Extraction and Characterization of α -Amylase and α-Glucosidase Enzymes for Ethanol Preparation

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Abstract

In the research work, two enzymes (α -amylase and α-glucosidase) were extracted from germinated wheat grains and ungerminated flint corn by ammonium sulphate precipitation method for the bioethanol preparation. Moreover, their activities were determined by using Nelson-Somogyi Method. Qualitatively examination of α -amylase and α -glucosidase were done by iodine staining method. The surface morphology of sorghum was determined by scanning electron microscope and the physicochemical properties were also determined. α-Amylase was used for liquefaction of starch and α -glucosidase was used in saccharification step.
Key words: bioethanol, sorghum, amylase, glucosidase, surface morphology

Introduction

Bioethanol is ethanol (alcohol) that is derived exclusively from the fermentation of plant starches such as sugar cane, grains, potato, corn and agricultural waste. Bioethanol is a form of renewable energy that can be produced from agricultural feedstocks. The energy and pollution balance of the whole cycle of ethanol production, especially from corn. Renewable energy means energy which comes from natural resources which are renewable (naturally replenished). 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). Glucose (a simple sugar) is created in the plant by photosynthesis.

$$6CO_2 + 6H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2$$

Ethanol production relying on fermentation processes is based on carbohydrate rich raw materials. Starch– based crops (rye, wheat, oat, millet, rice) and other carbohydrate sources (potato, sorghum) have been used as raw material for food industry and pharmacy.

Modern technologies enable complex converting of cereal materials to ethanol, providing high yields of ethanol, high quality feed, energy, and high – value biological fertilizers. Utilizing enzymatic and fermentation technology ethanol can be produced from low cost raw material such as agricultural waste, agro-industrial waste, woody, crops, sorghum, potato, and sago (Patle and Lal, 2008). Enzymatic preparation of ethanol involved the following steps. There are (1) Starch liquefaction, (2) Starch saccharification, (3) Fermenation and (4) Distillation and Dehydration. Starch is a polymer of glucose. Cereal grain starch is normally a mixture of two types of polymers: amylase, a linear glucose polymer, and amylopetin, a branched polymer. Starch is the main carbohydrate storage in many plants. Starch from all plant sources occurs in the form of water soluble granules which differ in size and physical characteristics from species to species. The hydrolysis of starch to glucose requires two types of enzymes. The alpha-amylase is a bacterial thermostable endo-amylase. It hydrolyzes α-1,4-bonds at random points in the starch molecule to rapidly reduce the viscosity of gelatinized starch solutions. Glucoamylase, produced by fungi, is an exo-amylase. It hydrolyse the maltose and dextrins from the non-reducing end of the molecule. Glucoamylase hydrolyzes both and α-1, 4 and α-1, 6-bonds to completely degrade the dextrins to glucose (Dicko *et al*., 2006). The enzyme is optimally active at pH 3.5-4.5. So pH adjustment after saccharification is not needed for the yeast fermentation. The yeast fermentation takes place at pH 3.7-4.

Materials and Methods

Determination of Chemical Composition of Starch

Sorghum starch powder (2 g) was placed in a 500 mL round bottomed flask to which 100 mL of distilled water and 20 mL of 25 % hydrochloric acid were added. The mixture was then boiled gently under water bath for 2 hr. Next the solution was cooled down to room temperature and neutralized with 25 % sodium hydroxide solution. Then the solution was filtered and diluted to 500 mL with distilled water in a volumetric flask. The amount of reducing sugar in this solution was determined by Lane-Eynon method (1923). Moreover, moisture content, ash content, protein content and fat were also determined by oven drying method, muffle furnace, Kjeldahl digestion method, and soxhlet extraction method respectively.

Study on Surface Morphology of Sorghum Powder

Surface morphology of prepared sorghum grain powder was investigated by Scanning Electron Microscopy (SEM) employing scanning electron microscope (Model JSM-5610 LV, JEOL. Japan) operating at 15 kV and 1000x magnification.

Extraction of α-Amylase from Germinated Wheat Grains

Wheat seeds 500 g were wet with water and kept at room temperature. After 4^{th}, 5^{th} days germinated seeds (100 g) were taken and mixed with 400 mL solution mixture of 0.01 M sodium chloride solution and 0.1 M acetate buffer (1:1). The mixture was blended in a blender for 10 min. The solution was stirred with magnetic stirrer for 1 hr at room temperature and heated to 70 °C for 10 min to inactivate β-amylase and then filtered by using thin cotton cloth. Filtration was continued by using the centrifuge at 3000 rpm for 20 min. The filtrate was mixed with ammonium sulphate (80 % saturation). Then the solution was stirred and kept over-night. Finally, it was filtered and enzyme precipitate was obtained.

Extraction of α-Glucosidase from Flint Corn

Flint corn (ungerminated seeds) 500g were ground by crushing machine and then the flint corn powder was obtained. Then the flint corn powder (100g) were taken and mixed with 200 mL of 0.1 M acetate buffer. The mixture was stirred with magnetic stirrer for 3 hr at room temperature and then filtered by using thin cotton cloth. Filtration was continued by using the centrifuge at 3000 rpm for 20 min. The enzyme precipitate was also prepared to the above procedure.

Determination of Amylase/ Glucosidase Activities by Nelson Somogyi Method

A 0.5 mL of standard maltose solution I was pipetted into a test tube containing 1 mL of alkaline copper reagent solution and the contents were mixed well (Whistler, 1965 and Oser, 1976). The test tube was heated on a vigorously boiling water-bath for 10 min. Next the tube was cooled under running tap water for 1 min and 1 mL of arsenomolybdate colour reagent solution was added into the test tube. The solution mixture gave a bluish green colour after shaking vigorously. This solution was diluted to 10 mL with distilled water and mixed by inversion. Similary, 0.5 mL of standard maltose solutions II, III, IV and V were prepared according to the above procedure. Standard glucose solution I, II, III, IV and V were also prepared to the above procedure. The range of wavelength was fixed between 705 and 780 nm. Then the wavelength of maximum absorption of arsenomolybdate chromogenic compound was found at 750 nm.

Qualitative Examination of α-Amylase and α-Glucosidase Activities by Iodine Staining Method

A test tube containing 1.0 mL of enzyme solution was labeled as tube A and another tube containing 1.0 mL of distilled water was labeled as tube B for blank solution. 3 mL each of 2 % starch solution was added into test tubes A and B, then the contents were mixed well and stand for 30min. After this, 1.0 mL each of iodine solution was added into tubes A and B, respectively.

Construction of Calibration Curve for Arsenomolybdate Chromogenic Compound

The absorbance of five arsenomolybdate chromogenic solutions obtained from standard maltose solutions I, II, III, IV and V were measured at 750 nm against the blank solution with a UV-visible spectrophotometer. Then the plot of absorbance against concentrations of standard glucose solutions was done. Standard glucose solution I, II, III, IV and V were also prepared to the above procedure.

Determination of α-Amylase Activity during Germination of Wheat Seeds

α-Amylase activity was determined by measurement of maltose from starch according to Nelson Somogyi method (Nelson and Somogyi, 1973). Wheat seeds 500 g were wet with water and kept at room temperature. After 1 day germinated seeds (10 g) were taken and mixed with 40 mL solution mixture of 0.01 M sodium chloride solution and 0.1 M acetate buffer (1:1). The mixture was blended in a blender for 10 min. The solution was stirred with magnetic stirrer for 1 hr at room temperature, and heated to 70 °C for 10 min to inactivate contaminated β-amylase and then filtered by using thin cotton cloth. The solution was continued by using the centrifuge at 3000 rpm for 20 min. Finally crude enzyme extract was obtained. Similarly, same procedure was carried out for 2, 3, 4, 5, 6 and 7 days. The prepared enzyme solutions were kept in refrigerator at 40 °C.

Results and Discussion

Examination of Surface Morphology of Sorghum Powder

Figure 1 shows the SEM image of sorghum powder to be used for preparation of bioethanol. Sorghum powder showed foam-like structure with irregular shape. Thus, it was easily attacked by enzyme.

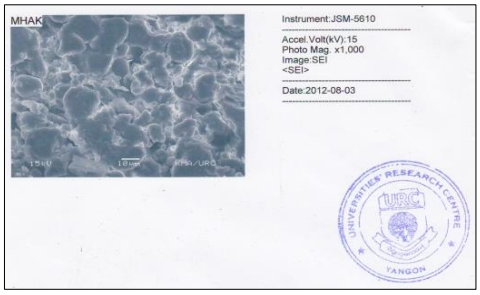

Figure 1. SEM image of sorghum powder

Physicochemical Properties of Sorghum

Physicochemical properties of sorghum grain is shown in Table 1. Moisture content was found to be 17.53 % and ash content was 3.53 %. Sorghum grain was found to be 45.01 % starch, 9.33 % protein and 0.70 % crude fat. Being a rich source of starch and it is suitable for preparation of bioethanol.

Table 1. Analysis of sorghum starch

No.	Parameter determined	Percent (%)
1	Moisture	17.53
2	Ash	3.53
3	Starch	45.01
4	Protein	9.33
5	Fat	0.70

Wavelength of Maximum Absorption of Arsenomolybdate Chromogenic Compound in Nelson-Somogyi Method

Starch is hydrolyzed by α- amylase to maltose. By using the Nelson-Somogyi reagent reducing sugar was determined by measuring the absorbance of arsenomolybdate chromogenic compound formed from reduction by sugar. Thus the Nelson reagent also termed as arsenomolybdate colour reagent is used to measure the reducing power of maltose. In this research, the absorption spectrum of arsenomolybdate chromogenic compound was recorded in the range from 705-780 nm (figure 2) and the wavelength of maximum absorption was found at 750 nm.

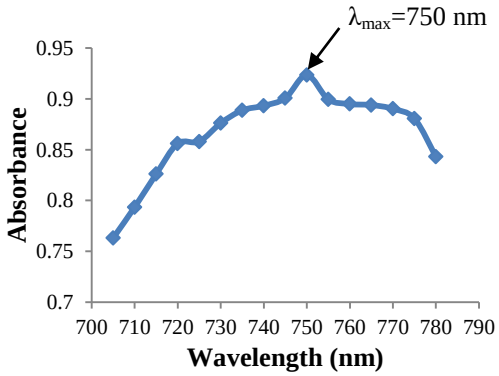

Figure 2. Wavelength of maximum absorption of arsenomolybdate chromogenic compound for standard maltose solution

Calibration curve for Standard Maltose by Nelson – Somogyi Method

In this research, the absorbance values of different maltose concentrations ranging from 1.11×10^{-4} to 5.55×10^{-4} M measured at 750 nm by using a visible spectrophotometer (Smart Spectro). The nature of the plot of absorbance Vs concentration of maltose was straight line (correlation coefficient R^2 of 0.9962) passing through the origin showing that this calibrate curve was obeyed Beer's Law (Table 2 and figure 3).

Table 2. Relationship between absorbance of arsenomolybdate and concentration of maltose

Concentration × 10^{-4} /M	Absorbance (λ_{max}=750 nm)
1.11	0.223
2.22	0.3994
3.33	0.6003
4.44	0.8819
5.55	1.0765

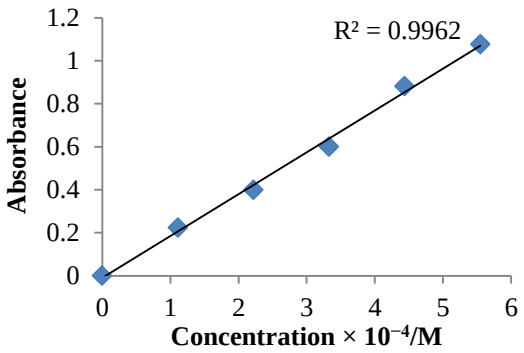

Figure 3. Standard curve for standard maltose solution at 750nm

Table 3. Relationship between absorbance and activity of α-amylase

No.	Absorbance	Activity (μ mol ml^{-1} min^{-1})	Mean (μ mol mL^{-1} min^{-1})
1	1.619	8.981	9.0
2	1.622	8.998	
3	1.625	9.014	

The activity was calculated as

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

Wavelength of Maximum Absorption of Arsenomolybdate Chromogenic Compound in Nelson – Somogyi Method

Starch is hydrolyzed by maltose to glucose. By using the Nelson – Somogyi reagent reducing sugar was determined by measuring the absorbance of arseno-molybdate chromogenic compound formed

from reduction by sugar. In this research, the absorption spectrum of arsenomolybdate chromogenic compound was recorded in the range from 705-785 nm (Figure 4) and the wavelength of maximum absorption was found at 750 nm.

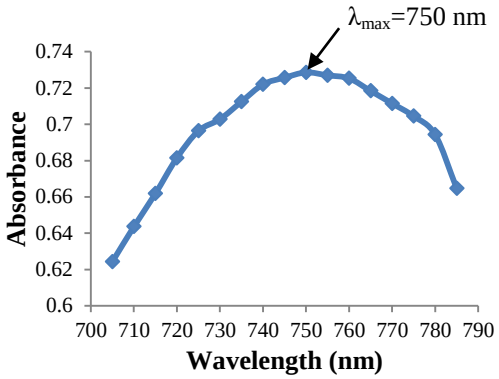

Figure 4. Wavelength of maximum absorption of arsenomolybdate chromogenic compound for standard glucose solution

Calibration curve for standard Glucose by Nelson – Somogyi method

In this research, the absorbance values of different glucose concentrations ranging from 1.11×10^{-4} to 5.55×10^{-4} M are measured at 750 nm by using a visible spectrophotometer (smart spectro). The nature of the plot of absorbance vs concentration of glucose was straight line correlation coefficient R^2 of 0.9989) passing through the origin showing that this calibrate curve was obeyed the Beer's Law (Table 4 and Figure 5).

Table 4. Relationship between absorbance of arsenomolybdate chromogenic compound and concentration of glucose

Concentration × 10^{-3} /M	Absorbance (λ_{max}=750 nm)
0.555	0.2059
1.111	0.4621
1.666	0.7074
2.222	0.9703
2.777	1.1817

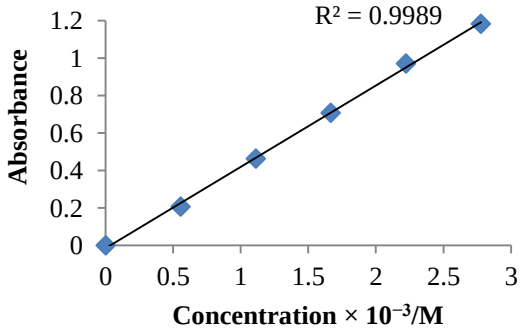

Figure 5. Standard curve for standard glucose solution at 750 nm

Table 5. Relationship between absorbance and activity of α-glucosidase

No.	Absorbance	Activity (μ mol mL^{-1} min^{-1})	Mean (μ mol mL^{-1} min^{-1})
1	2.2957	54.07	54.03
2	2.2903	53.94	
3	2.2957	54.07	

The activity was calculated as

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

Variation of α-Amylase in Germinated Wheat

In this work the activity of α-amylase vs day of germination was shown in Figure 6. It was found that the α-amylase activity was increased day by day. The maximum α-amylase activity was found to be (6.485) EU of 5 days. After 5 days the activity decreased with the increase in germination day.

Table 6. Variation of α-amylase activity with day of germination

Day of germination	Absorbance	Activity (μ mol mL^{-1} min^{-1})
1	0.395	2.191
2	0.725	4.022
3	0.789	4.427
4	0.899	4.990
5	1.162	6.485
6	1.102	6.113
7	0.926	5.137

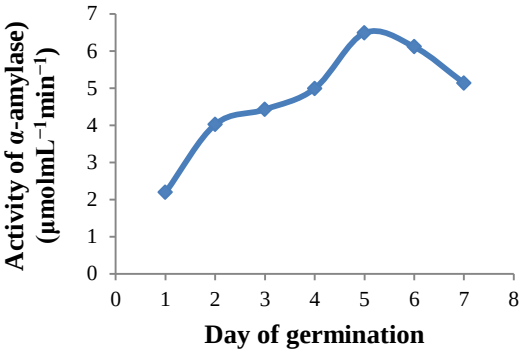

Figure.6 Changes of α-amylase activity during germination

Figure 7. Degradation of starch by enzymes extracts of α-amylase

Conclusion

The enzymes used for bioethanol preparation *viz*. α-amylase and α-glucosidase were extracted from germinated wheat and ungerminated flint corn. The activity of α-amylase of wheat seed was found to be the highest (6.485 μ mol mL^{-1} min^{-1}) at fifth day of germination. α-Amylase and α-glucosidase were confirmed by qualitative examination using iodine staining method. The activity of extracted α-amylase and α-glucosidase were found to be 9.0 μ mol ml^{-1} min^{-1} and 54.03 μ mol mL^{-1} min^{-1} respectively. Enzymatic hydrolysis of sorghum starch was carried out to prepare bioethanol in this study. Firstly, some chemical compositions of starch were determined and sorghum starch was found to have 17.53 % of moisture, 3.53 % of ash, 45.01 % of starch, 9.33 % of protein and 0.70 % of crude fat. SEM image showed the sorghum powder as irregular shape with foam-like structure which can easily be hydrolyzed by enzyme. α-Amalyse extracted from germinated wheat grain having the activity of 9.0 μ mol mL^{-1} min^{-1} and α-glucosidase extracted from ungerminated flint corn having activity of 54.03 μ mol mL^{-1} min^{-1} were used for liquefaction and saccharification of sorghum starch.

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