PREPARATION OF BIOETHANOL FROM SORGHUM STARCH AND ITS CHARACTERIZATION

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Abstract

Bioethanol was prepared from powder of sorghum (*Sorghum biocolor* L. Moench) grain by enzymatic hydrolysis. α–Amylase from germinated wheat grains was used for liquefaction of starch and α–glucosidase from ungerminated flint corn was used in saccharification step. After liquefaction and saccharrification, the solution was tested for glucose by using Benedict solution and Fehling solution. During fermentation of liquid glucose by *Saccharomyces cerevisiae* yeast for 6 days, the changes of physicochemical properties such as pH (5.2 to 3.7), acid content (0.093 % to 0.423 %), glucose content (155 to 63 mg L^{-1}) and specific gravity (0.994 to 0.975) during fermentation were determined. After distillation the yield percentage of alcohol in fermented solution is 10 % and the physicochemical properties such as specific gravity, refractive, colour, free acid, free base and alcohol content of the hydrated bioethanol and dehydrated bioethanol were comparatively studied with absolute ethanol. Moreover, the functional groups of bioethanol were analysed by FT IR spectroscopy.

Keywords: sorghum, bioethanol, α-amylase, α-glucosidase, *Saccharomyces cerevisiae*, glucose, physicochemical properties

Introduction

Bioethanol is derived exclusively from the fermentation of plant starches such as sugar cane, grains, potato and corn and agricultural waste. Though ethanol can be extracted as a by-product from a chemical reaction with ethylene and other some products, these sources are not considered renewable. The interest for renewable biofuels has increased significant over the past few years. It is important to find an alternative to oil, due both to the limited supply and the effect from the greenhouse gases that are released from oil use. One possible biofuel is bioethanol. The world's leading manufacturers and industries are seeking to substitute petrochemical-based petroleum supplies continue to decline (Zhan *et al*., 2003). Great attention has been given to ethanol production using various substrates which can be classified into three main types of materials, which are sugars from sugarcane, sugar beet, sweet sorghum, molasses and fruits), starches (from sweet sorghum grain, cassava, corn, potato and root crops) and cellulose materials (from agricultural residue, wood and paper mills) (Lin and Tanaka, 2006), because of the increase in demand for ethanol which is considered as an alternative biochemical source (Lynd *et al*., 1991). Steps involved in enzymatic preparation of ethanol include starch liquefaction, starch saccharification, fermentation, distillation and dehydration. In liquefaction step, gelatinization is required to increase the rate of hydrolysis as the native starch is slowly degraded by α-amylase (Nadir *et al*., 2009). Liquefaction process is employed to loosen the structure of starch polymer and reduce the viscosity of the gelatinized starch and ease the next hydrolysis processing. α-amylase is employed due to its active actions (1) degrade the long starch chains so that starch will not form a gel at lower temperature and, (2) produce more chain ends, as glucoamylase, the enzyme used in the saccharification step, will cleave glucose molecules only from the nonreducing ends of the chains. In liquefaction, pH is not allowed to drop below 4.5 otherwise the α-amylase

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will be denatured (Nigam and Singh, 1995). Saccharification step is important to further hydrolyze the liquefied starch. α-Glucosidase is used in the saccharification step. The glucosidase breaks the α-(1,6) glycosidic bonds in the liquefied starch chains.

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6$$

Fermentation step is to convert glucose to ethanol. *Saccharomyces cerevisiae* (yeast) is used for fermentation of sugar solution.

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2 + \text{heat}$$

For the ethanol to be used as usable fuel, water must be removed. Most of the water is removed by distillation. The most widely used purification method is a physical absorption process using a molecular sieve. Another method, azeotropic distillation, is achieved by adding hydrocarbon benzene which also denatures ethanol. Last method involves use of calcium oxide as desiccant.

Materials and Methods

Sample Collection

Sorghum grains were collected from Meiktila Township, Mandalay Region. Wheat grains were collected from Butalin Township, Sagging Region, after harvesting crops. Flint corn was collected from Shan-Ywa Village, Kyaukse Township, Mandalay Region.

Preparation of Bioethanol by Liquefaction and Saccharification

Substrate

Sorghum grains were blended into small size to enhance the hydrolysis process.

Enzymes

α-Amylase was extracted from germinated wheat grains and α-glucosidase was extracted from ungerminated flint corn by ammonium sulphate precipitation method. The activities of α-amylase and α-glucosidase were determined by spectrophotometric method at 750 nm employing Nelson-Somogyi Method.

Yeast

Saccharomyces cerevisiae yeast was obtained from a local market in dry form. For inoculum, 100 mL of distilled water was heated to 40 ^{o}C. After that, 0.5 % (w/w) of *Saccharomyces cerevisiae* yeast was added into the warm water to activate the yeast. The mixture was left for 5-10 min at 150 rpm.

Liquefaction and saccharification of sorgum starch

Sorghum (200 g) was added to a 1 L beaker and 900 mL of distilled water was then added into it. The mixture was heated while stirring for 1 h-and then cooled down to 55 ^{o}C. After that 100 mL of 1 % (w/v) α-amylase solution was added and stirred for 1 h. After 1 h liquefaction, the solution was cooled down to 50 ^{o}C and the pH of the solution was adjusted to 5 with hydrochloric acid. Next, 100 mL of 1 % (w/v) α-glucosidase solution was added and the mixture was left for 2 h. After 2 h saccharification, the solution was filtered into a glass bottle with a thin layer cotton cloth. After liquefaction and saccharification, the solution was tested for glucose by using Benedict solution and Fehling solution.

Fermentation

Into the liquid glucose solution 0.5 % (w/w) of urea and 0.05 % (w/w) potassium dihydrogen phosphate were added. After 10 min, the activated yeast solution was added. The mixture was mixed thoroughly to disperse uniformly. The bottle was loosely closed and fermented for 6 days at room temperature. After 6^{th} day, the fermented solution was filtered into a glass bottle with a thin layer cotton cloth. The physicochemical properties such as pH, specific gravity, refractive index, colour, free acid content, free base content of the fermented solution were investigated during fermentation.

Distillation

The fermented solution was filtered with a thin layer cotton cloth and then put into a 1L round-bottomed flask. During distillation the distillate at 78ºC was collected as bioethanol in a receiver. The yield percentage of alcohol is 10% obtained from fermented solution.

Dehydration of bioethanol

Bioethanol (100 mL) was put into a 1L beaker and then calcium oxide (15 g) was added into it. After constant stirring for 2 h, the mixture was transferred to a 1 L round-bottomed flask and heated. At 78 ºC, dehydrated bioethanol was liberated and collected in a receiver.

Characterization of Prepared Bioethanol

Qualitative test

Ethanol obtained after distillation was tested by using Lucas test and iodoform test.

Determination of the physicochemical properties of hydrated Bioethanol, dehydrated bioethanol and absolute ethanol

Specific gravity was determined by using a density bottle and refractive index was determined by a refractometer. Colour of bioethanol was determined by using Lovibond Tintometer. Free acid (as acetic acid) and free base (as ammonia) were determined by titrimetric method.

FT IR analysis of prepared bioethanol

Attenuated total reflection Fourier transform infrared (ATR-FT IR) spectra of the prepared bioethanol (both hydrated and dehydrated) were recorded on a Perkin Elmer FT IR spectrometer in a range of wave number from 4000 to 550 cm^{-1}. For comparison purpose the spectrum of absolute ethanol from Pure Chemical Industries, was also recorded.

Results and Discussion

Calculation of Enzyme Activities

α-Amylase was extracted from 5^{th} day germinated wheat grains by ammonium sulphate precipitation method. Sorghum starch was degraded into maltose by α-amylase. The amount of maltose liberated was determined by Nelson Somogyi method at 750 nm (Hatanaka and Kobara, 1980). The activity was calculated as the following:

$$\text{Activity} = \frac{\text{amount of maltose liberated}}{\text{volume of enzyme} \times \text{time}}$$

Moreover, α-glucosidase was extracted from flint corn by ammonium sulphate precipitation method. α-Glucosidase degraded maltose into glucose and the amount of glucose liberated was determined by Nelson-Somogyi method at 750 nm. The activities of α-amylase and α-glucosidase are shown in Table 1.

$$\text{Activity} = \frac{\text{amount of glucose liberated}}{\text{volume of enzyme} \times \text{time}}$$

Table 1 Activities of α-Amylase and α-Glucosidase

No	Enzyme	Activity (μmol mL^{-1} min^{-1})
1	α-Amylase	9.0
2	α-Glucosidase	54.03

Liquefaction and Saccharification of Sorghum Starch Powder

In the liquefaction step, hydrolysis of starch was carried out by α- amylase enzyme at 55 ºC for 1 h. This condition was chosen because plant amylases were found to have optimum temperature of 55 ºC (Nerkar, *et al*., 2011). In the saccharification step α-glucosidase breaks down starch to glucose. Saccharification temperature was chosen as 50 ºC at pH 5 because the optimum temperature of sweet corn was reported to be 50 ºC and the maximal activity was found in the range of pH 4.6 to 5 (Chaw Ei Phyu, 2010). The reported maximum saccharification was occurred at 45 ºC (Aggarwal *et al*., 2001). The glucose produced in the liquefaction and saccharification steps were confirmed by the Benedict test and Fehling test. Brick red precipitates were observed in these tests (Figure 1).

(a) (b)

Figure 1 Tests for glucose (a) Benedict (b) Fehling

Fermentation of liquid glucose solution

In this step activated *Saccharomyces cerevisiae* was added to ferment the liquid glucose solution. After fermentation for 6 days the physicochemical properties of the fermented solution were determined. The results are shown in Table 2.

Table 2 Physicochemical Properties of Fermented Solution During Fermentation

No.	Parameters determined	Fermentation Time					
		1st day	2nd day	3rd day	4thday	5th day	6th day
1	pH	5.2	5.1	4.5	3.9	3.8	3.7
2	Specific gravity	0.994	0.990	0.986	0.980	0.978	0.975
3	Acid content (%)	0.093	0.177	0.252	0.327	0.399	0.423
4	Glucose (mg L^{-1})	155	140	130	125	112	63

Distillation of Fermented Solution

After fermentation for 6 days, the fermented solution was distilled and the distillate was collected at 78 ^{o}C. The alcohol content of the hydrated bioethanol was 20 %. In the distillation process, the alcohol content was increased up to 95 % by repeat distillation (Table 3).

Dehydration of Bioethanol

Since prepared bioethanol contained 5 % water, dehydration was carried out by using calcium oxide as desiccant.After dehydration the alcohol content increased up to 98 % (Table 3).

Qualitative Tests for Bioethanol

Bioethanol was qualitatively examined by Lucas test and iodoform test. No oily layer was obtained when treated bioethanol with anhydrous zinc chloride and hydrochloric acid. When ethanol was treated with chloroform, iodine and sodium hydroxide in water bath, yellow precipitate of iodoform were obtained (Figure 2).

(a) (b)

Figure 2 Qualitative test for bioethanol (a) Lucas test (b) iodoform test

Comparison of Some Physicochemical Properties of Hydrated, Dehydrated and Absolute Ethanol from Pure Chemical Industries

Physicochemical properties of hydrated, dehydrated and absolute ethanol from Pure Chemical Industries, were compared and the results are shown in (Table 3). The specific gravity of distilled water is, by definition, equal to one. Pure ethanol has a specific gravity of 0.79. Therefore, it follows that a mixture of water and ethanol must have a specific gravity that is less than one, the more alcohol the lower the specific gravity. Specific gravity of the fermented solution was found to be 0.994 after fermentation for 6 days. After distillation the specific gravity decreased

to 0.803 indicating that the increase in ethanol content. It means that yield of ethanol content after 6 days fermentation increased from 20 % to 95 % after distillation. After dehydration the ethanol content increased to 98 %. The specific gravity of absolute ethanol from Pure Chemical Industries was found to be 0.793 and ethanol content was 99 %. Refractive index is the ratio of velocity of light in air to that in the substance. Refractive index of the hydrated bioethanol was 1.382 while that of dehydrated one was 1.37. These values are slightly higher than that of absolute ethanol. Refractive index has been found to provide a reliable indication of the dry weight of the solids in the solution. All the colours of the hydrated and dehydrated bioethanol were colourless by measuring Lovibond tintometer as the absolute ethanol. Free acids and free base percentages were determined by titrimetric method. Free acid as acetic acid contents were found to be nearly the same, i.e., 0.006 % and 0.007 %. Free base as ammonia contents in hydrated ethanol (0.006 %) was higher than those of dehydrated ethanol (0.003 %) and absolute ethanol (0.0025 %).

Table 3 Comparison of the Physicochemical Properties of Hydrated, Dehydrated and Absolute Ethanol

No	Parameters	Hydrated bioethanol	Dehydrated bioethanol	Absolute ethanol
1	Specific gravity	0.803	0.801	0.793
2	Refractive index	1.382	1.371	1.361
3	Colour	colourless	colourless	colourless
4	Free acid (acetic acid) (%)	0.007	0.006	0.006
5	Free base (as ammonia) (%)	0.006	0.003	0.0025
~~6~~	Alcohol content (%)	95	98	99

FT IR Spectral Data of Dehydrated Bioethanol, Hydrated Bioethanol and Absolute Ethanol

FT IR spectra of dehydrated bioethanol, hydrated bioethanol and absolute ethanol from Pure Chemical Industries were shown in (Figure 3) and the spectral data are shown in Table 4. All the spectra show –OH stretching vibration at between 3500-3300 cm^{-1}. However, the intensity of –OH stretching peak for hydrated ethanol was found to be higher than those of dehydrated and absolute ethanol. It is due to the coalescent peak which is caused by the –OH peak from water and that from ethanol. All spectra show that the –CH stretching peaks were between 2983-2973 cm^{-1}.The –OH bending and –CH bending vibrations were observed at ~1650 cm^{-1} and 1455 cm^{-1} respectively. The C-C-O stretching vibrations were observed at ~1275 and 1087 cm^{-1}. Among the spectra the spectrum of dehydrated bioethanol was similar to that of absolute ethanol.

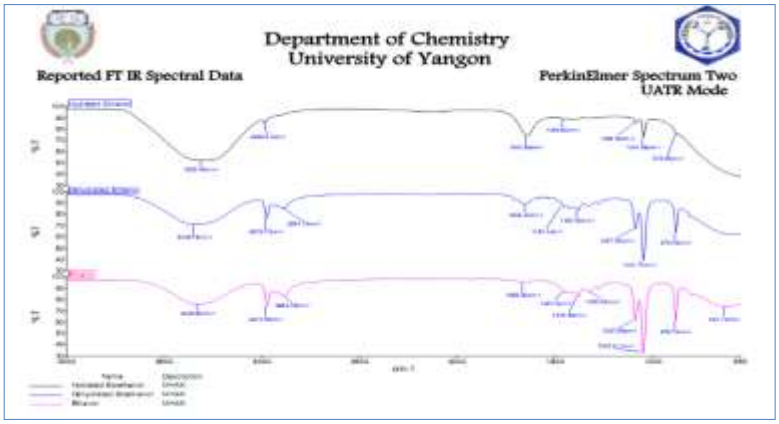

Figure 3 FT IR spectra of dehydrated bioethanol, hydrated bioethanol and absolute ethanol from Pure Chemical Industries

Table 4 FT IR Spectral Data for Dehydrated Bioethanol, Hydrated Bioethanol and Absolute Ethanol

Sr No	Wave number (cm^{-1})			Remark
	Hydrated bioethanol	Dehydrated bioethanol	Absolute ethanol	
1	3306	3339	3325	O-H stretching
2	2983	2975, 2894	2973, 2883	C-H stretching of CH_3 and CH_2
3	1455	1455	1455	-OH bending
4	1272	1275	1275	C-C-O bending
5	1085	1087	1087	C-O stretching

Conclusion

Enzymatic hydrolysis of sorghum starch was carried out to prepare bioethanol in this study. α-Amylase was extracted from germinated wheat grain having the activity of 9.0 μmol mL^{-1} min^{-1} and α-glucosiadse was extracted from ungerminated flint corn having activity of 54.03 μmol mL^{-1} min^{-1} were used for liquefaction and saccharification of sorghum starch. *Saccharomyces cerevisiae* yeast was used for the fermentation of glucose solution. After saccharification the obtained glucose solution was confirmed by using Benedict solution and Fehling solution which gave red precipitate. After fermentation the pH of the fermented solution was 3.7 and specific gravity was 0.975. Distillation of the fermented solution at 78°C gave hydrated bioethanol which contain the alcohol content (95%). After dehydration with calcium oxide the alcohol content increase to 98 % in dehydrated ethanol. The physicochemical properties of the hydrated bioethanol were specific gravity (0.803), refractive index (1.382), colour (colourless), free acid as acetic acid (0.007 %), free base as ammonia (0.006 %) and alcohol content (95 %). After dehydration, the physicochemical properties of dehydrated bioethanol were specific gravity (0.801), refractive index (1.371), colour (colourless), free acid as acetic acid (0.006 %), free base as ammonia (0.003 %) and alcohol content (98 %). FT IR spectra of dehydrated bioethanol, hydrated bioethanol and absolute ethanol from Pure Chemical Industries were comparatively studied. In hydrated bioethanol, the intensity of OH- stretching peak was found to be higher than those of dehydrated and absolute ethanol. This peak is a coalescent peak which is caused by OH- group from water and that from ethanol. All spectral data show the characteristic peaks of ethanol. Thus, the spectrum of dehydrated bioethanol was similar to that of absolute bioethanol.

Acknowledgements

We would like to thank to the Department of Higher Education, Ministry of Education in Myanmar, for giving the opportunity to do this research. Our deepest gratitude is expressed to Dr Ba Han, Rector, Meiktila University, for his encouragement and kind help to do this research. Thanks, are also extended to all teachers Department of Chemistry, Meiktila University, for their helpful advice.

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