and dissolution pretreated celluloses. Hydrolysis condition: 20mg/ml cellulose, Cellulase AP3 10FPU/g cellulose, and 50°C. NU-, N-, B-, and P-celluloses are cellulose regenerated from NaOH/urea, NMMO, [BMIM]Cl, and 85% phosphoric acid dissolution, respectively [26].

1.2.1.5 Combination pretreatment

Choi et al. (2012) [27] studied a two-stage fractionation process using sodium hydroxide and sulfuric acid to remove lignin and hemicelluloses from rapeseed (Brassica napus) straw. This process consists of two stages; using sodium hydroxide, the first stage solubilized 35.54% of lignin and increased the glucan content in the treated solid from 32.86% to 38.13%. The second stage solubilized 85.85% of the lignin and 91.56% of the XMG (xylan+mannan+galactan) into the hydrolyzate. After the two-stage process, merely 2.53% of glucan and 3.81% of XMG were lost due to excessive decomposition. Despite the reduction in enzyme loading by 50%, the enzyme digestibility with the two-stage treated straw was approximately 23% higher than that of the single stage acid fractionation process.

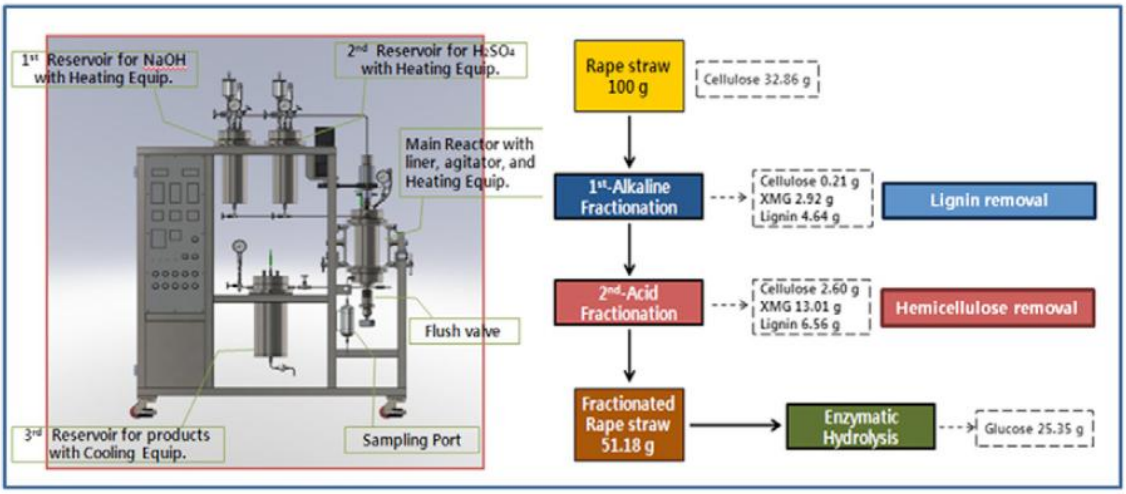


Fig. 8 Two-stage fractionation process using sodium hydroxide and sulfuric acid [27].

Table 9 Compositional analysis for the two-stage fractionation of rapeseed straw [27].

Sample	Solid remaining	solid (%)				liquid (%)		
	(%)	glucan	XMG	K.lignin	ash	glucan	xylan	pH
Raw straw	100	32.86	17.06	13.09	7.95	-	-	-
1st Fractionated	85.62	38.13	16.52	9.87	5.83	0.21	2.61	12.25
1st Fractionated[a]		32.65	14.14	8.45	4.99			
2nd fractionated	51.18	57.73	2.21	3.69	0.70	1.77	12.70	1.39
2nd fractionated[a]		30.12	1.13	1.93	0.37			
Component retention (%)		91.67	8.44	14.15	4.78			

The straw were fractionated sequentially with 0.5%(w/v)NaOH at 140°C for 15min and with 3%H_2SO_4 at 150°C for 20min

[a] Data are based on the oven dry raw straw

Kim et al. (2011) [28] developed a two-stage pretreatment process using aqueous ammonia and dilute acid in a percolation mode to improve the production of fermentable sugars from rice straw. Aqueous NH_3 was used in the first stage which removed lignin selectively but left most of cellulose (97%) and hemicelluloses (77%). Dilute acid was applied in the second stage which removed most of hemicelluloses, partially disrupted the crystalline structure of cellulose, and thus enhanced enzymatic digestibility of cellulose in the solids remaining. Under the optimum pretreatment conditions, the enzymatic hydrolysis yields of the two-stage treated samples were 96.9% and 90.8% with enzyme loadings of 60 and 15 FPU/g of glucan, respectively. The overall sugar conversions of cellulose and hemicelluloses into glucose and xylose by enzymatic and acid hydrolysis reached 89.0% and 71.7%, respectively.

Table 10 Effect of reaction time during the two-stage pretreatment on the solid composition of rice straw, sugar concentration in the liquid fraction, and degree of lignin removal[a] [28].

Time (min)	SR[b] (g)	cellulose (g, solid)	glucan (g, liquid)	cellulose remaining (g, solid)	hemicellulose (g, solid)	xylose (g, liquid)	xylose recovery (g, liquid)	K. lignin[c] (g, solid)	Delignification (%)
Untreated	100	39.5	0	100	24.4	0	0	15.9	0
After 1st stage	79.9	38.3	0.5	97.0	18.9	4.8	-	4.9	69.2
10	78.5	37.8	1.0	95.7	11.2	9.9	40.6	4.3	72.9
15	70.4	35.1	1.3	88.9	5.6	14.4	59.0	4.0	74.8
20	62.7	32.9	1.8	83.3	0.8	17.5	71.7	3.3	79.2
25	59.2	31.5	5.3	79.7	0.5	16.3	66.8	3.0	81.1
30	57.1	28.9	9.0	73.2	0.3	15.5	63.5	2.6	83.6

[a] All sugar and lignin contents are based on 100g dry untreated rice straw. Pretreatment conditions: 3wt% of sulfuric acid, 5ml/min of flow rate, 130°C of reaction temperature

[b] SR: solid remaining after reaction

[c] Klason lignin

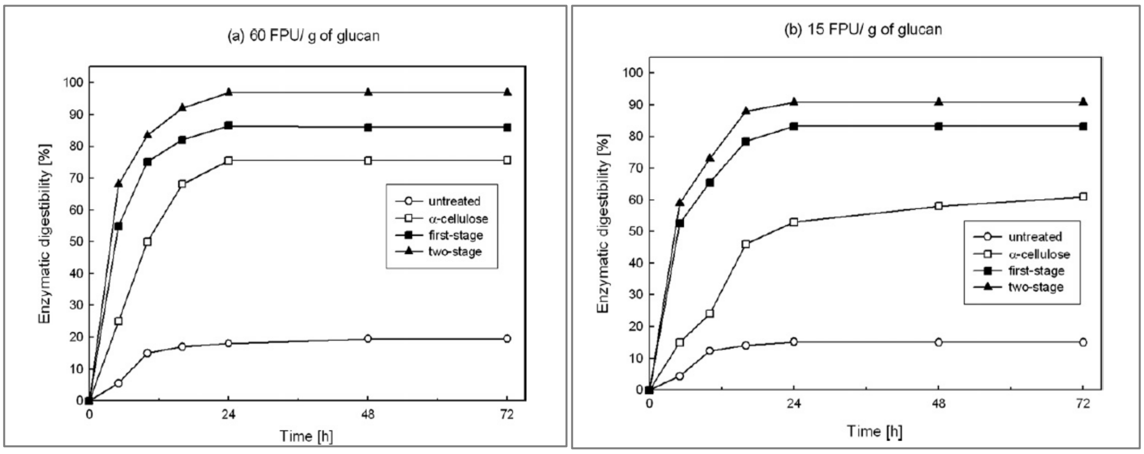


Fig. 9 Enzymatic digestibility of pretreated samples by the percolation reaction with different cellulose loadings. The first stage pretreatment conditions: 15 wt% of aqueous ammonia, 5 ml/min of flow rate, 20 min of reaction time at 130°C. The second stage pretreatment conditions: 3 wt% of dilute acid, 5ml/min of flow rate, 20 min of reaction time at 130°C followed by the first stage. Enzymatic hydrolysis conditions: 72 h, pH 4.8, 50°C [28].

1.2.2 Enzymatic Hydrolysis

Enzymatic hydrolysis is the second step in the production of ethanol from lignocellulosic materials. It involves cleaving the polymers of cellulose and hemicelluloses using enzymes. Consequently, the main hydrolysis product of cellulose is glucose, whereas the hemicellulose gives rise to several pentoses and hexoses [11].

Saha and Cotta (2007) [16] studied alkaline H_2O_2 pretreatment and enzymatic saccharification of rice hull. The yield of sugars from diluted alkaline peroxide pretreated (7.50% H_2O_2, v/v; pH 11.5; 35^oC; 24 h) rice hulls (15.0%, w/v) after enzymatic saccharification (45^oC, pH 5.0, 72 h) by three commercial enzyme preparations *(cellulase, β-glucosidase, and xylanase*) was 428±12 mg/g (90% yield). The almost complete conversion (96%) of rice hulls to sugars was achieved by saccharifying the liquid and solid fractions separately after alkaline peroxide pretreatment.

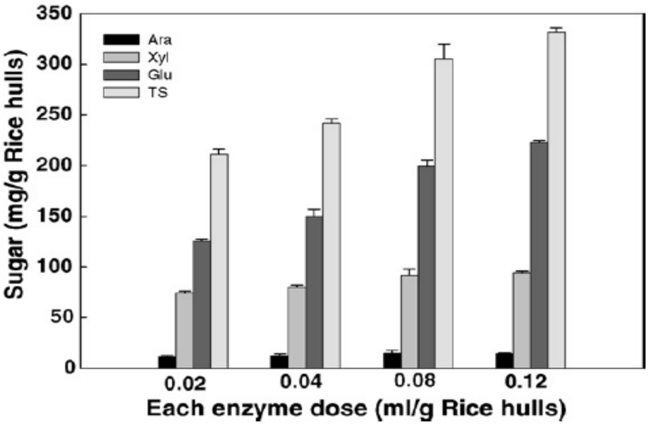


Fig. 10 Effect of each enzyme dose on the release of sugars at 72 h from alkaline H_2O_2 pretreated (7.5%, w/v; pH 11.5; 35^oC, 24 h) rice hulls (15.0%, w/v). A cocktail of three commercial enzyme preparations (cellulase, β-glucosidase and xylanase) was used. The data presented are the averages of two separate experiments [16].

MacIntosh and Vancov (2010) [13] studied the enzymatic saccharification of wheat straw pretreated by dilute alkaline pretreatment (NaOH). The combination of three commercial enzyme preparations (cellulase, β-glucosidase and xylanase) was found to maximize monomeric sugar release, particularly for substrates with higher xylan contents. The combined enzyme activities increased total the reducing sugar release 1.65-fold and effectively reduced cellulase enzyme loadings by 3-fold. Prehydrolysate liquors contained 4-fold more total phenolics compared to enzyme saccharification mixtures. Harsher pretreatment conditions provide saccharified hydrolysates with reduced phenolic content and greater fermentation potential.

Table 11 Specific activity of commercial enzymes used in NaOH pretreated wheat straw saccharification [13].

Enzyme	Specific activity (U/mg protein)		
	NS50013	NS50010	NS50030
Endoglucanase	14.2	0.11	0.02
Exoglucanase	1.51	0.07	0.05
Xylanase	7.05	75	129.5
β-glucosidase	1.07	10.08	0.04
Pectinase	0.03	0.4	ND
Cellulase	70	ND	ND
Protein (mg/ml)	135	150	33

ND Not determined

a At pH 5.0 and 50 °C

b Measured as filter paper units/g protein

c Concentration of Novozymes preparation

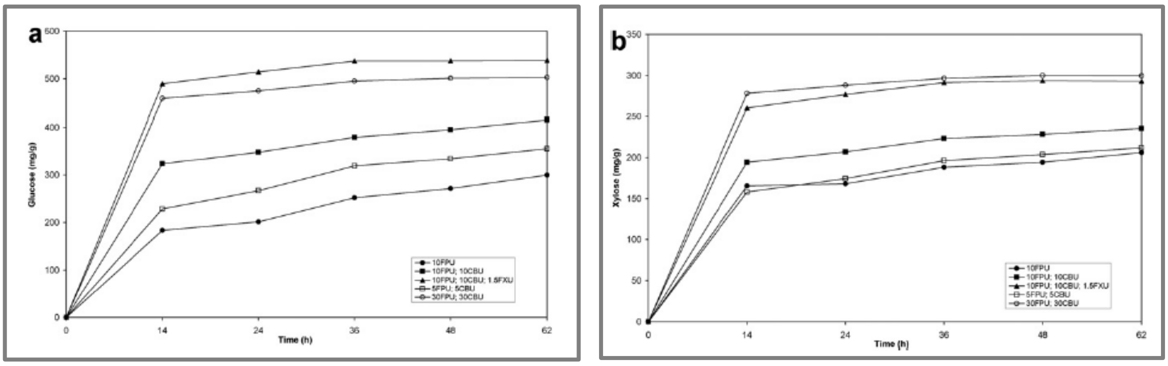


Fig. 11 Time course of (a) glucose and (b) xylose released by enzymatic saccharification (5%, w/v; 50°C, pH 5.2) of alkaline pretreated (1.0% NaOH; 60 min; 121°C) wheat straw using five enzyme combinations. Glucose and xylose yields are pretreated as mg/g pretreated material. Data represents averages of three separate experiments. The average l.s.d. (p=0.05) = 12.9 (glucose) and 9.6 (xylose) [13].

1.3 Research objectives

1.3.1 To study the effects of alkaline catalysts on the organosolv pretreatment of rice straw.

1.3.2 To find the optimum conditions of the alkaline-catalyzed organolv pretreatment process i.e. alkaline types, solvent types, temperature, acetone content, alkaline concentration, reaction time, and recycling of liquid fraction after pretreatment.

1.3.2 To evaluate enzymatic digestibility of the pretreated biomass obtained from alkaline-catalyzed organosolv pretreatment under different conditions.

1.4 Scope of research work

In the present work, an alkaline-catalyzed organolsolv pretreatment process for rice straw was developed. The effects of organic solvents, temperature, residence time, solid loading and concentration of alkaline were investigated for maximizing the sugar yield obtained from enzymatic hydrolysis. The work provides an altenative pretreatment process with high efficiency and selectivity of increasing digestibility of the lignocellulosic biomass for application in biorefinery process.

CHAPTER 2
THEORIES

2.1 Biofuel generalities

Considering the very probable depletion of liquid fossil fuels by 2090 and the start of a decline in oil production from 2020 to 2030, humankind will face the huge challenge of maintaining its economic growth and stable technological development without compromising the welfare of future generations (sustainable development). Furthermore, man should develop and implement technologies for reducing the environmental pollution and CO_2 emissions. For these reason, the renewable energies may partially or totally replace the fossil fuels, especially if humankind does not choose the dangerous pathway toward the global development of nuclear energy as a primary source of energy.

The renewable energy sources correspond to the kind of energy obtained from natural sources, which are practically inexhaustible due to the huge amount of energy that they contain and their ability to regenerate themselves by natural means. The global potential of bioenergy is represented by the energy-rich crops and lignocellulosic biomass. The conversion of these feedstocks into biofuels, either for electricity generation or for their use in vehicles, is an important option for exploitation of alternative energy sources and reduction of polluting gases, mainly CO_2 [5]. The lignocellulosic biomass includes agricultural, forestry, and municipal solid residues as well as different residues from agro-industry, the food industry, and other industries. The lignocellulosic biomass is made up of complex biopolymers that are not for food purposes. For their conversion into a liquid biofuel such as ethanol, a pretreatment process is required in order to efficiently convert the carbohydrate polymers (cellulose and hemicellulose) into fermentable sugars [1].

2.2 Bioethanol

Bioethanol (ethyl alcohol, fuel ethanol) is the most-used liquid biofuel in the world. It is obtained from energy-rich crops, such as sugar cane and corn. Ethanol can be directly employed as the sole fuel in vehicles or as gasoline oxygenate for increasing the fuel's oxygen content and allowing better hydrocarbon oxidation that reduces the amount of aromatic compounds and carbon monoxide released into the atmosphere.

The fuel ethanol can be obtained from lignocellulosic biomass as well, but its production is much more complex. Nowadays, great efforts are being made to diminish the production costs of lignocellulosic ethanol. It is expected that the evolution of biomass conversion technologies will allow the massive replacement of gasoline with fuel ethanol and make possible the substitution of a significant portion of fossil fuels considering the huge availability of lignocellulosic biomass worldwide [1].

2.3 Lignocellulosic biomass

Lignocellulose is by far the major component of biomass, comprising around half of the plant matter produced by photosynthesis and representing the most abundant renewable organic resource in the world. It consists of three types of polymers; cellulose, hemicelluloses, and lignin, which are strongly intermeshed and chemically bonded by noncovalent forces and by cross-linkages. Compared with starchy biomass, it is considered as a quite recalcitrant material due to its highly lignified and crystalline structure. Only a small amount of the cellulose, hemicelluloses, and lignin produced as byproducts in agriculture or forestry is used, the rest being considered waste.

Cellulose and hemicelluloses are macromolecules constructed from different sugars, whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and proportions of these compounds vary between plants [2].

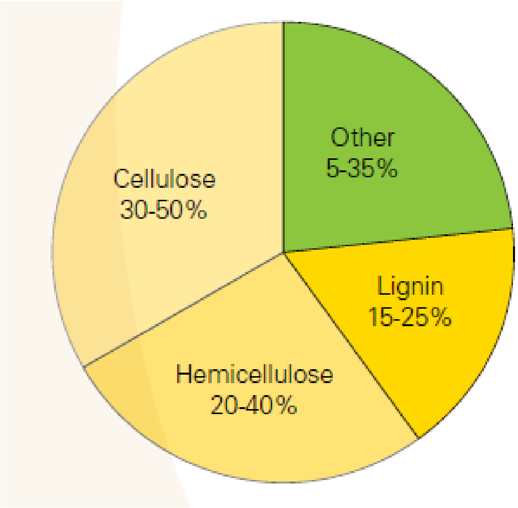


Fig. 12 General composition of lignocellulosic biomass feedstocks [19].

2.3.1 Lignin

Lignin comprises 10 to 25% of lignocellulosic biomass. This component is a very complex phenolic polymer composed of phenyl propane units linked by C-C and C-O-C bonds forming a three-dimensional amorphous structure. The structural units of lignin are the cinnamyl alcohol, which are differentiated by the various substitutions that the aromatic ring presents. Thus, p-hydroxyphenyl units are derived from the p-coumaryl alcohol, the guaiacyl units are derived from the coniferyl alcohol, and the syringyl units are derived from the sinapyl alcohol. The lignin has hydrophobic character and its main function is as incrusted material of a cell wall, i.e., as a sort of cement between the cells [1].

2.3.2 Hemicellulose

Hemicellulose comprises 20 to 40% of lignocellulosic biomass and consists of short and highly branched chains of sugars (200 sugars on average). Among these sugars are in their order, xylose and arabinose (either 5-carbon sugar or pentoses), and galactose, glucose, and mannose (these latter sugars are hexose). Furthermore, hemicellulose contains, in a lower proportion, acetyl groups esterified to some OH groups of its different sugars. Due to the predominance of xylose, hemicelluloses can be considered as a xylan. For lignocellulosic materials derived from hardwood, the xylan backbone is composed of xylose units linked by β (1, 4) bonds that branch through α (1,2) bonds with the methyl glucuronic acid. Considering its branched structures, hemicellulose does not form crystalline structure, but amorphous ones. Thus, this biopolymer is more soluble in water and has a higher susceptibility to the hydrolysis [1].

2.3.3 Cellulose

Cellulose is a β-glucan, i.e., a polymer composed of glucose molecules linked by β(1,4) bonds. Cellulose can be seen as a linear polymer made up of cellobiose monomers as shown in Fig.13.

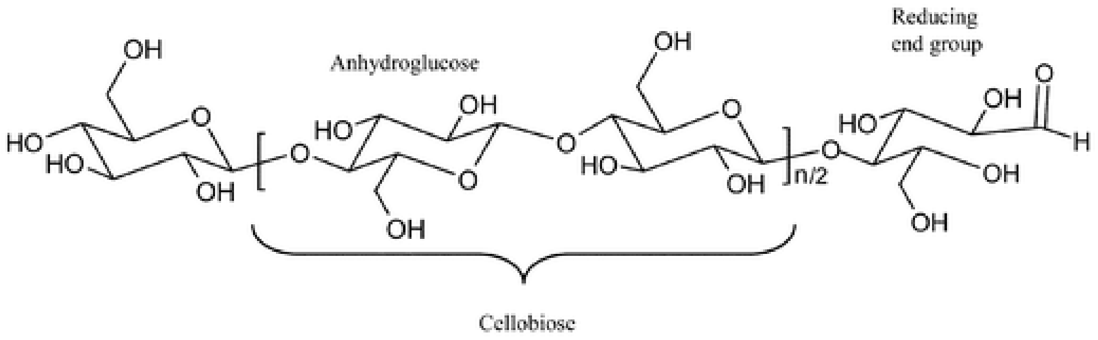


Fig. 13 Cellobiose monomer [30].

The polymerization degree of cellulose is about 7,000 to 15,000. Due to its linear nature and to the interactions by hydrogen bonds between the OH groups of the same chain or of different chains, cellulose molecules are oriented by length leading to the formation of very stable crystalline structures. These structures allow the bundles of cellulose chains to form rigid, difficult to break microfibers. For this reason, the main function of cellulose in plants is structural, which explains its majority presence in the cell wall. In general, the cellulose composes 40 to 60% of dry matter of lignocellulosic biomass [1].

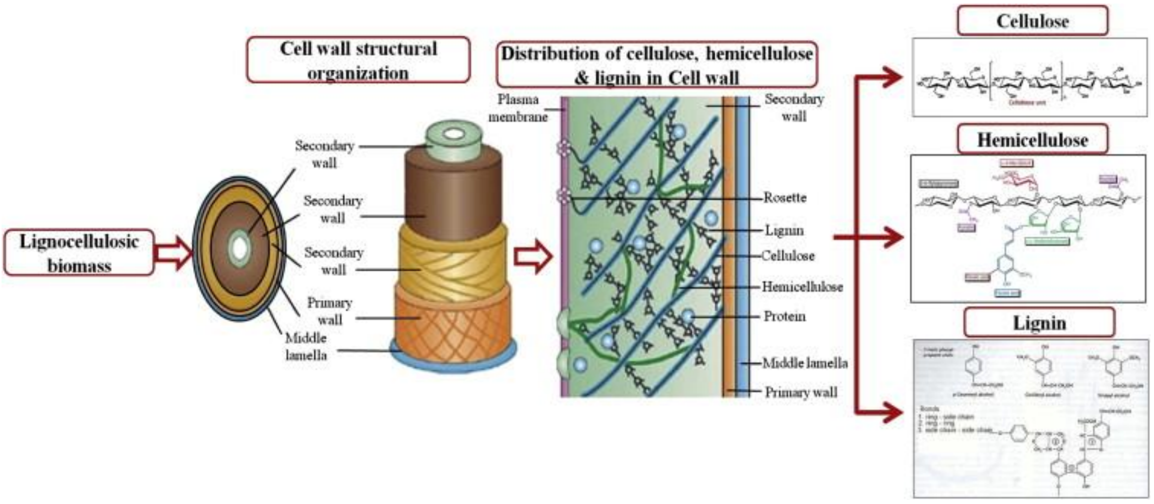


Fig. 14 Diagrammatic illustration of the framework of lignocelluloses [24].

2.4 Rice straw

Rice straw has several characteristics that make it a potential feedstock for fuel ethanol production. It has high cellulose and hemicellulose content that can be readily hydrolyzed into fermentable sugars. In terms of chemical composition, the rice straw predominantly contains cellulose (32-47%), hemicelluloses (19-27%) and lignin (5-24%). The pentoses are dominant in hemicelluloses, for which xylose is the most important sugar (14.8-20.2%). The carbohydrate composition and theoretical ethanol yields of rice straw are shown in Table 12.

Table 12 Carbohydrate composition and theoretical ethanol yield of rice straw [12].

Carbohydrate composition of rice straw	***%composition***
Cellulose	38.60%
Hemicellulose	19.70%
Ethanol yield of rice straw	***yield***
Theoretical ethanol yield (L/kg dry)	0.42
Theoretical ethanol yield (gal/MT dry)	110

*It is assumed that the hemicelluloses fractions are all polymers of xylose

The chemical composition of feedstock has a major influence on the efficiency of bioenergy generation. Table 13 lists the chemical properties of rice straw, rice husk, and wheat straw to highlight the particular differences in feedstock.

Table 13 Proximate composition and selected major elements of ash in rice straw, rice husk and wheat straw [12].

Proximate analysis (%dry fuel)	**Rice straw**	**Rice husk**	**Wheat straw**
Fixed carbon	15.86	16.22	17.71
Volatile matter	65.47	63.52	75.27
Ash	18.67	20.26	7.02
Elemental composition of ash (%)	**Rice straw**	**Rice husk**	**Wheat straw**
SiO_2	74.67	91.42	55.32
CaO	3.01	3.21	6.14
MgO	1.75	<0.01	1.06
Na_2O	0.96	0.21	1.71
K_2O	12.3	3.71	25.6

The low feedstock quality of rice straw is primarily determined by high ash content (10-17%) as compared to wheat straw (around 3%) and also high silica content in ash (SiO_2 is 75% in rice and 55% in wheat). On the other hand, rice straw as feedstock has the advantage of having a relatively low total alkali content (Na_2O and K_2O typically comprise <15% of total ash), whereas wheat straw can typically have >25% alkali content in ash.

Straw quality varies substantially within seasons as well as within regions. If straw is exposed to precipitation in the field, alkali and alkaline compounds are leached, improving the feedstock quality. Thus, the preferred use of this material for bioethanol production is related to both quality and availability [12].

2.5 Cellulosic ethanol production

The use of sugar or starch as a raw material for fuel production competes with their use as foods, and the supply is not expected to be sufficient to face the increasing demand for ethanol fuel. Lignocellulosic biomass is an attractive alternative material for bioethanol production. Lignocellulose is the most abundant renewable resource on earth, and it constitutes a large component of the wastes originating from municipal, agricultural, forestry, and some industrial resources. The more widespread geographical distribution of lignocelluloses sources, as compared to fossil reserves, can provide security of supply by using domestic sources of energy. The use of lignocellulosic material would minimize the conflict between land use for food (and feed) production and energy feedstock production. This raw material is less expensive than conventional agricultural feedstock and can be produced with lower input of fertilizers, pesticides, and energy. Biofuels from lignocelluloses generate low net GHG emissions, reducing environmental impact, particularly on climate change.

The reduction of bioethanol costs depends mainly on the purchase prices of feedstock and the costs of feedstock processing. At recent prices for corn, sugarcane, and lignocellulosic biomass, the latter feedstock is the least expensive. However, order of conversion cost using current technology is the opposite of that of feedstock purchase cost: cellulosic biomass > corn > sugarcane. Taking both purchase price and current conversion technology into account, the near-term fuel cost is sugarcane ethanol > corn ethanol > cellulosic ethanol, whilst in the long term, incorporating advanced technological improvements, the projected selling price of cellulosic ethanol is less than the purchase cost of the other feedstocks considered. Cellulosic biomass is the best candidate for large-scale energy production in the long term among the feedstock types, for its potential in low fuel production price, large scale production, and environmental benefits. However, cellulosic biofuels are not produced at a competitive level yet due to the high cost of processing with the currently available technology [2].

2.6 Pretreatment method

The lignocellulosic biomass represents the most abundant source of fermentable sugar in nature, but to utilize this renewable resource in the production of most of these products, the lignocellulosic biomass must be pretreated, i.e., the lignocellulosic materials should be suitably processed in such a way that their constituent sugars and polysaccharides are susceptible to the action of hydrolytic enzymes as well as of fermenting microorganisms [1].

The pretreatment of the raw material depends on the type of feedstock selected. However, some procedures are common in most cases e.g. washing in order to remove impurities; grinding to reduce size; pressing to extract the juice and separate the solid fraction, usually called bagasse [2].

The first step in converting biomass to ethanol is pre-treatment which involves the cleaning and breakdown of materials. A combination of physical and chemical processes is typically applied, which allows separation of the biomass into its cellulose, hemicelluloses and lignin components. Some hemicelluloses can be converted to sugar in this step, and lignin removed [3].

The complexity of the structure of lignocellulosic biomass is recognized considering that the lignin and hemicelluloses form a sort of seal covering the polysaccharide with the highest potential to release glucose, which is the cellulose. In addition, it should be emphasized that most of cellulose in biomass has a crystalline structure derive from the longitudinal alignment of its linear chains. In the crystalline cellulose, the polysaccharide-polysaccharide interactions are favored and the polysaccharide-water interactions are reduced so this biopolymer is insoluble in water. A minor fraction of cellulose has an amorphous structure. The hemicelluloses chains establish hydrogen bonds with the cellulose microfibers forming a matrix reinforced with lignin. The lignin presence makes it so the lignocellulosic complex cannot be directly hydrolyzed with enzymes. In this way, factors such as the crystallinity degree of cellulose, available surface area (porosity of the material), protection of cellulose by the lignin, pod-type cover offered by the hemicelluloses to the cellulose, and heterogeneous character of the biomass particles contribute to the recalcitrance of the lignocellulosic materials to hydrolysis. In addition, the relationship between the biomass structure and its composition adds a factor implying even more variability exhibited by these materials regarding their digestibility. Therefore, the pretreatment step of the lignocellulosic complex has the following goals:

- Breakdown of the cellulose-hemicellulose matrix
- Reduction of the crystallinity degree of cellulose and increase of the fraction of amorphous cellulose
- Hydrolysis of hemicelluloses
- Release and partial degradation of lignin
- Increase of the biomass porosity

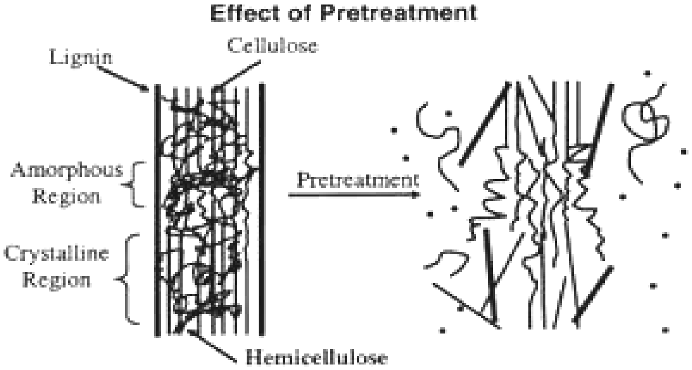


Fig. 15 Schematic representation of the effect of pretreatment [31].

In addition, the pretreatment should contribute to the formation of sugars (hexoses and pentoses) through the hemicelluloses hydrolysis or to the ability to form glucose during the subsequent enzymatic hydrolysis of cellulose. The pretreatment should also avoid the formation of by-products inhibiting the subsequent bioprocesses. As a complement, the pretreatment avoids the need of reducing the biomass particle size, a very energy-consuming process is evidenced by the fact that, when the biomass is not pretreated, glucose yields during the following cellulose hydrolysis step are less than 20% of theoretical yields, whereas the yields after the pretreatment often exceed 90% of the theoretical yields. In this way, the pretreatment is a crucial step during the overall process for fuel ethanol production from lignocellulosic materials. However, the pretreatment is the one of the most expensive step. Nevertheless, the improvement of pretreatment has a great potential to reduce its costs and increase the efficiency of all process.

Different methods have been developed for the pretreatment of lignocellulosic biomass, which can be of a physical, chemical, physical-chemical, or biological nature. The evaluation of each one of these methods is related to whether it meets all the goals mentioned above, in addition to other features involving techno-economic criteria, such as the cost of the agent or catalyst employed, the possibility of recycling the agents or catalysts involved, the degree of technological maturity of each method, the possibility of generating lignin as a co-product, and the ability of each method to be applied to the maximum possible amount of lignocellulosic materials [1].

2.6.1 Physical pretreatment

The mechanical or physical process is usually the first step in biomass pretreatment and aims to reduce the size of the material, breaking the physical structure. It can be performed by milling, grinding, or chipping. The particle size after the physical treatment is an important parameter to be optimized. The use of very small particles may not be desirable due to higher energy consumption in the milling stage as well as imposing negative effects on the subsequent pretreatment method. This process can demand a lot of energy since it requires equipment, such as knives and hammer mills [2].

2.6.2 Physicochemical pretreatment

The aim of pretreatment is to promote the separation of the lignocellulosic components, which are, lignin, hemicelluloses, and cellulose, reduce the crystallinity of cellulose, and disrupt the structure of biomass. This treatment should improve the formation of fermentable sugars, avoid the degradation of carbohydrates and, of course, be cost effective. Fernandes et al. (2009) concluded that a physicochemical pretreatment utilizing alkali or acid enhanced the biodegradability of lignocellulosic materials when the lignin content was high. To determine the best pretreatment process for a particular feedstock and product, rigorous technical and economic analysis is necessary [2].

2.6.2.1 Steam explosion

Steam explosion is one of the most common methods for the pretreatment of lignocellulosic materials. In this method, grinded biomass is treated with high pressure saturated steam, and then the pressure is quickly reduced, which makes the material undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160-260^{o}C (corresponding pressure of 0.69-4.83 MPa) for several seconds to a few minutes before the material is explosed to atmospheric pressure. The process causes the disrupting of the material’s structure, the degradation of hemicelluloses and lignin transformation due to the high temperature, thus facilitating the subsequent hydrolysis of cellulose. The steam treatment can be performed in the presence of catalyst (acid or alkali). Many parameters such as temperature, residence time, catalyst concentration, time of presoaking, and moisture content can be optimized to improve steam pretreatment [2].

2.6.2.2 Acid hydrolysis

Acid hydrolysis can be employed in the pretreatment of cellulosic material in order to cleave the interchain linkage in hemicelluloses and cellulose. Concentrated acid such as H_2SO_4 and HCl are good hydrolysis agents but, on the other hand, they are very corrosive and hazardous. Dilute acid hydrolysis has achieved high reaction rates and has improved cellulose hydrolysis significantly. During hot acid pretreatment, some of the polysaccharides are hydrolyzed, mostly hemicellulose. The resulting free sugars can degrade to furfural (from pentose) and to 5-hydroxy-methyl-furfural or HMF (from hexose). These compounds inhibit yeast cells and lead to decreased growth rate, ethanol production rate, and ethanol yield. In addition, their production means loss of fermentable sugars. Organic acids such as a maleic and fumaric have been suggested as alternatives to avoid HMF formation [2].

2.6.2.3 Alkaline hydrolysis

Alkaline hydrolysis can also be used for the pretreatment of lignocellulosic material. The effect of the pretreatment depends on the lignin content of the materials. The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin and other hemicelluloses. Alkaline pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies. It can largely improve the cellulose digestibility and sugar degradation is less significant than in acid treatment; however, the application is hindered by high cost of alkalis. Dilute NaOH treatment of lignocellulosic material causes swelling, leading to an increase of internal surface area, a decrease in the degree of polymerization, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure [2].

2.6.2.4 Organosolv

The organosolv process was originally developed as an alternative pulping process for paper making. Since then, it has been demonstrated as a potential and promising pretreatment strategy for lignocellulosic materials and successfully applied for biomass fractionation/pretreatment. First, the lignocellulosic biomass is separated into its three components (cellulose, hemicelluloses, and lignin). Second, by fractionation and the treatment during organosolv, the cellulose fraction can be made more susceptible for enzymatic hydrolysis to fermentable sugars.

Organosolv processes use an organic solvent or mixtures of organic solvents with water for the removal of lignin before enzymatic hydrolysis of the cellulose fraction. In addition to lignin removal, hemicellulose hydrolysis occurs leading to improved enzymatic digestibility of the cellulose fraction. High yield of xylose can usually be obtained with the addition of acids such as HCl, H_2SO_4, oxalic, or salicylic acids. Common solvents for the process include ethanol, methanol, acetone, and ethylene glycol, tetrahydrofurfuryl alcohol, and so on.

The solvent must be removed prior to fermentation, because the solvent itself can be an inhibitor for the enzymatic hydrolysis and the fermentation step. Generally, it is removed by evaporation and condensation, removal and recovery of the solvent is required for reducing its cost and environmental impact as well. Normally, low-molecular weight alcohols with lower boiling point, such as ethanol and methanol, are economically favored [4].

Benefits of organosolv pretreatment include:

- Lowering the enzyme costs improved production of cellulose
- Recovery of high-quality lignin, which make it possible to produce higher value derivative chemicals from lignin
- Minimum cellulose loss

2.6.2.5 Oxidative delignification

Delignification of lignocelluloses can also be achieved by treatment with an oxidizing agent, such as hydrogen peroxide, ozone, oxygen, or air. Lignin biodegradation could be catalyzed by the peroxidase enzyme in the presence of H_2O_2. The pretreatment with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis.

Wet oxidation was successfully applied for the treatment of lignocellulosic materials. In recent studies on alkaline wet oxidation of wheat straw, the main degradation products made from hemicellulose and lignin were carboxylic acid, CO_2, and H_2O. Compared to other pretreatment processes, wet oxidation is proved to be efficient for treating lignocellulosic materials, because the crystalline structure of cellulose is open during the process [4].

2.6.2.6 Ionic liquids (ILs)

The use of Ionic liquid (ILs) as solvents for the pretreatment of cellulosic biomass has recently received much attention. ILs are salts that are in the liquid phase typically at temperatures below 100^oC. Different kinds of ILs with common characteristics are available; which is that they usually comprise an organic anion and an organic cation of very heterogeneous molecular structure. The difference in the molecular structure renders the bonding of the ions weak enough for the salt to appear as liquid at room temperature. They do not produce any toxic or explosive gases so they are known as “green” solvents. ILs from hydrogen bond between the nonhydrated chloride ions of the IL and the sugar hydroxyl protons, thus leading to disruption of the complex network of non-covelent interactions among the polymers cellulose, hemicelluloses, and lignin. Only limited information is available on the different ILs and their effects and further research is needed to improve the economics of ILs pretreatment and their recycle [4].

2.6.3 Biological pretreatment

Biological pretreatment has low energy requirements and mild environmental conditions. However, most of these processes are too slow, which limits their application at an industrial level for ethanol production.

Fungi are enzyme producers when they grow on the surface of wood and other lignocellulosic materials. Brown-rot fungi mostly attack the cellulose, while white- and soft-rot fungi attack both cellulose and lignin. As many white-rot fungi degrade the lignin, they have been employed for the production of ligninases and the degradation of lignocelluloses [1].

2.7 Enzymatic hydrolysis

The hydrolysis of glucans is a main process for the production of fermentable sugars for fuel ethanol production. The most important glucans in the ethanol industry are starch and cellulose. In particular, the difficulties related to the enzymatic hydrolysis of cellulose are analyzed taking into consideration the enzyme complexes used and the presence of solid particles in the mixture.

Enzymatic hydrolysis converts cellulose to reducing sugars by the action of cellulases, so they can be fermented by yeasts or bacteria to ethanol. The enzymatic hydrolysis is a multistep reaction that takes place in a heterogeneous system, in which insoluble cellulose is initially broken down at the solid-liquid interface via the synergistic action of endoglucanases and exoglucanases/cellubiohydrolases. Subsequently, a liquid-phase of soluble intermediate product takes place, that is, short cellulo-oligosaccharides and cellobiose that are catalytically cleaved to produce glucse by action of β-glucosidases. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because it is usually conducted at mild conditions and does not cause corrosion problems. Both bacteria and fungi can produce cellulases for hydrolysis of lignocellulosic materials.

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulases to the surface of cellulase, hydrolysis of cellulose to glucose, and desorption of cellulases. The noncellulose components i.e.lignin and hemicelluloses and high crystallinity of cellulose make the adsorption of cellulase a rate-limiting step. Substrate concentration is one of main factors that affects the yield and initial rate of the enzymatic hydrolysis of cellulose. High substrate concentration can lead to accumulation of cellobiose which can inhibit activity of the upstream enzymes. Several methods have been developed to reduce the inhibition of hydrolysis, including the use of high concentrations of enzymes, the supplementation of β-glucosidases during hydrolysis, and removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (SSF) [2].

2.7.1 Enzyme system for cellulose hydrolysis

The microbial cellulolytic enzymes (cellulases) can overcome the disadvantages of acid hydrolysis of cellulose. Being specific biological catalysts, secondary products (or by products) of degradation are not formed and work only under mild condition. Nevertheless, the reaction times are more prolonged than in the case of acid hydrolysis [1].

2.8 Ethanol fermentation

The fermentation step is central to the overall fuel ethanol production process since it represents the actual transformation of the conditioned and pretreated raw materials into the main product, ethyl alcohol, using bioagents, such as yeast or other ethanol-producing microorganism. Ethanolic fermentation is one of the most studied biological processes. Nevertheless, the need of increasing the efficiency of ethanol production including the usage of alternative feedstock has led to the development of new fermentation method with better techno-economic and environmental indicators [1].

2.8.1 Fermentation of cellulose hydrolyzates

The classic configuration employed for fermenting biomass hydrolyzates involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out in different units [5]. This configuration is known as separated hydrolysis and fermentation (SHF). When this sequential process is employed, the solid fraction of pretreated lignocellulosic material undergoes hydrolysis (saccharification). This fraction contains the cellulose in a form accessible to acids or enzymes.

Once hydrolysis is complete, the resulting cellulose hydrolyzate is fermented and converted into ethanol. *Saccharomyces cerevisiae* is the employed microorganism for fermenting the hydrolyzates of lignocellulosic biomass. This yeast ferments the hexoses contained in the hydrolyzate, but not the pentoses. One of the main features of the SHF process is that each step can be performed at its optimal operating conditions (especially temperature and pH). The overall scheme of this process is presented in Fig. 12

Depending on the type of biomass pretreatment, the lignin can be separated in this step or remain in the stillage. This scheme involves the fermentation of hemicelluloses hydrolyzate by pentose-assimilating yeasts in a way parallel to the fermentation of glucose using *S. cerevisiae.*

S. cerevisiae has demonstrated its elevated resistance to the presence of inhibitors in the lignocellulosic hydrolyzate. In the case of more productive continuous regimes, one way to enhance this resistance is the increase in the cell retention to prevent washout and maintain high yeast cell density [1].

2.8.2 Pentose fermentation

To increase the amount of sugars converted into ethanol, yeast assimilating the xylose besides glucose can be used, but in this case the biomass utilization rates are lower relative to microorganisms that assimilate only hexoses. To offset this effect, sequential fermentations are employed in which *S. cerevisiae* utilizes the hexoses during the first days of cultivation, and later xylose-utilizing yeast is added in order to complete the conversion to ethanol [20], but achieved ethanol yields are not very high. Therefore, the most suitable configuration corresponds to the scheme of Fig. 16 where both fermentations are performed independently.

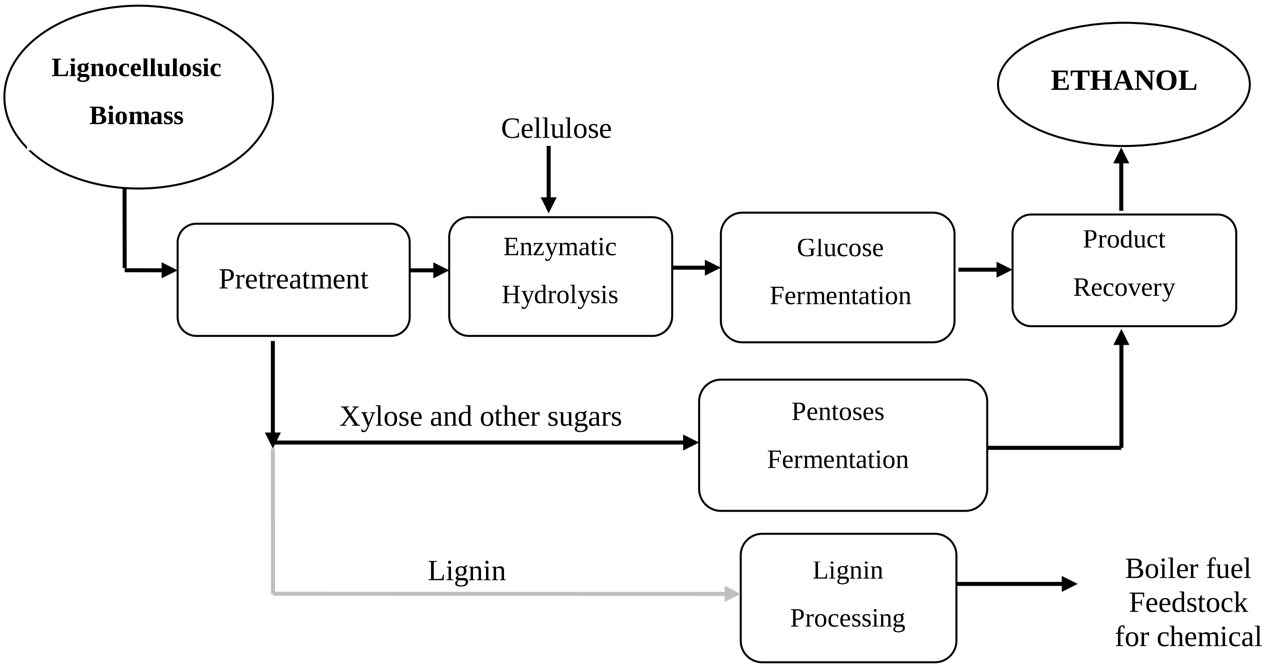


Fig. 16 Block diagram of fuel ethanol production from lignocellulosic materials by separate hydrolysis and fermentation (SHF). [1]

2.8.3 Simultaneous saccharification and fermentation

The simultaneous saccharification and fermentation (SSF) process has been extensively studied in order to reduce the inhibition of cellulases caused by end-products of hydrolysis fromglucose and short cellulose chains. In the process, reducing sugars produced in cellulose hydrolysis or saccharification is simultaneously fermented to ethanol, which greatly reduces the product inhibition in hydrolysis. The SSF process increases the yields of ethanol by minimizing product inhibition as well as eliminates the need for separate reactors for saccharification and fermentation. The SSF process is superior to saccharification and subsequent fermentation due to the rapid assimilation of sugars by yeast during SSF. The microorganisms used in the SSF are usually the fungus *Trichoderma reesei* and the yeast *S. cerevisise*. Hydrolysis is usually the rate-limiting process in SSF. Thermotolerant yeasts and bacteria have been used in the SSF to raise the temperature close to the optimal hydrolysis temperature [2].

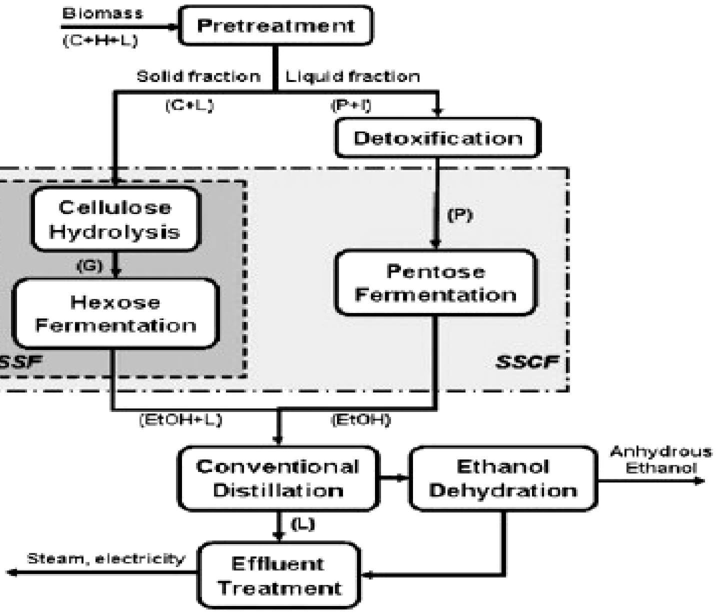


Fig. 17 Generic block diagram of bioethanol production from lignocellulose biomass. Possibilities for reaction–reaction integration are shown inside the shaded boxes: SSF – simultaneous saccharification and fermentation; SSFC – simultaneous saccharification and co-fermentation. Main stream components are: C – cellulose; H – hemicellulose; L – lignin; G – glucose; P – pentose; I – inhibitors; EtOH – ethanol.

2.8.4 Co-fermentation of lignocellulosic hydrolyzates

The co-fermentation of lignocellulosic hydrolyzates represents another technological option for utilizing all the sugars released during biomass pretreatment and cellulose hydrolysis. This kind of cultivation process is aimed at the complete assimilation of all the sugars resulting from lignocellulosic degradation by the microbial cells and consists of the employment of a mixture of two or more compatible microorganisms that assimilate both the hexoses and pentoses present in the medium. This means that the fermentation is carried out by a mixed culture. However, the use of mixed cultures presents a problem that microorganisms utilizing only hexoses grow faster than pentose-utilizing microorganisms leading to a more elevated conversion of hexoses into ethanol. To solve this problem, the utilization of respiratory-deficient mutants of the hexose-fermenting microorganisms has been proposed. In this way, the fermentation and growth activities of the pentose-fermenting microorganisms are increased as they grow very slowly when cultivated along with rapid hexose-fermenting yeasts. In addition, the presence of hexose-assimilating microorganisms allows the reduction of the catabolic repression exerted by glucose on the pentose consumption in pentose-assimilating microorganisms.

Another variant of co-fermentation consists of the utilization of a single microorganism capable of assimilating both hexoses and pentoses in an optimum way, allowing high conversion and ethanol yield. Although these microorganisms exist in nature, their efficiency and ethanol conversion rates are reduced for the implementation of an industrial process. Hence, the addition to the culture medium of an enzyme transforming the hexose into xylulose (xylose-isomerase) has been proposed. In this way, microorganisms exhibiting high rates of conversion to ethanol and elevated yields (such as *S. cerevisiae*) can assimilate the xylulose, involving it in the metabolic pathways leading to ethanol biosynthesis. On the other hand, high efficiency in the conversion to ethanol can be reached though the genetic modification of yeast or bacteria already adapted to the ethanolic fermentation. The microorganisms most commonly modified for this purpose are *S. cerevisiae* and *Z. mobilis* to which genes encoding the assimilation of pentose have been introduced. The other approach for genetic modification is the introduction of genes encoding the metabolic pathways for ethanol production to microorganisms that are capable of fermenting both hexoses and pentoses in their native form. The “design” of ethanologenic bacteria, such as *Escherichia coli* or *Klebsiella oxytoca* is an example of such a type of modification. Using these recombinant microorganisms allow implementing the co-fermentation process intended to the more complete utilization of the sugars contained in the hydrolyzates of lignocellulosic biomass.

CHAPTER 3
METHODOLOGY

3.1 Materials

Rice straw (RS) was collected from a local field in Pathum Thani province, Thailand. It was physically processed using a cutting mill (Retsch ZM2000, Haan, Germany) and sieved to retain particles 250-420 µm in diameter. The processed biomass was then used as a starting material for experimental studies. According to the standard NREL analysis [22], the rice straw contained 35.80 wt% cellulose, 21.50 wt% hemicelluloses, 24.40 wt% lignin, and 15.0 wt% ash [29] Analytical grade organic solvents and chemicals were purchased from major chemical suppliers i.e. Sigma-Aldrich, Merck, and Fluka.

3.2 Pretreatment

3.2.1 Organosolv pretreatment in the absence of alkaline

Organosolv pretreatment of rice straw was performed using an organolsolv method in the absence of alkaline catalyst. The reaction was operated in a 600-ml stainless steel reactor that installed with a thermocouple and cooling system (Parr Reactor 4560, Parr instrument, USA). The mixture contained 2% (w/v) of rice straw in 200 ml of different organic solvents (acetone, ethanol and ethyl acetate) and heated at 80°C for 30 min at 20 bars with mixing 250 rpm. The solid and liquid fractions after pretreatment were separated by vacuum filtration. After that, the solid fraction was washed by deionized water until pH equal 7 and dried it in oven at 60^{o}C overnight.

3.2.2 Effect of alkaline promoter on organosolv pretreatment

The alkaline-catalyzed in organosolv pretreatment of rice straw contained 2% w/w of dried rice straw (4 g) in deionized water or organic solvents (i.e. acetone, ethanol and ethyl acetate) in the presence of three different alkaline promoters: 40 ml of 25% NH_3 solution/160 ml of solvent, 10 ml of triethylamine (TEA)/190 ml of solvent and 40 ml of 25%w/v of NaOH/160 ml of solvent, which are equivalent to final concentrations at 5% (v/v) ammonia, 5% (v/v) TEA, and 5% (w/v) NaOH. All of reaction was performed at

80^oC for 30 min under a pressurized condition at 20 bars using N_2 with mixing at 250 rpm. The solid and liquid fractions after pretreatment were separated by vacuum filtration. After that, the solid fraction was washed by deionized water until pH equal 7 and dried it in oven at 60^oC prior to enzymatic hydrolysis.

3.2.3 Effects of pretreatment temperature

In this step, the method was divided into 2 parts: organosolv pretreatment with and without alkaline promoters. For the non-catalyzed organosolv process, the reaction contained 2% w/v of dried rice straw in 200 ml of the organic solvent to mix and pretreated for 30 min at 20 bars 250 rpm at varying reaction temperature from 80°C to 100°C. The alkaline-catalyzed reaction, 5% v/v NH_3, 5% v/v TEA and 5% w/v NaOH was added as the promoters. The reaction was treated at vary temperature from 50°C to 100°C. After that, the solid and liquid fractions were separated by vacuum filtration and tested for enzymatic digestibility

3.2.4 Effects of acetone content

The effects of the volume of acetone was tested at varying amounts of acetone in the alkaline/organosolv system as other parameters were kept constant i.e. reaction temperature (80°C), pressure (20 bars), reaction time (30 min), and mixing at 250 rpm. Volume of 50% (w/v) NaOH and volume of acetone ratio were varied from 40: 160, 30: 170, and 20: 180.

3.2.5 Effects of NaOH concentration

3.2.5.1 Acetone fixed at 160 ml

The reaction contained 2% w/w of dried rice straw at a fixed volume of 160 ml acetone. Reaction time was 30 min for all experiments with the initial pressure of 20 bars by nitrogen and mixing at 250 rpm. The effect of alkaline concentration was tested by varying the ratio between volume of 50% (w/v) NaOH solution and volume of deionized water at 20: 20, 30:10, and 40:0.

3.2.5.2 Acetone fixed at 180 ml

The reaction contained 2% w/w of dried rice straw at a fixed volume of 160 ml acetone. Reaction time was 30 min for all experiments with the initial pressure of 20 bars by nitrogen and mixing at 250 rpm. The determination of the optimum concentration of NaOH was studied using 50% (w/v), 55% (w/v), 60% (w/v), 65% (w/v) and 70% (w/v) NaOH solution, which was equal to the final alkaline concentrations at 5% (w/v), 5.5% (w/v), 6% (w/v), 6.5% (w/v), and 7% (w/v), respectively. The pretreatment reaction was carried out under the same conditions as described in the previous section and with the volume of acetone fixed at 180 ml.

3.2.6 Effects of solid loading

The reaction was done following the procedure as explained in the previous section (using 50% w/v NaOH, 80°C, 30 min, 20 bars at 250 rpm). The solid loading was varied from 2% until 9%.

3.2.7 Effects of pretreatment time

The optimum reaction temperature (80°C), percent solid loading (4% (w/v)) and volume of acetone (180 ml) were used in this section. The influence of the reaction time was studied by varying from 0 min to 60 min. The reaction time was determined at the start of the isothermal phase. The pretreatment study was performed using 50% w/v and 70% w/v NaOH. Both of two cases were operated under the optimum conditions previously described.

3.2.8 Effects of solvent reusability

The reaction was carried out at 80°C for 30 min with 180 ml of acetone, 4% solid loading, and 20 ml 50% w/v NaOH solution. After pretreatment, the solvent was separated from the solid residues and reused again by adjusting the pH to be 11.33 and the total volume to 200 ml (by adding 50% w/v NaOH and acetone). Then, the solvent was reused for rice straw pretreatment again for 4 times.

3.3 Enzymatic hydrolysis

The pretreatment efficiency and selectivity were determined based on biomass digestibility by using a commercial enzyme mixture. The enzymatic hydrolysis was performed using 5% (w/v) loading of pretreated rice straw in 1-ml hydrolysis vessel containing 20 FPU/g Accellerase® *1500* (cellulase from *Trichoder a reesei*, Dupont, Rochester, USA), 50 mM sodium citrate buffer (pH 4.8) and 50 µl of 5% sodium azide. The reaction was incubated at 50^{o}C for 72 hours with vertical rotation at 30 rpm. Enzymatic hydrolysis experiments were performed in 2 times replication. The released total reducing sugar concentration was analyzed based on the amount of liberated reducing sugars using 3,5-dinitrosalisylic acid (DNS) method. The fermentable sugars were analyzed by high performance liquid chromatography (HPLC).

3.4 Analysis

3.4.1 Reducing sugar analysis by DNS method

The concentration of released reducing sugars from biomass hydrolysis was analyzed by using the DNS method (dinitrosalicylic acid) (Miller, 1959), which measures absorbance at 540 nm on a micro plate UV-Vis spectrophotometer. (Thermo Scientific Multiskan Ascent, Thermo Scientific, USA). The amount of reducing sugars was calculated based on a standard curve of glucose.

3.4.2 High Performance Liquid Chromatography (HPLC)

Samples from enzymatic hydrolysis were centrifuged at 6,720 xg for 10 min and the supernatant was separated for analysis. A high performance liquid chromatography, HPLC (SPD-M10A DAD, Shimadzu, Japan) was used to analyze the sugar components of the samples. Refractive index (RI) detector was used as a detector equipped with Bio-rad Aminex HPX-87H column (Bio-rad, Hercules, CA, USA). 5 mM H_2SO_4 was used as a mobile phase at a flow rate 0.5 ml/min and operating at 65°C.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Enzymatic hydrolysis of native rice straw

According to the composition analysis based on the standard NREL method, the native rice straw used in this study contained cellulose (35.80%), hemicellulose (21.50%), and lignin (24.40%) as the major constituents with a high ash content (15.00%) [29]. Composed mainly of silica. The high silica content of rice straw is a unique characteristic compared with other lignocellulosic agricultural residues which may affect pretreatment efficacy.

Firstly, the enzymatic hydrolysis of 5% (w/v) of dried rice straw was performed by using Accellerase® 1500 at 20 FPU/g (The enzyme used in this study contained 22.9 U/ml of FPase, 1157.4U/ml of CMcase, 277.8 U/ml of Xylanase, 62.1 U/ml of β-glucosidase, and 28.6U/ml of β-xylosidase) in the reaction containing 50 mM sodium citrate buffer (pH 4.8) and 0.25% sodium azide with total volume 1 ml. Released sugar yield of the un-pretreated rice straw after enzymatic hydrolysis was low (187 mg/g). Glucose was found to be the major sugar (136 mg/g) while xylose and arabinose were found as minor sugars from the hydrolysis reaction (Fig. 18).

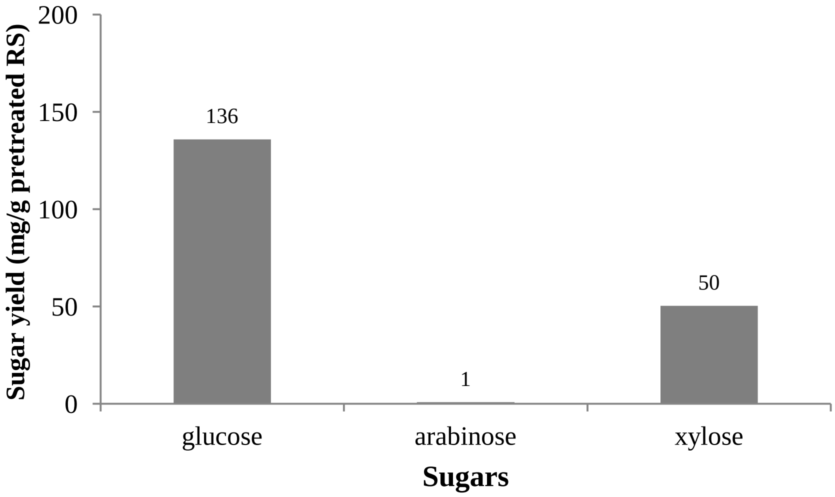


Fig. 18 Sugar recovery from enzymatic hydrolysis of un-pretreated rice straw. The reaction contained 2% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

The low digestibility was due to the highly recalcitrant nature of intact lignocellulose with a high lignin content and waxy surface which minimizes enzyme accessibility to the carbohydrates and results in poor yields of fermentable sugar [9]. Therefore, a pretreatment is required to disrupt the structure of lignocellulosic materials in order to produce a feasible sugar yield.

4.2 Organosolv pretreatment in the absence of alkaline

In this step, the pretreatment of rice straw was performed using an organolsolv method in the absence of an alkaline catalyst. The reaction contained 2% (w/v) of rice straw in 200 ml of different organic solvents and heated at 80°C for 30 min prior to enzymatic hydrolysis. As shown in Figs. 19 and 20, it was found that among three different solvents, the highest total sugar yield was 171 mg/g pretreated biomass when used ethyl acetate as a medium.

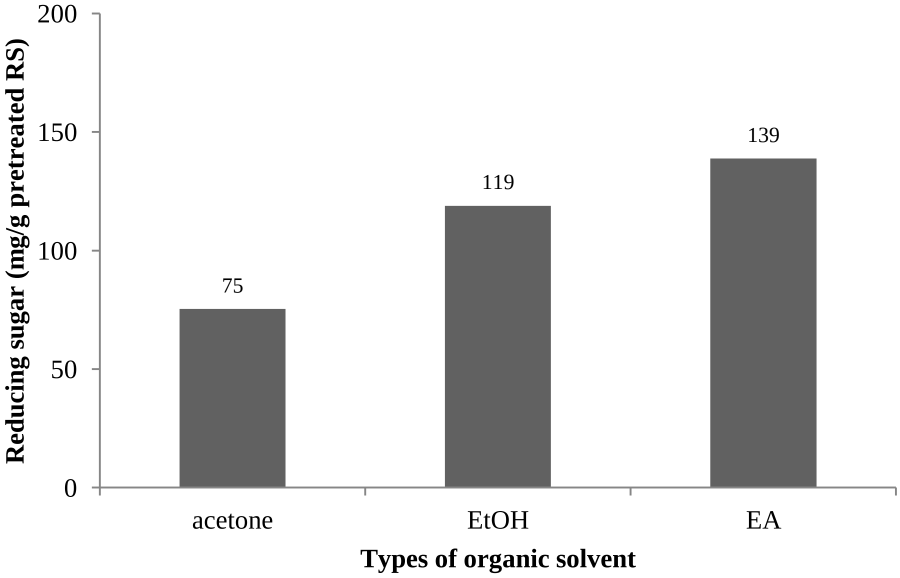


Fig. 19 Effects of types of organic solvent in organosolv pretreatment on total reducing sugar by the DNS method

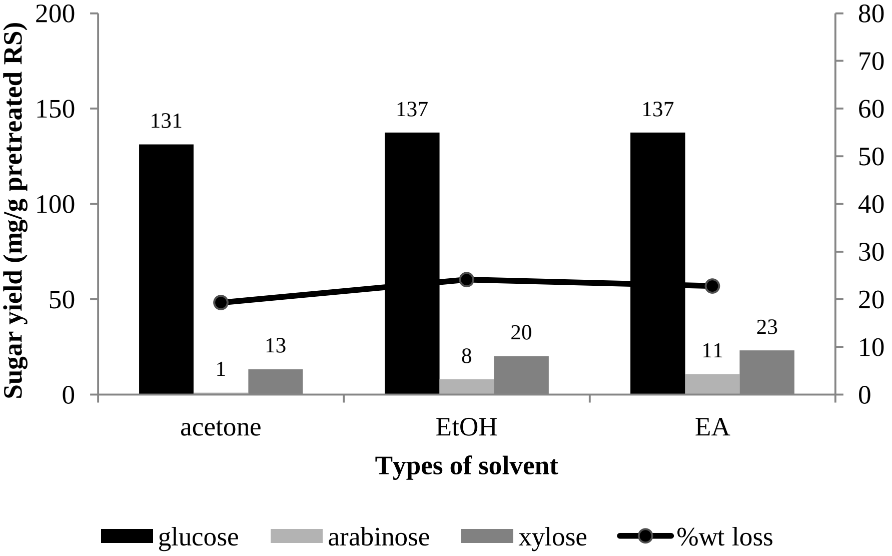


Fig. 20 Effects of solvents on organosolv pretreatment of rice straw in the absence of alkaline promoters. The pretreatment reaction contained 2% (w/v) rice straw in different solvents and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

Glucose was the major product with substantial cross contamination of pentoses. With 23% weight loss, this resulted in a significant increase in glucose recovery from the native rice straw (132 mg/g native) (Table 14). It is known that the organosolv pretreatment of rice straw generally resulted in enrichment of cellulose content as a result of partial solubilization of hemicellulose and lignin [18]. This resulted in the higher glucose yield and lower pentose yield obtained from hydrolysis of the pretreated biomass. This was equivalent to the glucose recovery yield of 27% from the native biomass (Table 14).

Table 14 Sugar recovery from organosolv pretreatment of rice straw using different organic solvents in the absence of alkalines at 80°C

Organic solvent	**Total sugar yield[a] (mg sugar/g pretreated RS)**	**Total sugar yield (mg sugar/g native RS)**	**Glucose recovery yield**	**%wt loss**
acetone	146	118	27	19
ethanol	166	126	26	24
ethyl acetate	171	132	27	23

[a] glu+xyl+ara

4.3 Effect of alkaline promoters on organosolv pretreatment

The alkaline-catalyzed organosolv pretreatment of rice straw was studied using 2% w/w of dried rice straw in 160 ml of deionized water or organic solvents (i.e. acetone, ethanol and ethyl acetate) in the presence of three different alkaline promoters i.e. 5% (v/v) ammonia, 5% (v/v) tri-ethyl amine, and 5% (w/v) NaOH.

4.3.1 NH_3 addition

The effect of NH_3 addition over various organic solvents, including deionized water (DI), acetone, ethanol (EtOH), and ethyl acetate (EA) on sugar yield from enzymatic hydrolysis was investigated. It can be seen from Figs. 21 and 22 that NH_3/deionized water pretreatment operated at 80°C for 30 min showed the highest total reducing sugar yield of 338 mg/g (comprising 284 mg/g of glucose, 3 mg/g of arabinose and 51 mg/g of xylose) from pretreated rice straw.

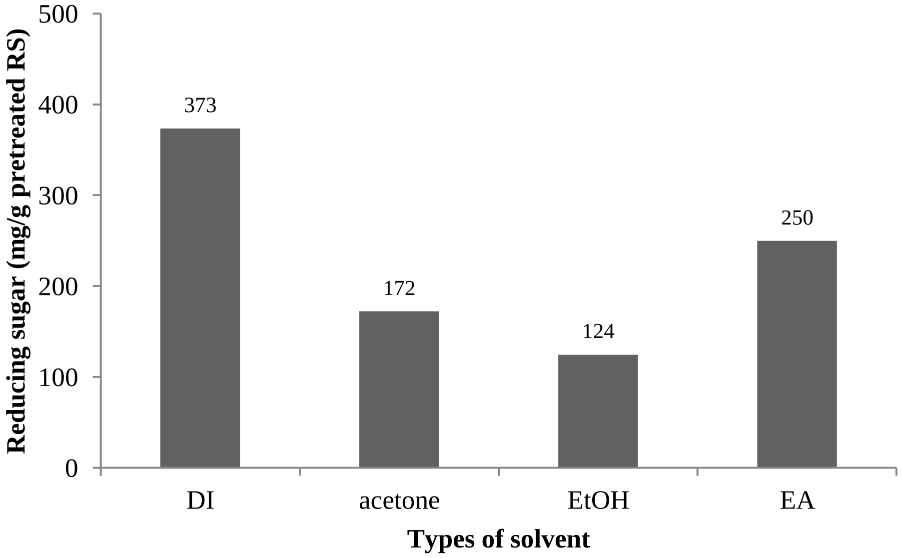


Fig. 21 Effect of organosolv pretreatment in the presence of NH_3 solution on total reducing sugar by the DNS method

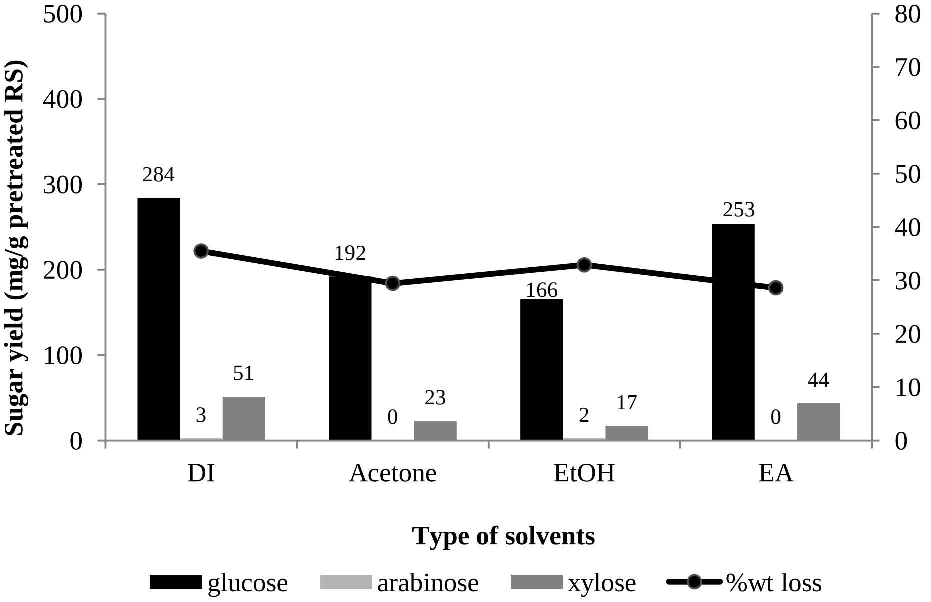


Fig. 22 Effects of organosolv pretreatment using different solvents in the presence of NH_3 on enzymatic hydrolysis of rice straw. The pretreatment reaction contained 2% (w/v) rice straw in different solvents containing 5% NH_3 and treated at 80 °C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% pretreated rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

This led to an increased glucose yield to 218 mg/g native equivalent to a glucose recovery yield of 47% which was higher than those obtained from the un-treated rice straw (Table 15).

Table 15 Sugar recovery of alkaline/organosolv pretreatment using NH_3 solution as a catalyst at different solvents

Type of solvents	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
deionized water	338	218	47	35
acetone	214	151	34	29
ethanol	186	125	28	33
ethyl acetate	296	211	46	29

[a] glu+xyl+ara

4.3.2 Triethylamine addition

The effect of tri-ethylamine (TEA) addition on the organosolv pretreatment was studied under the same pretreatment condition for NH_3. The result revealed that the use of TEA in deionized water system also showed the highest reducing sugar yield of 328 mg sugar/g pretreated biomass. This led to an increased glucose yield to 230 mg/g native equivalent to a glucose recovery yield of 49%, which was higher than that obtained using NH_3 (Figs. 23, 24 and Table 16).

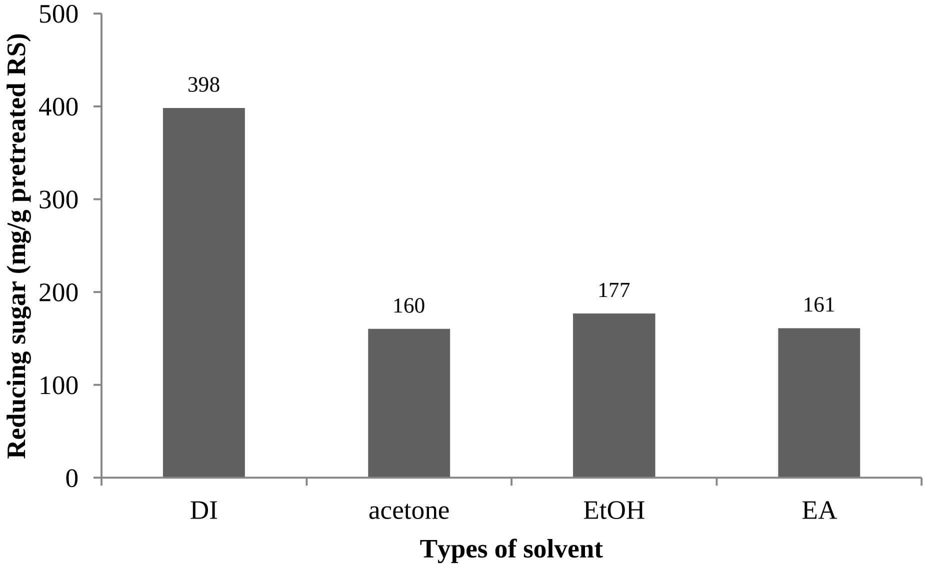


Fig. 23 Effect of organosolv pretreatment in the presence of tri-ethyl amine (TEA) on total reducing sugar by the DNS method

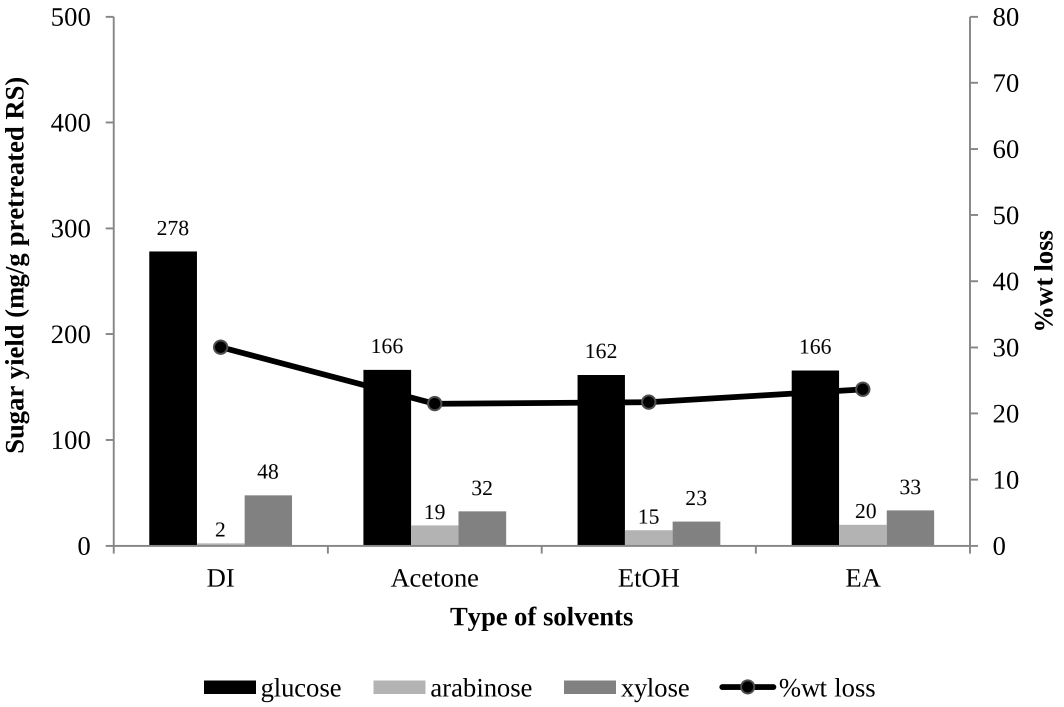


Fig. 24 Effects of organosolv pretreatment using different solvents in the presence of TEA on enzymatic hydrolysis of rice straw. Enzymatic hydrolysis of rice straw was pretreated by organosolv in the presence of TEA. The pretreatment reaction contained 2% (w/v) rice straw in different solvents with 5% TEA and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

Table 16 Sugar recovery of alkaline/organosolv pretreatment of rice straw using TEA as a catalyst at different solvents

Type of solvents	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
deionized water	328	230	49	30
acetone	218	171	33	21
ethanol	199	156	32	22
ethyl acetate	219	167	32	24

[a] glu+xyl+ara

4.3.3 NaOH addition

The effect of NaOH addition on the organosolv pretreatment using different organic solvents was studied. It was found that the presence of NaOH significantly promoted the yield of sugar production, particularly when using DI, acetone, and EtOH as the medium (Fig. 25 and Fig. 26). Among the solvents, the use of EA showed the lowest reducing sugar, whereas the pretreatment of rice straw in acetone with NaOH led to the highest glucose yield of 834 mg/g pretreated biomass with very little contamination of xylose and arabinose (Table 17). Although a slightly lower glucose recovery based on native biomass was obtained, this result indicated the advantage of using NaOH and acetone for biomass pretreatment in terms of high yield and glucose selectivity achievements, from which the highest total sugar recovery of 305 mg/g native rice straw that equivalents to 76% of glucose recovery from native biomass could be obtained (Table 17).

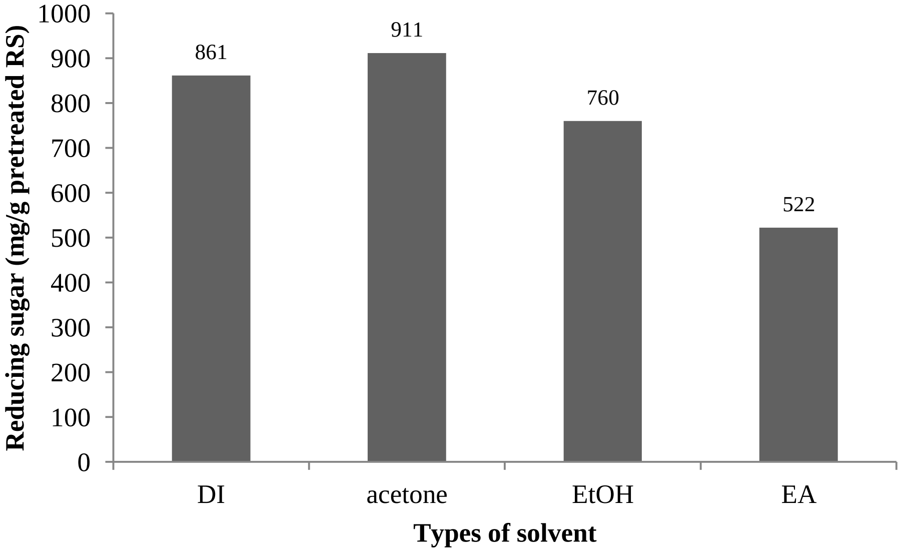


Fig. 25 Effect of organosolv pretreatment in the presence of NaOH solution on total reducing sugar by the DNS method

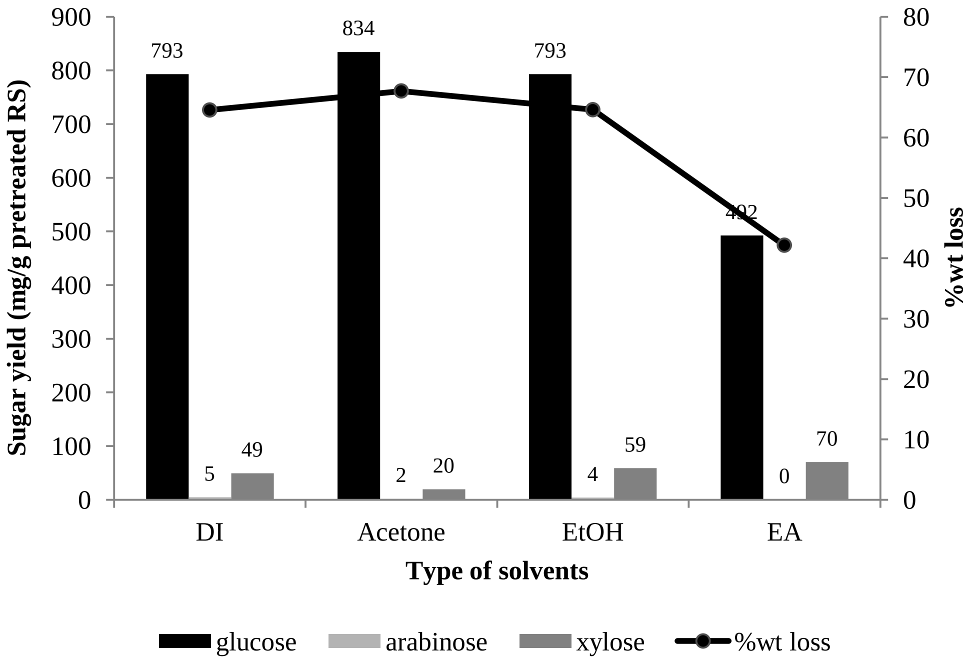


Fig. 26 Effects of organosolv pretreatment using different solvents in the presence of NaOH on enzymatic hydrolysis of rice straw. The pretreatment reaction contained 2% (w/v) rice straw in different solvents with 5% NaOH and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

Table 17 Sugar recovery from organosolv pretreatment of rice straw using NaOH solution as a catalyst in different solvents

Type of solvents	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
deionized water	847	300	71	65
acetone	856	305	76	64
ethanol	856	303	71	65
ethyl acetate	562	237	72	42

[a] glu+xyl+ara

4.4 Effects of pretreatment temperature

The effect of pretreatment temperature on the degree of enzymatic digestibility of rice straw was studied by varying the pretreatment temperature from 80°C to 90°C and 100°C while keeping other conditions constant (i.e. using 2% (w/v) rice straw mixed with organic solvents in the presence and absence of the alkaline promoter for 30 min 20 bars 250 rpm). The solid residue obtained after pretreatment was saccharified by the enzymatic hydrolysis process under the standard conditions in this study.

4.4.1 Effects of temperature on organosolv pretreatment without alkaline

As shown in Figs. 27 and 28 (A-C), the temperature showed no marked effects on the sugar yield and selectivity in all solvent systems. Higher temperature led to slightly increasing weight loss in the acetone system; however, an opposite trend was observed with the ethanol and ethyl acetate systems where lower weight loss was observed with increasing temperature (Table 18).

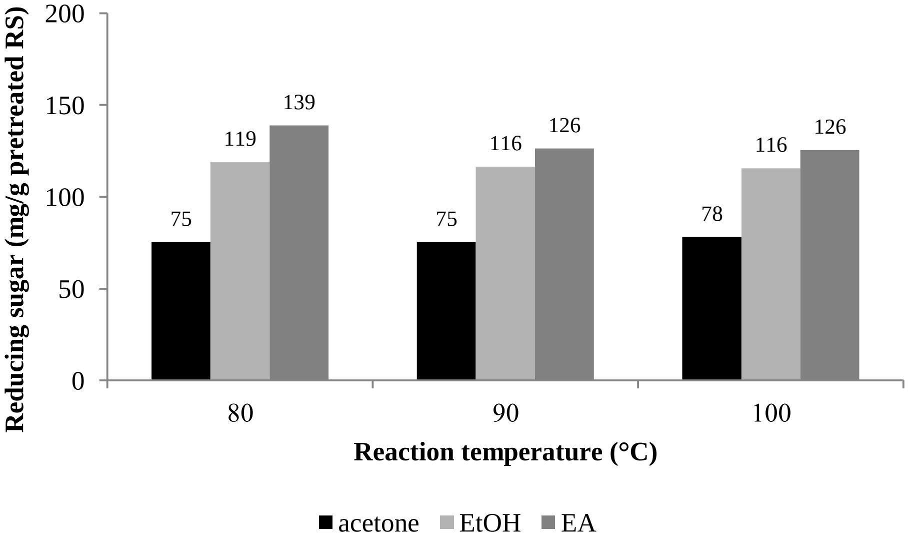


Fig. 27 Effect of reaction temperature of organosolv pretreatment on the total reducing sugar by the DNS method

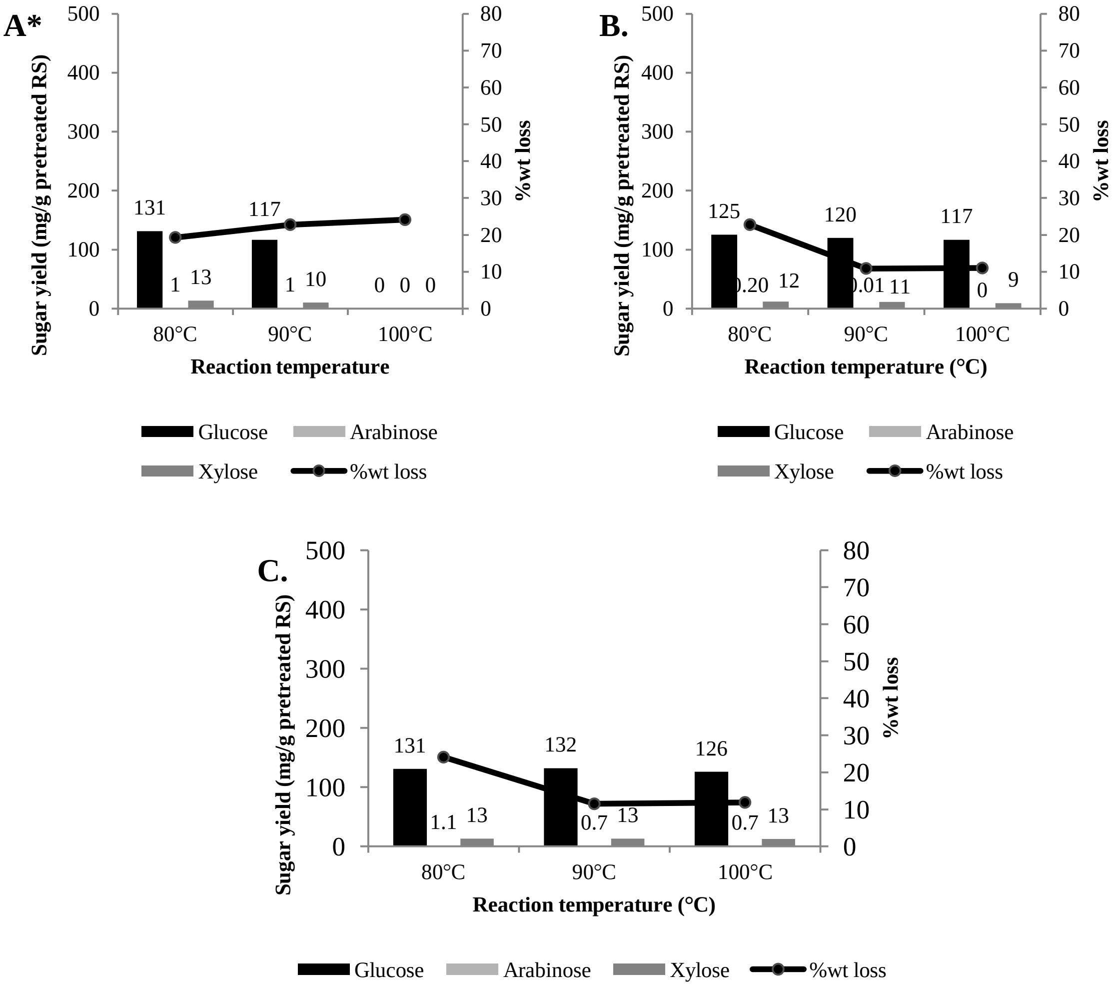


Fig. 28 Effects of temperatures on organosolv pretreatment of rice straw in the absence of the alkaline promoter. The reaction contained 2% (w/v) rice straw in 200 ml of organic solvents at varying operating temperatures for 30 min. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72h. (A) Acetone (B) Ethanol and (C) Ethyl acetate

Table 18 Effects of temperature of organosolv pretreatment of rice straw in the absence of alkaline promoter at different temperatures

Organic solvents	Temp (°C)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
Acetone	80	146	118	27	19
	90	128	117	27	23
	100	**ND***	**ND**	**ND**	24
EtOH	80	137	106	25	23
	90	131	117	27	11
	100	126	112	26	11
EA	80	145	110	25	24
	90	146	129	30	12
	100	139	122	28	12

[a] glu+xyl+ara / *ND: not detectable

4.4.2 Effects of temperature on organosolv pretreatment in the presence of organic alkaline promoters

The effects of reaction temperature on organosolv pretreatment in the presence of organic alkalines i.e. NH_3 or TEA were investigated by varying the temperature between 70-100°C. It is noted that deionized water was also used as a solvent for comparison. As shown in Figs. 29-30 and Tables 19-20, the yield of sugar and glucose recovery trend to increase with increasing temperature. Among all solvents, the use of NH_3 or TEA in deionized water appeared to enhance the highest sugar yield (Fig. 30), from which the highest glucose yield of 476 mg/g pretreated biomass was obtained from NH_3/deionized water system at 100^{o}C with low cross contamination of xylose and arabinose. This was equivalent to the glucose recovery of 64%. Overall, NH_3 was more effective at enhancing enzymatic digestibility of the biomass as compared to TEA in all solvent systems.

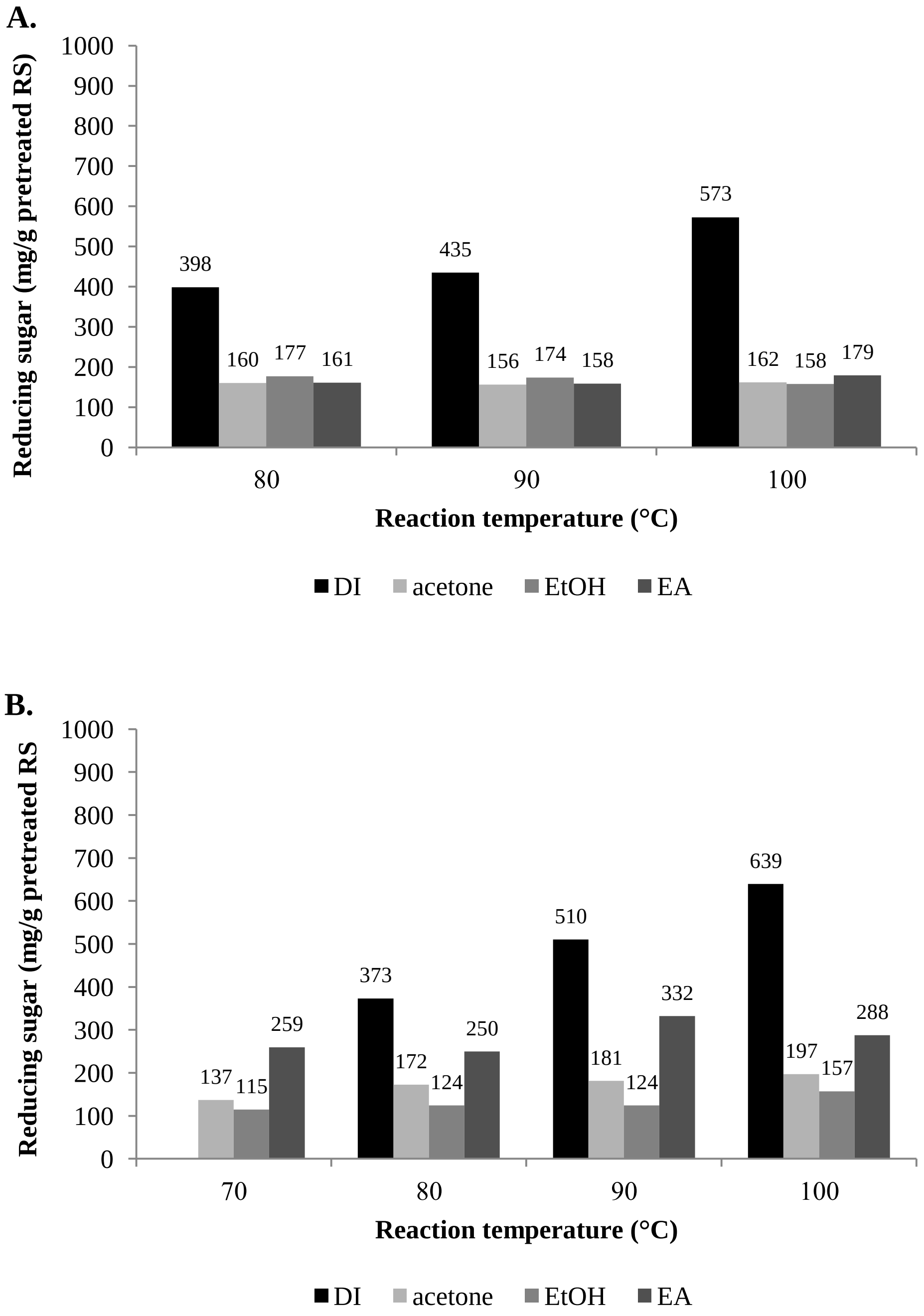


Fig. 29 Effects of reaction temperature of organosolv pretreatment in the presence of NH_3 and/or TEA solutions on total reducing sugar by the DNS method. (A) NH_3 and (B) TEA

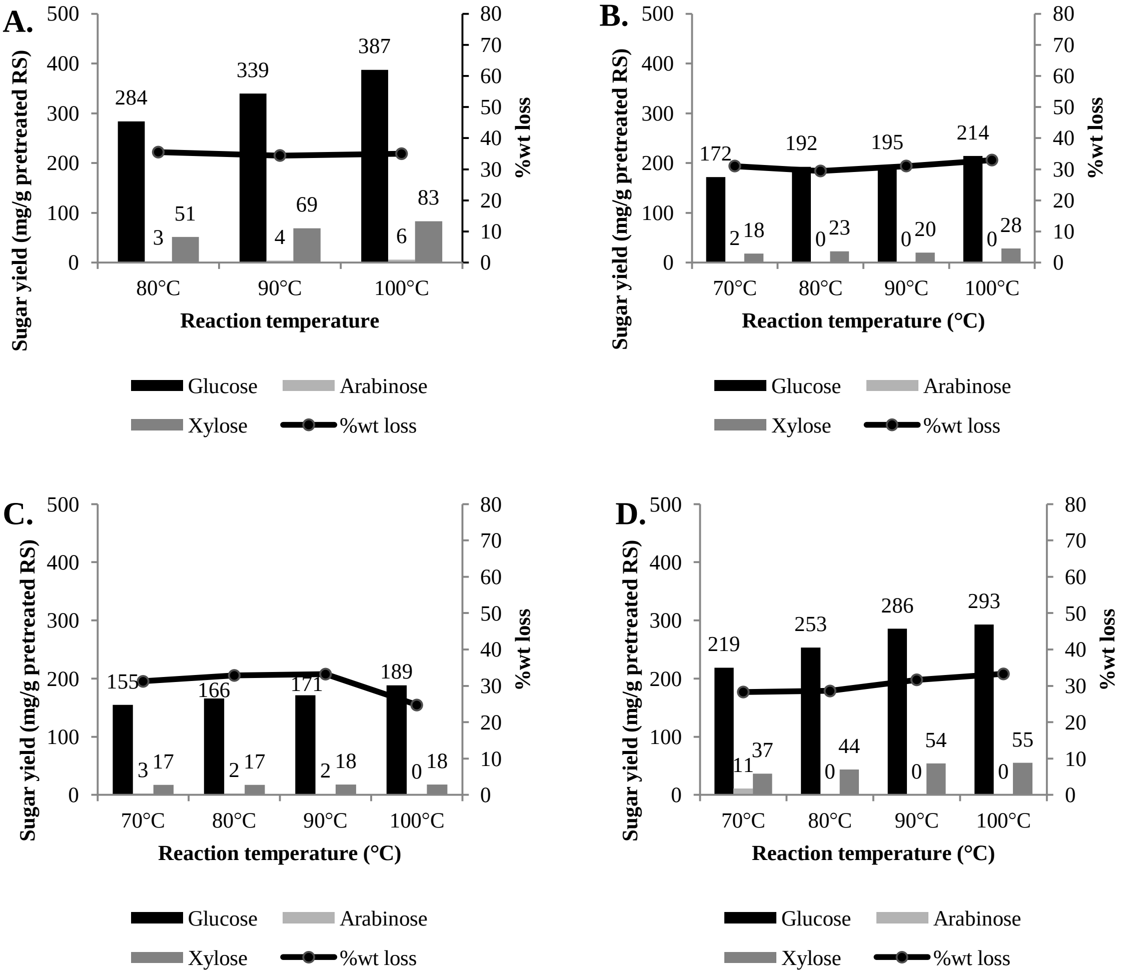


Fig. 30 Effects of temperatures on organosolv pretreatment of rice straw in the presence of NH_3. The reaction contained 2% (w/v) rice straw in 160 ml of solvent and 40 ml of NH_3 solution at varying operating temperatures for 30 min. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72h. (A) NH_3 in deionized water; (B) NH_3 in acetone; (C) NH_3 in ethanol; (D) NH_3 in ethyl acetate

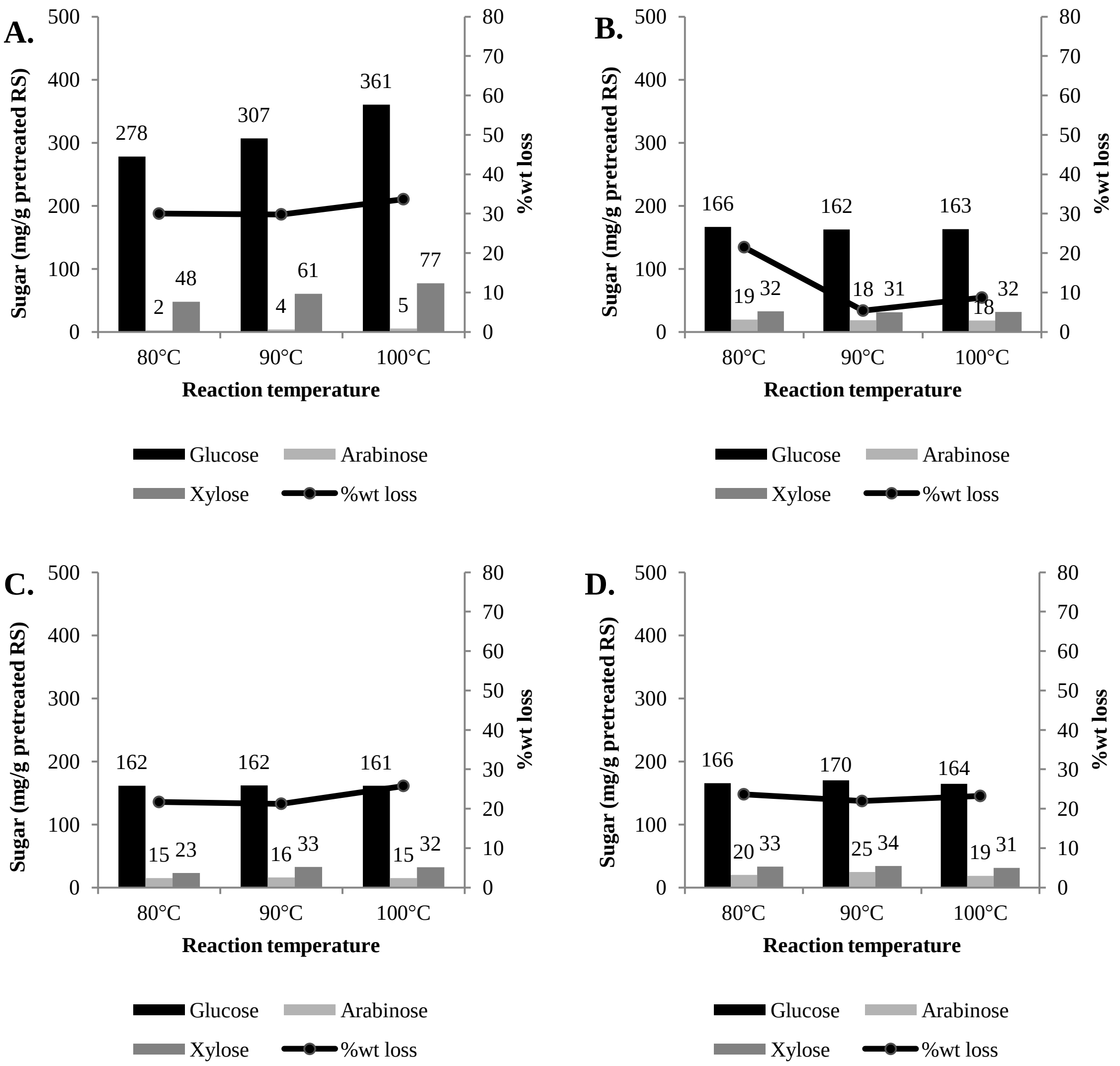


Fig. 31 Effects of temperatures on organosolv pretreatment of rice straw in the presence of TEA. The reaction contained 2% (w/v) rice straw in 190 ml of solvent and 10 ml of TEA at varying operating temperatures for 30 min. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h. (A) TEA in deionized water; (B) TEA in acetone; (C) TEA in ethanol; (D) TEA in ethyl acetate

Table 19 Sugar recovery of organosolv pretreatment of rice straw in the presence of 5% NH_3 at different temperatures

Pretreatment	Temp (°C)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
DI	80	338	218	47	35
	90	413	271	57	34
	100	476	310	64	35
Acetone	70	218	150	29	31
	80	214	151	34	29
	90	214	148	34	31
	100	241	162	36	33
EtOH	70	212	146	28	31
	80	186	125	28	33
	90	191	127	29	33
	100	206	155	36	25
EA	70	213	152	30	28
	80	296	211	46	29
	90	339	231	50	32
	100	347	232	50	33

[a] glu+xyl+ara

Table 20 Sugar recovery of organosolv pretreatment of rice straw in the presence of 5% TEA at different temperatures

Pretreatment	Temp (°C)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
DI	80	328	230	49	30
	90	371	260	55	30
	100	443	294	61	34
Acetone	80	218	171	33	21
	90	212	201	39	5
	100	213	194	38	9
EtOH	80	199	156	32	22
	90	211	166	32	21
	100	209	155	30	26
EA	80	219	167	32	24
	90	228	178	34	22
	100	214	164	32	23

[a] glu+xyl+ara

The results of this study are in good agreement with the literature. The pretreatment of lignocellulosic biomass by soaking in aqueous ammonia (SAA) at mild temperatures ranging from 40 to 90°C for longer reaction times (between 12 h and 11 weeks) has been reported to increase the enzymatic digestibility of the solid and preserve most of the glucan and xylan in the samples. The effectiveness of SAA process was found to be strongly dependent on the pretreatment temperature [11].

4.4.3 Effects of temperature on organosolv pretreatment in the presence of NaOH

The effect of reaction temperature on organosolv pretreatment of rice straw in the presence of NaOH was then investigated by varying the temperature from 50-100°C. As shown in Figs. 32-33, significantly higher sugar yields were achieved as compared to the pretreatment in the presence of organic alkalines. Furthermore, increasing temperature showed only a marginal effect on the sugar yield and glucose recovery (Table 21), which clearly highlights the benefit of using NaOH as a promoter in term of its low temperature requirement. Importantly, unlike the use of organic alkalines, the pretreatment with acetone in the presence of NaOH enhanced the higher sugar yield than the pretreatment with DI water in the presence of NaOH under the same pretreatment condition as shown in Fig. 33. The maximal glucose yield of 872 mg/g pretreated biomass was achieved in the NaOH/acetone system at 70^{o}C, which was equivalent to glucose recovery of 80%. The highest glucose recovery of 86% was obtained at 50^{o}C in the NaOH/acetone system at 50^{o}C due to the lower weight loss. Therefore, the benefit of NaOH/acetone system compared to NaOH/DI water system in term of possible solvent regeneration would offer good potential of alkaline/organosolv for biomass pretreatment.

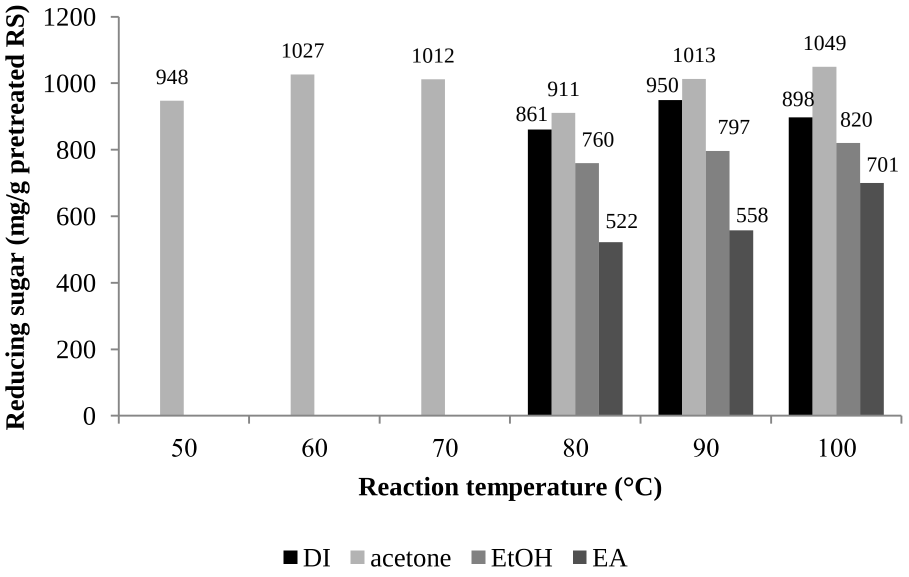
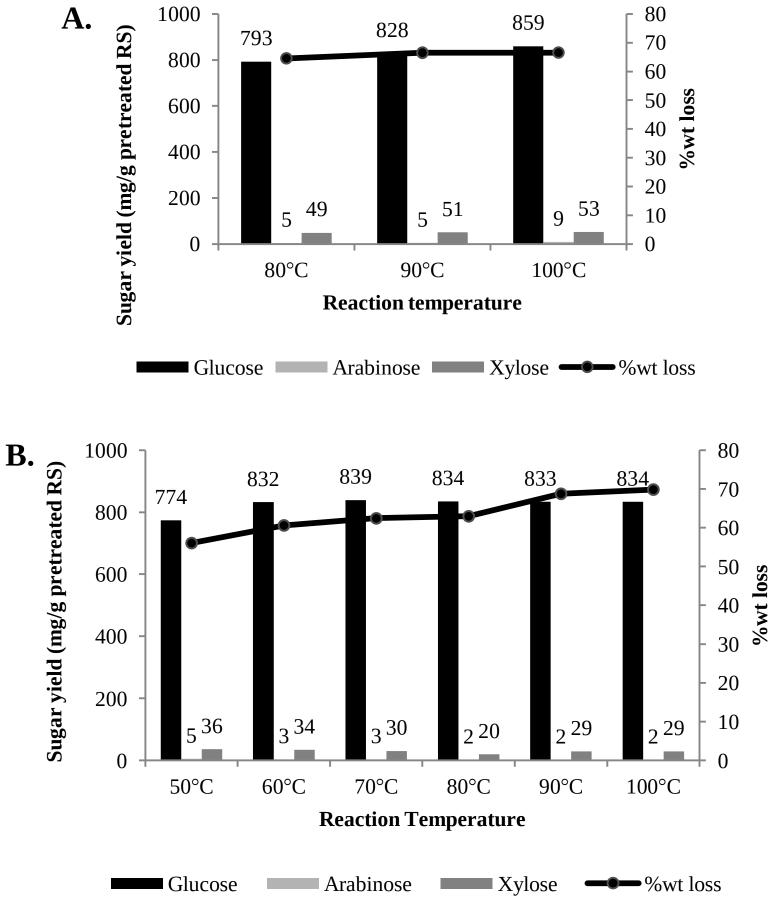


Fig. 32 Effect of reaction temperature of organosolv pretreatment using different organic solvents in the presence of NaOH solution on total reducing sugar by the DNS method



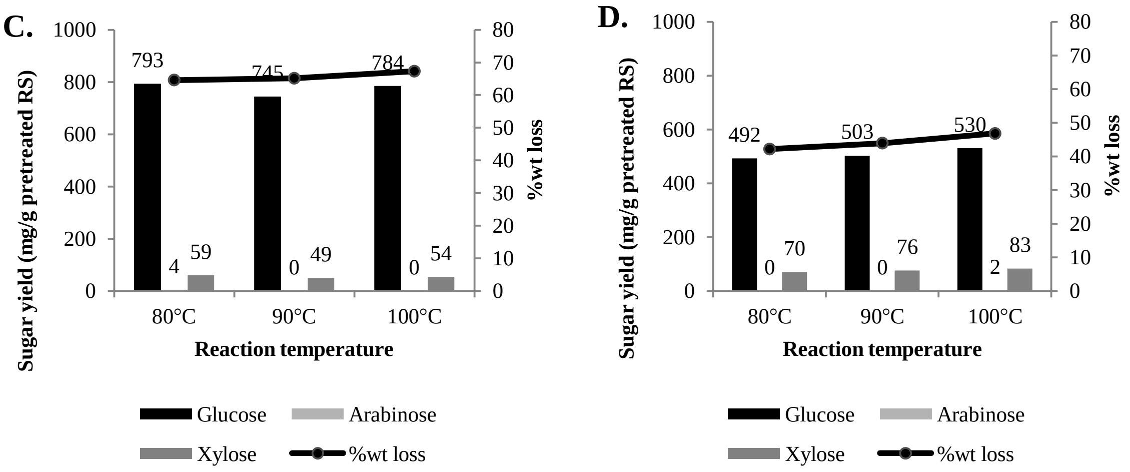


Fig. 33 Effects of temperatures on organosolv pretreatment of rice straw in the presence of NaOH. The reaction contained 2% (w/v) rice straw in 160 ml of solvent and 40 ml of 25%(w/v) NaOH solution at varying operating temperatures for 30 min. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72h. (A) NaOH in deionized water; (B) NaOH in acetone (C) NaOH in ethanol (D) NaOH in EA

Table 21 Sugar recovery of organosolv pretreatment of rice straw in acetone with NaOH at different temperatures

Pretreatment	Temp (°C)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
DI	80	847	300	71	65
	90	884	296	70	67
	100	921	308	73	67
Acetone	50	815	358	86	56
	60	870	343	83	61
	70	872	328	80	62
	80	856	305	76	64
	90	864	270	66	69
	100	865	261	64	70
EtOH	80	856	303	71	65
	90	793	277	66	65
	100	839	274	65	67
EA	80	562	237	72	42
	90	577	254	72	44
	100	616	288	72	47

[a] glu+xyl+ara

4.5 Effect of solvent content

The previous section described the use of NaOH/acetone system showing good potential for biomass pretreatment. The use of acetone gains the benefit in term of its easy recovery due to the low boiling point. Therefore, the effect of acetone content on the pretreatment efficiency was further optimized by varying the acetone content from 160-180 ml (80-90% solvent in the system) with the operating temperature of 80^{o}C in the presence of 5% (w/v) NaOH. As seen in Figs. 34-35, it is clear that the higher content of acetone promoted the reaction and produced more glucose. The highest total sugar yield was 933 mg/g pretreated biomass was achieved by using 90% acetone content, which was equivalent to the glucose recovery yield of 73% from the native biomass (Table 22). High glucose selectivity was achieved from the process.

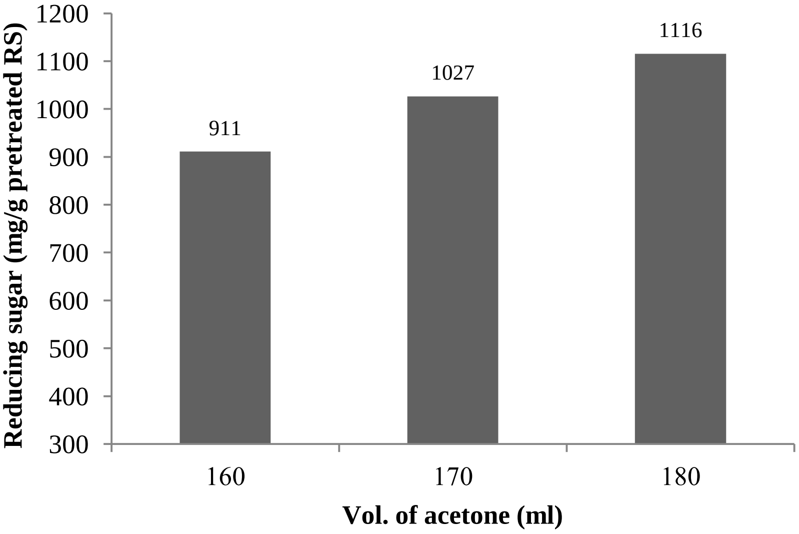


Fig. 34 Effects of volume of acetone in alkaline/organosolv system on total reducing sugar by the DNS method

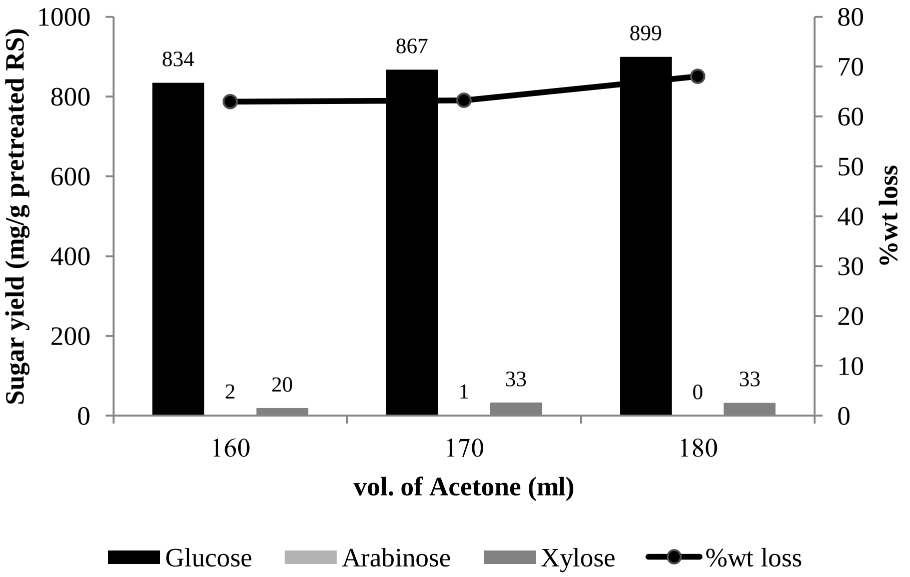


Fig. 35 Effects of acetone content on organosolv pretreatment of rice straw in the presence of NaOH. The pretreatment reaction contained 2% (w/v) rice straw in different content of acetone containing 5% NaOH concentration and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

Table 22 Sugar recovery from organosolv pretreatment of rice straw using different acetone contents in the presence of NaOH

vol. of acetone (ml)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
160	856	305	76	64
170	902	332	81	63
180	933	298	73	68

[a] glu+xyl+ara

4.6 Effects of NaOH concentration

The effects of NaOH concentration on the pretreatment efficiency was studied by varying the concentration of NaOH from 5-10%. As seen in Figs. 36-37, it is clear that the higher concentration of NaOH promoted the reaction efficiency as reflected by higher glucose from enzymatic hydrolysis.

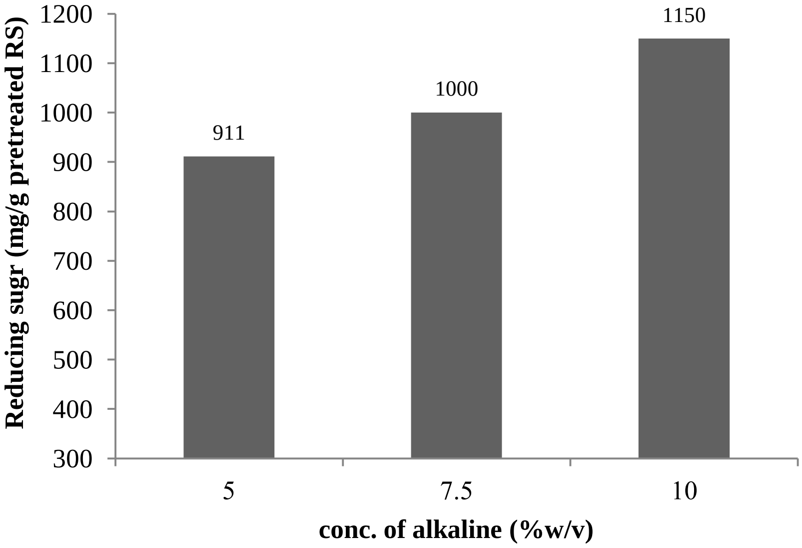


Fig. 36 Effects of NaOH concentration in alkaline/organosolv pretreatment using acetone on total reducing sugar by the DNS method

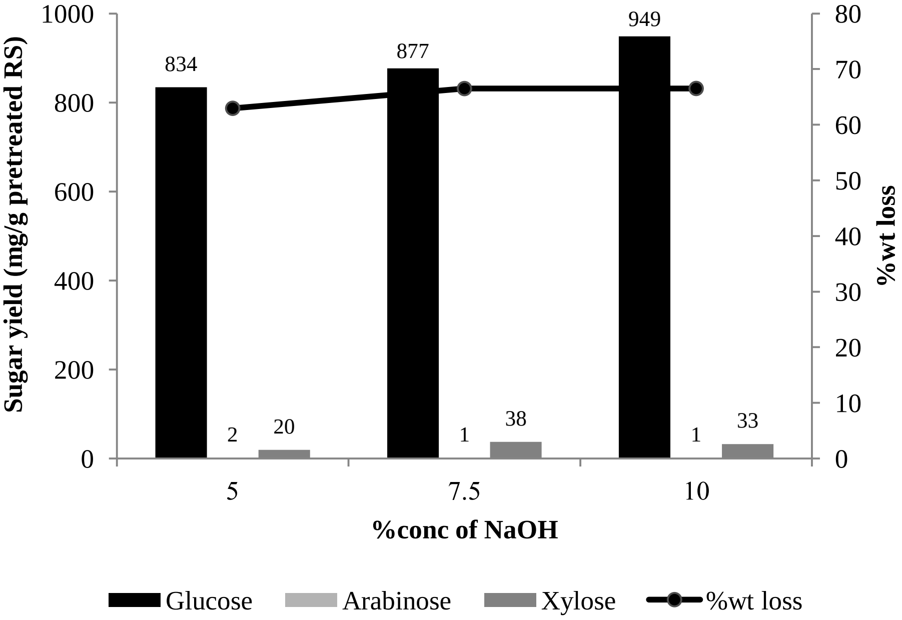


Fig. 37 Effects of NaOH concentration on organosolv pretreatment of rice straw. The pretreatment reaction contained 2% (w/v) rice straw in acetone with different alkaline concentrations and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at50°C for 72 h.

The highest total sugar yield was 982 mg/g pretreated biomass and was achieved by using 40 ml of 50% (w/v) NaOH/ 160 ml of acetone, which was equivalent to the final concentration of 10% (w/v) in the reaction. This was equivalent to the glucose recovery yield of 85% from the native biomass (Table 23). Furthermore, high glucose selectivity was achieved from the process as shown by low cross-contamination of pentoses in the enzymatic hydrolysate.

Table 23 Sugar recovery from organosolv pretreatment of rice straw using acetone with different NaOH concentrations

Concentration (%w/v)	**Total sugar yield (mg sugar/g pretreated RS)**	**Total sugar yield (mg sugar/g native RS)**	**Glucose recovery yield**	**%wt loss**
5	856	305	76	64
7.5	916	309	75	66
10	982	348	85	65

[a] glu+xyl+ara

The above results confirm that the higher NaOH concentration efficiently boosted up the glucose recovery yield from the enzymatic hydrolysis step. To further optimize the effects of NaOH, the higher concentration of NaOH between 5-7% was used. The pretreatment reaction was carried out under the same conditions and the sugar recovery yields at this high range of NaOH concentration are shown in Figs. 38-39 and Table 24. It was found that the use of high NaOH concentration did not further promote the sugar yield compared to the use of 10% NaOH concentration. The highest sugar product yield was observed at 7% (w/v) NaOH, from which 969 mg sugar/g native biomass with some cross contamination of xylose can be produced. This was equivalent to the glucose recovery of 101% from the native rice straw.

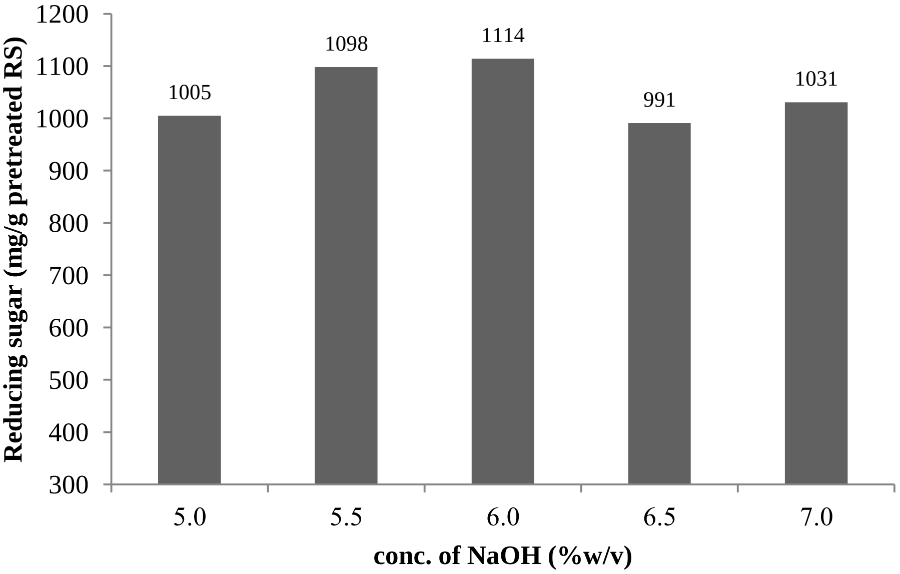


Fig. 38 Effects of NaOH concentration in alkaline/organosolv pretreatment using acetone on the total reducing sugar by the DNS method

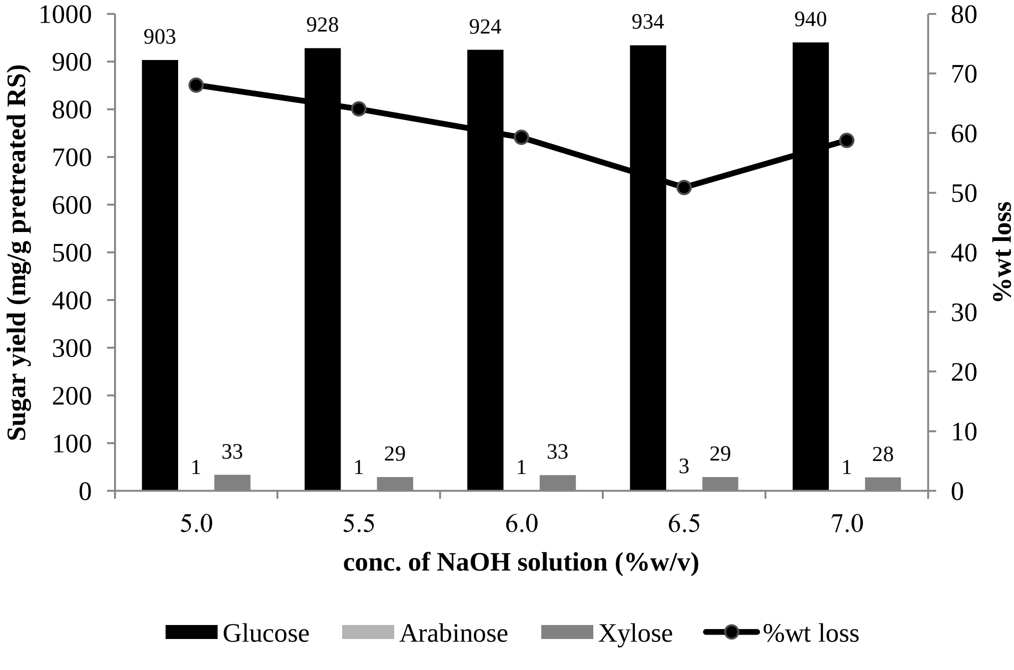


Fig. 39 Effects of NaOH concentration at high range on organosolv pretreatment of rice straw. The pretreatment reaction contained 2% (w/v) rice straw in acetone different NaOH concentration and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at50°C for 72 h.

Table 24 Sugar recovery from organosolv pretreatment of rice straw using acetone in the presence of alkaline at different NaOH concentrations

conc. of NaOH (%w/v)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
50	938	300	75	68
55	958	344	88	64
60	958	390	99	59
65	966	475	117	51
70	969	399	101	59

[a] glu+xyl+ara

4.7 Effect of biomass loading

The effects of rice straw loading on sugar production yield from organosolv pretreatment using the NaOH/acetone system was studied. Increasing solid loading from 2 to 9% led to slightly decreasing glucose yields from 938 to 865 mg/g. This could be due to the inefficient mixing in the system. However, no marked trend on glucose recovery from the native rice straw was observed. The highest glucose recovery of 86% was achieved at 6% solid loading. In addition, markedly increased xylose yield was also observed when higher solid loading was applied. From the study, the optimum solid loading percentage that gave the highest glucose concentration was 4%solid loading (Figs.40-41 and Table25).

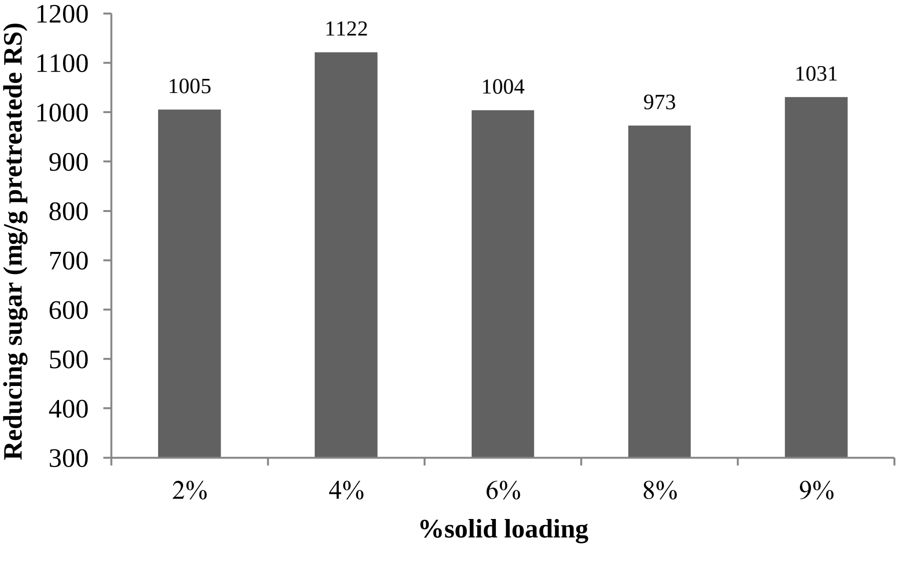


Fig. 40 Effects of percent solid loading in alkaline/organosolv system using acetone on the total reducing sugar by the DNS method

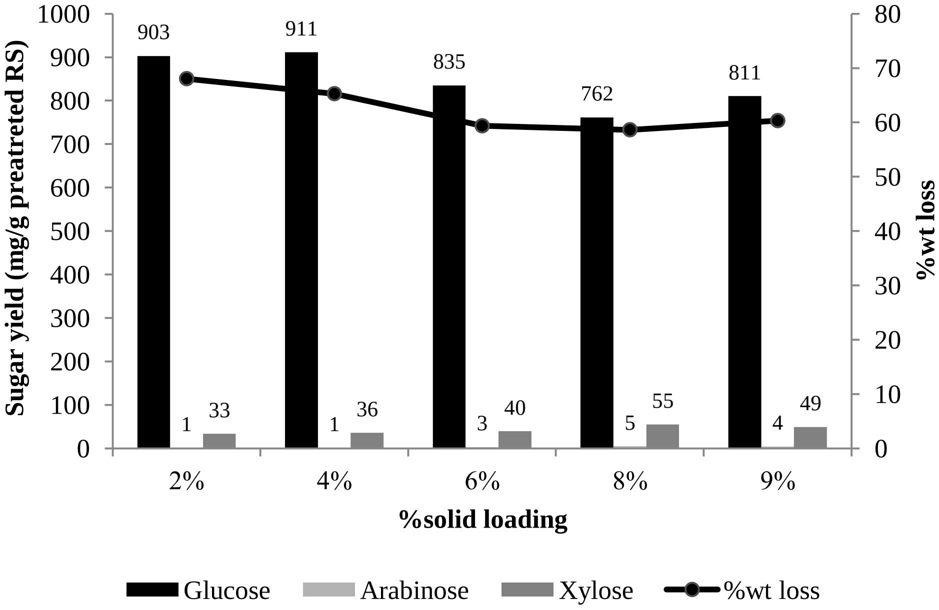


Fig. 41 Effects of solid loading on sugar yield from organosolv pretreatment of rice straw using the NaOH/ acetone system. The pretreatment reaction contained varying loading of rice straw in 180 acetone/ 20 ml 50% NaOH and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

Table 25 Sugar recovery from organosolv pretreatment of rice straw in NaOH/acetone system at different % solid loadings

Solid loading (%)	**Total sugar yield[a] (mg sugar/g pretreated RS)**	**Total sugar yield (mg sugar/g native RS)**	**Glucose recovery yield**	**%wt loss**
2	938	300	75	68
4	948	316	77	67
6	878	357	86	59
8	822	340	80	59
9	865	343	82	60

[a] glu+xyl+ara

4.8 Effect of pretreatment time

The effect of operating time was studied at the optimum reaction temperature (80°C) with percent solid loading of 4% w/v and acetone volume of 180 ml. It should be noted that two NaOH concentrations (50% w/v and 70% w/v) were applied in this study. As shown in Fig. 43(A) and 43(B), the total glucose yield slightly increases with increasing the pretreatment time and reaches the highest level in 20-30 min, where the glucose yield of 943-948 mg sugar/g pretreated biomass could be obtained (Tables 26-27). This was equivalent to the glucose recovery of 77-83% from the native rice straw. It was noted that at pretreatment time 0 (quenching the reaction immediately after reaching the target temperature), a relatively high glucose yield was obtained. This could be due to the effects of pretreatment reaction during the heating up step (approx. 10 min).

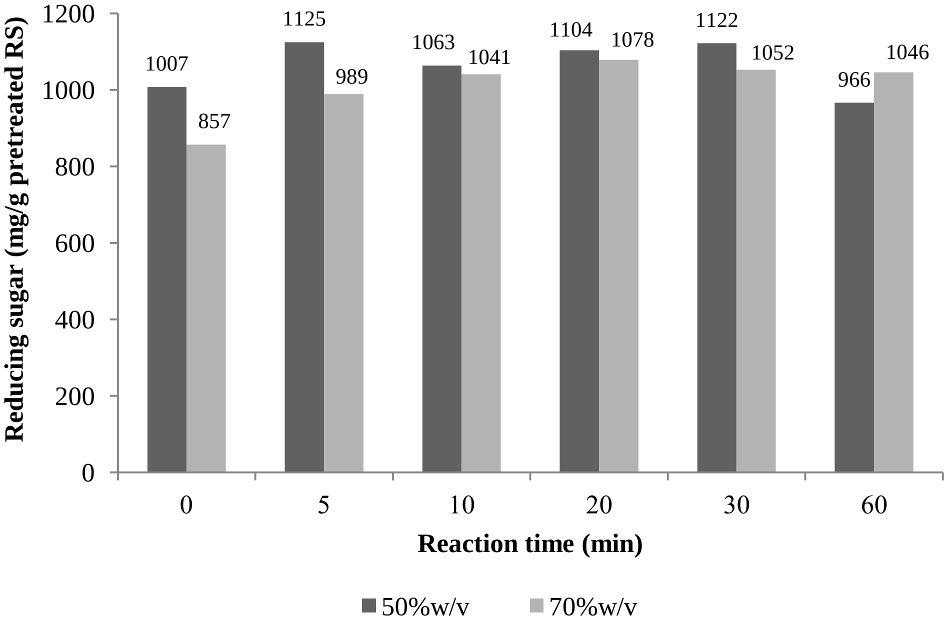


Fig. 42 Effect of reaction time in alkaline/organosolv system on the total reducing sugar by the DNS method

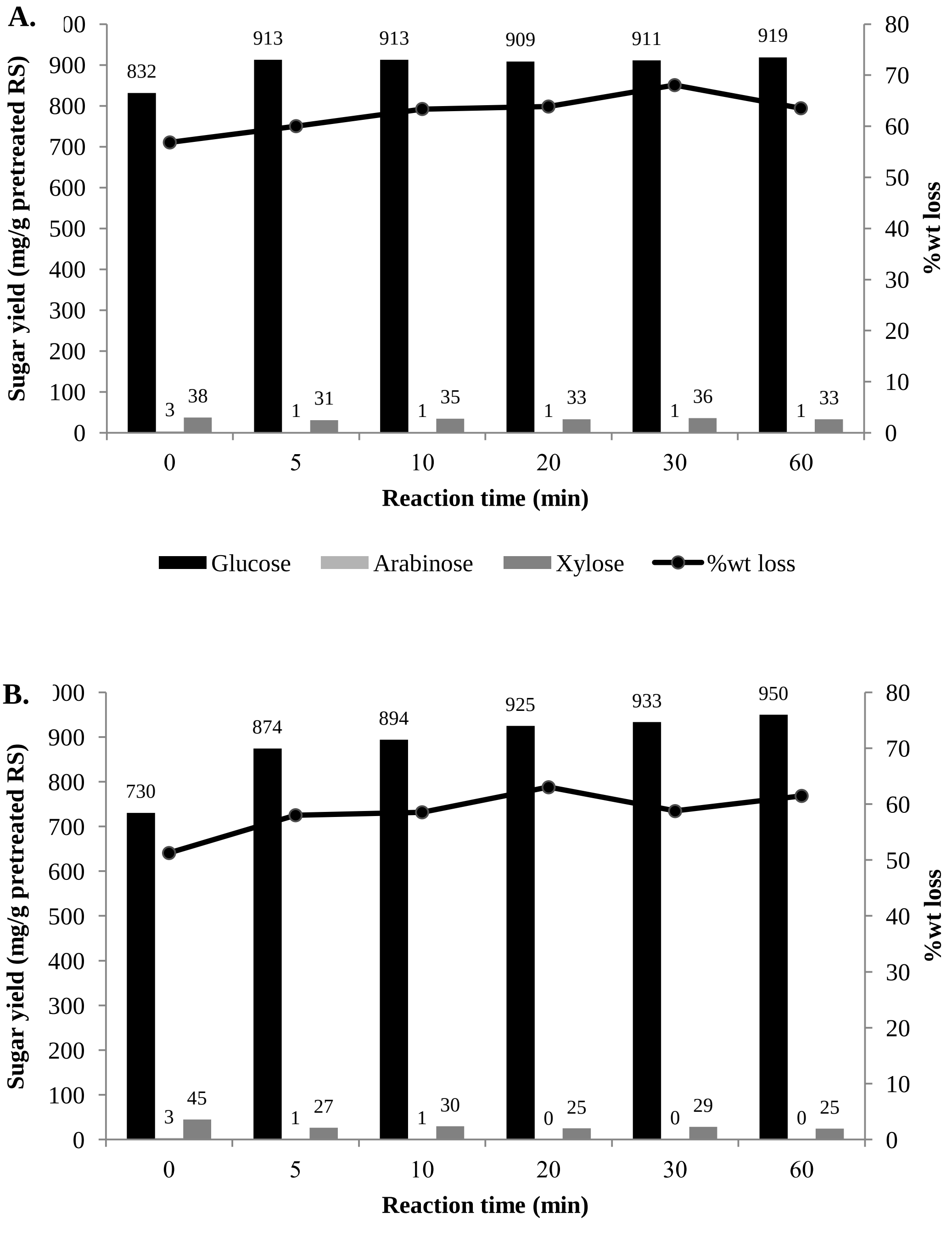


Fig. 43 Effects of operating time on sugar yield from organosolv pretreatment of rice straw using the NaOH/ acetone system. The pretreatment reaction contained rice straw in 180 ml acetone with 20 ml 50-70% (w/v) NaOH and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°Cfor 72h. (A) 50% w/v NaOH and (B) 70% w/v NaOH

Table 26 Sugar recovery from organosolv pretreatment of rice straw using NaOH/acetone system at different reaction times (50% w/v NaOH)

Reaction time (min)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
0	873	377	91	57
5	945	378	93	60
10	949	347	85	63
20	943	341	83	64
30	948	316	77	67
60	953	348	85	64

[a] glu+xyl+ara

**Reaction time 0 refers to immediate quenching of the reaction after reaching the target temperature

Table 27 Sugar recovery from organosolv pretreatment of rice straw using NaOH/acetone system at different reaction times (70% w/v NaOH)

Reaction time (min)	Total sugar yield[a] (mg sugar/g pretreated RS)	Total sugar yield (mg sugar/g native RS)	Glucose recovery yield	%wt loss
0	779	379	90	51
5	902	379	93	58
10	925	383	94	59
20	950	351	87	63
30	962	397	98	59
60	974	375	93	61

[a] glu+xyl+ara

**Reaction time 0 refers to immediate quenching of the reaction after reaching the target temperature

4.9 Effects of solvent reusability

The effects of recycled organic solvent after pretreatment was investigated under the optimum conditions in which the reaction was carried out at 80°C for 30 min with 180 ml of acetone, 4% solid loading, and 20 ml 50% w/v NaOH solution. After pretreatment, the solvent was separated from the solid residues and reused again by adjusting the pH to be 11.33 and the total volume to be 200 ml (by adding 50% w/v NaOH and acetone). Then, the solvent was reused for rice straw pretreatment again for 4 times. As seen in Fig. 45 and Table 28, sugar yield and glucose recovery yield markedly dropped, which indicates the limitations of the solvent and alkaline promoter recycling under these experimental conditions. Therefore, further study is required to regenerate the solvent before reuse.

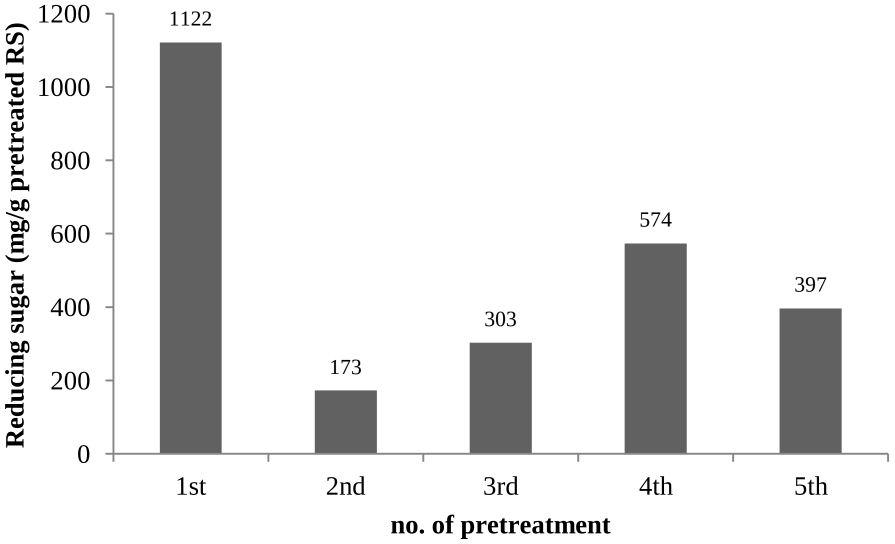


Fig. 44 Determination of pretreatment efficiency using recycled liquid fraction

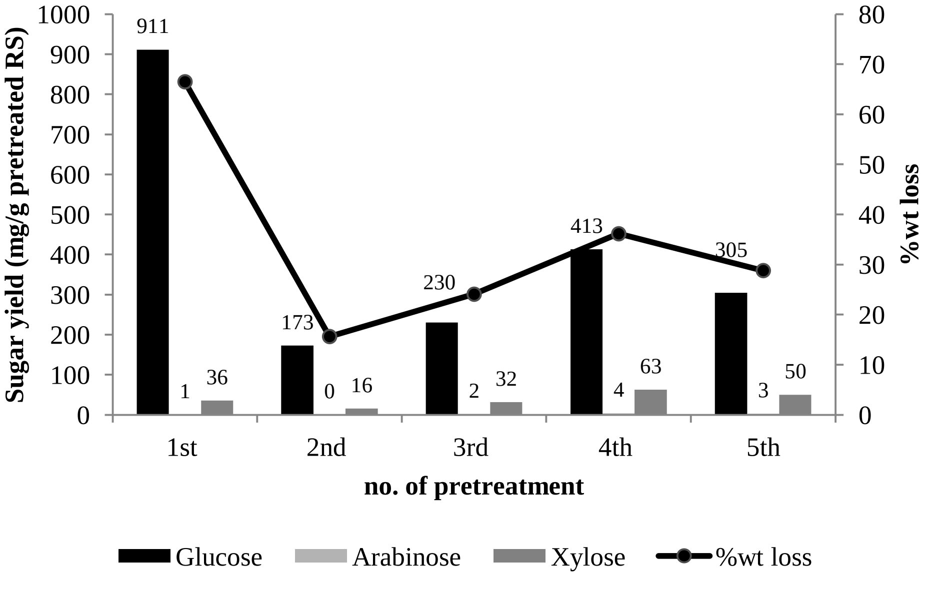


Fig. 45 Effects of solvent and alkaline promoter recycling on organosolv pretreatment of rice straw using NaOH/acetone system. The reaction contained 4% rice straw in 180 ml acetone/ 20 ml 50% (w/v) NaOH and treated at 80°C for 30 min at 20 bars with mixing at 250 rpm. The hydrolysis reaction contained 5% native rice straw in 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide with 20 FPU/g Accellerase® 1500 and was incubated at 50°C for 72 h.

Table 28 Sugar recovery from enzymatic hydrolysis of rice straw pretreated by organosolv using NaOH/acetone system with recycled liquid phase

Pretreatment (time)	**Total sugar yield[a] (mg sugar/g pretreated RS)**	**Total sugar yield (mg sugar/g native RS)**	**Glucose recovery yield**	**%wt loss**
1st	948	316	77	67
2nd	189	159	37	16
3th	264	200	44	24
4th	480	306	67	36
5th	358	255	55	29

[a] glu+xyl+ara

CHAPTER 5
CONCLUSION AND SUGGESTIONS

5.1 Conclusion

In this study, the effects of alkaline promoters on enhancing the efficiency of the organosolv pretreatment of rice straw were demonstrated. The addition of different alkalines into organic solvent systems showed enhancing glucose recovery and glucose selectivity in the final enzymatic hydrolysates. Among them, NaOH was found to be the most efficient promoter compared to organic alkalines. Addition of NaOH resulted in invreased efficiency in removal of lignin and a part of the hemicelluloses, and efficiently increase the accessibility of enzyme to the cellulose resulting in a sharp increase in saccharification yield [20]. Acetone was found to be a promising solvent for the alkaline-catalyzed organosolv pretreatment compared to ethanol and ethyl acetate. The alkaline-catalyzed pretreatment of rice straw in acetone led to higher product yield and selectivity compared to the conventional alkaline-catalyzed hydrothermal pretreatment in aqueous solution. This could be due to combination between organic solvent and alkaline to separate hemicelluloses and lignin during preserve cellulose in one pretreatment step. The effects of acetone in this study agrees well with the effects of organosolv pretreatment that enhances the enzymatic digestibility mainly by delignification and hemicelluloses removal leaving a cellulose-rich residue [20].

Alkaline promoters are commonly used in the hydrothermal pretreatment of lignocelluloses. The major effects of alkalines are the increase of internal surface by swelling, decrease of polymerization degree and crystallinity, destruction of links between lignin and other polymers, and breakdown of lignin, which leads to enhancing digestibility of the biomass in subsequent enzymatic hydrolysis [22]. Organosolv pretreatment is considered an efficient pretreatment method for improving digestibility of lignocellulose with the advantage on separating of high quality lignin for further processing. The most commonly used organic solvent in organosolv were methanol, ethanol, ethylene glycol, triethylene glycol and acetic acid [19] while there is only a limited number of study on the use of acetone. The use of acid promoters on improving efficiency and product selectivity in organolv pretreatment has been extensively studied [14] while the use of alkaline promoters has been very limited. In this study, the optimal alkaline-catalyzed pretreatment reaction contained 4 % (w/w) of solid loading with 50% (w/v) NaOH solution in 180 ml of acetone at 70°C for 30 min with the initial pressure at 20 bars and mixing 250 rpm. Under these conditions, almost all hemicellulose is extracted into the organic phase and cellulose remained in the solid fraction. This led to the highest glucose yield of 872 mg/g pretreated biomass which was equivalent to the maximal glucose recovery of 80.0% based on available glucan in the native biomass. The sugar yield and glucose selectivity achieved was superior to many previous reports on alkaline-catalyzed hydrothermal pretreatment of different biomass using various alkaline promoters (NaOH, lime, and NH_3).

The developed alkaline-catalyzed organolv pretreatment process using acetone developed in this study allows a design of a novel pretreatment method that involves the recycling of acetone at low temperatures (60^{o}C) and the regeneration of NaOH or other alkaline promoters. The process would be advantageous to the currently used alkaline-catalyzed hydrothermal pretreatment in terms of lower energy for operating temperature and solvent recycle. The high efficiency and selectivity of this alkaline/organosolv pretreatment provides a promising method for to production a high quality and purity sugar fraction for further processing. This would lead to the development of an alternative pretreatment process for sugar platform biorefinery.

5.2 Suggestions

Although the alkaline-catalyzed organosolv pretreatment process was found to be an efficient approach, further studies on the following issues are required:

- Solvent recycling: The separation and recycling of the applied solvent could reduce the operational costs of the process. It also requires strict controlled conditions due to the volatility of organic solvents during the reaction [19].
- Catalyst regeneration: The method of regeneration of NaOH or other alkaline should be further investigated to improve the pretreatment process and economic feasibility.
- Increasing solid loading: Further study on optimizing the reaction for higher solid loading is suggested in order to increase the process efficiency and to improve the economic feasibility of the developed process.

REFERENCES

[1]. Cardona, C. A., Sanchez, O. J., et al. (2010), **Process synthesis for fuel ethanol production**, 1 st ed. New York : Taylor & Francis group

[2]. Pandey, A., Larroche, C., et al. (2011), **Biofuels**, 1 st ed. USA: Acadamic Press

[3]. Mandil, C. (2004), **Biofuels for transport**, 1 st ed. France: IEA book

[4]. Vijai, K.G and Maria, G.T. (2013), **Biofuel Technologies**, 1 st ed. London: Springer

[5]. Poulomi, S., Stephen, J.M., Arther, J.R. (2010), "Effect of organosolv pretreatment and enzymatic hydrolysis on cellulose structure and crystallinity in Loblolly pine", **Carbohydr. Res**, Vol.345, pp. 965-970

[6]. Jinaporn, W., Kunn, K., Vorakarn, B., Ratanavalee, I., Pakorn W., Tikamporn, Y., Warawut, C. (2012), "The potential of cellulosic ethanol production from grasses in Thailand", **Biomedicine and Biotechnology**, Vol.2012, 10 pages, doi: 10.1155/2012/303748

[7]. Yueshu, G., Jingliang, X., Yu, Z., Qiang, Y., Zhenhong, Y., Yunyun, L. (2013), "Effects of different pretreatment methods on chemical composition of sugarcane basse and enzymatic hydrolysis", **Bioresource Technology**, Vol.144, pp. 396-400

[8]. Gaspar, M., Kalman, G., Reczey, K. (2007), "Corn fibre as a raw material for hemicelluloses and ethanol production", **Process Biochem**, Vol.42, pp.1135-1139

[9]. Ziyu, W., Deepak, R.K., Arthur, P.R., Jay, J.C. (2010), "Sodium hydroxide pretreatment and enzymatic hydrolysis of Coastal Bermuda grass", **Bioresource Technology**, Vol.101, pp.3583-3585

[10]. Jiele, X and Jay, J.C. (2010), "Pretreatment of switchgrass for sugar production with the combination of sodium hydroxide and lime", **Bioresource Technology**, Vol.102, pp.3861-3868

[11]. Rajesh, G and Y.Y, Lee. (2010), "Investigation of biomass degradation mechanism in pretreatment of switchgrass by aqueous ammonia and sodium hydroxide", **Bioresource Technology**, Vol. 101, pp.8185-8191

[12]. Parameswaran, B., Raveendran, S., Reeta, R.S., Surender, V., Lalitha, D., Satya, N., Noble, K., Rajeev, K.S., Ashok, P. (2009), "Bioethanol production from rice straw: An overview", **Bioresource Technology**, Vol.101, pp.4767-4774

[13]. S, McIntosh and T. Vancov. (2011), "Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw", **Biomass and bioenergy**, Vol.35, pp.3094-3103

[14]. Iwona, C., Grzegorz, P.B., Brett, R.H., James, L.J., Hanwu, L. (2012), "Catalyzed modified clean fractionation of switchgrass", **Bioresource Technology**, Vol. 127, pp.92-99

[15]. Farzad, M., Hamid, A., Sabihe, S-Z., Mohammad, R.E., Keikhosro, K. (2013), "Improvement of acetone, butanol and ethanol production from rice straw by acid and alkaline pretreatments", **Fuel**, Vol. 112, pp.8-13

[16]. Badal C. Saha and Michael A. Cotta. (2007), "Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol", **Enzyme and Microbial Technology**, Vol. 41, pp.528-532

[17]. Hamid, A., Keikhosro, K., Hamid, Z. (2013), "Organosolv pretreatment of rice straw for efficient acetone, butanol, and ethanol production", **Bioresour. Technol**. Vol. 152, pp.450-456

[18]. L, Mesa., E, Gonzalez., C, Cara., M, Gonzalez., E, Castro., S.I, Mussatto. (2011), "The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse", **Chem Eng Jour**. Vol. 168, pp.1157-1162

[19]. Sun, Y and Cheng, J., (2002). "Hydrolysis of lignocellulosic materials for ethanol production: A review". **Bioresour. Technol**. Vol.83 (1), pp. 1-11

[20]. Fan, L.T, Gharpuray, M.M, Lee, Y.H., (1987). *"Cellulose Hydrolysis*". **Biotechnology Monographs**, vol.3 Springer-Verlag

[21]. Oscar, J.S and Carlos, A.C., (2008). "Trend in biotechnological production of fuel ethanol from different feedstocks". **Bioresour Technol**. Vol. 99, pp. 5270-5295

[22]. Sluiter, A. et al., (2008). "Determination of structural carbohydrates and lignin in biomass". **National Renewable Energy Laboratory**, Golden, CO. Available at http://www.nrel.gov/biomass/pdfs/42618.pdf.

[23]. Xuan, L, Tae H.K, Nhuan, P.N., (2010). "Bioethanol production from corn stover using aqueous ammonia pretreatment and two phase simultaneous saccharification and fermentation (TPSSF)". **Bioresour Technol**. Vol. 101, pp. 5910-5916

[24]. Vishnu, M and Mala, R., (2012). "Trends in bioconversion of lignocelluloses: Biofuels, platform chemicals & biorefinery concept". **Progress in Eng and Comb scien**. Vol.38, pp. 522-550

[25]. Thomas, I, Kai, W, Juan, C.I.L, Vera, B, Garabed, A, Gerd, B, Irina, S., (2011). “Comparison of different pretreatment methods for lignocellulosic materials. Part I: Conversion of rye straw to valuable products”. **Bioresour Technol**. Vol. 102, pp. 5221-5228

[26]. Chia-Hung, K and Cheng-Kang, L., (2008). “Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments”. **Carbohyd Pol**. Vol. 77, pp.41-46

[27]. Chang, H.C, Byung, H.U, Young, S.K, Kyeong, K.O., (2012). “Improved enzyme efficiency of rapeseed straw through the two-stage fractionation process using sodium hydroxide and sulfuric acid”. **Applied Ene**. Vol.102, pp. 640-646

[28]. Jin-Woo, K, Kyoung, S.K, Jin-Suk, L, Sung, M.P, Hwa-Young, C, Jae, C.P, Jun, S.K., (2011). “Two-stage pretreatment of rice straw using aqueous ammonia and dilute acid”. **Bioresour Technol**. Vol. 102, pp. 8992-8999

[29]. Imman, S, Arnthong, J, Burapatana, V, Champreda,V, Laosiripojana, N., (2014). “Effect of acid and alkali promoters on compressed liquid hot water pretreatment of rice straw”. **Bioresour Technol**. Vol. 171, pp. 29-36

[30]. http://pubs.rsc.org/en/content/articlelanding/2006/cs/b601872f

[31]. http://www.omicsonline.org

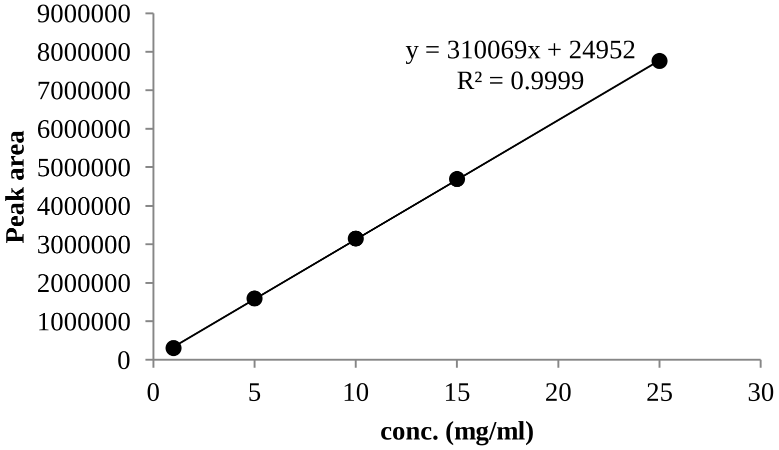
APPENDIX A

A.1 Standard calibration curve

- High Performance Liquid Chromatography (HPLC)

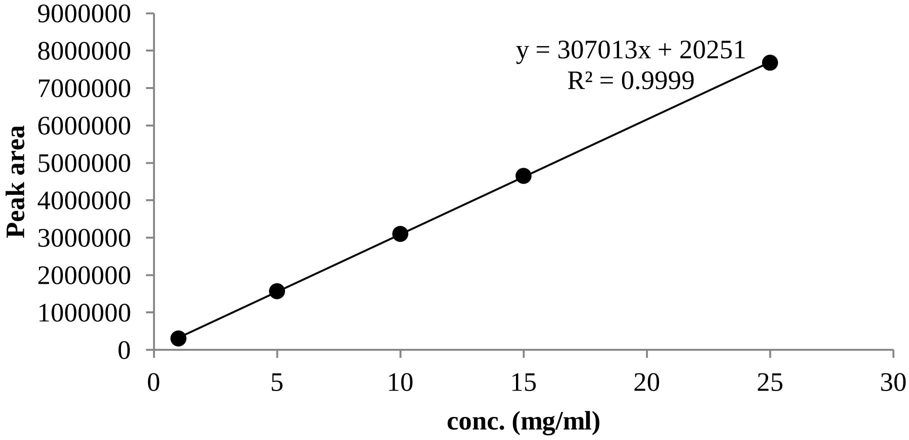
- **Glucose**

conc. (mg/ml)	Retention time (min	Peak area	Height
1	11.214	302995.8	14626.6
5	11.210	1590305.0	76724.1
10	11.215	3146363.7	152010.3
15	11.218	4691733.6	226491.7
25	11.226	7757205.4	371091.4



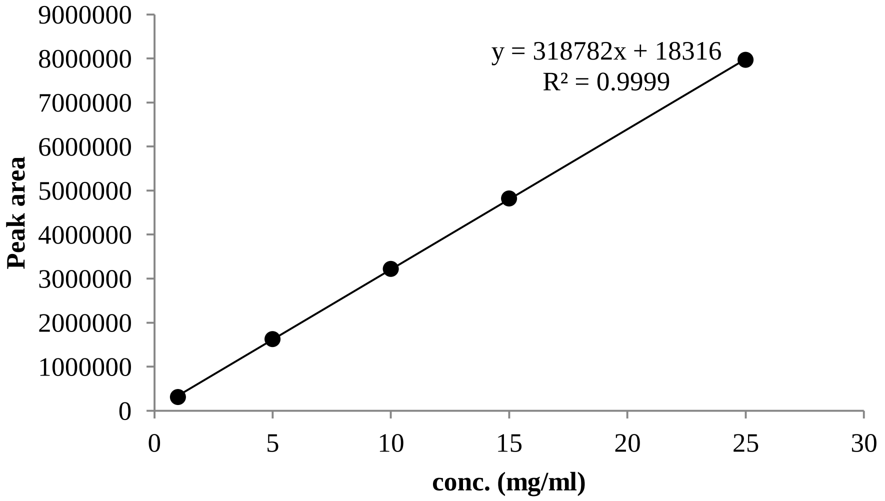
- **Arabinose**

conc. (mg/ml)	Retention time (min)	Peak area	Height
1	13.093	302876.2	13737.2
5	13.103	1566334.0	71056.4
10	13.097	3098498.1	140617.9
15	13.101	4649778.4	210635.5
25	13.107	7676511.9	344853.8



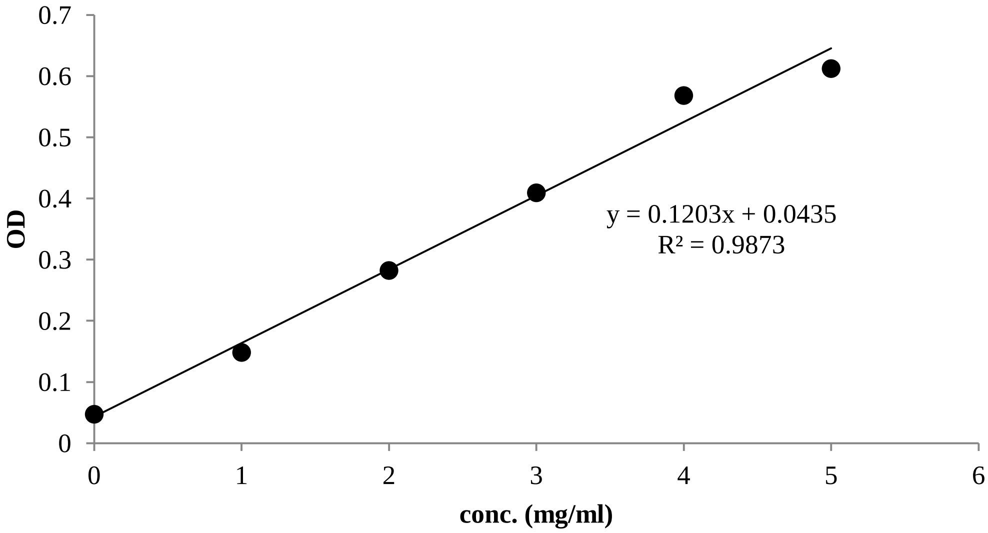
- **Xylose**

conc. (mg/ml)	Retention time (min)	Peak area	Height
1	12.004	309175.0	14649.5
5	12.007	1625538.4	77001.7
10	12.019	3219998.0	152549.6
15	12.015	4819666.6	227910.3
25	12.020	7969023.1	373313.6



- UV spectrophotometer (DNS method)

concentration (mg/ml)	OD
0	0.047
1	0.148
2	0.282
3	0.409
4	0.568
5	0.612



APPENDIX B

B.1 Raw data

- High Performance Liquid Chromatography (HPLC)

Enzymatic hydrolysis of native rice straw

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
non pretreat RS	11.251	13.115	12.151	2193007.2	49365.0	794252.1
	11.261	13.125	12.160	2068536.5	13181.9	843402.3

Organosolv pretreatment in the absence of alkalines

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
acetone	11.186	13.062	11.954	2059494.3	37225.4	230692.4
	11.186	13.062	11.954	2059494.3	37225.4	230692.4
EtOH	11.210	13.092	11.981	2159351.1	185671.3	386074.6
	11.204	13.085	11.977	2153786.7	189415.9	393011.7
EA	11.204	13.082	11.974	2154566.3	143003.3	341515.4
	ND*			**ND**		

*ND: non detect

Effect of alkaline promoters on organosolv pretreatment

- **NH_3 addition**

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
DI water	11.259	13.124	12.045	4359475.1	57401.5	814592.7
	11.258	13.123	12.043	4495179.0	65005.0	859649.9
acetone	11.213	13.058	11.992	2945005.7	3801.8	386092.9
	11.218	13.070	11.998	3064283.7	18033.2	377378.6
EtOH	11.215	13.065	11.990	2627670.1	59510.1	296427.2
	11.220	13.071	11.994	2571290.0	57453.6	288837.1
EA	11.222	13.067	12.004	4027204.5	4659.2	715848.4
	11.224	13.068	12.006	3872136.5	4562.0	713434.2

- **TEA addition**

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
DI water	11.260	13.125	12.045	4336621.9	57614.7	777671.4
	ND			ND		
acetone	11.234	13.118	12.009	2562840.2	325240.8	537085.8
	11.242	13.124	12.018	2643623.7	307789.1	533911.3
EtOH	11.250	13.124	12.020	2280608.2	7633.1	248825.1
	11.260	13.133	12.030	2777891.4	247528.5	516223.5
EA	11.254	13.133	12.031	2603579.8	329882.0	551712.0
	11.258	13.135	12.033	2585695.7	323873.9	546913.8

- **NaOH addition**

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
DI water	11.279	13.134	12.046	12210704.4	90146.8	793235.2
	11.281	13.135	12.048	12428945.4	96525.6	812926.8
acetone	11.207	13.074	11.959	12815359.9	55162.4	338770.4
	11.217	13.084	11.970	13234161.3	51280.6	355690.9
EtOH	11.206	13.062	11.960	12151276.0	77517.0	946046.7
	11.208	13.060	11.962	12488830.3	89467.8	979301.1
EA	11.223	13.059	11.999	7726640.7	2496.1	1149226.2
	11.222	13.062	11.999	7591020.6	2891.3	1132162.0

Effects of pretreatment temperature

- **Effects of temperature on organosolv pretreatment without alkaline**

Condition	Temp (°C)	Retention time			Peak area		
		Glucose	**Arabinose**	**Xylose**	**Glucose**	**Arabinose**	**Xylose**
acetone	80	11.186	13.062	11.954	2059494.3	37225.4	230692.4
		11.186	13.062	11.954	2059494.3	37225.4	230692.4
	90	11.184	13.06	11.951	1801225.9	28274.4	183595.0
		11.185	13.061	11.953	1864617.2	31332.8	185025.8
	100	11.184	**ND**		9309.2	**ND**	
		ND			**ND**		
EtOH	80	11.267	13.151	12.039	1960601.9	22858.9	201938.2
		11.277	13.161	12.047	1969403.4	23646.1	208223.5
	90	11.253	13.141	12.017	1915170.1	10514.9	188188.9
		11.244	13.134	12.008	1847192.5	30417.0	206136.3
	100	11.239	13.130	12.005	1824035.4	17642.7	177504.6
		11.239	13.133	12.007	1846848.1	17846.1	159610.7
EA	80	11.266	13.151	12.041	2081447.6	37709.2	233654.9
		11.265	13.150	12.040	2028631.4	36329.4	222533.2
	90	11.267	13.151	12.041	1927827.6	29234.2	217592.9
		11.268	13.151	12.043	2218743.8	34027.1	241576.8
	100	11.267	13.151	12.041	1965010.7	31354.7	219583.9
		11.268	13.152	12.044	1984287.7	31430.2	216012.2

- **Effects of temperature on organosolv pretreatment in the presence of organic alkaline promoters**

NH_3

Condition	Temp (°C)	Retention time			Peak area		
		Glucose	**Arabinose**	**Xylose**	**Glucose**	**Arabinose**	**Xylose**
DI water	80	11.259	13.124	12.045	4359475.1	57401.5	814592.7
		11.258	13.123	12.043	4495179.0	65005.0	859649.9
	90	11.261	13.126	12.046	5079042.5	79191.4	1082569.2
		11.260	13.125	12.045	5495153.7	91014.1	1160574.2
	100	11.262	13.128	12.046	5964878.3	100398.5	1296043.1
		11.262	13.127	12.046	6094909.6	117810.1	1391140.4
acetone	70	11.219	13.070	11.995	2581608.6	60108.0	321048.3
		11.220	13.072	11.995	2802809.3	31505.7	295139.9
	80	11.213	13.058	11.992	2945005.7	3801.8	386092.9
		11.218	13.070	11.998	3064283.7	18033.2	377378.6
	90	11.221	13.073	11.998	3096282.3	17437.5	357932.9
		11.217	13.068	11.995	2993202.2	4247.0	321036.7
	100	11.217	13.070	11.997	3252553.1	4848.3	477909.6
		11.217	13.066	11.996	3436615.4	4595.3	458573.8
EtOH	70	11.218	13.067	11.990	2466603.7	44779.3	283536.7
		11.219	13.070	11.992	2391096.9	77648.3	302038.1
	80	11.215	13.065	11.990	2627670.1	59510.1	296427.2
		11.220	13.071	11.994	2571290.0	57453.6	288837.1
	90	11.216	13.065	11.991	2617083.6	36764.8	288730.9
		11.217	13.070	11.993	2742176.3	49915.1	317644.5
	100	11.217	13.064	11.995	2926361.3	3873.9	300399.6
		11.218	13.068	11.996	2973405.9	4045.8	311097.5
EA	70	11.229	13.110	12.012	3425395.3	186764.1	593762.3
		11.232	13.115	12.015	3410698.8	187933.7	609203.9
	80	11.222	13.067	12.004	4027204.5	4659.2	715848.4
		11.224	13.068	12.006	3872136.5	4562.0	713434.2
	90	11.221	13.070	12.004	4443697.1	5662.0	888292.4
		11.222	13.074	12.004	4461138.1	6081.7	870119.0
	100	11.224	13.083	12.006	4583363.6	24102.5	896496.4
		11.222	13.075	12.005	4545101.6	5724.7	896031.1

TEA

Condition	Temp (°C)	Retention time			Peak area		
		Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
DI water	80	11.260	13.125	12.045	4336621.9	57614.7	777671.4
		ND			**ND**		
	90	11.262	13.127	12.047	4851422.1	85549.4	1012611.6
		11.261	13.126	12.046	4712351.6	67965.0	955510.1
	100	11.261	13.127	12.045	5639010.1	102445.6	1245957.9
		11.262	13.128	12.047	5589874.2	105278.7	1249515.6
acetone	80	11.234	13.118	12.009	2562840.2	325240.8	537085.8
		11.242	13.124	12.018	2643623.7	307789.1	533911.3
	90	11.249	13.130	12.020	2506239.2	309377.3	513104.7
		11.246	13.125	12.017	2581202.8	298751.6	516139.6
	100	11.247	13.124	12.018	2503947.2	305701.4	523930.0
		11.248	13.124	12.019	2599392.2	291275.4	517067.0
EtOH	80	11.250	13.124	12.020	2280608.2	7633.1	248825.1
		11.260	13.133	12.030	2777891.4	247528.5	516223.5
	90	11.257	13.129	12.028	2492523.1	264790.7	538795.9
		11.259	13.132	12.030	2584490.8	263148.3	533994.1
	100	11.251	13.123	12.022	2549102.9	254263.4	531179.0
		11.260	13.130	12.030	2501633.2	252794.3	530707.3
EA	80	11.254	13.133	12.031	2603579.8	329882.0	551712.0
		11.258	13.135	12.033	2585695.7	323873.9	546913.8
	90	11.260	13.137	12.036	2700771.1	491500.2	604200.0
		11.257	13.133	12.034	2617748.7	302265.6	515772.0
	100	11.256	13.132	12.032	2554190.0	310552.8	520043.1
		11.256	13.132	12.032	2588239.1	304686.2	512489.5

NaOH

Condition	Temp (°C)	Retention time			Peak area		
		Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
DI water	80	11.279	13.134	12.046	12210704.4	90146.8	793235.2
		11.281	13.135	12.048	12428945.4	96525.6	812926.8
	90	11.281	13.134	12.047	12838262.4	103493.7	840040.5
		11.281	13.135	12.048	12877536.5	101530.9	824930.4
	100	11.280	13.130	12.045	13326292.2	155362.5	844327.3
		11.282	13.133	12.048	13367812.4	166803.6	870917.5
acetone	50	11.241	13.112	12.007	11786949.5	90525.8	573315.7
		11.239	13.109	12.004	12257311.2	95956.6	609532.2
	60	11.244	13.110	12.008	12851149.5	70526.8	551981.8
		11.247	13.112	12.010	13010158.7	76555.4	566230.3
	70	11.241	13.110	12.004	12623128.2	59159.9	472073.3
		11.241	13.108	12.002	13444895.2	72005.0	534521.3
	80	11.239	13.108	12.000	12999407.1	57559.5	454169.3
		11.239	13.108	11.999	13090684.5	56418.1	448068.1
	90	11.236	13.106	11.998	13192473.5	52548.9	487719.2
		11.231	13.101	11.993	12692841.9	51824.1	473420.7
	100	11.241	13.110	12.004	12855718.6	40216.2	485713.3
		11.242	13.108	12.004	13054426.1	46508.3	487797.6
EtOH	80	11.206	13.062	11.960	12151276.0	77517.0	946046.7
		11.208	13.060	11.962	12488830.3	89467.8	979301.1
	90	11.212	13.068	11.980	10991011.8	20600.2	771445.6
		11.216	13.069	11.982	12148826.5	13127.4	826711.6
	100	11.220	13.075	11.986	12353872.5	27639.0	882498.7
		11.224	13.075	11.990	12017138.6	26343.8	873567.9
EA	80	11.223	13.059	11.999	7726640.7	2496.1	1149226.2
		11.222	13.062	11.999	7591020.6	2891.3	1132162.0
	90	11.225	13.058	12.002	7840472.7	2198.3	1222029.3
		11.225	13.063	12.002	7794996.7	2506.5	1235837.8
	100	11.271	13.134	12.051	8227488.0	48366.7	1348208.2
		11.269	13.132	12.049	8270394.1	48435.0	1344050.1

Effect of solvent content

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
160 ml	11.207	13.074	11.959	12815359.9	55162.4	338770.4
	11.217	13.084	11.970	13234161.3	51280.6	355690.9
170ml	11.263	13.114	12.025	13549905.5	39707.5	547623.1
	11.265	13.117	12.028	13394944.4	39182.1	546455.0
180ml	11.260	13.106	12.021	14091403.6	26528.4	539051.4
	11.261	13.108	12.023	13847347.0	27638.3	538608.5

Effect of NaOH concentration

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
5%(w/v)	11.213	13.058	11.952	12985016.8	33045.3	132845.7
	11.208	13.054	11.949	12636005.1	30408.8	134734.4
7.5%(w/v)	11.278	13.125	12.042	13334491.9	38606.9	626090.5
	11.280	13.126	12.043	13908663.6	30606.2	608723.3
10%(w/v)	11.297	13.143	12.055	14815947.2	36150.3	553181.6
	11.272	13.119	12.028	14647512.8	35747.2	527192.1

Effect of NaOH solution

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
50% (w/v)	11.255	13.132	12.017	14126898.3	34814.9	560492.3
	11.247	13.124	12.009	14030740.9	39264.9	560893.5
55% (w/v)	11.248	13.125	12.008	14246162.2	36386.7	475657.3
	11.250	13.126	12.009	14576115.6	36190.9	491920.5
60% (w/v)	11.250	13.127	12.011	14395172.9	32474.2	539625.8
	11.252	13.129	12.014	14314334.8	33985.5	554815.4
65% (w/v)	11.255	13.133	12.014	14421541.6	95343.1	523966.5
	11.265	13.136	12.025	14590998.1	33575.4	443217.9
70% (w/v)	11.281	13.146	12.041	14639954.6	30985.8	474756.2
	11.280	13.142	12.040	14556206.3	29397.7	463256.6

Effect of biomass loading

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
2%	11.260	13.106	12.021	14091403.6	26528.4	539051.4
	11.261	13.108	12.023	13847347.0	27638.3	538608.5
4%	11.252	13.114	12.015	14032285.8	39468.4	611417.0
	11.253	13.114	12.015	14260641.6	41921.3	630410.8
6%	11.250	13.114	12.015	12852229.5	66427.8	651238.5
	11.252	13.116	12.017	13095329.8	69709.3	656334.2
8%	11.244	13.111	12.015	11740077.4	97214.8	913747.4
	11.247	13.114	12.017	11928641.0	90563.1	886961.1
9%	11.256	13.122	12.024	12605851.2	85041.3	796173.4
	11.256	13.123	12.025	12584048.3	91112.8	815419.2

Effect of pretreatment time

- **50% NaOH solution**

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
0 min	11.285	13.151	12.051	12927610.6	76621.3	623706.9
	11.281	13.146	12.047	12908152.5	68910.5	610223.0
5 min	11.286	13.147	12.049	14150177.2	44682.0	525515.9
	11.290	13.150	12.051	14194872.6	41243.1	505718.3
10min	11.289	13.148	12.052	14084260.9	34990.7	564150.0
	11.290	13.149	12.053	14263013.2	38952.8	582376.0
20min	11.291	13.150	12.054	14081626.7	36153.0	540122.5
	11.291	13.150	12.054	14140918.3	39502.3	559984.5
30min	11.291	13.148	12.054	14139612.9	27964.5	559912.4
	11.295	13.151	12.059	14186006.7	25628.7	551845.5
60min	11.294	13.153	12.056	14353735.8	38188.4	552638.3
	11.294	13.152	12.056	14183215.0	37682.1	539793.8

- **70% NaOH solution**

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
0 min	11.285	13.151	12.057	11268805.9	68262.6	719290.5
	11.285	13.150	12.057	11422756.7	75966.3	749687.8
5 min	11.294	13.155	12.055	13632334.3	38517.3	442322.5
	11.292	13.152	12.053	13525474.7	38604.6	439937.8
10min	11.292	13.151	12.052	14022048.7	42750.1	513853.9
	11.293	13.153	12.055	13739618.8	34725.5	482105.7
20min	11.294	13.150	12.052	14142895.5	21676.2	396624.7
	11.295	13.151	12.052	14591324.2	23931.9	432687.3
30min	11.295	13.153	12.054	14448290.5	26143.7	466478.5
	11.294	13.152	12.053	14541944.4	28326.8	481596.4
60min	11.296	13.153	12.052	14804433.0	24879.4	404874.0
	11.295	13.152	12.050	14686838.9	27916.7	412950.5

Effects of solvent reusability

Condition	Retention time			Peak area		
	Glucose	Arabinose	Xylose	Glucose	Arabinose	Xylose
1st	11.296	13.153	12.054	14608589.1	47574.3	554109.0
	11.293	13.151	12.053	14211199.2	35011.9	516481.3
2nd	11.268	13.147	12.046	2389477.8	20348.4	238241.9
	11.270	13.148	12.046	3017829.0	26588.4	297796.9
3rd	11.270	13.150	12.054	3747218.3	52078.6	560360.2
	11.270	13.149	12.054	3435826.5	43653.6	492065.4
4th	11.276	13.152	12.060	6363609.1	83376.3	1030479.9
	11.275	13.151	12.059	6489675.8	80128.3	1004022.4
5th	11.273	13.151	12.059	4766734.8	62900.9	828482.1
	11.271	13.150	12.057	4727516.1	62404.5	810783.0

- UV-Vis spectrophotometer (DNS method)

Enzymatic hydrolysis of native rice straw

Condition	OD		
	1st	2nd	AVG
non pretreat RS	0.139	0.140	0.140

Organosolv pretreatment in the absence of alkalines

Condition	OD		
	1st	2nd	AVG
acetone	0.068	0.083	0.076
EtOH	0.113	0.117	0.115
EA	0.134	0.120	0.127

Effect of alkaline promoters on organosolv pretreatment

- **NH_3 addition**

Condition	OD		
	1st	2nd	AVG
DI water	0.279	0.257	0.268
acetone	0.144	0.147	0.146
EtOH	0.113	0.109	0.111
EA	0.209	0.194	0.202

- **TEA addition**

Condition	OD		
	1st	2nd	AVG
DI water	0.281	0.285	0.283
acetone	0.132	0.142	0.137
EtOH	0.136	0.162	0.149
EA	0.149	0.126	0.138

- **NaOH addition**

Condition	OD		
	1st	2nd	AVG
DI water	0.635	0.652	0.644
acetone	0.533	0.533	0.533
EtOH	0.571	0.570	0.571
EA	0.391	0.406	0.399

Effects of pretreatment temperature

- **Effects of temperature on organosolv pretreatment without alkaline**

Organic solvents	**Condition**	**OD**		
		1st	**2nd**	**AVG**
acetone	80°C	0.068	0.083	0.076
	90°C	0.077	0.074	0.076
	100°C	0.082	0.073	0.078
EtOH	80°C	0.113	0.117	0.115
	90°C	0.118	0.109	0.114
	100°C	0.117	0.109	0.113
EA	80°C	0.134	0.120	0.127
	90°C	0.118	0.121	0.120
	100°C	0.115	0.123	0.119

- **Effects of temperature on organosolv pretreatment in the presence of organic alkaline promoters**

NH_3

Organic solvents	**Condition**	**OD**		
		1st	**2nd**	**AVG**
DI	80°C	0.279	0.257	0.268
	90°C	0.314	0.387	0.351
	100°C	0.417	0.439	0.428
acetone	70°C	0.114	0.126	0.120
	80°C	0.144	0.147	0.146
	90°C	0.155	0.149	0.152
	100°C	0.169	0.158	0.164
EtOH	70°C	0.109	0.099	0.104
	80°C	0.113	0.109	0.111
	90°C	0.116	0.106	0.111
	100°C	0.101	0.168	0.135
EA	70°C	0.217	0.200	0.209
	80°C	0.209	0.194	0.202
	90°C	0.256	0.266	0.261
	100°C	0.279	0.179	0.229

TEA

Organic solvents	Condition	OD		
		1st	2nd	AVG
DI	80°C	0.281	0.285	0.283
	90°C	0.318	0.292	0.305
	100°C	0.391	0.385	0.388
acetone	80°C	0.132	0.142	0.137
	90°C	0.129	0.139	0.134
	100°C	0.137	0.139	0.138
EtOH	80°C	0.136	0.162	0.149
	90°C	0.147	0.146	0.147
	100°C	0.131	0.139	0.135
EA	80°C	0.149	0.126	0.138
	90°C	0.135	0.136	0.136
	100°C	0.145	0.156	0.151

NaOH

Organic solvents	Condition	OD		
		1st	2nd	AVG
DI	80°C	0.635	0.652	0.644
	90°C	0.686	0.729	0.708
	100°C	0.665	0.675	0.670
acetone	50°C	0.599	0.628	0.614
	60°C	0.655	0.667	0.661
	70°C	0.624	0.680	0.652
	80°C	0.643	0.656	0.650
	90°C	0.650	0.656	0.653
	100°C	0.676	0.673	0.675
EtOH	80°C	0.571	0.570	0.571
	90°C	0.579	0.615	0.597
	100°C	0.631	0.597	0.614
EA	80°C	0.391	0.406	0.399
	90°C	0.440	0.409	0.425
	100°C	0.528	0.527	0.528

Effect of solvent content

Condition	OD		
	1st	2nd	AVG
160ml	0.643	0.605	0.624
170ml	0.644	0.678	0.661
180ml	0.722	0.707	0.715

Effect of NaOH concentration

Condition	OD		
	1st	2nd	AVG
5% (w/v)	0.643	0.605	0.624
7.5% (w/v)	0.686	0.604	0.645
10% (w/v)	0.759	0.711	0.735

Effect of NaOH solution

Condition	OD		
	1st	2nd	AVG
50% (w/v)	0.721	0.643	0.682
55% (w/v)	0.679	0.729	0.704
60% (w/v)	0.704	0.723	0.714
65% (w/v)	0.665	0.614	0.640
70% (w/v)	0.610	0.717	0.664

Effect of biomass loading

Condition	OD		
	1st	2nd	AVG
2%	0.599	0.628	0.614
4%	0.745	0.718	0.732
6%	0.620	0.675	0.648
8%	0.637	0.620	0.629
9%	0.678	0.649	0.664

Effects of pretreatment time

- **50% NaOH solution**

Condition	OD		
	1st	2nd	AVG
0 min	0.649	0.650	0.650
5 min	0.709	0.731	0.720
10 min	0.727	0.639	0.683
20 min	0.738	0.677	0.708
30 min	0.726	0.683	0.705
60 min	0.675	0.574	0.625

- **70% NaOH solution**

Condition	OD		
	1st	2nd	AVG
0 min	0.578	0.540	0.559
5 min	0.625	0.652	0.639
10 min	0.697	0.642	0.670
20 min	0.659	0.725	0.692
30 min	0.665	0.688	0.677
60 min	0.656	0.689	0.673

Effects of solvent reusability

Condition	OD		
	1st	2nd	AVG
1st	0.662	0.635	0.649
2nd	0.128	0.167	0.148
3rd	0.236	0.215	0.226
4th	0.379	0.398	0.389
5th	0.278	0.286	0.282