Production and Identification of Glucose from Rice Straw by Hydrolyzing with Cellulase Extracted from *Lycopersiconesculentum* Mill. (Tomato) Fruit

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Abstract

Rice straw is an agriculture by-product. Glucose is a simple sugar ($C_6H_{12}O_6$) and it circulates in the blood of animals as blood sugar. Cellulase (EC 3.2.1.4) was identified as one of the key enzyme degrading cellulose. So, this research was performed to produce glucose (valuable product) from rice straw (by-product) by using cellulase enzyme extracted from tomato fruits (plenty in local). Firstly, cellulase was extracted from tomato fruits. The cellulasecan hydrolyze cellulose to produce glucose. Therefore, the glucose concentration in the reaction mixture wasdetermined by glucose-oxidase enzyme reagent method. Then standard calibration curve was constructed using standard glucose solution at the absorbance of 505 nm. The optimum conditions on cellulase-catalyzed reaction were determined.Secondly, hydrolyzing of cellulose present in rice straw was carried out by cellulase at optimum conditions. The changes of the concentration of glucose with hydrolyzing time were studied by UV-visspectrophotometry and the percent yield of glucose was calculated. Finally, the qualitative analysis of glucose produced from rice straw was performed. According to FTIR result, the hydrolyzing product of rice straw was glucose.It validates that glucose can be produced from rice straw by using cellulase enzyme.

Keywords: Cellulase,Rice straw,Glucose,Glucose-oxidase enzyme reagent, FT IR

Introduction

Tomato

In 1753, Linnaeus placed the tomato in the genus*Solanum* (alongside the potato) as *Solanumlycopersicum*. In 1768, Philip Miller moved it to its own genus, naming it *Lycopersiconesculentum*.This name came into wide use, but was in breach of the plant naming rules(Website 1).The scientific name of tomato is *Lycopersicon esculentum* Mill. It is classified as follows:

Kingdom	:	Plantae
Phylum	:	Angiospermophyta
Class	:	Magnoliopsida
Order	:	Gentianales
Family	:	Solanaceae
Genus	:	*Lycopersicon*
Species	:	*L. esculentum*Mill.
Scientific name	:	*Lycopersiconesculentum* Mill.
English name	:	Tomato
Myanmar name	:	Khayanjin

Figure1 Tomatofruits

Cellulase

Cellulase(EC 3.2.1.4) refers to a class of enzyme produced chiefly by fungi, bacteria, and protozoan that catalyze cellulolysis (i.e. the hydrolysis of cellulose).Cellulase is also found in tomato, mango, avocado,and pea epicotyls (Website 2).

Reaction Mechanism of Cellulases

The three types of reaction (Figure 2) catalyzed by cellulases: (1) breakage of the non-covalent interactions present in the crystalline structure of cellulose, endo-cellulase (2) hydrolysis of the individual cellulose fibers to break it into smaller sugars, exo-cellulase (3) hydrolysis of disaccharides and tetrasaccharides into glucose, β-glucosidase (Website 2).

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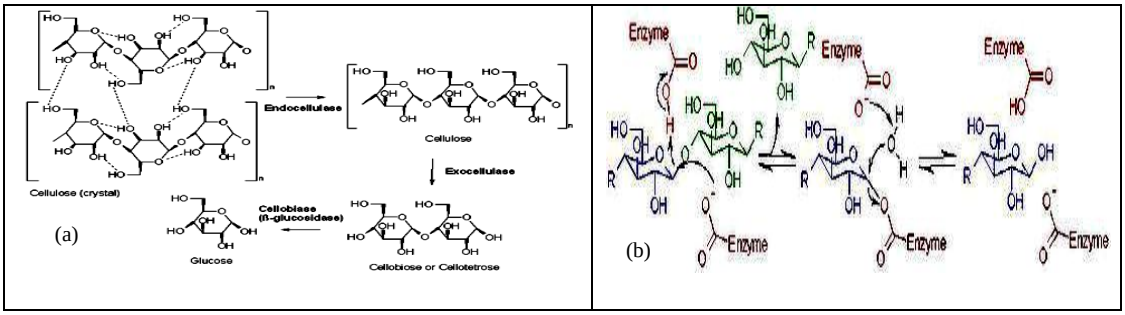

Figure2 Cellulolysis(a) three types of cellulase enzyme attack on cellulose crystal (b)mechanistic details of beta-glucosidase activity of cellulase

Cellulose and Rice Straw

Cellulose is the world's most abundant natural biopolymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals.Rice straw is composed of 35% cellulose, 18 % hemicellulose, and 15 % lignin(Kotchoni*et al.*, 2003).

Glucose

Glucose is a simple sugar with the molecular formula$C_6H_{12}O_6$. Glucose circulates in the blood of animals as blood sugar. It is made during photosynthesis from water and carbon dioxide, using energy from sunlight. The reverse of the photosynthesis reaction, which releases this energy, is an important source of power for cellular respiration. Glucose is stored as a polymer, in plants as starch and in animals as glycogen, for times when the organism will need it(Website 3).

Materials and Methods

Extraction of Cellulase from Tomato

The tissue of 1 Kg of tomato fruits was cooled in a beaker placed in a refrigerator and then blended for 2 min in a blender at high speed. The macerate of tomato fruits was adjusted to pH 8.0 by addition of 1M sodium hydroxide solution. After 45 min placed in a refrigerator to allow adequate desorption of the enzyme, the macerate was centrifuged at 20,000 U $min^{-1}$for 2 min. The filtrate was collected and kept in the refrigerator for 45 min and then centrifuged again. After repeated cooling and centrifugation, the solution was filtered by a filter paper to remove other suspensions. The supernatant fluid containing cellulase activity was collected and kept in refrigerator at 4ºC to use directly for enzyme assays.

Determination of Wavelength of Maximum Absorption of Quinoneimine Chromogenic Compound in Glucose-oxidase Enzyme Reagent Method

The standard glucose solution I was obtained by added 3mL of stock standard glucose solution (3.028 $x10^{-4}$M) into a test tube. Then 1 mL of glucose-oxidase enzyme reagent solution was pipetted into this test tube. This solution mixture gave a pale red color. This solution mixture was kept in an incubator at 37˚C for 10 minutes. The solution was diluted to 5 mL with distilled water and quinoneimine chromogenic solution was obtained. Similarly, by taking 2.5, 2.0, 1.5 and 1 mL of standard stock glucose solution ($3.028x10^{-4}$ M), standard glucose solution II ($2.523 x10^{-4}$ M), III ($2.018 x10^{-4}$ M), IV ($1.514 x10^{-4}$ M) and V ($1.000 10^{-4}$ M) were obtained. Then these were prepared according to the above procedure.A blank solution was prepared by carried out the procedure as describe above except that 1 mL of distilled water was used instead of 1 mL of standard glucose solution.The absorption spectra were recorded from 400 to 600 nm by using UV-visible spectrophotometer and the wavelength of maximum absorption was determined. The absorbance of quinoneimine chromogenic solutions obtained from standard glucose solutions I, II,

III, IV and V were measured at 505 nm against the blank solution with a UV-visible spectrophotometer. Then the plot of absorbance against the concentration of standard glucose solution was drawn.

Determination of Cellulase Activity

A 0.3 mL of distilled water was pipetted into a test tube containing 0.1 mL of cellulose solution, then 0.1 mL of extract enzyme solution was added and the contents were mixed well. After 10 min at room temperature, 1 mL of glucose-oxidase enzyme reagent was added and incubated at 37˚C. Then the reaction was interrupted by adding 3mL of distilled water. The contents were then mixed thoroughly.In the case of blank solution, 0.1 mL of distilled water was used, instead of enzyme solution. The absorbance was measured at 505 nm against the blank solution.

Determination of Reaction Time of Cellulase-catalyzed Reaction

A 0.3 mL of distilled water was pipetted into a test tube containing 0.1 mL of cellulose solution, then 0.1 mL of enzyme solution was added and the contents were mixed well. After 5 min at room temperature, 1 mL of glucose-oxidase enzyme reagent was added and incubated at 37˚C. Then cellulase activity was determined by spectrophotometry. The whole of the above procedure was repeated for the reaction times of 10, 15, 20, 25, 30, 45 and 60 min by varying the mixing times of cellulose with cellulase enzyme.

Determination of Optimum pH of Cellulase-catalyzed Reaction

The reaction mixture consist of the 0.1 mL of enzyme solution, 0.3 mL of 0.1M sodium acetate buffer solution (pH 5.0) and 0.1mL of cellulose solution was mixed well and kept for 10 min at room temperature. After that, 1mL of glucose-oxidase enzyme reagent solution was added to this reaction mixture and incubated at 37˚C for 10 min. Then 3 mL of distilled water was added to terminate the reaction.For the blank solution 0.1 mL of distilled water was used instead of enzyme. The enzyme activities at pH 6.0, 7.0, 7.5, 8.0, 9.2 and 10 were carried out asabove procedure using appropriate buffer solutions.

Determination of Optimum Temperature of Cellulase-catalyzed Reaction

The reaction mixture consist of the 0.1 mL of enzyme solution, 0.3 mL of 0.1 M of sodium phosphate buffer solution (pH 7.5) and 0.1 mL of cellulose solution was mixed well and kept in water bath at 25˚C for 10 min. After that, the remaining procedure was carried out the same as the procedure in pH determination.The whole of the above procedure was repeated at 30, 35, 40, 45, 50, 55, 60, 65 and 70 ˚C respectively.

Production of Glucose from Rice Straw by Hydrolyzing withCellulase Enzyme

The mixture of 10 g of rice straw powder, 500 mL of 1 M phosphate buffer (pH 7.5) and 50 mL of crude enzyme solution was added to the 1 L conical flask and shake at 50˚ C. After 15 min, 0.5 mL of the reaction mixture was taken and centrifuged for 10 min to remove insoluble parts. Then 0.3 mL of the filtrate was pipette into a test tube containing 1 mL of glucose-oxidase enzyme reagent and incubated at 37˚ C for 10 min. Then 3 mL of distilled water was added into the solution. The contents were mixed thoroughly and absorbance was measured at 505 nm.Same procedures were carried out for hydrolyzing times of 20, 30, 40, 50, 60, 90, 120, 150, 180 min. The glucose contents in the filtrates were determined by using glucose-oxidase enzyme reagent method. From the resulting absorbance, the concentration of glucose produced from rice straw as a function of hydrolyzing times was calculated.

Qualitative Analysis of Glucose Produced from Rice Straw

Each 1 mL of the solution produced from hydrolyzed rice straw was examined with Benedict's Test, Barfoed'sTest and Fehling's Test.

FT IR Assignment of Glucose Produced from Rice Straw

The hydrolyzing product of rice straw was structurally identified by FT IR spectroscopy at West Yangon University. It is shown in Table 6 and Figure 8.

Results and Discussion

Calibration Curve for Standard Glucose Solutions

To construct the calibration curve, the standard glucose solution was used. Glucose is oxidized by glucose-oxidase reagent to gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts in the presence of peroxides with phenol and amino-4-antipyrine forming a red quinoneimine dye. The intensity of the colour formation is proportional to the glucose concentration. In the determination of concentration of glucose by measuring the absorbance of reduced a red quinonemine dye compound in the glucose-oxidase enzyme reagent method (Caraway, 1976).Then standard calibration curve was constructed using standard glucose solution at the absorbance of 505 nm.The different absorbance values were obtained for different concentrations by using a UV-visible spectrophotometer. It was found that the nature of the plot of absorbance at 505 nm *vs.* concentration of glucose was in straight line passing through the origin showing that Beer's Law was obeyed.

Cellulase Activity

The cellulase activity was determined by spectrophotometric method. According to enzyme specificity, the enzyme that can hydrolyzecellulose to glucose was cellulase. In the present research, the action of cellulase on cellulose was studied by measuring glucose concentration. The cellulase enzyme activity was calculated by the following equation.The unit of cellulase activity was $\mu molmL^{-1}min^{-1}$.

$$\text{Activity} = \frac{\mu\text{mole of glucose}}{\text{Reaction time} \times \text{Volume of enzyme solution}}$$

Reaction Time of Cellulase-catalyzed Reaction

Each enzyme has its own particular reaction time to get the maximum. In the present research, the amount of glucose liberated during the various reaction times of 5, 10, 15, 20, 25, 30, 45and 60 min were determined by glucose-oxidase enzyme reagent method. The plot of velocity *vs.* reaction times revealed that the velocity decreased linearly with time up to 10 minutes and after that velocity decreased very slowly (Figure 3). Therefore, reaction time of 10 min was chosen in this research.

Optimum pH of Cellulase-catalyzed Reaction

Each enzyme has a characteristic optimum pH. At which the velocity is maximum, and on each site of this optimum the rate of enzyme-catalyzed reaction is lower (Bell *et al.*, 1972). In the present research, The nature of the activity *vs.* pH curve of the enzyme (Figure 4) was obviously found to be symmetrical and it was found that the optimum pH for maximum cellulase activity was 7.5. This value is in agreement with the literature ranging between pH 3 to 9 for cellulase. From lower to optimum pH, the activities increased with pH gradually and over the optimum pH, the activities decreased rapidly due to the denaturing of enzyme protein.

Optimum Temperature of Cellulase-catalyzed Reaction

Temperature is an important factor in the regular of enzyme activity (Website 4). Cellulase preparations are effective between 40 to 50°C. The optimum pH generally lies between 40 and 50˚C (Beldmen*et al.*, 1985).

The effect of the temperature on the activity was investigated in the temperature range from 25 to 70°C. The optimum temperature for enzyme was found to be 50°C (Figure 4). It was obvious that the activity of cellulase was increased from 25 to 50°C and then decreased from 50 to 70°C.

Production of Glucose from Rice Straw by using Cellulase

Rice straw is a by-product of rice production and great bio resource. Rice straw predominally contains cellulose 32-47 %, hemicelluloses 19-27 % and lignin 5-24 %, and ashes 18.8 % (Yoswathana and Phuriphipot, 2010).

In the present research, preparation of glucose from rice straw powder was carried out by using cellulasewhich hydrolyzed the carboxymethyl cellulose and cellulose in rice straw powder (Adereml, *et al*., 2004). Table 4 showed relationship between hydrolyzing times and concentration of glucose liberated from rice straw. It was found that glucose concentration increased with reaction time up to 90 min, 9.716 $\times 10^{-4}$M glucose was obtained. After that increases of glucose concentration were very slow. After 180 min hydrolyzing time, 9.916 $\times$ 10^{-4} M glucose was obtained. So, the percent yield was calculated from 9.716 $\times 10^{-4}$M of glucose.Figure 5 showed a plot of concentration of glucose liberated as a function of hydrolyzing time.

Qualitative Analysis of Glucose Produced from Rice Straw

The extracted glucose from rice straw powder was analyzed qualitatively by using Test Solutions. In Benedict's Solution Test, reddish precipitate was observed and shown in Figure 7 (a). It was due to reduction of cupric hydroxide in alkaline solution to cuprous oxide by sugar from tested solution. In Barfoed's Solution Test, orange coloured precipitate was observed and shown in Figure 7(b). Barfoed's reagent was weakly acidic and it was reduced by monosaccharides from tested solution. In Fehling's Solution Test, brick red precipitate was observed and shown in Figure 7 (c). It was due to the reduction of cupric hydroxide present in Fehling's solution to cuprous oxide by the reducing sugar from tested solution.

Table 1 Relationship between Reaction Time and Velocity of the Cellulase-catalyzed Reaction

No.	Reaction time (min)	Absorbance at 505 nm	Velocity (10^{-5} M min^{-1})
1	**5**	**0.318**	**1.146**
2	10	0.184	0.331
3	15	0.159	0.191
4	20	0.169	0.152
5	25	0.176	0.127
6	30	0.197	0.118
7	45	0.158	0.063
8	60	0.150	0.045

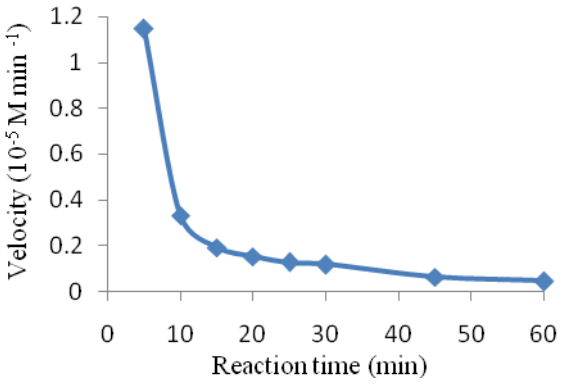

Figure 3 Plot of velocity of cellulase-catalyzed reaction as a function of reaction time

Table 2 Relationship between pH of the Solutions and Cellulase Activities

No.	Type of buffer	pH	Absorbance at 505 nm	Cellulase Activity (µmol $mL^{-1}min^{-1}$)
1	Sodium Acetate buffer	5.0	0.341	0.553
2		6.0	0.360	0.584
3	Sodium Phosphate buffer	7.0	0.392	0.635
4		**7.5**	0.533	**0.864**
5		8.0	0.401	0.650
6	Carbonate bicarbonate buffer	9.2	0.387	0.627
7		10	0.377	0.611

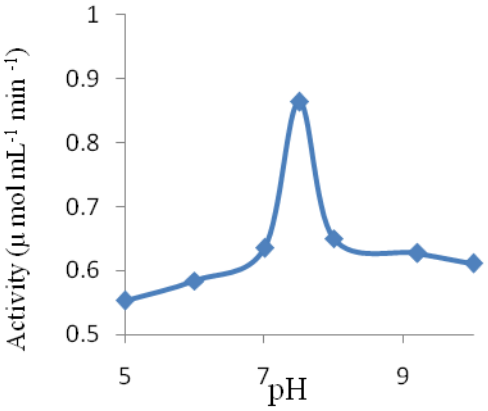

Figure 4 Plot of cellulase activity as a function of pH of the solution

Table 3 Relationship between Temperature and Cellulase Activities

No.	Temperatures (°C)	Absorbance at 505 nm	Cellulase Activity (µmol $mL^{-1}min^{-1}$)
1	25	0.453	0.734
2	30	0.487	0.789
3	35	0.559	0.906
4	40	0.641	1.039
5	45	0.694	1.124
6	**50**	0.738	**1.196**
7	55	0.695	1.127
8	60	0.646	1.047
9	65	0.596	0.966
10	70	0.526	0.852

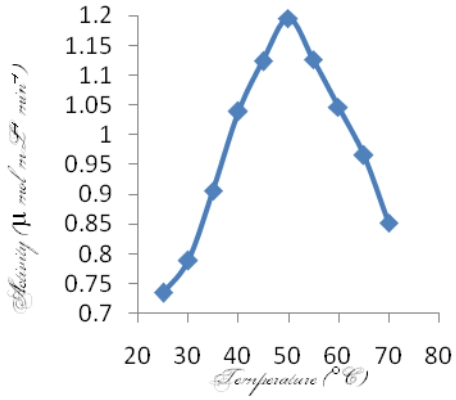

Figure 5 Plot of cellulase activity as a function of temperature

Table 5 Result of Chemical Testing of Glucose with Various Test Solutions

Reagent used	Appearances
Benedict's solution	Reddish precipitate
Barfoed's solution	Orange colored precipitate
Fehling's solution	Brick red precipitate

(a)

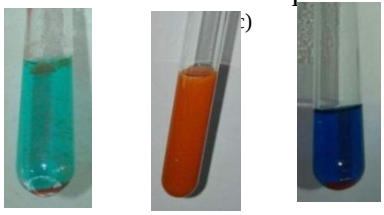

Figure 7 Confirmation tests for the presence of glucose

Table 4 Relationship between Hydrolyzing Times and Glucose Concentration

No.	Hydrolyzing time (min)	Absorbance at 505 nm	Glucose concentration (10^{-4} M)
1	10	3.388	6.103
2	20	4.060	7.313
3	30	4.370	7.871
4	40	4.597	8.280
5	50	4.770	8.592
6	60	4.978	8.966
7	90	5.394	9.716
8	120	5.432	9.784
9	150	5.490	9.889
10	180	5.505	9.916

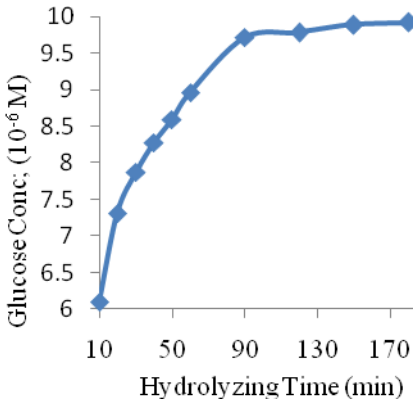

Figure 6 Plot of concentration of glucose produced as a function of hydrolyzing time

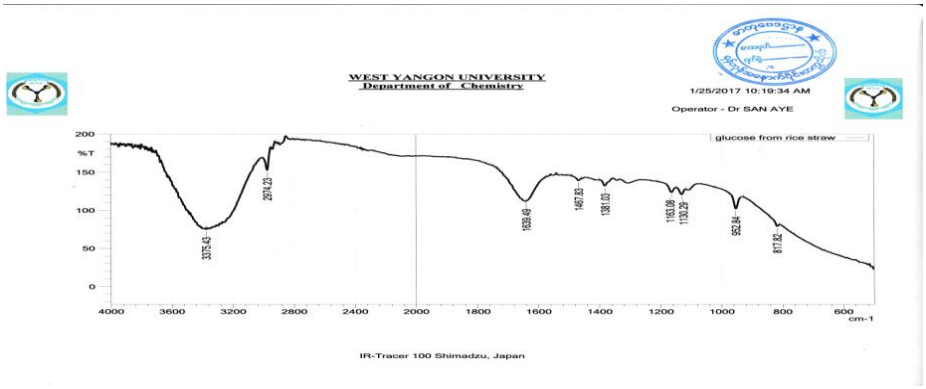

Figure 8 FT IR spectrum of glucose produced from rice straw

Conclusion

The cellulase (E.C. 3.2.1.4) was extracted from tomato fruits using sodium hydroxide solution (pH 8). The activity of extracted cellulase enzyme was determined by using cellulose solution which can be hydrolyzed into glucose. The glucose-oxidase enzyme reagent method was applied for the determination of glucose. According to enzyme specificity, the enzyme that can hydrolyzecellulose to glucose was cellulase. To determinewavelength of maximum absorption of red quinoneimine dye,the standard glucose solution was used. The wavelength of maximum absorption of red quinoneimine dye was found to be 505 nm. Then standard calibration curve was constructed using standard glucose solutions at the absorbance of 505 nm. The curve is straight line and passing the origin and therefore, Beer's law was obeyed.The plot of velocity *vs.* reaction times revealed and the reaction time of 10 min was chosen. The cellulase enzyme activity was found to be 0.789 µ mol mL^{-1} min^{-1}. The nature of the activity *vs.* pH curve of the enzyme was obviously found to be symmetrical and it was found that the optimum pH for maximum cellulase activity was 7.5. This value is in agreement with the literature.The optimum temperature ofcellulaseenzyme was found to be50°C. This value is also in agreement with the literature. At optimum conditions, the hydrolysis of rice straw by cellulase enzyme to produce glucose was performed. The glucose contents in the filtrates obtained from hydrolysis were determined by using glucose-oxidase enzyme reagent method.At 90 minute hydrolyzing time, the glucose concentration increased to 9.716×10^{-4} M and

the percent yield was found to be 0.96 %. The formation of brick red precipitate in Fehling Test, orange color in Barfoed's Test, reddish precipitate in Benedict's validated the product that produced from hydrolyzing of rice straw was reducing sugar, glucose. According to FT-IR result, this product was glucose. So cellulase should be used in the production of glucose in pharmaceutical industry.

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