Bioconversion of Banana Pseudostem Fiber to Ethanol: Optimization of Acid Pretreatment Conditions and Fermentation Yeast Selection

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Abstract-***The banana (Musa sapientum) pseudostem is a massive agricultural leftover in Taiwan. Conversion of such agricultural waste into cellulosic alcohol is an appealing idea and the aim of this study. This study optimized conditions for hydrolyzing banana pseudostem with sulfuric acid during the pretreatment process and selected a best strain of yeast (Saccharomyces cerevisiae) for fiber hydrolysate fermentation. Hydrolysis catalyzed by sulfuric acid reached a plateau at 5% concentration when measuring reducing sugar yield. Five % sulfuric acid also demonstrated slightly higher sugar to alcohol conversion rate than 4% sulfuric acid during the subsequent fermentation. The optimal fiber to acid ratio is 3:10 and 30-60 min boiling is sufficient. Under 3:10 fiber/acid ratio with 4% sulfuric acid and 1 hr boiling, YA yeast can achieve 46% sugar to alcohol conversion rate.***

Keywords: Banana Pseudostem, Bioethanol, Pretreatment, Fermentation.

1. INTRODUCTION

The increase of CO_2 concentration in the atmosphere has manifested the greenhouse effect and ushered in the global warming [1,2]; consequently, cutting CO_2 emission has become a primary goal in many countries around the world [3]. Biofuels, such as bioethanol and biodiesel, contain chemical energy derived from photosynthesis [4] and are excellent alternatives to fossil fuels because they are synthesized from atmospheric CO_2. When biofuels are combusted for work or energy, they simply return the carbon into the atmosphere as CO_2 and are thus carbon neutral [4]. Previous productions of bioethanol require fermentation of starch from food resource, *e.g.*, corn in the United States or sugarcane in Brazil [5]. Since diverting foodstock to transportation fuel may raise the price of food, the feedstock of bioethanol fermentation would be better if they are from nonfood sources such as agricultural wastes or cellulose of nonfood plants [6,7].

It has been estimated that nonfood-source biomass including agricultural wastes and lignocellulose can be converted to bioethanol to satisfy approximately half of transportation need in the United States [8]. Taiwan produces 5 million tons of agricultural wastes annually, and their disposals have been an issue. It is estimated that Taiwan produces approximately 1.2 million tons of agricultural waste from banana cultivation alone [9]. Therefore, the feasibility of turning agricultural wastes, such as banana pseudostem [10] or peels [11], into bioethanol should be investigated vigorously. Banana pseudostem is an agricultural waste that requires a long time to decompose in the field. During the decomposing period, the banana pseudostem may become a hot bed for insect growth; therefore, converting banana pseudostem into bioethanol is an ideal test for addressing these aforementioned issues in one move. While banana pseudostem fiber has been used as a reinforcing fiber for epoxy concrete [12], polyester composite [13], or as a possible source of paper pulp [14], we investigated the feasibility of converting banana pseudostem into bioethanol because of its relatively high cellulose (34.5%), low lignin (12%) and hemicelluloses (25.6%) contents [14].

Since the fermentation process requires simple sugars as the substrate, a pretreatment process that converts cellulosic fibers to simple sugars is necessary. Common pretreatment methods include, but not limited to, 1) lignocellulose hydrolysis catalyzed by acid [15] or alkaline [16,17], which may require a neutralization of pH before subsequent fermentation; 2) fiber explosion mediated by steam [18] or ammonia [19], which needs sophisticated equipments to execute a quick pressure relief; 3) enzymatic degradation by cellulases which are costly and slow [20]. We elected sulfuric acid hydrolysis because it is a simple method for initial depolymerization of lignocellulose [15] and does not require sophisticated equipment or expensive digestive enzymes. This study fine-tuned the sulfuric acid pretreatment conditions for banana pseudostem fiber and optimized hydrolysate fermentation conditions for bioethanol production.

2. MATERIALS AND METHODS

2.1 Preparation for Banana Pseudostem

Bananas (*Musa sapientum* L.) were cultivated at Taiwan Agriculture Research Institute in Wufong District of mid-Taiwan (24°4'N, 120°42'E) and their pseudostems were diced, weighed and separated into 2 batches. Batch 1, abbreviated as BPS-1, was baked directly at 85°C for 3 days, whereas batch 2 (BPS-2), was pressed to remove stem juice before the residue being baked in the same manner. Dry banana pseudostem fibers were weighed and ground to powder for storage.

2.2 Pretreatment Conditions

Three major variables that include the sulfuric acid concentration, the fiber to sulfuric acid ratio, and the duration of hydrolysis in the pretreatment process were optimized. To determine the optimal pretreatment conditions, powdered fibers were soaked in 0%-8% sulfuric acid and boiled under atmospheric pressure for 0.5-3 hr or mixed with 4% sulfuric acid at 1:10, 2:10, 3:10 or 4:10 (w/v) ratio and boiled for 0.5-3 hr. The hydrolysate was filtered through triple layer cheese clothes and centrifuged at 12,000x g for 15 min followed by one filtration through a 0.22 μm membrane. The reducing sugar content in the hydrolysate was analyzed by dinitrosalicylic acid (DNS) method [21].

2.3 Reducing Sugar Assayed by 3,5-Dinitrosalicylic Acid (DNS) Reagent

The DNS working solution is consisted of 1% 3,5-dinitrosalicylic acid, 0.2% phenol, 0.05% sodium bisulfite ($NaHSO_3$), 20% potassium sodium tartarate and 1% NaOH which should be prepared as 2% NaOH stock solution. The reaction is buffered by 50 mM citric acid, pH4.5. Glucose ranging from 0 to 2 g/L was used as the reference standard. The hydrolysate of pseudostem fiber was diluted serially to ensure one of the dilutions will fall within the standard reference range. An aliquot of 0.5 mL standard or sample was added with 1 mL 50 mM citrate buffer and 3 mL DNS reagent before being heated to 100°C in boiling water for 5 min followed by ambient cooling. Fifteen mL of cold water was added to each tube before a brief vortex. Spectral absorption (540 nm) of the reaction product, 3-amino-5-nitrosalicylic acid, was measured by a spectrophotometer. The standard reference line based on the glucose standard was established with the least square linear regression method.

2.4 Glucose Content by Hexokinase/Glucose 6-phosphate Dehydrogenase Assay

The glucose content in the hydrolysate was determined by coupling glucose phosphorylation with glucose 6-phosphate dehydrogenation in the presence of 5 mM ATP and 0.75 mM NADP. One mole of glucose gives rise to one mole of NADPH which has a molar extinction coefficient of 6220/M/cm at 340nm wavelength. [22]

2.5 Furfural Content by HPLC

The content of furfural was determined by HPLC with a reverse phase column and an aqueous mobile phase of 5% acetic acid and 10% acetonitrile at a flow rate of 1 mL/min. The elution was monitored with a diode array detector and the furfural standard has a retention time of about 12 min and a maximum absorbance at 292 nm. [23]

2.6 Saccharomyces cerevisiae Selection

Five yeast strains were obtained from the Bioresource Collection and Research Center (BCRC) at Food Industry Research and Development Institute, Hsinchu, Taiwan. YA is BCRC20581; YB is BCRC21809; YC is BCRC22728; YD is BCRC23000; Y36 is BCRC23007, all of which are *Saccharomyces cerevisiae* that have been used for wine or liquor productions at various locations in Taiwan.

For pH effect on ethanol yield, the pH of YMB media containing 10 g glucose/L were adjusted to pH2, 3, 4, or 5 and inoculated with YA, YB, YC, YD, or Y36 at 1% (v/v) ratio. Yeasts were grown at 25°C for 3 days while the fermentation media were sampled every 24 hr. The samples were filtered and stored at -20°C until their ethanol contents were determined. To determine temperature effect on yeast growth, 3 different strain of *S. cerevisiae* (YA, YB, YD) were first grown in yeast malt broth (YMB) before being spread out on yeast malt agar (YMA) plates at 20°C, 25°C, or 30°C for counting colony numbers.

2.7 Fermentation of Pseudostem Fiber Hydrolysate

The hydrolysate of pseudostem fiber were adjusted to pH4 before being inoculated with one of *S. cerevisiae* strains, YA, YB, or YD at 1% (v/v) ratio followed by a 4-day fermentation at 25°C. The fermented media were filtered before the ethanol contents were determined.

2.8 Determination of Ethanol Content

Ethanol in the fermented media was resolved by HPLC with an Aminex HPX-87H Fermentation Monitor Column (300 x 7.8 mm, 5 µm, BioRad) and measured by a refractive index detector (Gilson 133). The mobile phase is 1 mM sulfuric acid with a flow rate of 0.8 mL/min at 40°C. The ethanol content is estimated by regression on a series of ethanol standard ranging from 0%-10%

3. RESULTS

3.1 Optimization of pretreatment conditions

3.1.1 Pseudostem fiber preparation: Because banana pseudostem contains large amount of water (approximately 8.9%) [14], they were treated with 2 different drying methods, either dried directly (BPS-1) or juice pressed off before being dried (BPS-2). The initial test indicated that the drying process had little impact on the reducing sugar content in banana pseudostem fibers (data not shown); therefore, all remaining studies were carried out with the juice pressed off method (BPS-2).

3.1.2 Sulfuric acid concentration and duration of hydrolysis: Under 2 hr boiling and 1 part fiber per 10 part sulfuric acid ratio, the presence of sulfuric acid is critical for the hydrolysis of pseudostem fiber. The optimal concentration of sulfuric acid for this fiber hydrolysis is 5% where the resulting sugar plateaus out at approximately 22 g sugar/L hydrolysate, as shown in figure 1, top panel. Since 5% sulfuric acid is higher than most other reports of 0.5%-3% [24-28], we chose 4% sulfuric acid for subsequent experiments. In the presence of 4% sulfuric acid, the duration required for this acid hydrolysis is 2 hr when the resulting sugar reached 39 g/L. Prolonged boiling for 3 hr reduced the sugar level down to about 31 g/L, figure 1, middle panel.

3.1.3 Fiber to acid ratio: The fiber to acid (w/v) ratio can influence the resulting sugar content in the hydrolysate; under 4% sulfuric acid and boiling for 2 hr, the resulting sugar level was increased from 18 g/L to 53.4, 54.4, 39.2 g/L as the fiber/acid ratio was increased from 1:10 to 2:10, 3:10, or 4:10, respectively, figure 1, lower panel.

3.1.4 Effect of pretreatment conditions on hydrolysate content: To sort out which pretreatment condition may have a significant impact on the hydrolytic products, *e.g.*, reducing sugar, glucose, and furfural, we repeated the pretreatment process with fiber/acid ratio at 1:10 or 3:10 and hydrolyzed with 4% or 5% sulfuric acid for 30 min. The hydrolysate was recovered by centrifugation at 4000x g for 30 min and assayed for reducing sugar, glucose, and furfural content. As shown in figure 2, under the same condition when compared to 10% fiber, 30% fiber has a more pronounced impact on reducing sugar yield (more than 2 fold increase, top panel) but less significant on glucose (50-60% increase, middle panel) or furfural (30-40% increase, lower panel), while the increment of 4% sulfuric acid to 5% has a near proportional impact on glucose content but less significant effect on reducing sugar and furfural contents.

3.2 Fermentation conditions

3.2.1 pH and yeast strains: Since pH can influence yeast growth and subsequent fermentation result dramatically, pH effect on alcohol yield was examined. Based on the alcohol yield from fermenting glucose in the yeast malt broth, strain YA, YB, and YD were less influenced by pH when compared to YC and Y36, figure 3. Because the fermentation process is more susceptible to bacterial contamination at pH above 4, the selected yeast strain should have an optimal pH preference at pH4 or below so as to maintain its population dominance during the fermentation. Because YC has a narrow optimal pH range for growth while Y36 does not grow well at acidic pH, subsequent studies will focus on YA, YB, and YD only.

3.2.2 Temperature effect: Since temperature can influence yeast growth significantly, the optimal temperature for yeast culturing was investigated. When subjected to different temperature during colony formation on yeast agar plates, YA and YD grew better at temperature between 25°C and 30°C than at 20°C, while YB showed larger variation and slower colony formation at 25-30°C, figure 4.

3.3 Alcohol yield from fermentation of pseudostem fiber hydrolysate

3.3.1 Strain of yeast: Fermentation by YA, YB, and YD were tested to select a best yeast strain for alcohol production. YA is a better yeast strain for initial fermentation, because not only it has a higher alcohol yield than YB or YD but also is less affected by different batches of banana pseudostem fiber (figure 5).

3.3.2 Sulfuric acid concentration and duration of hydrolysis: When pseudostem fiber was subjected to 4% or 5% sulfuric acid hydrolysis, 5% sulfuric acid resulted slightly better sugar to alcohol conversion, but the increment is small (figure 6,

top panel). Therefore, 5% sulfuric acid seems optimal but 4% sulfuric acid is an economical alternative. In the presence of 4% sulfuric acid, 1 hr of boiling is sufficient, since prolonged boiling offered no substantial increment in alcohol yield, figure 6, middle panel.

3.3.3 Fiber to acid ratio: To determine the optimal fiber acid ratio for yeast fermentation, dried pseudostem fiber powder was mixed with 4% sulfuric acid at 1:10, 2:10 or 3:10 (w:v) ratios and boiled for 1 hr before a 4-day fermentation. As illustrated in figure 6, lower panel, the sugar to alcohol conversion reached 41-44% with 2:10 ratio and 46-47% with 3:10 ratio. Among those 3 yeast strains tested, YA performed slightly better than YB or YD, except that YD at 3:10 ratio performed just barely better than YA and YB, figure 6, lower panel. A concurrent group with fiber to acid ratio of 4:10 was also carried out, but the hydrolysate resulted in no fermentation (data not shown).

4. DISCUSSION

4.1 Yeast strain

The most intriguing part of this study is perhaps the uncertainty of selecting a clear cut yeast strain that is better at producing cellulosic ethanol from fermentation of banana pseudostem fiber. It would seem YB and YD to be better for alcohol production at pH4 when they were grown in yeast malt broth (figure 3). However, when fermenting the juice or the hydrolysate of banana pseudostem instead of yeast malt broth, YA consistently produced more alcohol than YB or YD as shown in figure 5 and top panel of figure 6, except that YD at 5% sulfuric acid had a marginally higher conversion rate than YA (figure 6, top panel). Since the purpose of this study is to select a best yeast strain for fermenting pseudostem hydrolysate, it would seem YA is a better choice for this task.

4.2 Pretreatment conditions

4.2.1 Concentration of catalytic acid: In this study, we found 5% sulfuric acid is the optimal concentration to use, as shown in top panels of figure 1 and 6. Since other reports cited uses of sulfuric acid between 0.5-3.0% during their hydrolyses of corncobs [24], wheat straw [25], sugarcane bagasse [26], red sage [27], or agricultural residues [28], as summarized in table 1, we do not know exactly why our acid requirement is higher than others. However, one possible explanation is that our hydrolysis was carried out under atmospheric pressure and the sulfuric acid may have evaporated during the process. As a result, our hydrolysis requires more sulfuric acid to reach the optimal result.

4.2.2 Hydrolysis duration and furfural formation: The optimal duration for acid catalyzed hydrolysis depends on which parameter is being evaluated. When measuring reducing sugar content, 2 hr heating generated the highest amount of reducing sugar within the hydrolysate (figure 1, middle panel); however, when evaluating alcohol yield by yeast fermentation (g alcohol/g of reducing sugar), 1 hr heating is as good as 2 or 3 hr heating, *i.e.*, prolonged heating does not warrant any additional alcohol yield (figure 6, middle panel). The reason for this discrepancy is probably due to the formation of furfural and 5-hydroxymethyl furfural (HMF) during the heated hydrolytic process. Furfural and HMF, derived from dehydration of pentose or hexose during the acid hydrolysis, are furan aldehydes that possess reducing potential, so that they may be recognized as reducing sugars by DNS reagent. Since furfural compounds are known for their inhibitory potential on subsequent yeast fermentation [29], boiling beyond 1 hr would provide no benefit on subsequent fermentation or alcohol yield. Other reports cited 20-45 min heating [24-27] during their acid hydrolysis process; however, 60 min was employed by Martin et al. [28] during their investigation, which concluded that low levels (mid mg/L) of furfural compounds were formed. When the duration was shorted to 30 min, we found the furfural levels to be in the low mg/L levels (figure 2 lower panel); therefore, it would seem 30-60 min of heating is sufficient.

4.2.3 Fiber to acid ratio: The fiber/acid ratio during the hydrolysis of pseudostem fiber is another factor that can influence the alcohol yield. Although 2:10 and 3:10 ratios would result in similar levels of reducing sugar content in the hydrolysate (figure 1, lower panel), 30% fiber (3:10 ratio) would consistently edge out 20% fiber when comparing the sugar to alcohol conversion rate (figure 6, lower panel). As 40% fiber, the reducing sugar yield is lower by one quarter (39g/L *vs* 54 g/L, figure 1, lower panel) and the hydrolysate resulted in no fermentation. Therefore 3:10 fiber/acid ratio would seem to be the optimal ratio for banana pseudostem fiber fermentation.

5. CONCLUSION

The optimal pretreatment condition for hydrolyzing banana pseudostem fiber with diluted sulfuric acid is 5% sulfuric acid at fiber/acid ratio of 30% and heated for 30 min at atmospheric conditions.

Table 1. Comparative list of sulfuric acid catalyzed pretreatment process of agricultural wastes

Feedstock	Sulfuric acid	Temperature	Duration	Reference
Corncobs	0.5 %	122 °C	20 min	24
Wheat straw	0.75 %	121 °C	60 min	25
Sugarcane bagasse	3 %	121 °C	30 min	26
Red sage	3 %	120 °C	45 min	27
Sugarcane bagasse, rice hulls, peanut shells, cassava stalks	2 %	122 °C	20-60 min	28

6. ACKNOLEDGMENT

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8. FIGURES

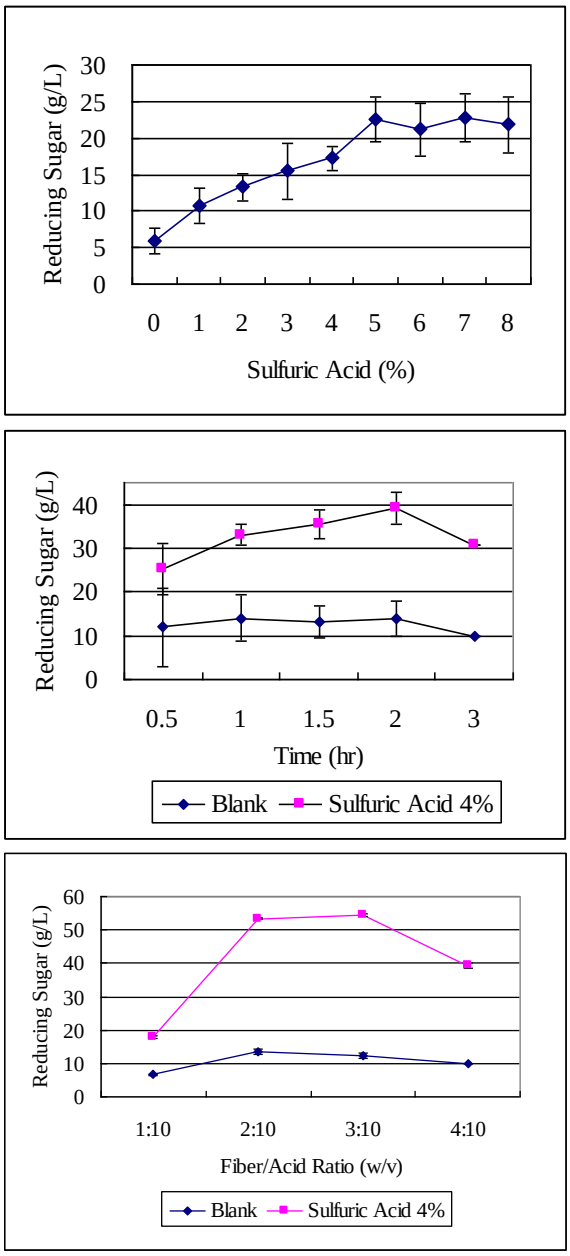

Figure 1. Optimization for the pretreatment process. The reducing sugar content in the hydrolysate was measured by 3,5-dinitrosalicylic acid (DNS) reaction assay, see Methods for details. Top panel, banana pseudostem fiber was soaked in various concentrations of sulfuric acid at 1:10 fiber/acid ratio and boiled for 2 hr. Each point indicates an average of 3 or more determinations and error bars indicate standard errors. Middle panel, banana pseudostem fiber was mixed with 4% sulfuric acid at 1:10 ratio and boiled for various durations. Each point indicates an average of 2 or more determinations and error bars indicate 1/2 the difference or standard errors. Lower panel, various amount of pseudostem fiber was soaked in 4% sulfuric acid, followed by a 2 hr heating. Each point indicates an average of 3 determinations and error bars indicate standard deviations.

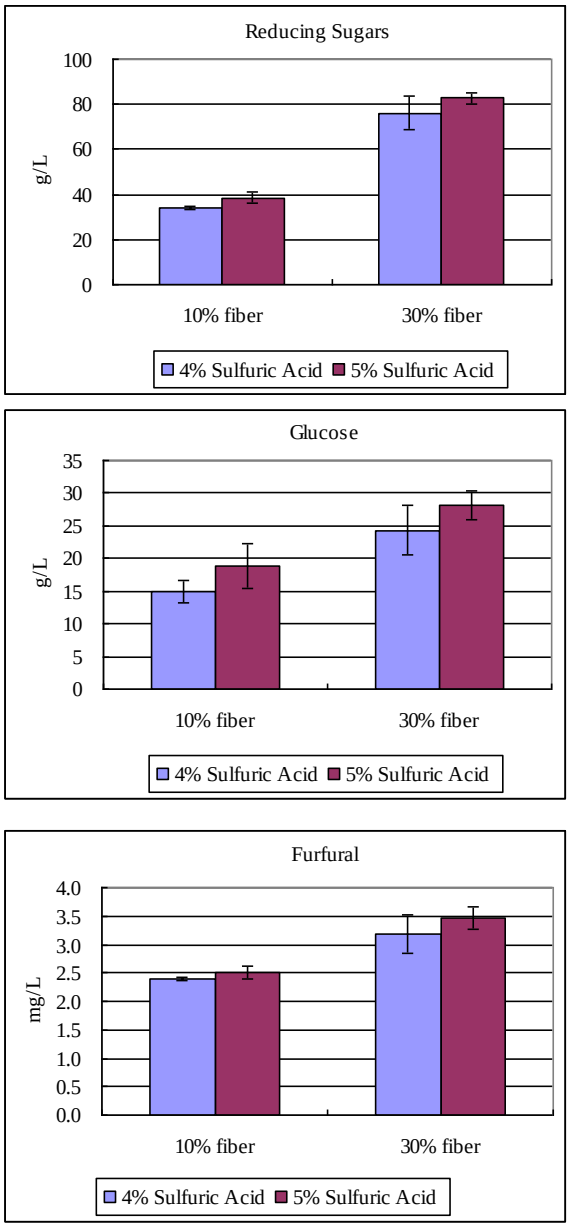

Figure 2. Effect of pretreatment conditions on the hydrolysate content. Banana pseudostem fiber was hydrolyzed with 4% or 5% sulfuric acid at 10:1 or 10:3 acid to fiber ratio for 30 min. The hydrolysate was recovered by centrifugation and the content was assayed for reducing sugar, glucose, and furfural content, see Methods for details. Top panel, reducing sugar was determined by DNS method. Each bar indicates an average of 4 distinct experiments and the error bars indicate standard errors. Middle panel, glucose content was determined by HK/G6PDH method. Each bar indicates an average of 4 distinct experiments and the error bars indicate standard errors. Lower panel, furfural content was determined by reverse phase HPLC with chemical grade furfural as the standard. Each bar indicates an average of 4 distinct experiments and the error bars indicate standard errors.

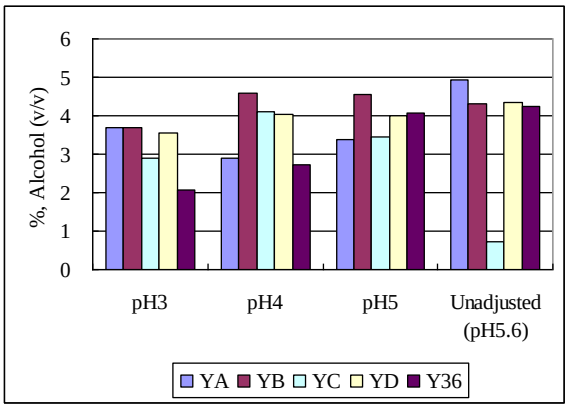

Figure 3. Effect of pH on final alcohol content after a 3-day fermentation by various yeasts. YMB medium containing 10 g glucose /L was acidified to pH 3, 4, or 5 and inoculated with 1% (v/v) stationary phase yeast before a 3-day fermentation. The fermented hydrolysate was filtered and analyzed by HPLC for alcohol content, which is expressed as % concentration. Bars indicate single point determinations.

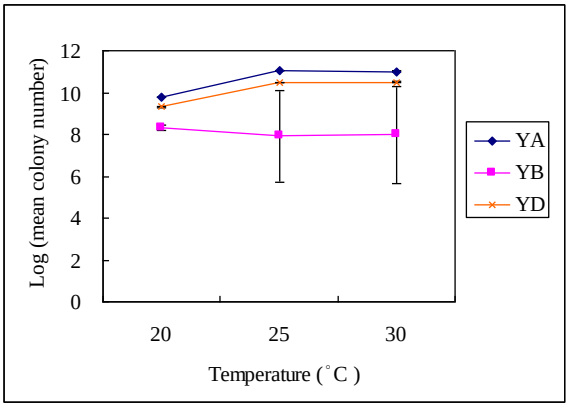

Figure 4. Temperature effect on yeast growth in yeast malt broth (YMB) medium. Various strains of yeasts were allowed to grow into the log phase before being diluted serially and plated out on yeast malt agar (YMA) plates. Those plates are grown at various temperatures for 2 days before being counted for colony numbers. Each point indicates an average of 6 determinations and error bars indicate standard error.

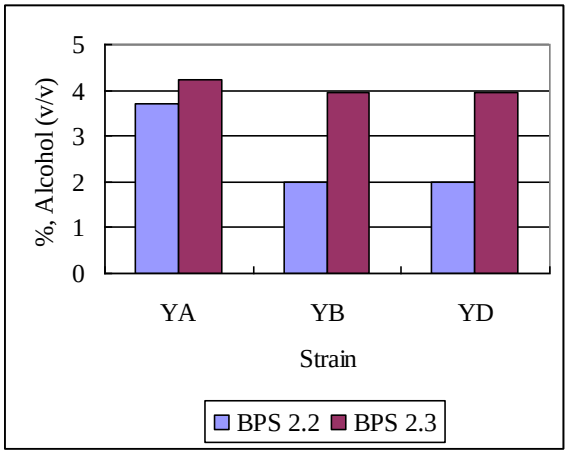

Figure 5. Effect of yeast strain and batch of banana pseudostem juice on the final alcohol content after fermentation. BPS 2.2 and BPS 2.3 are two different batches of banana pseudostem juice. YA consistently produced more alcohol than YB or YD, regardless different batches. The bars represent single point determinations.

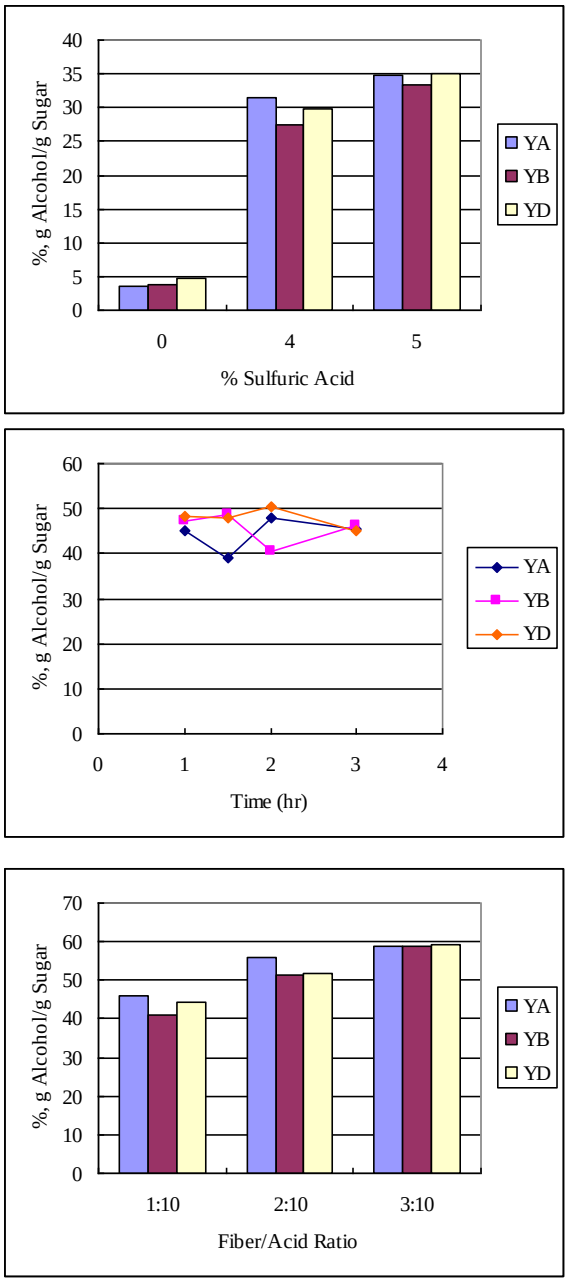

Figure 6. Optimization of sugar to alcohol conversion rate. Various pretreatment conditions were tested for optimal sugar to alcohol conversion. The hydrolysate was adjusted to pH4 prior to yeast inoculation and fermented for 4 days at 25°C. The reducing sugar was determined by DNS method prior to the fermentation, while the alcohol was determined by reverse phase HPLC, see Method for details. Top panel, banana pseudostem fiber was mixed with 4 or 5% sulfuric acid at 1:10 (w:v) fiber/acid ratio. Each Bar is a single point determination. Middle panel, pseudostem fiber was mixed with 4% sulfuric acid at 1:10 fiber/acid ratio and boiled for various durations. Each point in the chart represents a single point assay. Lower panel, banana pseudostem fiber was mixed with 4% sulfuric acid at different ratios and boiled for 1 hr. The 4:10 fiber to acid ratio group resulted in no fermentation and therefore its alcohol content was not determined. The results are single point determinations.