BIOSUGARS PRODUCTION FOR ETHANOL FERMENTATION FROM RICE STRAW

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Abstract

Rice straw is available in large quantities in Thailand and is attractive as a lignocellulosic material resource for biosugars production. Dilute acid was used to pretreated the rice straw before enzymatic hydrolysis. The maximum overall sugar yield (62.59 g/100 g dry rice straw) was achieved at 4% (v/v) H_2SO_4 for 30 min, representing 80% of the total sugar in the rice straw. Under this condition, the concentrations of acetic acid, furfural and HMF in the hydrolysate were 1.72, 0.16 and 0.06 g/l respectively. For ethanol production, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) were employed using *Pichia stipitis* JCM 10742 with acid pretreatment. Results indicated that both ethanol concentration and productivity using SSF were higher than the SHF process. Ethanol concentration and productivity for SSF attained 9.21 g/l and 0.30 g/l h in 30 h fermentation respectively, while the SHF process achieved 6.10 g/l and 0.16 g/l h in 36 h. Results indicated that dilute acid pretreatment can be successfully applied to rice straw for biosugars production.

Keywords : Rice straw, Lignocellulosic material, Dilute acid, Enzymatic hydrolysis, Biosugars

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Introduction

The development of biofuel is an important objective of a country's development strategy to reduce fuel imports and increase energy self-dependence. Biofuel produced from renewable lignocellulosic material is one of the most important renewable fuels and widely used as a partial gasoline replacement in Thailand. Ethanol is the commonest liquid biofuel and used as a fuel or a gasoline enhancer. Ethanol can be produced from sugar (sugarcane and molasses) and starch (cassava, rice and corn). However, these raw materials may become insufficient for future ethanol production because they are edible crops. Lignocellulosic materials such as agricultural softwood and hardwood residues are potential sources of biosugars that interest scientists for ethanol production. Biosugars are derived from hemicellulose and cellulose that can be fermented to ethanol. Ethanol produced from lignocellulose materials is an attractive alternative as they do not compete with the food supply and are less expensive. A major lignocellulosic material found in large quantities in Thailand is rice straw.

Rice straw is an abundant lignocellulosic material worldwide. In 2013, Thailand produced 19 million tons of rice straw (Department of Alternative Energy Development and Efficiency, Ministry of Energy, 2013). Rice straw has several characteristics as a potential biomass for ethanol production. The carbohydrates in the rice straw are first hydrolysed into sugars predominantly by enzymes and these sugars are then fermented to ethanol by specialised microorganisms. The natural structure of lignocellulosic biomass is extremely recalcitrant to enzymatic hydrolysis. Therefore, a pretreatment step is necessary to remove hemicellulose and lignin, reducing cellulose crystallinity and increasing the porosity of the biomass. Many methods have been studied and evaluated for biomass pretreatment such as using diluted acid, lime, sodium hydroxide, steam explosion, ammonium fibre explosion and organic solvents. Among the various pretreatment methods, dilute sulphuric acid has received the most attention as an inexpensive, economically feasible process which is effective for a wide range of lignocellulosic materials (softwood, hardwood and herbaceous).

Objectives

This study examined the production of biosugars from rice straw using dilute sulphuric acid pretreatment, followed by hydrolysis of the cellulose with a commercial enzyme. Ethanol fermentation was conducted by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) techniques to determine the feasibility of rice straw in bioethanol production.

Materials and methods

Raw material and preparation

The rice straw was collected from a locality in Yasothon Province, Thailand, was dried in sunlight and then cut into small pieces. The dried rice straw was ground and sieved to obtain 20-60 mesh fractions and stored in plastic bags at room temperature until required for used. The composition of the rice straw was glucan 38.51%, xylan 22.91%, galactan 2.89%, arabinan 5.04%, acid-insoluble lignin 19.09%, extractives 6.21% and ash 8.52% on a dry weight basis.

Dilute acid pretreatment

The rice straw was prepared for sulphuric acid pretreatment by presoaking the particles at 10% (w/v) solid loading at room temperature in a sulphuric acid solution of 0, 1, 2, 3 and 4% (v/v) overnight. Deionised water was used as the pretreatment control. The mixture was then pretreated at 121 $^{\circ}$C, 1.5 bar pressure in an autoclave for 30, 60 and 90 min. The pretreated solid was washed with tap water until the pH was neutral, dried in an oven at 60 $^{\circ}$C for 3-4 days and stored in plastic bags at room temperature prior to enzymatic hydrolysis. The prehydrolysate was analysed by high-performance liquid chromatography (HPLC) to determine the concentration of monomeric sugars (glucose, xylose, galactose, arabinose and mannose) and the inhibitors (acetic acid, furfural and hydroxymethylfurfural (HMF)).

Enzymatic hydrolysis of rice straw

Enzymatic hydrolysis was conducted at 5% (dry matter, w/v) solid loading in a laboratory bottle (50 ml). A sample of pretreated rice straw was soaked in 50 mM sodium citrate buffer (pH 4.8). The enzyme loading was set at 15 FPU/g dry pretreated solid and used a commercial cellulase (Celluclast® 1.5L, Novozymes A/s, Bagsvaerd, Denmark) was used with a filter paper activity of 58 FPU/ml. The laboratory bottles were placed in a shaking water bath at 50 $^{\circ}$C and 200 rpm for 24 h. The samples were then placed in boiling water for 5 min and centrifuged. The monomeric sugar concentrations in the enzymatic hydrolysate were analysed by HPLC.

Ethanol fermentation

1. Microorganisms

Pichia stipitis JCM 10742, obtained from the Japan Collection of Microorganisms, Riken Bioresource Centre, Japan was used for fermentation in the SSF and SHF experiments. The seed culture was inoculated with 100 ml of YM medium for 24 h, 150 rpm at 30 $^{\circ}$C. An inoculum size of 5% v/v was used to initiate the experiments.

2. Simultaneous saccharification and fermentation (SSF)

The condition for the maximum yield of glucose from enzymatic hydrolysis was carried forward to ethanol fermentation. The SSF experiment was performed in a 250 ml Erlenmeyer flask with a working volume of 150 ml. The culture medium contained rice straw at

5% (w/v) supplemented with 1.50 g/l yeast extract, 3 g/l peptone, 2 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$ and 0.5 g/l Mg $SO_4 \cdot 7H_2O$. The medium was sterilised by autoclaving at 121 °C for 15 min. The enzyme loadings of cellulase (Celluclast® 1.5L, Novozymes A/s, Bagsvaerd, Denmark) and beta-glucosidase (Novozym 188, Novozymes A/s, Bagsvaerd, Denmark) were 15 FPU/g dry rice straw and 7.50 IU/g dry rice straw respectively. The SSF experiment was carried out using *P. stipitis* JCM 10742 at 30 °C and 150 rpm. Samples were taken periodically for 72 h and analysed for glucose, xylose and ethanol concentration by HPLC. Viable cell were determined using plate count agar technique.

3. Separate hydrolysis and fermentation (SHF)

Enzymatic hydrolysis of the pretreated rice straw was performed in a 250 ml Erlenmeyer flask using 5% (w/v) solid loading and 50 mM sodium citrate buffer (pH 4.8). The enzyme loadings of cellulase (Celluclast® 1.5L) and beta-glucosidase (Novozyme 188) were 15 FPU/g dry rice straw and 7.50 IU/g dry rice straw respectively. The mixture was incubated at 50 °C for 12 h with shaking water bath at 200 rpm. The hydrolysate obtained from the enzymatic hydrolysis supplement with 1.50 g/l yeast extract, 3 g/l peptone, 2 g/l KH_2PO_4, 1g/l $(NH_4)_2SO_4$ and 0.5 g/l $MgSO_4 \cdot 7H_2O$ was used as a fermentation medium. The same fermentation condition as for the SSF process was used. Samples were taken periodically for 72 h and analysed for glucose, xylose and ethanol concentration by HPLC. Viable cells were determined by plate count agar.

Analytical methods

The carbohydrate compositions of the raw material and the pretreated solid residue were determined according to the National Renewable Energies Laboratory (NREL) protocols (Sluiter *et al*. 2012). The chemical composition of the raw material and the pretreated solid residue were determined according to the Technical Association of the Pulp and Paper Industry procedure (TAPPI., 1992). The sugar content (glucose, xylose, galactose, arabinose and mannose) was determined by HPLC using a Aminex HPX-87P (Bio-Rad Labs, Hercules, CA) with a refractive index detector, operated at 80 °C and eluted at 0.60 ml/min Milli-Q water. Furfural and HMF were determined by HPLC with a UV/Visible detector. An Aminex HPX-87H (Biorad Labs, Hercules, CA) operating at 60 °C with 5 mM H_2SO_4 as a mobile phase (0.6 ml/min) was used for separation. Detection was performed at 280 nm. Ethanol and acetic acid were determined by HPLC with a refractive index detector and an Aminex HPX-87H column maintained at 60 °C and flow rate of 0.6 ml/min, with 5 mM H_2SO_4 as the mobile phase.

Statistical analyses

All experiments were conducted in duplicate. The experimental data were ratified using a factorial test. The effects of acid pretreatment and pretreatment time were analysed.

Results and discussion

Substrate character and pretreatment

The chemical compositions of untreated and pretreated rice straw are shown in Table 1. The chemical composition of pretreated solid changed from the raw untreated material. The acid pretreatment solubilised most of the hemicellulose and a small amount of lignin, but had little effect on the cellulose fraction. Between 34% and 49% of the total hemicellulose of the rice straw was removed. The cellulose content increased to between 52.16% and 58.84% compared with the initial value of 47.22%. The acid-insoluble lignin recovered in the pretreated solid reached 34.21%, higher than the lignin content of the raw material. The higher cellulose and acid-insoluble lignin in the acid pretreated solid residue may be due to the removal of hemicellulose fraction (Gupta *et al.*, 2011).

Table 1. Chemical analysis of raw untreated material and pretreated rice straw

Time (min)	H_2SO_4 (%v/v)	Solid composition		
		Cellulose	Hemicellulose[a]	Acid-insoluble lignin
Raw untreated material		48.44	33.37	19.09
30	0	47.22	33.92	22.03
	1	57.94	17.46	29.52
	2	58.84	17.12	31.07
	3	57.28	17.31	31.08
	4	55.71	17.94	32.72
60	0	46.01	34.17	21.77
	1	57.05	17.75	30.06
	2	55.75	18.16	31.85
	3	56.18	18.20	33.18
	4	56.14	17.70	34.21
90	0	46.49	33.88	21.49
	1	57.57	17.65	30.61

Time (min)	H_2SO_4 (%v/v)	Solid composition		
		Cellulose	Hemicellulose[a]	Acid-insoluble lignin
	2	55.05	19.61	32.99
	3	53.02	20.52	33.25
	4	52.16	21.97	34.03

[a]: Hemicellulose = holocellulose-cellulose

Sugars and inhibitors in the prehydrolysate

Table 2 presents the composition of the prehydrolysate obtained after the pretreatment of 100 g dry rice straw. Xylose was the main sugar in the prehydrolysate. The amounts of xylose released were mainly soluble hemicellulose, because xylose represented the most abundant hemicellulose sugar monomer in rice straw. Xylose yields increased when the acid concentration increased from 1% to 4% (v/v) in the range 30-60 min pretreatment time. Under severe pretreatment conditions (such as 4% (v/v) H_2SO_4, 90 min) yields of xylose dramatically decreased. The maximum xylose yield was 20.29 g/100 dry rice straw (80% of xylose in rice straw) at 4% (v/v) H_2SO_4 for 30 min. The xylose yield during the hydrolysis of rice straw was reported in the range of 77-89% (Roberto *et al.,* 2003; Hsu *et al.,* 2010). Moreover, glucose, galactose and arabinose in the prehydrolysate released from both cellulose and hemicellulose during acid pretreatment. The amount of glucose release was below 9.25 g/ 100 g dry rice straw at 4% (v/v) H_2SO_4 for 30 min. The amount of glucose released during pretreatment may result in the lower cellulose content remaining in the pretreated solid and thus a reduction in the yield of glucose during the enzymatic hydrolysis step (Wei *et al.,* 2012). Under the pretreated conditions, a maximum total sugar yield of 36.58 g/ 100 g dry rice straw (47% of total sugar in rice straw) was obtained at 4% (v/v) H_2SO_4 for 30 min. Under the same conditions, the total hemicellulosic sugar yield (xylose, galactose and arabinose) in the prehydrolysate reached 27.33 g/100 g dry rice straw (77% of total hemicellulosic sugar in rice straw).

All experimental results were analysed by factorial design. The statistical analysis showed that pretreatment time and acid concentration had a significant effect on xylose, galactose and arabinose ($p<0.05$). In contrast, the pretreatment time did not have a significant effect on the glucose yield ($p>0.05$).

For the inhibitory compounds, the acid pretreatment generated significant amounts of acetic acid, furfural and HMF. All these compounds not only caused lower sugar yield, but also could potentially inhibit the growth of yeast and reduce ethanol production. Acetic acid was produced from the hydrolysis of the acetyl group in hemicellulose, while furfural and HMF were generated from pentose and hexose sugar degradation respectively. As seen in Table 2,

the concentration of acetic acid and furfural in the prehydrolysate increased with increased acid concentration and pretreatment time because of the degradation of the sugars. This was supported by the fact that more acetic acid and furfural were detected in the prehydrolysate at 4% (v/v) H_2SO_4 for 90 min. The HMF concentration in the prehydrolysate was low, indicating limited degradation of glucose. Under the maximum total sugar yield in prehydrolysate, the concentration of acetic acid, furfural and HMF were 1.72, 0.16 and 0.06 g/l respectively. These results were lower than reported by Palmqvist and Hahn-Hägerdal (2000). Therefore, the detoxification step is not required to diminish the inhibition effect of inhibitor compound in the step of fermentation.

Table 2. Composition of the prehydrolysate from dilute acid pretreatment at different pretreatment conditions

Pretreatment Condition		Prehydrolysate composition						
Time (min)	H_2SO_4 (%v/v)	Sugar analysis (g/100 g dry rice straw)				Inhibitor analysis		
		Glu	Xyl	Gal	Ara	Ace (g/l)	HMF (mg/l)	Furfural (mg/l)
30	Control	0.50	0.26	0.20	ND	ND	ND	ND
	1	5.05	14.58	1.42	2.98	1.68	52.25	28.45
	2	7.11	15.06	1.32	2.64	1.45	58.25	79.70
	3	8.47	18.23	1.96	3.99	1.58	99.80	201.10
	4	9.25	20.29	2.82	4.22	1.72	58.10	157.00
60	control	0.74	0.25	0.18	ND	ND	ND	ND
	1	5.46	17.50	3.16	4.83	1.48	51.10	44.75
	2	6.78	19.07	3.31	5.14	1.35	100.90	214.95
	3	7.73	19.31	3.28	5.16	1.50	41.15	256.85
	4	7.71	19.03	3.28	5.21	1.79	21.45	408.55
90	Control	0.60	0.24	0.17	0.39	ND	ND	ND
	1	6.41	18.52	3.27	4.90	1.89	110.85	152.8
	2	6.41	17.69	2.98	4.46	1.86	49.20	317.40
	3	6.54	16.70	2.60	4.00	2.44	50.85	501.00
	4	6.12	14.89	2.65	3.82	3.90	28.70	722.65

Glu: Glucose, Xyl: Xylose, Gal: Galactose, Ara: Arabinose, Ace: Acetic acid, HMF: 5-Hydroxymethylfurfural

Enzymatic hydrolysis of acid pretreated residues

Figure 1 shows the yields of sugars in enzymatic hydrolysis at different pretreatment conditions. Glucose and xylose were produced during enzymatic hydrolysis, with glucose the main product. The acid pretreatment samples showed higher glucose yield in the enzymatic hydrolysate. The glucose yield was increased by 32% compared to control. The higher glucose yield can be explained as follows: (1) hemicellulose removal by acid pretreatment increased the enzymatic digestibility of cellulose, and (2) the increased porosity and surface area of the cellulose provided greater accessibility for the enzyme (Zhang and Wu, 2014).

In this study, the maximum glucose yield in the hydrolysate was 24.41 g/ 100 g dry rice straw (57% of glucose in rice straw) at 1% (v/v) H_2SO_4 and 30 min. Nevertheless, the enzymatic hydrolysis yield of pretreated rice straw under this condition was low (40%), even though the removal of hemicellulose reached 80%. This indicated that the enzymatic digestibility may not be related to hemicellulose removal. These results were in agreement with a previous study (Laureano-Perez *et al.*, 2005; Cara *et al.*, 2008) which reported that not only hemicellulose solubilisation, but some other factors such as cellulose crystallinity, surface area accessibility or the lignin content in the pretreated solid may also affect enzymatic hydrolysis. The amount of xylose release was below 2.39 g/ 100 g rice straw (9% of xylose in rice straw) which implied that there was almost complete hydrolysis of xylose during acid pretreatment.

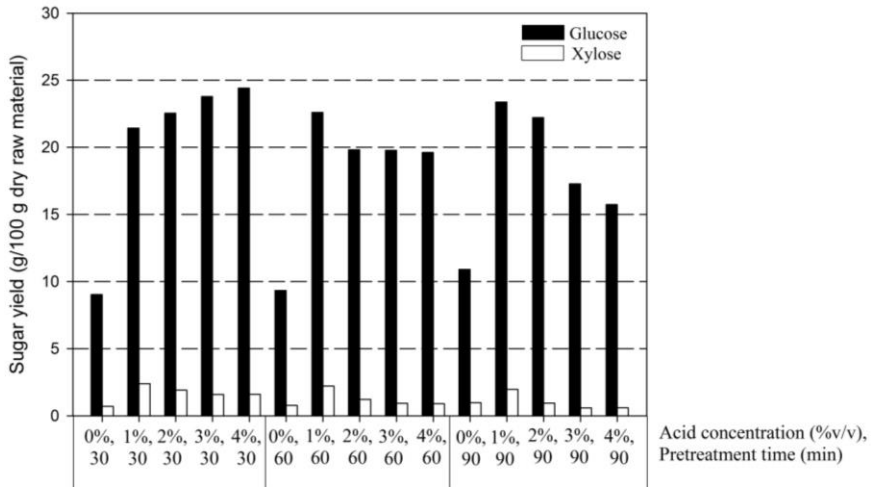


Figure 1. Glucose and xylose yield (g/100g dry rice straw) in the enzymatic hydrolysate after 24 h of enzymatic hydrolysis at different pretreatment conditions

Overall sugar yields

The overall sugar yield is presented in Figure 2. The maximum overall sugar yield of 62.59 g/ 100 g rice straw (80% of total sugars in rice straw) was obtained at 4% (v/v) H_2SO_4 for 30 min. The overall sugar obtained in this study was close to the yield obtained by Hsu *et al.* (2010) at 83% of sugar content in the rice straw when pretreated at 1% (w/w) H_2SO_4, 160 °C or 180 °C and 1-5 min pretreatment time, followed by enzymatic hydrolysis. Thus, dilute acid pretreatment followed by enzymatic hydrolysis appeared to be the most promising technology for biosugars production from rice straw. This is not only cost effective, but also practically adaptable to industrial production.

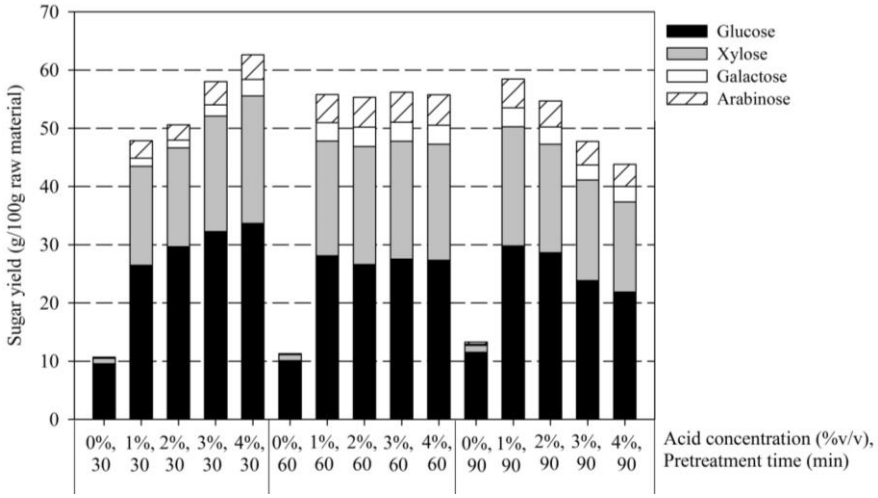


Figure 2. Overall sugar yield (g/ 100 g dry rice straw) from pretreatment and enzymatic hydrolysis at different pretreatment conditions

Ethanol fermentation by *P. stipitis* JCM 10742

The pretreatment condition (4% (v/v) H_2SO_4 for 30 min pretreatment time) was selected in both SSF and SHF for ethanol production. For SSF and SHF, a native pentose and hexose sugars fermenting yeast *P. stipitis* JCM 10742 was employed under batch fermentation.

1. Separate hydrolysis and fermentation (SHF)

In the SHF process (Figure 3A), the amount of glucose present in the hydrolysate was assimilated with 18 h of fermentation. Xylose was completely consumed after 24 h of fermentation. Xylose was also fermented during fermentation with glucose but at a slow rate (Buaban *et al.,* 2010). Yeast cell growth continued until the completion of fermentation. The maximum ethanol concentration at 24 h of fermentation time was 6.10 g/l, corresponding to a volumetric ethanol productivity 0.24 g/l h with ethanol yield 0.23 g ethanol/ g available

fermentable sugars (glucose and xylose). However, the ethanol productivity was 0.16 g/l/h when calculated as the highest ethanol concentration divided by total time process (12 h enzymatic hydrolysis and 24 h fermentation).

2. Simultaneous saccharification and fermentation (SSF)

In the SSF process (Figure 3B), glucose accumulation in the fermentation medium was only observed during the first 6 h of fermentation time and the glucose concentration was close to 0 g/l at 12 h of fermentation time. This indicated that the yeast cells were metabolically active during the whole course of the fermentation. Enzymatic hydrolysis was therefore the rate-limiting step in ethanol production from rice straw. Xylose concentration increased during the first 12 h and xylose was not detected after 24 h fermentation. Yeast cell growth continued until the complete of fermentation. The maximum ethanol concentration at 30 h of fermentation time was 9.21 g/l with an ethanol yield of 0.35 g ethanol/ g available fermentable sugars and ethanol productivity of 0.30 g/l h.

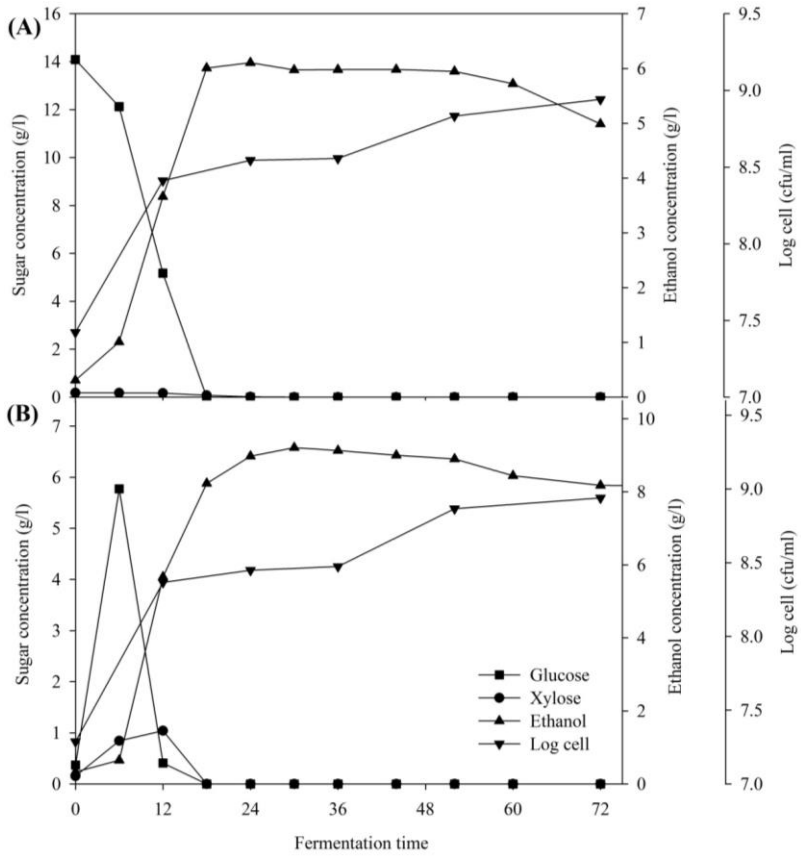
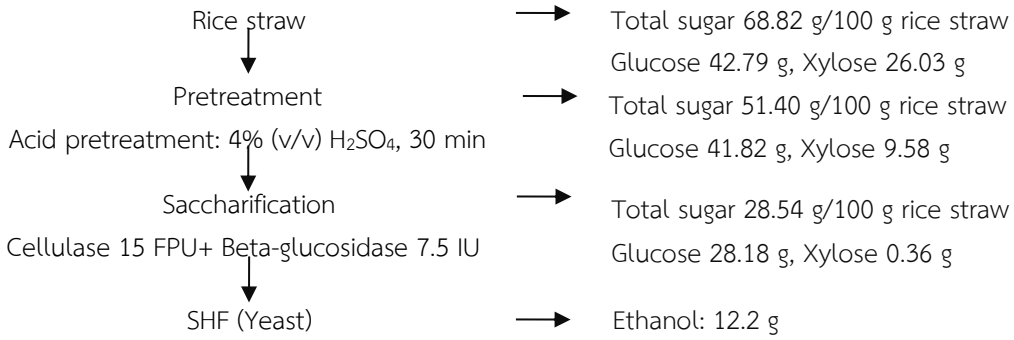


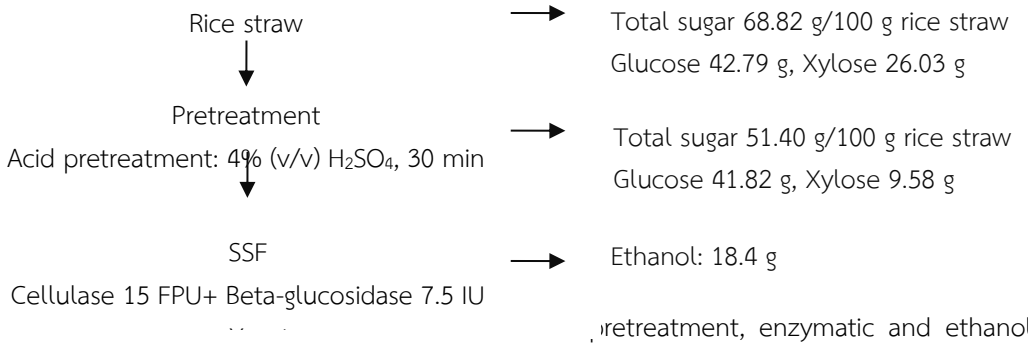
Figure 3. Time course of ethanol production by *P. stipitis* JCM 10742 from 5% (w/v) pretreated rice straw at 30 °C, 150 rpm and 72 h using separate hydrolysis and fermentation (SHF) (A) and simultaneous saccharification and fermentation (SSF) (B).

The overall mass balance was presented including the acid pretreatment, enzymatic hydrolysis and ethanol fermentation steps (Figure 4). After the acid pretreatment step, glucose and xylose /100 g rice straw decreased by 0.97 g and 16.45 g respectively, which indicated that xylose was the main sugar hemicellulose that was hydrolysed. Total sugar after pretreatment was 51.40 g/ 100 g dry rice straw which was lower than 25% of the theoretical maximum at 68.82 g/ 100 g dry rice straw. In the SHF process, after 12 h of enzymatic hydrolysis, a glucose concentration of 28.18 g/ 100 g dry rice straw and a xylose concentration of 0.36 g/ 100 g dry rice straw were obtained at 5% (w/v) solid loading with 15 FPU of cellulase and 7.5 IU of beta-glucosidase per 100 g of dry rice straw. Fermentation of enzymatic hydrolysate by *P. stipitis* JCM 10742 resulted in 0.124 g ethanol/ g biomass after 24 h fermentation time. In the SSF process, ethanol concentration of 0.184 g ethanol/g biomass was obtained after 30 h fermentation time. The ethanol yield obtained here was low compared to previous studies (Li *et al.,* 2011; Yadav *et al.,* 2011). The lower ethanol concentration was probably due to the low solid loading or low enzyme concentration. To increase the ethanol concentration, SSF must be carried out at high substrate loading and enzyme concentration to obtain higher sugar concentration in the fermentation broth.

(A) Separate hydrolysis and fermentation (SHF)



(B) Simultaneous saccharification and fermentation (SSF)



pretreatment, enzymatic and ethanol fermentation by *P. stipitis* JCM 10742

The results determined that the SSF process exhibited notable advantages over the SHF process. Ethanol concentration and ethanol productivity using SSF increased by 51% and 76% respectively compared to the SHF process. The result indicated that SSF was preferred to SHF in the hydrolysis and fermentation of lignocellulosic biomass with more rapid ethanol production and higher concentration of produced ethanol.

Conclusions

Rice straw is the most abundant agricultural waste in Thailand and therefore a potential biomass for biosugars production. A maximum overall sugar yield in the hydrolysate of 62.59 g/100 g raw material (80% of total sugar in rice straw) was obtained at 4% (v/v) H_2SO_4 for 30 min. Under the same conditions, the total hemicellulosic sugar yield reached 27.33 g/100 g dry rice straw (77% of total hemicellulosic sugar in rice straw). The results indicated that dilute acid pretreatment combined with enzymatic hydrolysis is a suitable process to produce sugar from rice straw for further processing to ethanol. In ethanol production, *P. stipitis* JCM 10742 produced 9.21 g/l of ethanol in 30 h fermentation by SSF and 6.10 g/l of ethanol in 36 h by SHF. From the results, SSF was considered as a better process than SHF because of the more rapid ethanol production and a higher concentration of ethanol produced.

Acknowledgements

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