CHAPTER IV
RESULTS AND DISCUSSION

4.1 Particle size analysis of mission grass

Dry mission grass underwent a physical pretreatment through milling. Milling decreases the particle size and essentially the crystalline structure, which results in an enhancement of surface area. The increase of surface area allows mission grass to be further pretreated and digested more easily (Quintero *et al.*, 2011). After milling with 60 mesh sieving size, the size of the powdered mission grass was determined using a particle size analyzer. According to Sluiter, the optimized size for lignocellulosic biomass hydrolysis for ethanol production should stay in between -20/+80 mesh particle size. A larger mesh size would cause inefficient hydrolysis of carbohydrates, and lead to lower amount of sugar released. Alternatively, over-hydrolysis of carbohydrates would occur if smaller mesh size is used, results in an increment of sugar degradation products (Sluiter *et al.*, 2010). The degradation products may potentially cause detrimental effects on fermenting yeasts as well as ethanol yield.

The average size of the milled mission grass obtained from Tak Province is approximately 300 μm. The milled size of Tak mission grass is comparable to size of the milled grass in a study by Tatijarern, where his mission grass from Nakornratchasima Province has particle size of 330 μm after using 60 mesh sieving size (Tatijarern *et al.*, 2013).

4.2 Chemical composition of mission grass

The milled mission grass was used to find the chemical composition by following the method from the National Renewable Energy Laboratory (NREL) (Sluiter *et al.*, 2010). The chemical composition of mission grass in this study (obtained from Tak

Province, Thailand) is compared to that in the previous study (obtained from Nakornratchasima Province, Thailand) in **Table 4.1** (Tatijarern *et al.*, 2013).

Table 4.1 The chemical compositions of mission grass obtained from Tak and Nakornratchasima Provinces, Thailand

Composition (%)[a]	Mission grass (*Pennisetum polystachion*)	
	Tak Province	Nakornratchasima Province (Tatijarern *et al.*, 2013)
Cellulose	47.2	39.8
Hemicellulose	27.3	29.2
Lignin	18.2	14.6
Ash	2.56	3.3

[a] Dry weight percentages

The mission grass from Tak Province presents a better candidate for ethanol production due to its higher percentage of cellulose. The higher percentage of cellulose is more preferable as cellulose is the main source of sugar for fermentation. However, Tak mission grass is comprised of higher percentage of lignin which could become degraded during the pretreatment process to produce inhibitory compounds and hinder ethanol fermentation (Palmqvist *et al.*, 2000). Thus, further research could be developed into optimizing the pretreatment method of Tak mission grass.

4.3 Alkaline pretreatment of mission grass

Milled mission grass was chemically pretreated with 3% w/v NaOH, and hydrolyzed using 1% v/v H_2SO_4 with 15:1 liquid-to-solid ratio according to the optimized grass pretreatment method (Boonmanumsin *et al.*, 2012, Tatijarern *et al.*, 2013). Then, it was further hydrolyzed by cellulase from *Trichoderma reesei ATCC*

26921 to ensure complete saccharification. **Figures 4.1a-e** illustrates the scanning electron microscope (SEM) images of mission grass at various stages of pretreatment and hydrolysis processes. The images have been magnified 1000x.

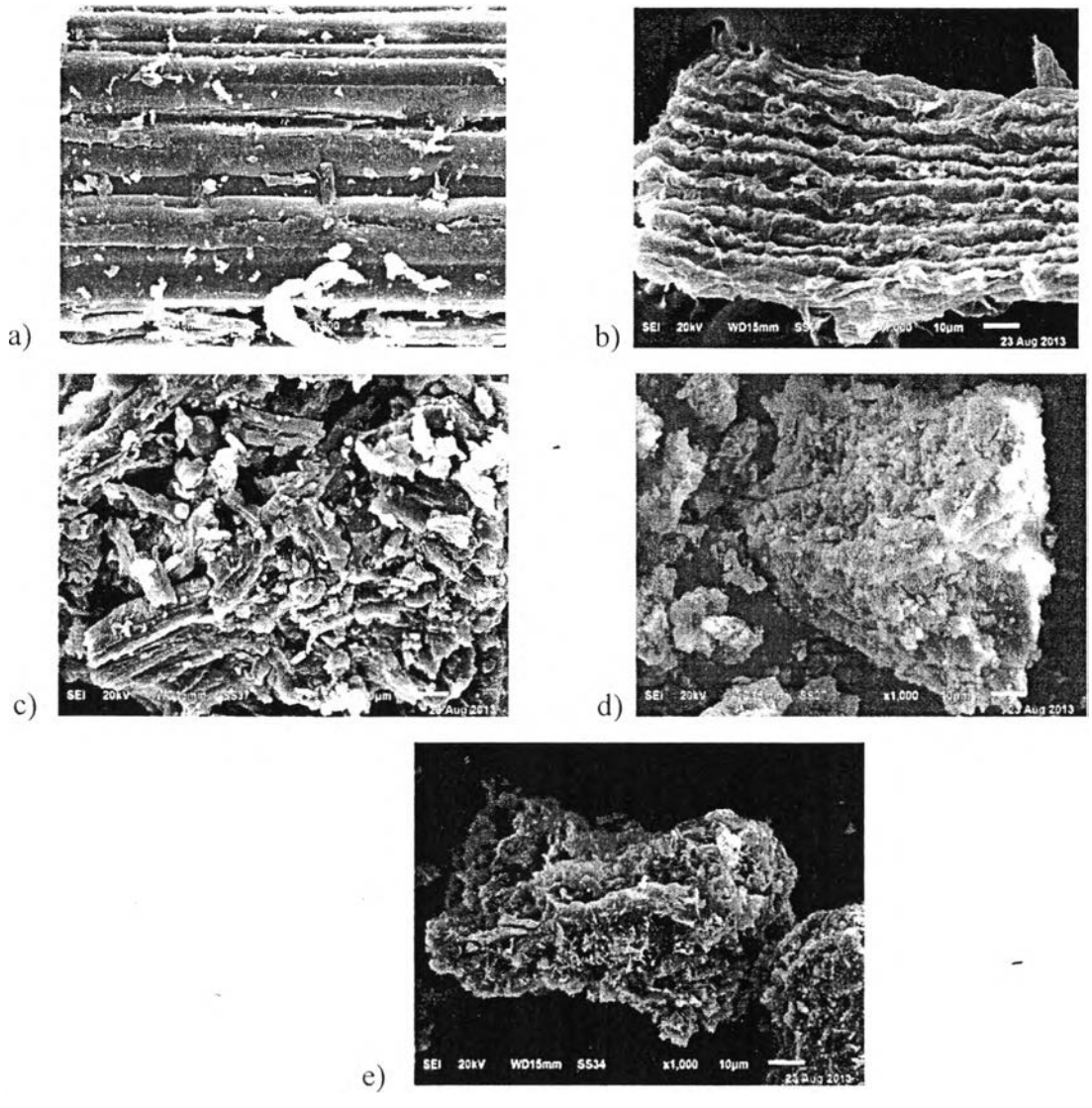

Figure 4.1 The SEM images in each treatment stage of mission grass (1000x). **a**) Milled raw mission grass **b**) Mission grass after alkaline pretreatment assisted by microwave **c**) Mission grass after acid hydrolysis assisted by microwave **d)** Mission grass after enzymatic hydrolysis **e**) Mission grass after overliming process at pH 10

The SEM image of milled raw mission grass in **Figure 4.1a** shows the highly fibrillar structure covered by thin waxy layer on the surface of that is frequently found in herbaceous biomass (Hu *et al.*, 2008). In comparison to the SEM image of untreated raw switchgrass, mission grass possesses a more ordered structure with less waxy film. After treating the grass in dilute alkaline, the crystalline structure of mission grass fibrils is noticeably disrupted **(Figure 4.1b)**. The waxy layer on the surface is partially removed, which indicates the breaking down of the lignin (Hu *et al.*, 2008).

4.4 Acid hydrolysis of mission grass

The breakdown of fibrils in mission grass after dilute acid hydrolysis assisted by microwave can easily be observed in **Figure 4.1c**. The dilute acid primarily solubilizes and ruptures hemicellulose. The structure of crystalline cellulose can be seen in the SEM image. Acid hydrolysis causes chemical changes in hemicellulose which disrupts covalent bonds, hydrogen bonds, and van der Waals forces (Li *et al.*, 2010). As the result, the treatment allows cellulose and hemicellulose to become more susceptible to enzymatic hydrolysis. In addition to having higher cellulose content than switchgrass (47% of cellulose in mission grass, and 34% in switchgrass (Hu *et al.*, 2008)), the lower lignin content further ensures that mission grass would be a better candidate for ethanol production. The presence of lignin that is localized on the grass' surface is evidently less in mission grass than that in the switchgrass. The image of residual lignin condensing on the surface of the cellulose confirms that acid treatment possesses only minimal effect on lignin removal (Yu *et al.*, 2011). This step forms degradation products which could negatively affect enzymatic hydrolysis and fermentation processes (Eliana *et al.*, 2014).

4.5 Enzymatic hydrolysis of mission grass

The SEM image of mission grass after being treated with cellulase shows a thorough destruction of cellulose fibrils **(Figure 4.1d)**. The cellulose crystalline structure can no longer be observed after enzymatic hydrolysis. Compared to enzymatic hydrolysis of pretreated eucalyptus, sorghum bagasse, and sugarcane bagasse, mission grass can be more efficiently hydrolyzed when enzyme treatment is used (Wang *et al.*, 2012). The SEM image also suggests that the current pretreatment of mission grass is efficient. Sugars including glucose are released in this step (Wongwatanapaiboon *et al.*, 2012). Cellulase can liberate twice the amount of glucose in comparison to the glucose released by acid hydrolysis alone, as shown in **Figure 4.2**.

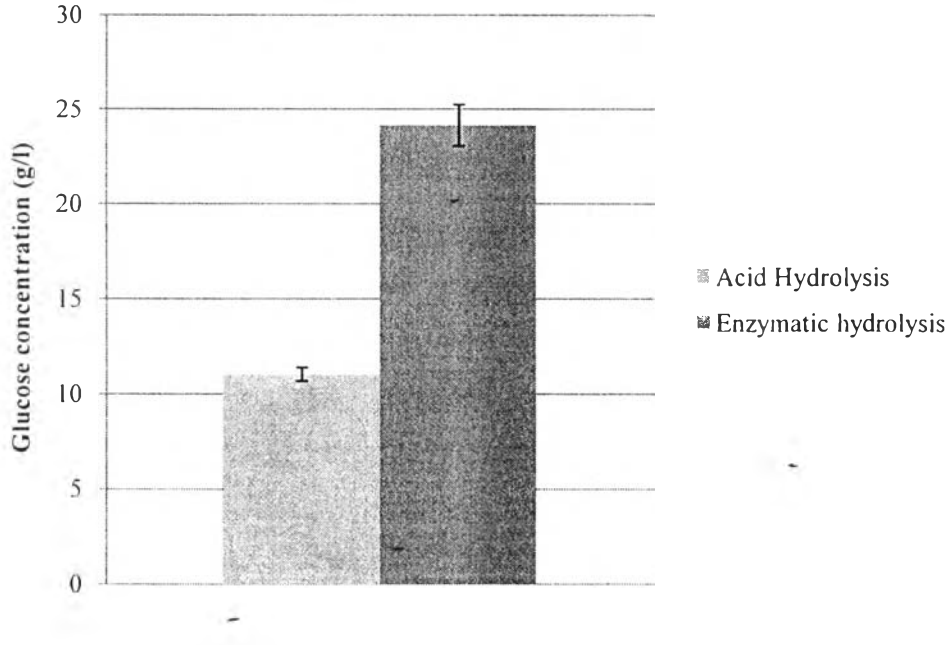

Figure 4.2 Glucose concentration obtained after acid hydrolysis and after enzymatic hydrolysis

From the graph, the glucose concentration increases by two folds after using cellulase enzyme. The amount of glucose obtained in this study is comparable to other studies where cellulase from *Trichoderma reesei* is used (Saha, 2003, Wongwatanapaiboon *et al.*, 2012). Enzymatic hydrolysis becomes a very attractive

method because no inhibiting compounds such as furfural and hydroxymethylfurfural are created from this pretreatment step (Wongwatanapaiboon *et al.*, 2012).

4.6 Optimization of detoxification process on mission grass hydrolyzate

4.6.1 Physical detoxification

Physical detoxification involves no addition of other chemicals into the lignocellulosic hydrolyzate. Some common physical detoxification methods include evaporation and membrane separation (Chandel *et al.*, 2011). Evaporation was chosen as the method of physical detoxification in this study due to its low cost and ease of operation.

Vacuum evaporation

Vacuum evaporation could remove inhibiting volatile compounds such as furfural, and acetic acid (Taherzadeh *et al.*, 2011). Another advantage of evaporation is that glucose concentration in the hydrolyzate can be regulated. A study on the production of ethanol from post-harvest sugarcane residue shows that it could take up to 12 days for the fermenting yeast to produce the maximum ethanol if evaporation is not used, and the maximum concentration of ethanol produced from the study is 336 mg/l (Dawson *et al.*, 2007). The long fermenting time and low yield indicate that the sugar in the hydrolyzate is extremely diluted.

In this study, approximately 60% of water was evaporated to increase the concentration of glucose. According to Larsson *et al.*, 10% of hydrolyzate evaporation could remove more than 40% of furfural, one of the major glucose degradation products and inhibiting compounds in the fermentation process (Larsson *et al.*, 1999). Further evaporation could remove more toxic compounds including acetic acid, formic acid, and other lignin degradation products. However, fermentative microorganisms like yeasts still require water to regulate their metabolism. If water is scarce, they would produce glycerol to regulate their cells. The production of glycerol could hinder the production of

ethanol because the carbon source that could be utilized to produce ethanol is redirected into the production of glycerol (Pagliardini *et al.*, 2013). However, too much water would dilute glucose concentration and result in lower ethanol production. Thus, the adjustment has to be made between water level and initial glucose concentration to optimize ethanol production.

4.6.2 Chemical detoxification

Many effective chemical detoxification techniques are available to remove inhibiting compounds from lignocellulosic hydrolyzate. A study from Larsson et al. involves the comparison between various methods of detoxification. According to his find, conditioning with calcium hydroxide, treatment with laccase, addition of sulfite at pH 10, and anion exchange at pH 10 are the most effective detoxification techniques (Larsson *et al.*, 1999). However, each method possesses various advantages and disadvantages in regards to amount of sugar loss, time, and cost. Therefore, an appropriate method of detoxification has to be decided prior the study by considering the resources available.

Combined detoxification: Overliming and addition of sodium sulfite

Combined detoxification was performed in the study because each detoxification method offers diverse capabilities in the removal of particular inhibiting compounds. Overliming was chosen as one of the detoxification techniques for mission grass hydrolyzate. A traditional overliming method involves addition of calcium hydroxide directly into the hydrolyzate to increase the pH. More degradation products can be removed as the overliming pH increases (Millati *et al.*, 2002). The overlimed hydrolyzate would then be filtered; and the pH would be adjusted for fermentation (Leonard *et al.*, 1945, Millati *et al.*, 2002). In this study, after overliming, sodium sulfite was subsequently added into the hydrolyzate, and the solution was filtered and pH-adjusted. A study by Telli-Okur *et al.* shows an increase in ethanol yield when the lignocellulosic

hydrolyzate is detoxified by combining overliming and sodium sulfite methods compared to overliming alone (Telli-Okur *et al.*, 2008).

Overliming proves to be a competent detoxification method. Despite its efficiency in removing inhibitory compounds and other degradation products as the pH increases, sugar loss also occurs (Mohagheghi *et al.*, 2006). **Figure 4.3** shows the amount of sugar loss at various overliming pH.

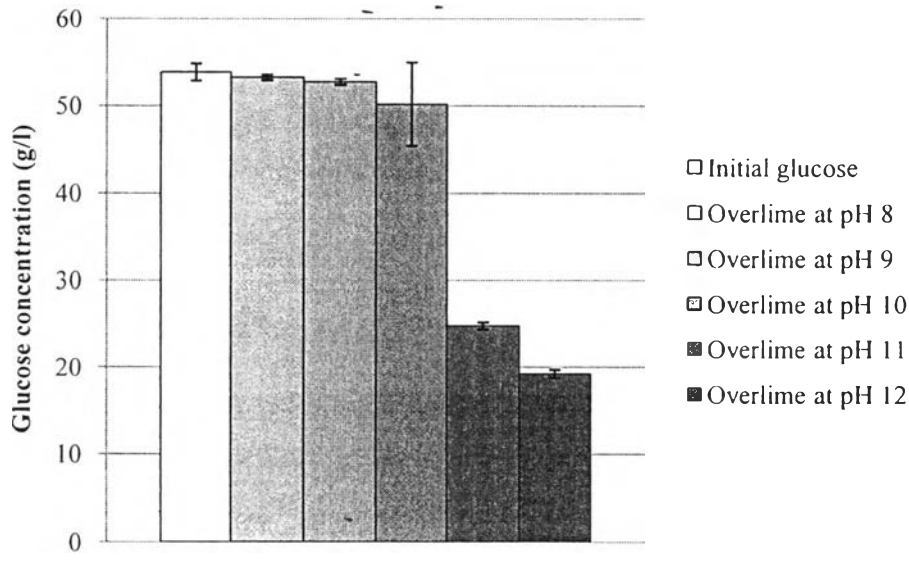

Figure 4.3 The effect of overliming in comparison to the hydrolyzate before overliming

Mohagheghi et al. claims that about 7%, 12%, and 14% of sugar is loss during conditioning at pH 9, 10, and 11, respectively (Mohagheghi *et al.*, 2006). The results of this study share the same trend as that of *Mohagheghi et al.* where glucose loss is inversely proportional to overliming pH. However, as low as 2% and 7% of glucose concentration is lost at pH 9 and 10 overliming, respectively, while up to 53% is lost at pH 11. The results are similar to that of *Millati et al.* where the glucose concentration does not reduce significantly when overliming is performed at pH 10, but is halved at pH 12. The sugars that are lost during overliming could be converted into lactic acid

(Millati *et al.*, 2002). With drastic sugar loss at pH 12, the concentration of the major inhibitors such as hydroxymethylfurfural and furfural almost reaches 0 g/l (Millati *et al.*, 2002).

Conversely, other inhibitors such as acetic acid and phenol products are not removed. The possible explanation is that during overliming with calcium hydroxide, acetic acid is combined with calcium to form soluble calcium acetate which cannot be taken out by filtration. Phenols from lignin degradation products are the inhibitors that cannot be eliminated by overliming, thus, they are the only inhibiting compounds that are still present and would be harmful to fermenting organisms. However, the phenol products only shows their inhibiting abilities when they reach over 1 g/l (Millati *et al.*, 2002). Therefore, overliming technique would be particularly suitable for lignocellulosic biomass with low lignin content. Consequently, the optimization of overliming pH is essential in order to obtain the highest concentration of ethanol.

4.7 Fermentative microorganisms

A variety of microorganisms ranging from fungi, bacteria, and yeasts could be exploited into producing ethanol from grass hydrolyzate. Baker's yeast, *Saccharomyces cerevisiae*, is one of the most popular candidates for ethanol fermentation. In addition to producing ethanol as the main product, yeasts *S. cerevisiae* possess high tolerance against inhibiting compounds compared to other microorganisms (Almeida *et al.*, 2007). One sole disadvantage of employing *S. cerevisiae* as the ethanol fermenter is their incapability to produce ethanol from other sugars beside glucose. **Figure 4.4** shows the growth of Baker's yeast (*Saccharomyces cerevisiae* TISTR 5049) in mission grass hydrolyzate at various range of overliming pH.

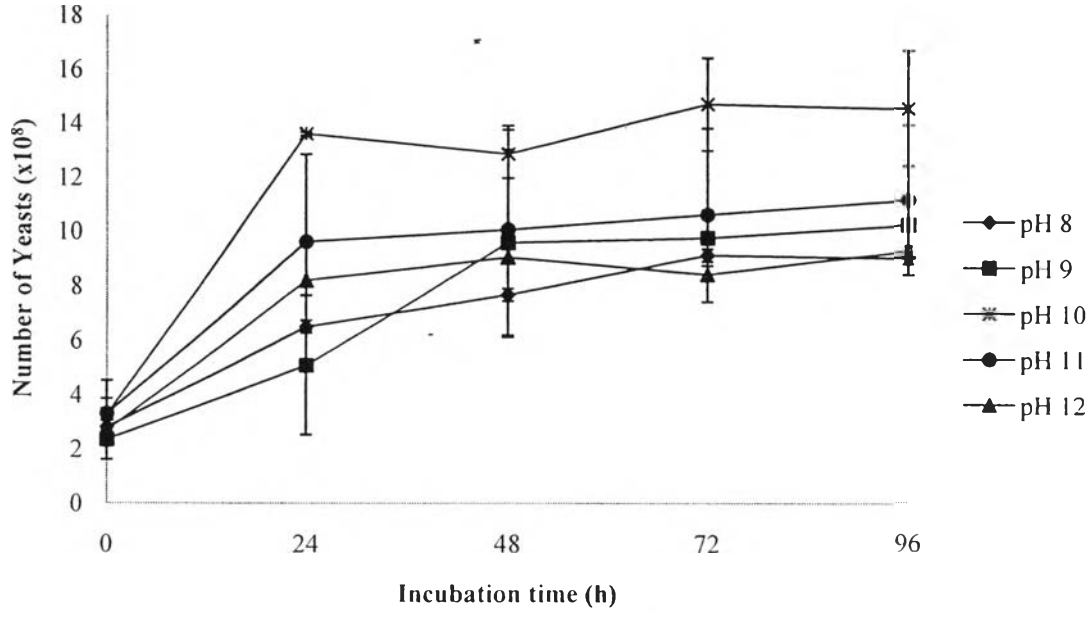

Figure 4.4 Yeast (*S. cerevisiae* TISTR 5049) population at various overliming pH per incubation time

The number of *S. cerevisiae* in **Figure 4.4** begins to rise rapidly within 24 hours. After 48 hours, the number of *S. cerevisiae* remains relatively stable. This may be due to exhaustion of sugar as the population of yeast increases. Overliming at pH 10 produces the highest number of yeasts, which implies that pH 10 can adequately eliminate degradation products while still maintaining sufficient amount of glucose for the yeasts' growth. In addition, the SEM image of the mission grass hydrolyzate after overliming at pH 10 **(Figure 4.1e)** shows no presence of lignin residues as could be observed in **Figures 4.1c and d**. Overliming at pH 11 produces the second highest yeast count. This could perhaps be due to the elimination of many toxic compounds despite the loss of utilizable glucose. The similar explanation also applies when overliming is performed at pH 12. Even though overliming at pH 8 and 9 does not diminish as much glucose compared to that at higher pH, the process does not take away enough degradation and inhibitory compounds. The remaining compounds become toxic to the fermenting

yeasts, which results in lower number of yeasts and potentially lower ethanol yield (Eliana *et al.*, 2014).

The consumption of glucose by baker's yeast *S. cerevisiae* TISTR 5049 at various overliming pH is shown in **Figure 4.5**.

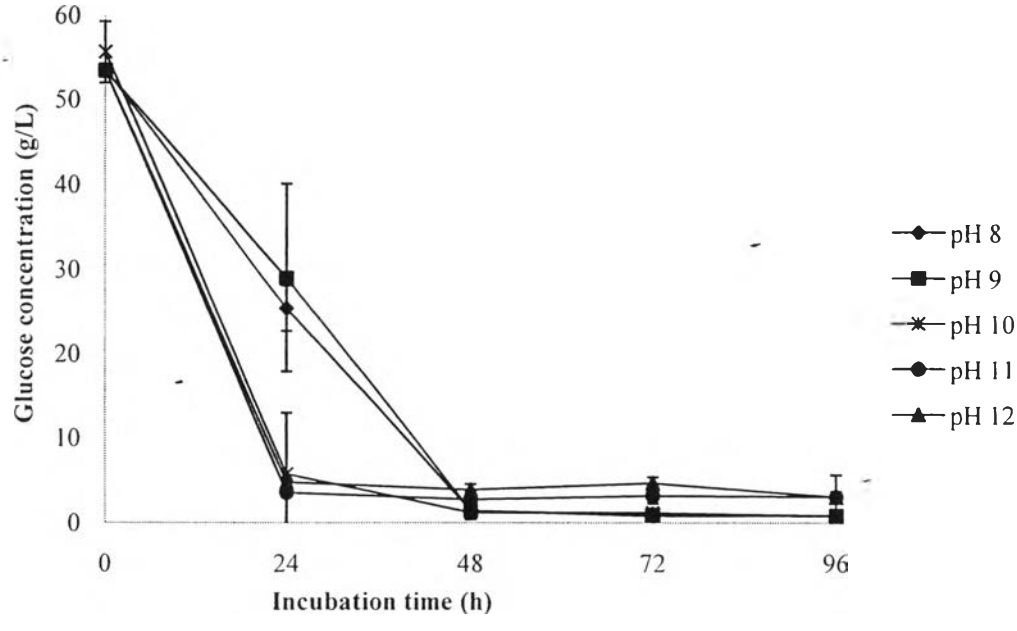

Figure 4.5 Glucose consumption of *S. cerevisiae* TISTR 5049 per incubation time

After 24 hours, glucose was almost used up when overliming was carried out at pH 10, 11, and 12. The trend indicates that overliming at pH 10 or higher can effectively remove inhibitory products from the hydrolyzate, enabling the yeast to immediately use the glucose. The graph further confirms the result in **Figure 4.4**. In contrast, some glucose still remained within 24 hours when conditioning at pH 8-9, which signifies that some glucose was not able to be utilized at once by the yeasts. This could possibly be due to residual inhibitory compounds such as furfural and hydroxymethylfurfural in the hydrolyzate (Mohagheghi *et al.*, 2006). Furfural and hydroxymethylfurfural inhibit enzymes such as alcohol dehydrogenase, pyruvate dehydrogenase, aldehyde dehydrogenase, and hexokinase which are vital to glycolysis and metabolism in yeasts (Taherzadeh

et al., 2011). Moreover, furfural causes damages in yeasts' vacuoles, mitochondria, and cell membranes (Almeida *et al.*, 2007, Taherzadeh *et al.*, 2011).

Another probable explanation of slower glucose utilization is osmotic stress caused by large concentration of glucose and other toxic compounds that are still present when overliming at pH 8 and 9 (Saint-Prix *et al.*, 2004). Afterward, the steady drop of glucose concentration in pH 8 and 9 overliming hydrolyzates could be detected. The drop in glucose concentration could plausibly be due to the consumption by the remaining yeasts that were capable of naturally adapting themselves to higher concentration of toxic products (Taherzadeh *et al.*, 2011). The yeasts then consumed the glucose until the concentration reached to almost 0 g/l after 48 hours. The result from **Figure 4.5** gives the evidence that pH 8 to12 could be a suitable overliming pH range for *S. cerevisiae*.

The production of ethanol is recorded every 24 hours for 96 hours. **Figure 4.6** is the result of ethanol production at a various range of overliming pH.

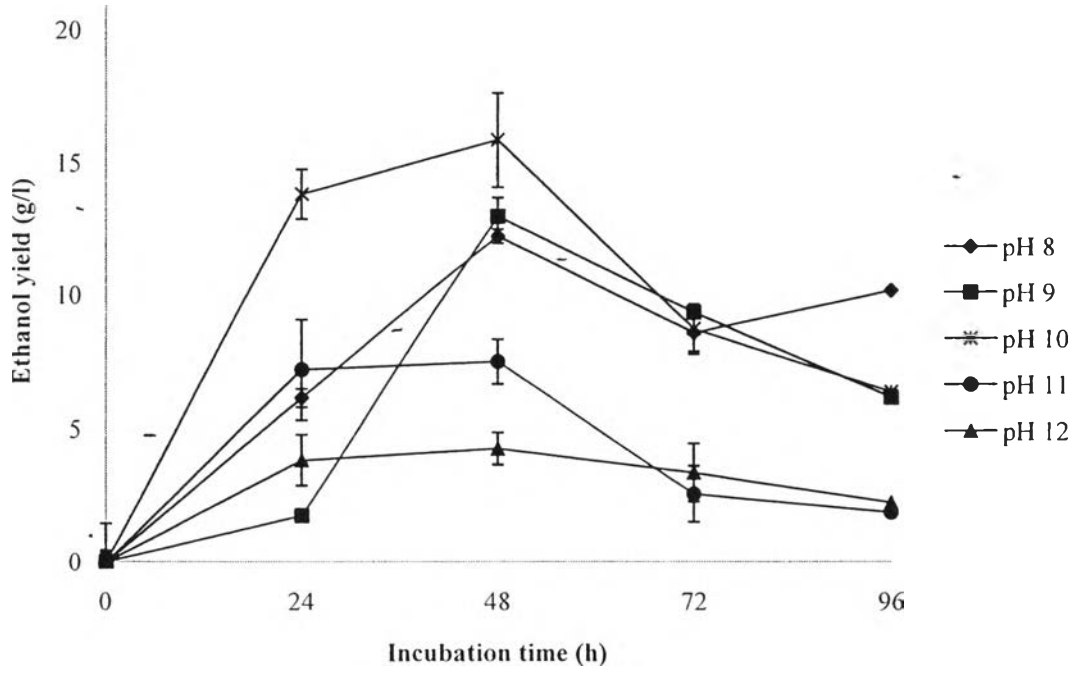

Figure 4.6 Ethanol production of mission grass hydrolyzate by *S. cerevisiae* TISTR 5049 at overliming pH 8-12

The highest amount of ethanol was produced within 48 h of incubation time. The sharp drop of ethanol concentration after 48 h of incubation suggests that ethanol may be oxidized into acetaldehyde or acetate (Zakhari, 2006). Another feasible explanation is the fact that ethanol, the product from fermentation, is also the inhibitor for ethanol production. Ethanol molecules are small enough to diffuse through cell membranes and slow down glucose metabolisms (Taherzadeh *et al.*, 2011). The inhibition caused slower ethanol production from the microorganisms, which resulted in lower ethanol concentration after 48 hours. The rapid decrease of ethanol production after the maximum yield of ethanol could also be justified by high osmotic stress caused by the remaining glucose, ethanol, and other inhibiting compounds in the hydrolyzate (Saint-Prix *et al.*, 2004, Taherzadeh *et al.*, 2011).

At the starting glucose concentration of 53 g/l, overliming at pH 10 gave the highest concentration of ethanol followed by pH 9, 8, 11, and 12, respectively. The lowest yields of ethanol occurred at pH 11 and 12 which suggested that a lot of glucose was lost during the overliming process. When overliming was done at pH 8 and 9, the inhibitory compounds were not completely removed, but a higher concentration of glucose were still present in the hydrolyzate where naturally adapted yeasts could used to ferment ethanol. The overliming process is an effective method in removing toxic compounds from lignocellulosic hydrolyzate, but using too much lime also causes sugar loss and affects ethanol yield. Consequently, a compromise has to be made between the amount of inhibitory compounds being removed and sugar loss from overliming process in order to obtained the highest amount of ethanol (Mohagheghi *et al.*, 2006).

4.8 Optimization of *Saccharomyces cerevisiae* strains for ethanol production

Baker's yeast (*Saccharomyces cerevisiae*) with different strains can produce varying amount of ethanol. Despite being the same type of yeast, each strain of *S. cerevisiae* acquires diverse characteristics such as glycolysis production speed, resistance against inhibitory compounds, and tolerance to osmotic stress. Four strains of *S. cerevisiae* were studied in this project: TISTR 5049, TISTR 5339, TISTR 5596, and TISTR 5606. *S. cerevisiae* TISTR 5330, 5596, and 5606 have been used in ethanol production studies (Srinorakutara *et al.*, 2008, Jutakanoke *et al.*, 2012, Vaithanomsat *et al.*, 2013) whereas no journal has been reported on the ethanol performance of *S. cerevisiae* TISTR 5049. **Figure 4.7** demonstrates the number of various strains of yeasts in mission grass hydrolyzate at pH 10 overliming. The standard deviation shown is in triplicates.

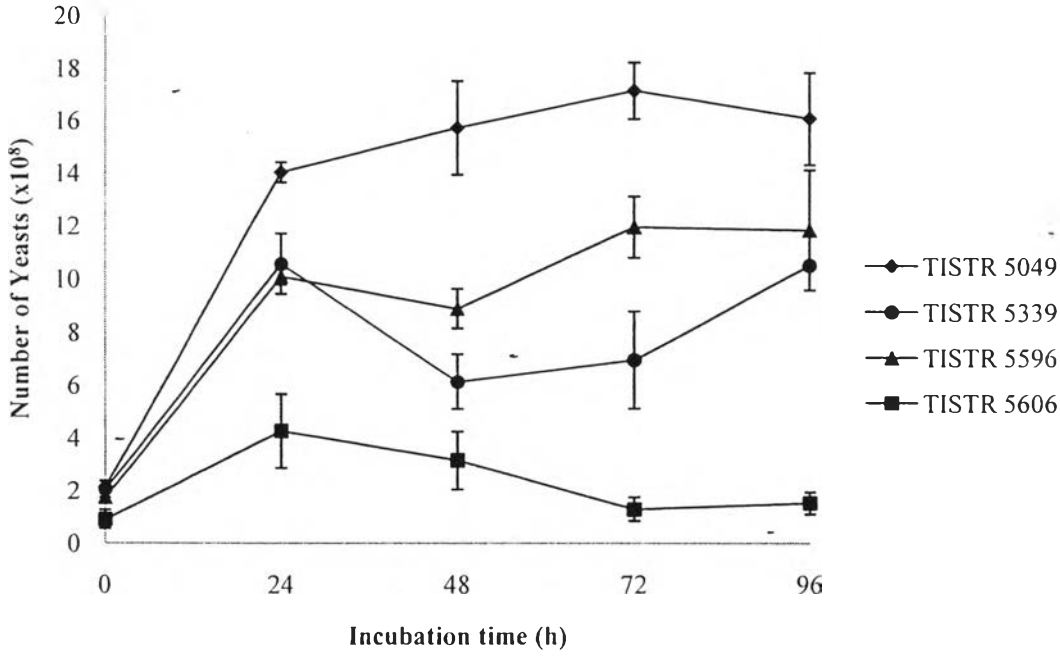

Figure 4.7 Various strains of baker's yeast (*S. cerevisiae*) count per incubation time at pH 10 overliming

S. cerevisiae TISTR 5049 could reproduce very quickly compared to other strains of *S. cerevisiae*. Similar to **Figure 4.4**, the fastest population growth occurred within 24 hours. Then, the number of yeasts began to remain stable which may be due to lower availability of glucose after 24 hours. According to **Figure 4.7**, *S. cerevisiae* TISTR 5606 had the least yeast population. This was because the difficulty in counting the number of yeasts under a microscope since *S. cerevisiae* TISTR 5606 aggregated into a big group and stacked on top of one another. The yeasts *S. cerevisiae* TISTR 5339 and 5596 showed similar growth patterns where their numbers rose rapidly within 24 hours and began to stay constant afterwards.

The detection of glucose concentration per incubation time for the strains of *S. cerevisiae* is illustrated in **Figure 4.8**.

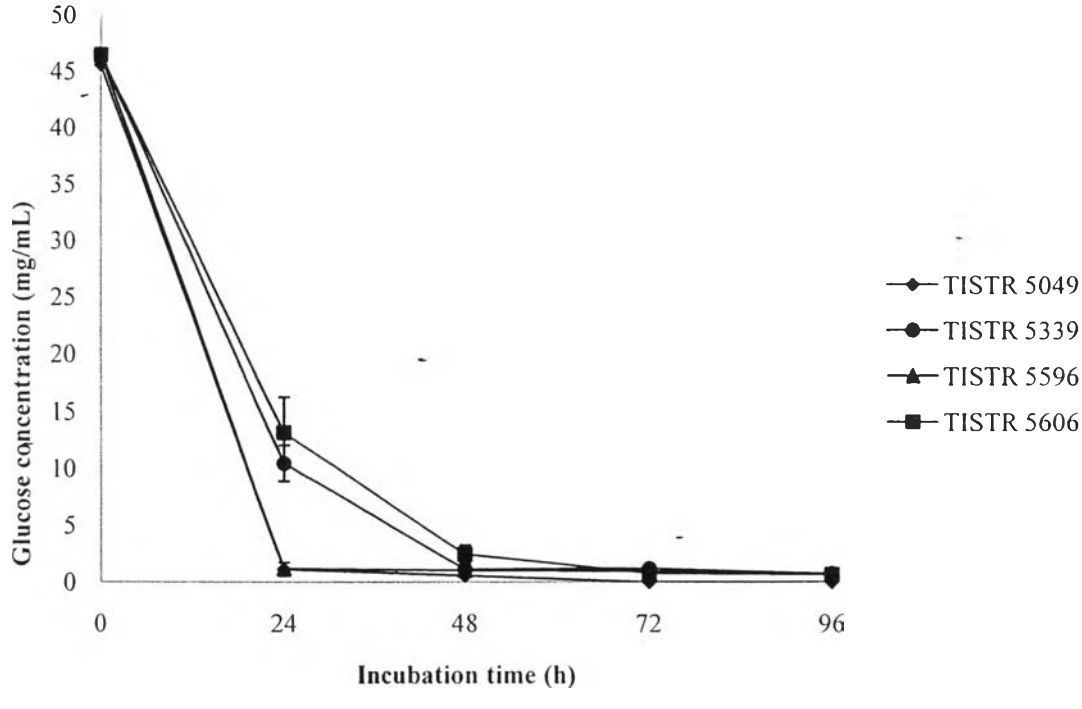

Figure 4.8 Glucose concentration per incubation time for various strains of baker's yeast *S. cerevisiae* at pH 10 overliming

Glucose concentration declined very quickly within 24 h, which correlated to the rapid increase of yeast population in **Figure 4.7**. *S. cerevisiae* TISTR 5049 and 5596 consumed almost all glucose within 24 h, while larger concentration of glucose, 10 g/l and 13 g/l, could still be detected in the flasks of *S. cerevisiae* TISTR 5339 and 5596, respectively. This could possibly be due to their lower tolerance to osmotic stress compared to their yeast counterparts (Navarro-Aviño *et al.*, 1999), causing some yeast cells to burst. The explanation corresponds to the lower number of yeasts for *S. cerevisiae* TISTR 5539 and 5606 after 24 hours in **Figure 4.7**. The remaining yeast cells that were capable of enduring the osmotic stress underwent through natural adaptation and consumed the leftover glucose (Taherzadeh *et al.*, 2011). Glucose concentration dropped to almost 0 g/l after 72 h of incubation in all samples. The result confirms that pH 10 is the most suitable overliming pH because all strains of *S. cerevisiae* could utilize the glucose in the hydrolyzate.

In comparison, another type of yeast, *Pichia stipitis*, takes up to 200 h to consume all sugar in the hydrolyzate when the concentration of starting sugar is approximately 45 g/l (Telli-Okur *et al.*, 2008). *P. stipitis* is among one of the most common types of yeasts used to produce ethanol due to its ability to utilize variety of sugars (Lee *et al.*, 2000). However, its low tolerance to inhibitory products and slow ethanol production cause the yeast to be less preferable as the ethanol fermenter.

Ethanol production from each strain of *S. cerevisiae* is shown in **Figure 4.9**.

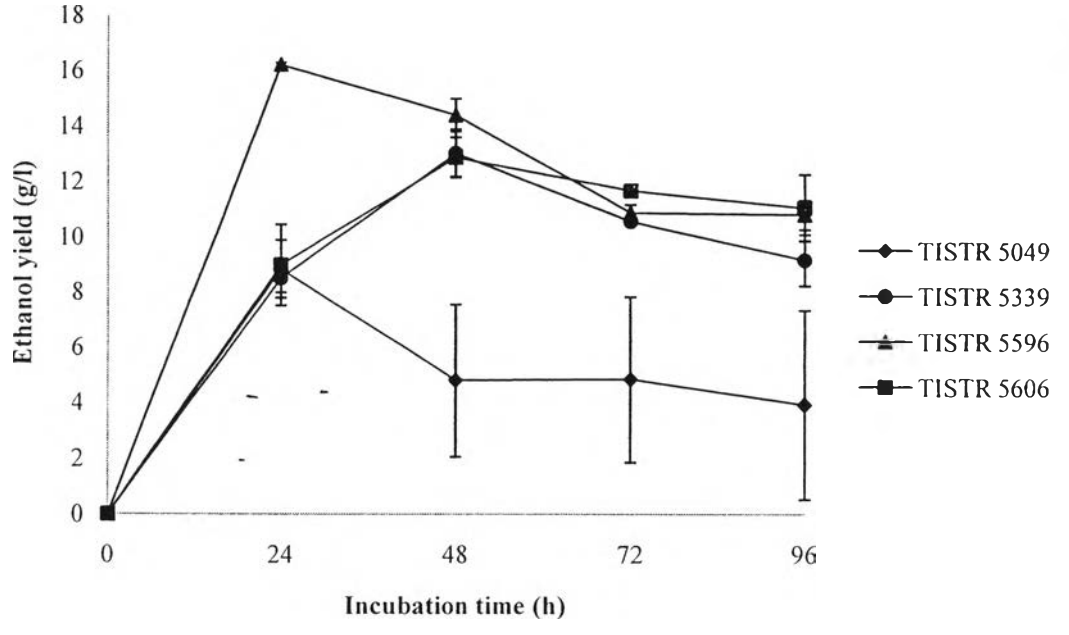

Figure 4.9 The production of ethanol from various strains of *S. cerevisiae* in 96 h

The starting glucose concentration for the fermentation process was 45 g/l. *S. cerevisiae* TISTR 5596 produced the most ethanol at 16 g/l in 24 h. The same yeast strain also produced the maximum ethanol production within 24 hours in sugarcane leaves hydrolyzate (Jutakanoke *et al.*, 2012). The amount of ethanol produced correlates with the results from Srinorakutara et al., where the performance in ethanol production from *S. cerevisiae* TISTR 5596 is more superior than that from *S. cerevisiae* TISTR 5606 (Srinorakutara *et al.*, 2008). The lower ethanol production of *S.cerevisiae* TISTR 5339 and 5606 could be due to their lower tolerance to osmotic stress, which corresponded to the yeast population and sugar consumption results in **Figures 4.7 and 4.8**, respectively. Moreover, their maximum ethanol production occurred after 48 h which further ascertained the ability of the adapted yeasts to produce ethanol.

According to the graph, *S. cerevisiae* TISTR 5049 produced the least ethanol out of all four strains. This could be due to its low tolerance to acetate since some of the ethanol produced gets oxidized into acetate (Verduyn *et al.*, 1990). Another explanation that could justify the difference of ethanol production performance of *S. cerevisiae*

TISTR 5049 in **Figures 4.6 and 4.9** is the yeast's low resistance to osmotic stress. When the yeast is under stress due to the toxic compounds in the hydrolyzate, the yeast would utilize the glucose present in the hydrolyzate to produce glycerol (Pagliardini *et al.*, 2013). As the result, less glucose could be utilized for ethanol production. According to the graph, more glucose was left to produce ethanol when the starting glucose was 53 g/l **(Figure 4.6)**, so the yeast could utilize the remaining glucose to further produce ethanol. On the other hand, if the initial glucose concentration was 45 g/l **(Figure 4.9)**; not as much ethanol could be produced when certain amount of glucose was necessarily hoarded to produce glycerol. Consequently, *S. cerevisiae* TISTR 5049 could not produce more ethanol after 24 h with the limited glucose left in the hydrolyzate.

In Telli-Okur's study on the production of ethanol from sunflower seed hull, the maximum ethanol produced from *P.stipitis* NRRL Y-124 is 8.5 g/l in 150 h where combined detoxification process of overliming and sodium sulfite is used (Telli-Okur *et al.*, 2008). With equal initial sugar substrate, this study produced higher yield of ethanol under a shorter period of time (16 g/l in 24 h).