CHAPTER IV

RESULTS

4.1 *Panicum maximum* cv TD 53 (purple guinea grass)

Chemical composition of purple guinea grass analyzed by Department of Science service using TAPPI method are shown in Table1.

Table1 Chemical compositions of purple guinea grass

Components	% (w/w) dry matter
Holocellulose	68.8
Alpha-cellulose	41.7
Beta-cellulose	12.0
Gramma-cellulose	15.1
Lignin	10.4

4.2 Pretreatment optimization

Pretreatment condition to improve on cellulose susceptibility of purple guinea grass were optimized by varying concentration of sulfuric acid, concentration of calcium hydroxide, substrate loading and autoclaving period. Reducing sugar liberated in supernatant was analyzed. The condition which released maximum reducing sugar was selected for the next experiments.

4.2.1 Effect of sulfuric acid concentration on pretreatment

Pretreatment of purple guinea grass (3% w/v , DS) in various concentrations of H_2SO_4 (1.0-3.5 %w/v) at 121 $^{\circ}C$, 15 lb/inc^2 for 30 min revealed that pretreated purple guinea

grass residue was most susceptible to cellulase after it was pretreated by 3%(w/v) H_2SO_4 as shown in Figure 18. The reducing sugar obtained after cellulase hydrolysis was 163 mg/g (DS) of substrate.

4.2.2 Effect of calcium hydroxide (lime) concentration on pretreatment

Pretreatment of purple guinea grass (3% w/v , DS) in various concentration of lime (1.5-3.0%w/v) at 121 °C , 15 lb/inc^2 for 30 revealed that pretreated purple guinea grass residue was most susceptible to cellulase after it was pretreated by 2%(w/v) $Ca(OH)_2$ or by $Ca(OH)_2$ at 1.5g(DS) substrate/g $Ca(OH)_2$ as shown in Figure 19. The reducing sugar obtained after cellulase hydrolysis was 30 mg/g (DS) of substrate.

4.2.3 Effect of substrate loading on pretreatment

Pretreatment of purple guinea grass by 3%(w/v) H_2SO_4 at 121 °C , 15 lb/inc^2 for 30 min, reducing sugar released by cellulase hydrolysis was maximum at substrate loading of 6%(w/v) (Figure 20A). The reducing sugar obtained was 165 mg/g (DS) of substrate. Substrate loading at 6% (w/v) was selected for further experiments.

While pretreatment of purple guinea grass by $Ca(OH)_2$ at 1.5 g(DS) substrate/g $Ca(OH)_2$ at 121 °C, 15 lb/inc^2 for 30 min, increase of substrate loading resulted in higher reducing sugar released. The reducing sugar released was maximum at 84 mg/g (DS) when substrate loading was 6% (w/v) as shown in Figure 20B. Substrate loading at 6% (w/v) was selected for further experiments.

4.2.4 Effect of autoclaving period on pretreatment

Reducing sugar released after cellulase hydrolysis was maximum when purple guinea grass (6% w/v) was pretreated by 3%(w/v) H_2SO_4 at 121 °C, 15 lb/inc^2 for 30 min as shown in Figure 21A. The reducing sugar released was 173 mg/g (DS) of substrate.

Pretreatment of purple guinea grass by $Ca(OH)_2$ at 1.5 g (DS) substrate/g $Ca(OH)_2$ and 6% (w/v) substrate loading, reducing sugar released after cellulase hydrolysis was maximum when autoclaving period was 5 min as shown Figure 21B. The reducing sugar obtained was 111 mg/g (DS) of substrate.

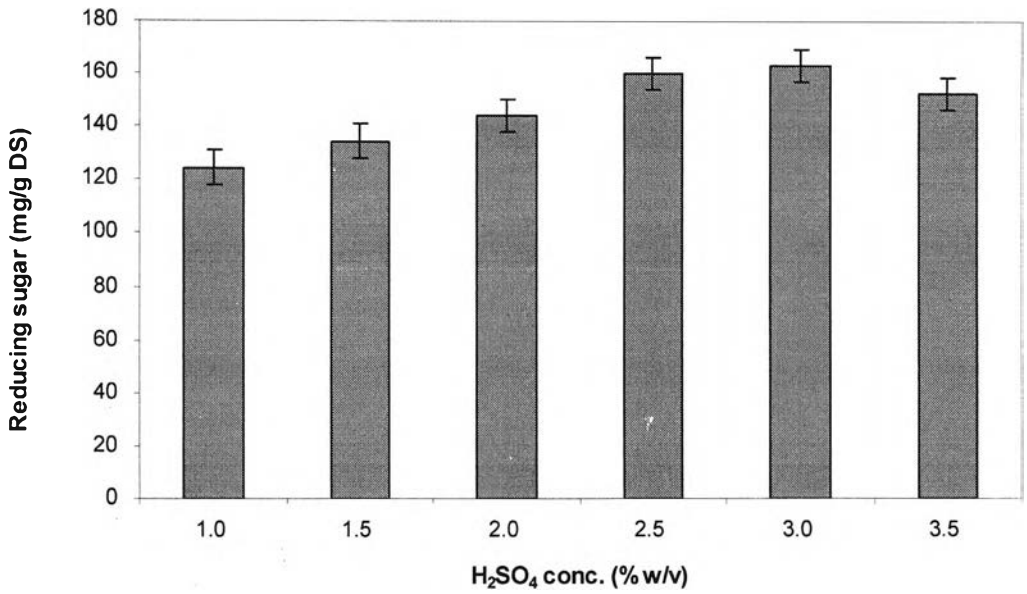


Figure 18 Effect of sulfuric acid concentration on cellulose susceptibility.

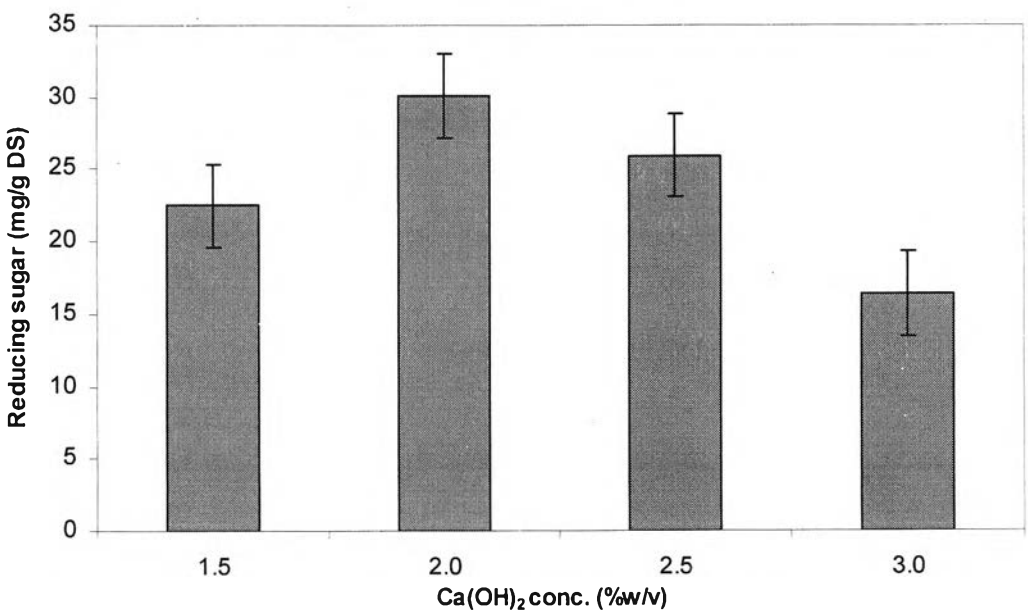
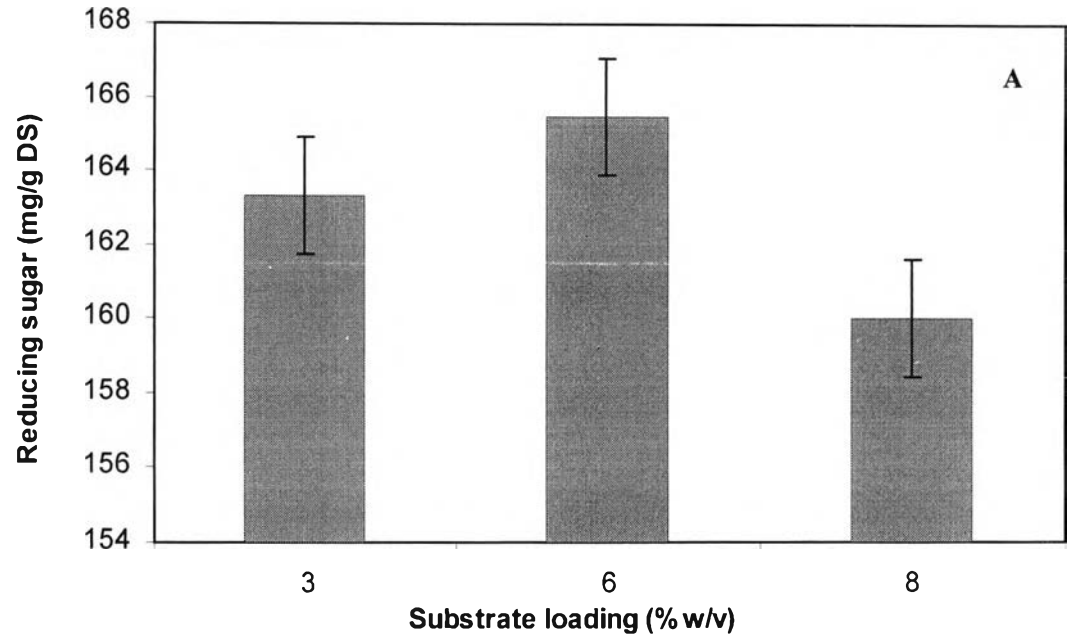


Figure 19 Effect of calcium hydroxide (lime) concentration on cellulose susceptibility.



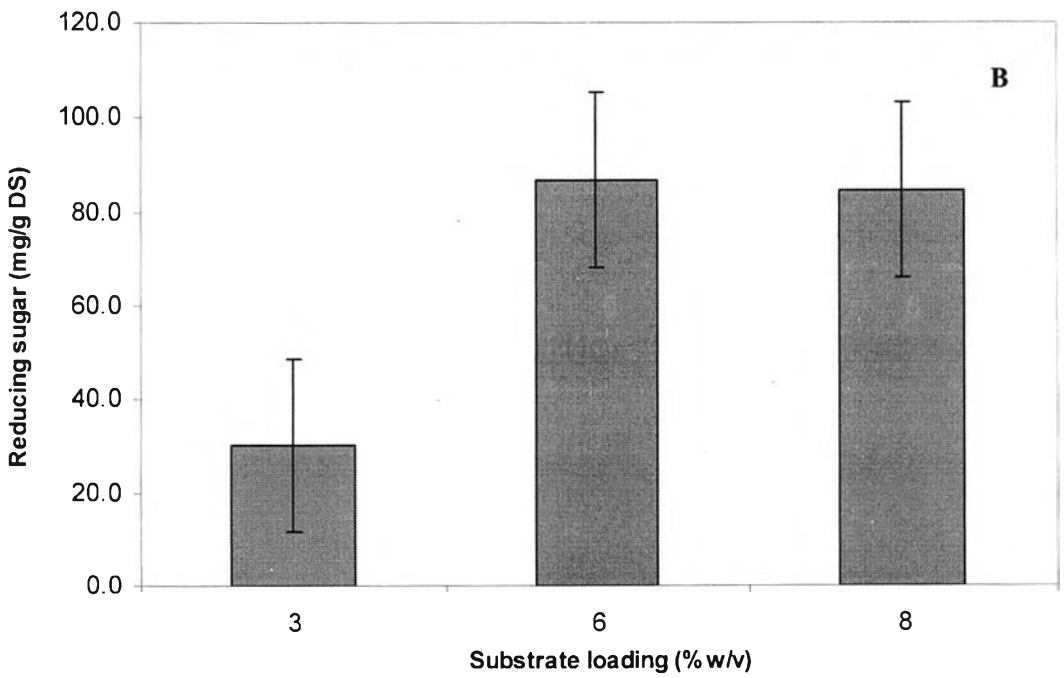
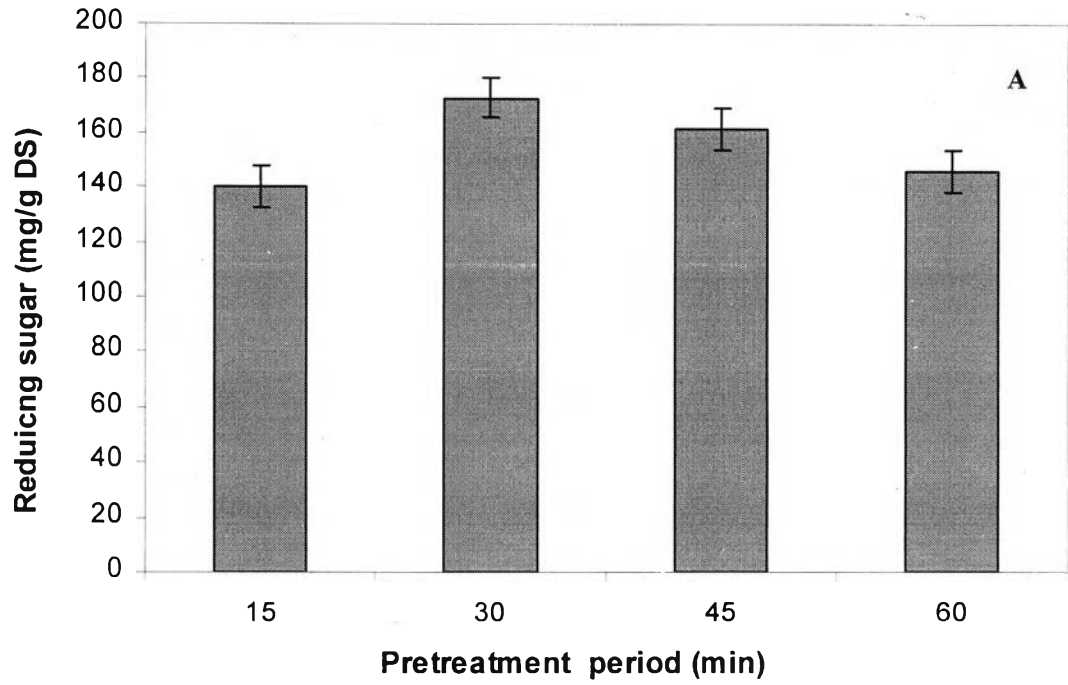


Figure 20 Effect of substrate loading on sulfuric acid pretreatment (A) calcium hydroxide or lime pretreatment (B) on cellulose susceptibility



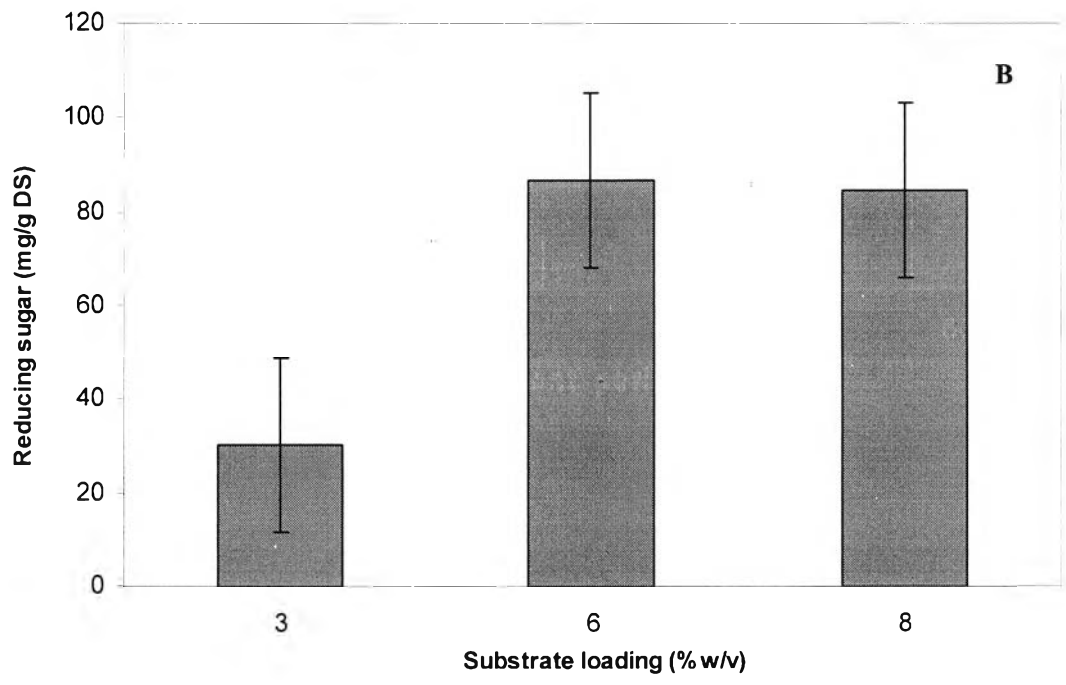


Figure 21 Effect of sulfuric acid pretreatment period (A) and calcium hydroxide or lime pretreatment period (B) on cellulose susceptibility of purple guinea grass residue.

4.3 Sugars and byproducts in pretreatment hydrolysate

Hydrolysate obtained from pretreatment of purple guinea grass by sulfuric acid and calcium-hydroxide at their optimal pretreatment conditions were pH adjusted to 7.0 and analyzed for following byproducts (furfural, hydroxymethylfurfural, 4-hydroxybenzaldehyde, syringaldehyde , vanillin) and sugars (xylose and glucose). As shown in Table 2, the concentration of all the byproducts analyzed was lower than the concentration which inhibited growth and ethanol fermentation of *S. cerevisiae* (Olsson and Hahn-Hagerdal, 1996; Palmqvist *et. al.*, 2000a). The sulfuric acid pretreatment hydrolysate contained xylose 4.6 mg/ml which was 3 times higher than glucose (1.5 mg/ml).

Table 2 Byproducts and sugars in an optimized pretreatment hydrolysate.

Pretreatment / By products	H_2SO_4 (mg/g DS)	$Ca(OH)_2$ (mg/g DS)
Hydroxymethylfurfural (HMF)	0.124	ND
Furfural	0.303	ND
4-Hydrobenzaldehyde	0.024	0.010
Vanillin	0.035	0.008
Syringaldehyde	ND	0.017
Xylose	71.51	ND
Glucose	22.98	ND

ND = Non detectable

4.4 Cellulase hydrolysis

Purple guinea grass was pretreated by H_2SO_4 and $Ca(OH)_2$ at the optimized condition (result of 4.2.1-4.2.4), then hydrolysed by Accellerase ™ 1000 (45 FPU/g DS of substrate or 400 units of β-glucosidase/g DS of substrate) at 53, 106, 159, 212 FPU/g DS of substrate (471, 942, 1413, 1884 units of β-glucosidase /g DS of substrate) for 6 hours. Reducing sugar released by H_2SO_4 pretreated purple guinea grass residue (261 mg/g (DS)) was higher than $Ca(OH)_2$

pretreated purple guinea grass residue (254 mg/g (DS)). But cellulose susceptibility of purple guinea grass residue determined from glucose released indicated that cellulose susceptibility of purple guinea grass residue pretreated with H_2SO_4 was lower than these pretreated with $Ca(OH)_2$. Glucose released by purple guinea grass which was pretreated with H_2SO_4 and $Ca(OH)_2$ was 168.3 and 198.3 mg/g DS, respectively. So, purple guinea grass was pretreated by $Ca(OH)_2$ in next experiments.

In cellulase hydrolysis experiment, purple guinea grass (6% w/v) was pretreated by $Ca(OH)_2$ (1.5 g (DS)/g $Ca(OH)_2$) at 121 °C, 15 lb/inc^2 for 5 min and saccharified by Accellerase™ 1000 (45 FPU/g DS of substrate or 400 units of β-glucosidase /g DS of substrate). Glucose liberated when enzyme dose (mg/g (DS) substrate) and saccharification time were varied are shown in Table 3.

Table 3 (A) Effect of enzyme dose on glucose liberated in cellulose hydrolysate of $Ca(OH)_2$ pretreated purple guinea grass residue.

Saccharification Time / Enzyme (FPU/g DS)	6 h	
	Glucose (mg/ml)	Glucose (mg/unit)
53	11.90	11.23
106	12.50	5.90
159	13.10	4.12
212	12.40	2.92

Table 3 (B) Effect of saccharification time on glucose liberation of $Ca(OH)_2$ pretreated purple guinea grass residue.

Saccharification Time / Enzyme (FPU/g DS)	6h	12h	18h
	Glucose (mg/h)	Glucose (mg/h)	Glucose (mg/h)
53	99.17	53.33	35.14

As shown in Table 3(A), glucose liberated after 6 hours hydrolysis was maximum when the enzyme (159 FPU/g (DS) or 1413 units of β-glucosidase/g DS of substrate) was used. But an efficiency of enzyme on glucose liberation was maximum (11.23 mg glucose/unit) at an enzyme dose of 53 FPU/g (DS)(471 units of β-glucosidase/g DS of substrate). From this result, the $Ca(OH)_2$ pretreated purple guinea grass residue was saccharified by the Accellerase ™ 1000 (53 FPU/g (DS) or 471 units of β-glucosidase/g DS of substrate) and glucose liberated was analyzed after 6, 12 and 18 hours (Table 3B). Glucose liberated was maximum at 640 mg after hydrolysis for 12 hours. But glucose liberation rate was decreased from 99.17 mg/h during the 6 hours to 53.33 mg/h during 6^{th} to 12^{th} of hydrolysis. To reduce a risk of glucose lost due to contamination during cellulase hydrolysis, saccharification time (6 hours) was used in further experiments.

4.5 Ethanol production

4.5.1 Ethanol production by Separate Hydrolysis and Fermentation (SHF) method

Cellulose hydrolysate obtained from saccharification of $Ca(OH)_2$ pretreated purple guinea grass residue by Accellerase™1000 (53 FPU/g (DS) substrate or 471 units of β-glucosidase/g DS of substrate) for 6 hours was used as substrate for ethanol fermentation by *S. cerevisiae*. The fermentation was performed at pH 4.5, 30°C, oxygen limit condition.

4.5.1.1 Effect of fermentation period on ethanol production by SHF method

In this experiment, ethanol produced in the fermentation broth was analyzed every 12 hours. Maximum ethanol (5.90 g/l) was produced at 48 hours. Therefore, the incubation period of 48 hours was used for ethanol production by SHF method in next experiments (Figure 22).

4.5.1.2 Effect of $(NH_4)_2SO_4$ supplementation on ethanol production by SHF method

Hydrolysate obtained after cellulase hydrolysis supplemented by various concentration of $(NH_4)_2SO_4$ (0, 0.2, 0.4, 0.6 %w/v) was used as substrate for ethanol fermentation. At 48 hours, maximum ethanol produced was 5.90 g/l without $(NH_4)_2SO_4$ supplementation as shown in Figure 23.

4.5.1.3 Effect of nutrient supplementation on ethanol production by SHF method

Hydrolysate obtained after cellulase hydrolysis supplemented by yeast extract 0.3% (w/v) and $(NH_4)_2HPO_4$ 0.25% (w/v) was used as substrate for ethanol fermentation. At 48 hours, maximum ethanol produced in the hydrolysate with and without the supplementation was 5.77 and 5.90 g/l respectively (Figure 24).

4.5.1.4 Effect of inoculum medium on ethanol production by SHF method

In this experiment, cells of *S. cerevisiae* inoculum (3.6.2) harvested by centrifugation, resuspended in YPD medium or YPD without glucose (2% (w/v)) was used as an inoculum. The hydrolysate obtained after cellulase hydrolysis which contained 11.90 g/l glucose without any supplementation was used as substrate for ethanol fermentation. At 48 hours, maximum ethanol (5.90 and 5.24 g/l) was produced in the cellulose hydrolysate inoculated with *S. cerevisiae* cell suspended in YPD and in YPD without glucose, respectively (Figure 25). This result indicated that glucose liberated from cellulase hydrolysis of pretreated purple guinea grass

residue (11.90 g/l) gave ethanol by SHF process at 0.087 g/g (DS) of purple guinea grass or ethanol 0.44 g/g glucose.

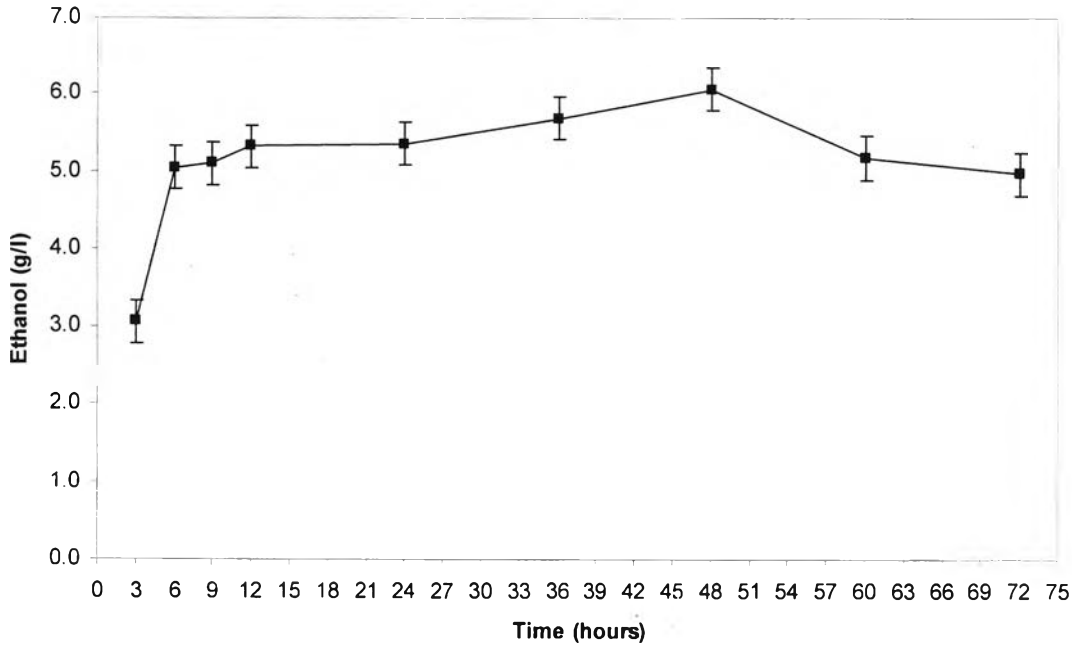


Figure 22 Effect of fermentation period on ethanol production by SHF method.

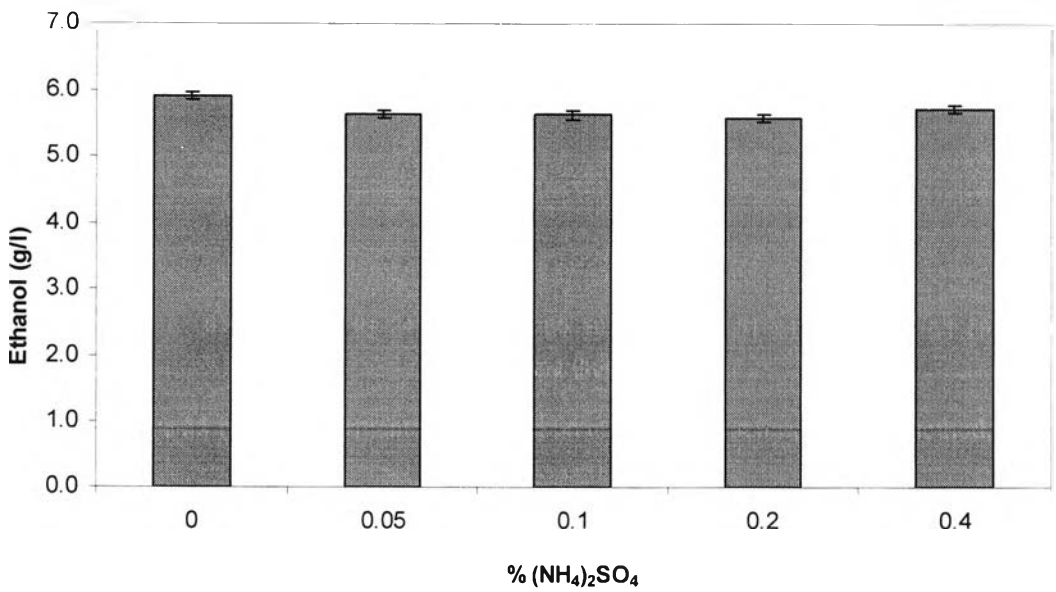


Figure 23 Effect of $(NH_4)_2SO_4$ supplementation on ethanol production by SHF method after 48 hours.

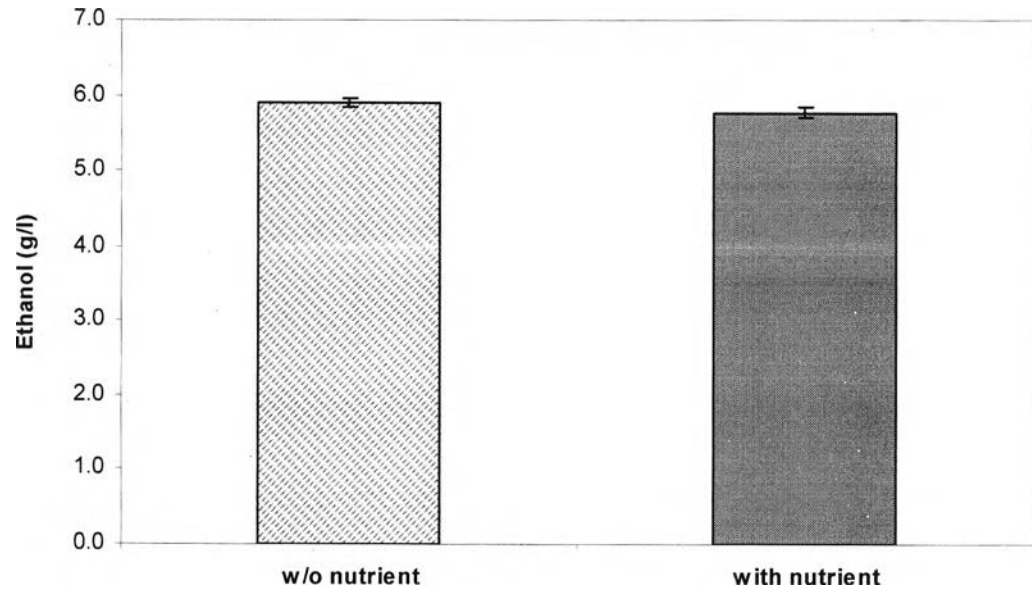


Figure 24 Effect of nutrient supplementation on ethanol production by SHF method after 48 hours.

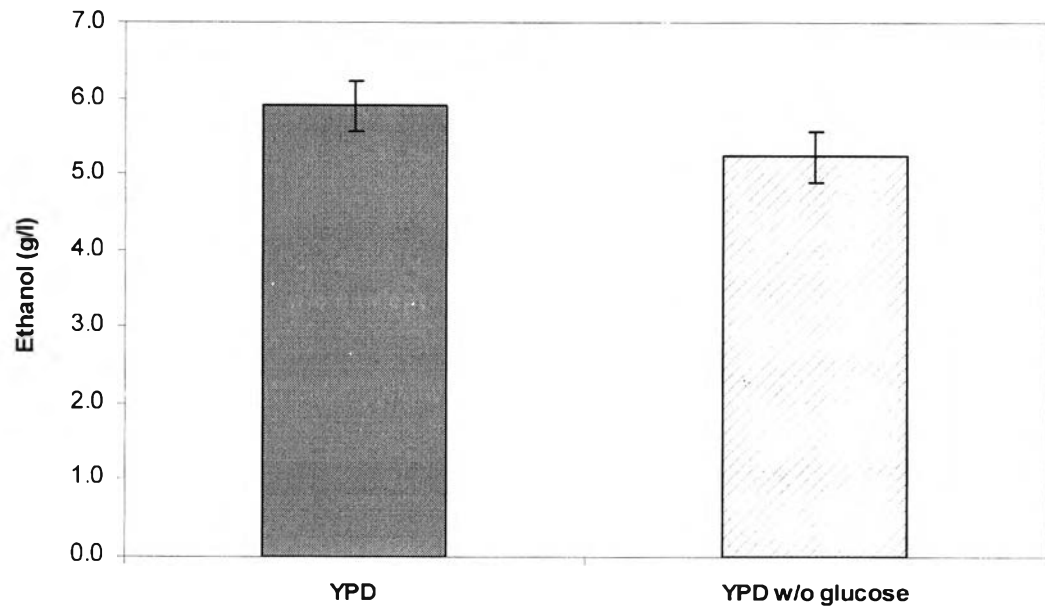


Figure 25 Effect of inoculum medium on ethanol production by SHF method after 48 hours.

4.5.2 Ethanol production by Simultaneous Saccharification and Fermentation (SSF) method

The $Ca(OH)_2$ pretreated purple guinea grass residue was washed with distilled water, resuspended in sodium citrate buffer pH 5.0 at original volume then simultaneous saccharified by Accellerase™1000 (53 FPU/g (DS) or 471 units of β-glucosidase/g DS of substrate) and ethanol fermented by *S. cerevisiae* at 30 °C for 72 hours.

4.5.2.1 Effect of temperature on ethanol production by SSF method

The $Ca(OH)_2$ pretreated purple guinea grass residue was simultaneous saccharified and ethanol fermented at various temperature (25, 30, 35, 40 and 50 °C). After 48 hours, maximum ethanol (3.85 g/l) was produced at 30 °C as shown in Figure 26.

4.5.2.2 Effect of pH on ethanol production by SSF method

In this experiment, the simultaneous saccharification and ethanol fermentation of $Ca(OH)_2$ pretreated purple guinea grass residue was performed at 30 °C, various pH (4.5, 5.0 and 5.5). After 48 hours, maximum ethanol (3.85 g/l) was produced at pH 5.0 as shown in Figure 27.

4.5.2.3 Effect of fermentation time on ethanol production by SSF method

The $Ca(OH)_2$ pretreated purple guinea grass residue was saccharified by Accellerase™ 1000 (53 FPU/g (DS) or 471 units of β-glucosidase/g DS of substrate) along with fermented by *S. cerevisiae* (10% v/v inoculum) at pH 5.0, 30°C for 120 hours. Ethanol produced was determined every 24 hours. Maximum ethanol (4.65 g/l) was produced at 96 hours after incubation as shown in Figure 28.

4.5.2.4 Effect of nutrient supplementation on ethanol production by SSF method

The hydrolysate obtained after cellulase hydrolysis supplemented by yeast extract 0.3 % (w/v) and $(NH_4)_2HPO_4$ 0.25% (w/v) was used as substrate for ethanol fermentation. At 96 hours, maximum ethanol (4.60 g/l and 4.65 g/l) was produced in the hydrolysate supplemented by yeast extract + $(NH_4)_2HPO_4$ and without supplementation, respectively (Figure 29).

4.5.2.5 Effect of inoculum medium on ethanol production by SSF method

In this experiment, cells of *S. cerevisiae* inoculum (3.6.2) harvested by centrifugation, resuspended in YPD with and without glucose (2% (w/v)) was used as an inoculum. The hydrolysate obtained after cellulase hydrolysis without any supplementation was used as substrate for ethanol fermentation. After 72 hours, maximum ethanol (4.65 and 4.45 g/l) was produced from the cellulose hydrolysate inoculated with *S. cerevisiae* cells suspended in YPD and YPD without glucose, respectively (Figure 30). The result indicated that glucose liberated from the cellulase hydrolysis of pretreated purple guinea grass residue gave ethanol 0.074 g/g (DS) of purple guinea grass by SSF method.

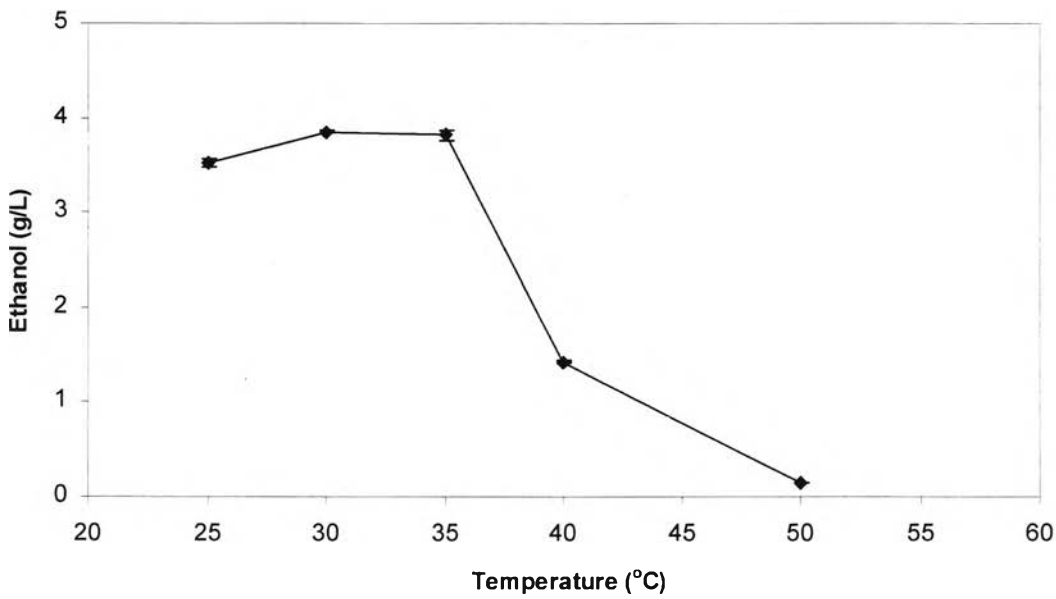


Figure 26 Effect of temperature on ethanol production by SSF method after 48 hours.

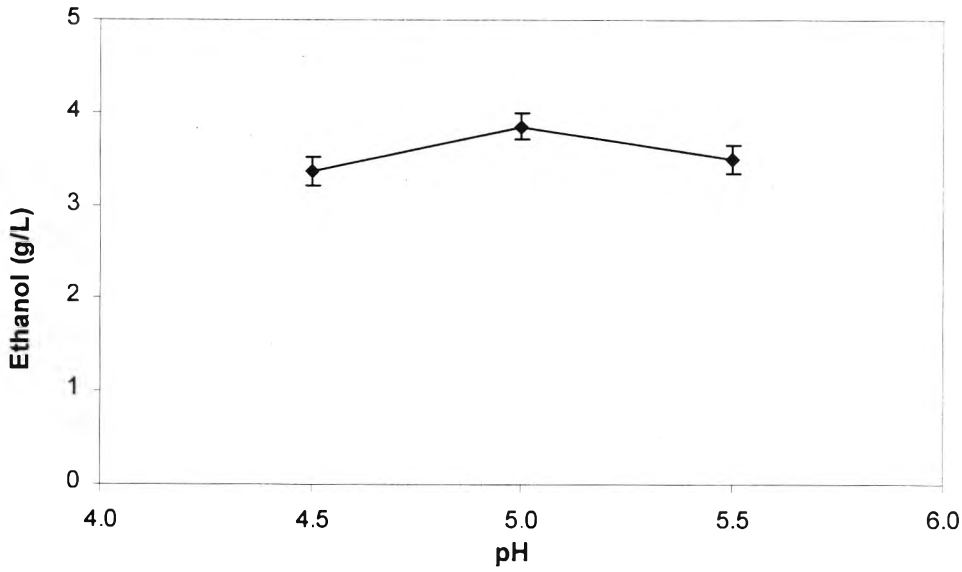


Figure 27 Effect of pH on ethanol production by SSF method after 48 hours.

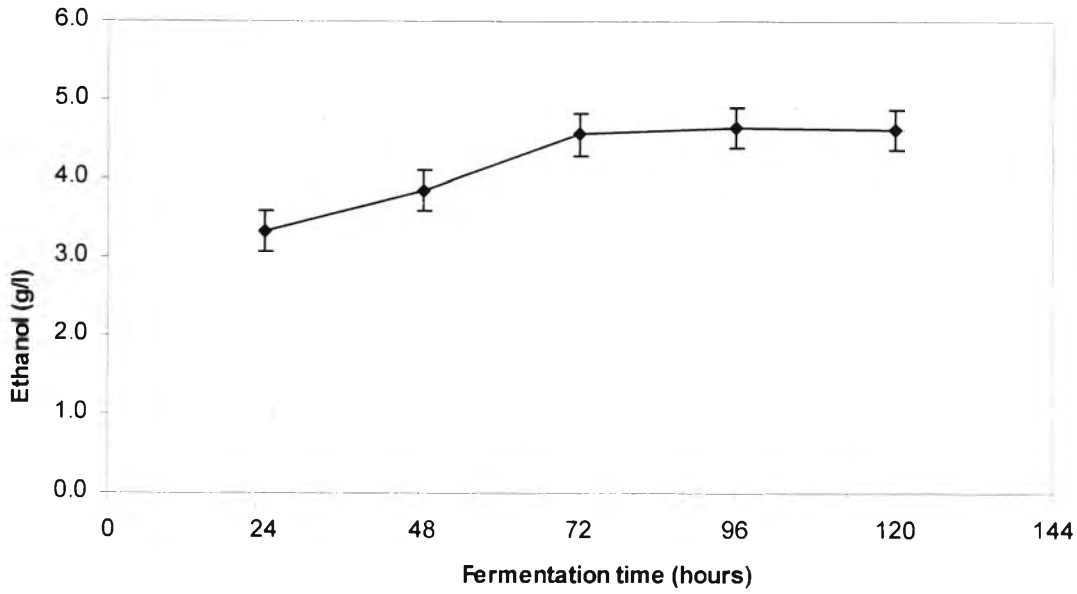


Figure 28 Effect of fermentation time on ethanol production by SSF method.

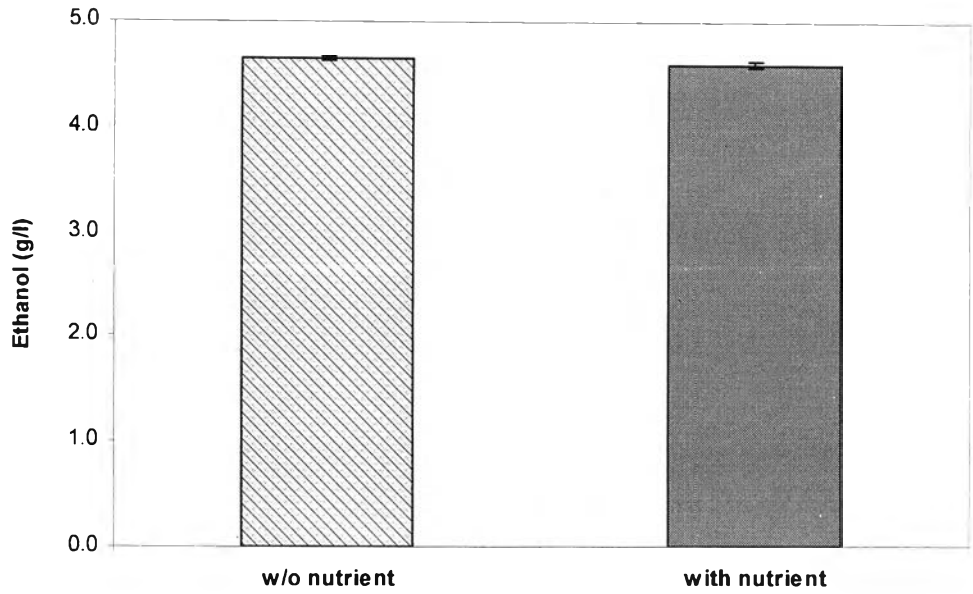


Figure 29 Effect of nutrient supplementation on ethanol production by SSF method after 96 hours.

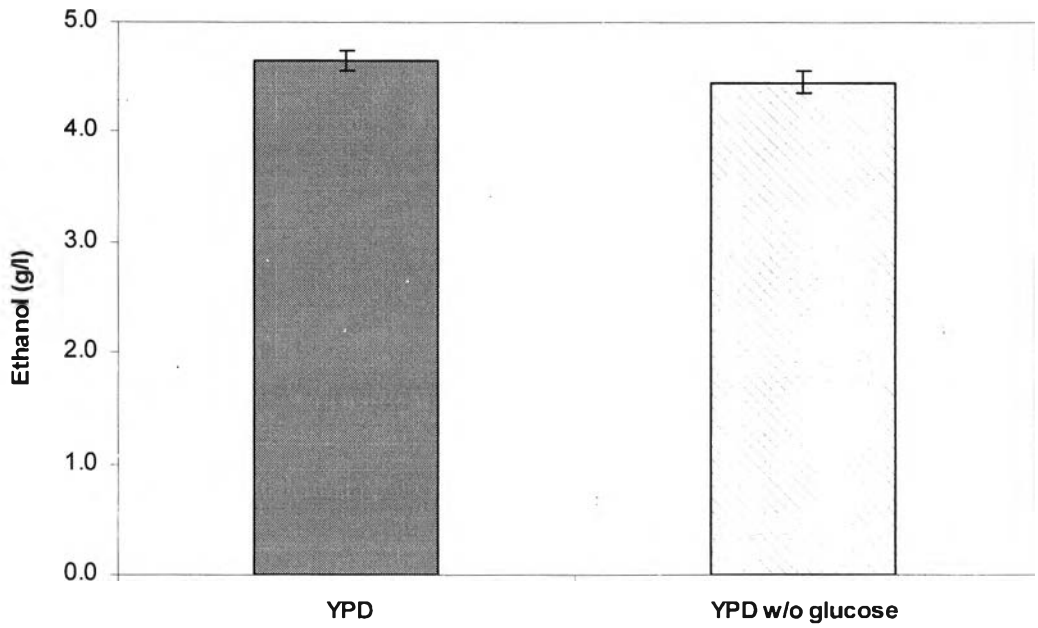


Figure 30 Effect of inoculum medium on ethanol production by SSF method after 96 hours.

4.6 Scale up of the ethanol fermentation

This experiment, SHF was performed in 5.0 L fermentor with working volume 3.0 L. Cells of *S. cerevisiae* prepared as described in 3.6.2 and harvested by centrifugation, resuspended in YPD without glucose (2% (w/v)) was used as an inoculum. The purple guinea grass (6% w/v) was pretreated by 2% $Ca(OH)_2$ (1.5 g DS substrate/g $Ca(OH)_2$) at 121 °C, 15 lb/inc^2 for 5 min then further hydrolyzed with 53 FPU/g (DS) substrate or 471 units of β-glucosidase/g DS of substrate of Accellerase ™1000 for 6 hours. The hydrolysate obtained after cellulase hydrolysis which contained 12.0 g/l glucose without any supplementation was used as substrate for ethanol fermentation. After 9 hours, maximum ethanol (5.92 g/l) was produced in the hydrolysate inoculated with *S. cerevisiae* cell suspended in YPD without glucose. This result indicated that glucose liberated from cellulase hydrolysis of pretreated purple guinea grass residue (12.0 g/l) gave ethanol by the SHF method at 0.099 g/g (DS) of purple guinea grass or ethanol 0.497 g/g glucose (Figure 31).

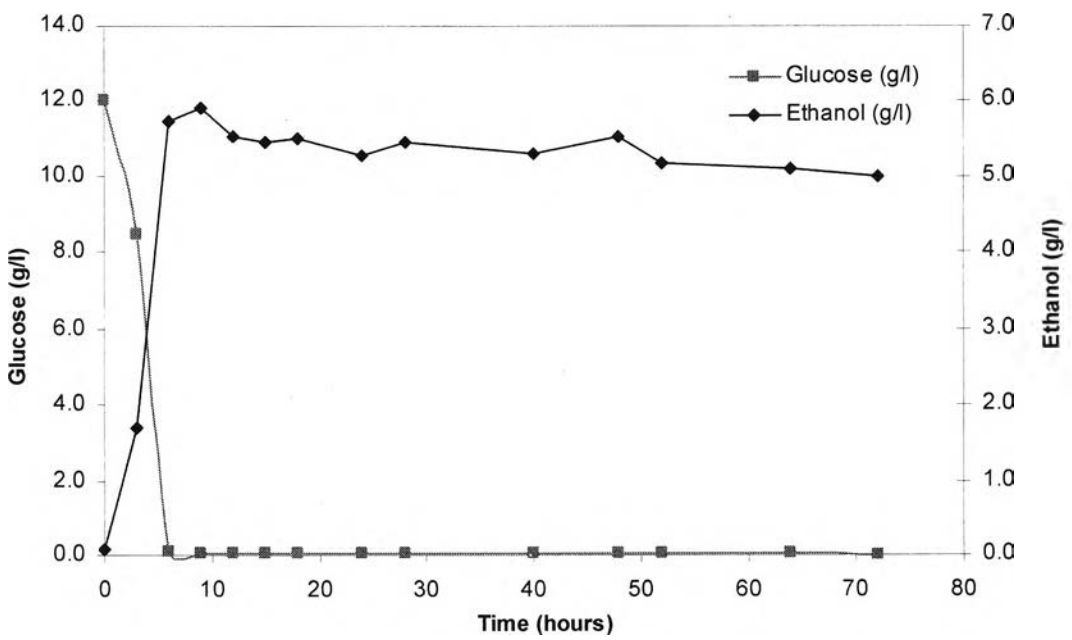


Figure 31 Separate hydrolysis and ethanol fermentation of purple guinea grass in 5L fermentor.