The production of alcohol by cellulase enzymes using cellulose material as a substrate

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Abstract

β-D Glucose can be produced from cellulose materials by cellulase enzymes. The cellulase enzyme breakdown β-1,4 glucosidic linkage and formed β-D Glucose. The conversion rate of cellulose to β-D glucose depends on the enzyme activity. In this study, two kinds of commercial cellulase namely Food Grade (FG) and Technical Grade (TG) enzymes were used for the saccharification of celluolosic materials such as rice straw, grass, water hyacinth, bagasse and corn cob and then studied on the determination of reducing sugar by Filter Paper Assay or Filter Paper Degrading (FPD) Method. In this study, the optimal parameters of enzyme concentration, weight of substrate, reaction time, temperature and pH of TG were found to be enzyme 0.6 ml (range from 0.2-1.0 ml), 0.4 g of substrates (range from 0.1-1.0 g), for 4 hours reaction time (range from 1-24 hr), at 50°C (range from 30-70°C) and pH 4 (range from 3.0-5.5) for rice straw, grass, water hyacinth, bagasse and corn cob, respectively. Similarly, enzyme 0.4 ml, 0.4 g of substrates, 4 hours reaction time, at 50°C and pH 4.5 were the optimal parameters of FG on rice straw, grass, water hyacinth, bagasse and corn cob, respectively. The effect of fermentation period of enzymes TG on the production of alcohol was studied using rice straw and water hyacinth and using rice straw and bagasse for FG, respectively. According to the results 48 hr fermentation period was the most suitable reaction time for both TG and FG. Lab scale alcohol fermentation production was conducted using rice straw with optimal parameters. The result showed that 48 hours fermentation period, temperature at 30°C and pH-5 and 5.5 by TG and FG, respectively, gave the best alcohol concentration.

Keywords: Alcohol, Cellulase Enzymes, Cellulose Materials

1. Introduction

Life is associated with waste production and the exploitation on these materials as a renewable resource for bioproduct development could be a major challenge for biotechnology (Van Wyk, 2001). There is an increasing interest in recycling useful components of wastes and in using certain fractions for production of energy or higher value materials such as useful chemicals of particular interest in large amount of cellulosic materials.

Cellulosic resources are mainly found in plant material and it is a major component in the primary and secondary cell wall of plants. Cellulose is the most abundant polymer in the biosphere with its estimated synthesis rate of 10^{10} tonnes per year (Schlesinger, 1991; Singh and Hayashi, 1995; Lynd *et al*., 2002). Cellulose-rich plant biomass is one of the foreseeable and sustainable sources of fuel, animal feed and feed stock for chemical synthesis (Bhat, 2000). Lignocellulosic wastes such as rice straw have a high polysaccharide content mainly cellulose and hemicellulose which have energy levels similar to corn (Ferrer *et. al*, 1994).

Myanmar is an agricultural developing country, rich in various kinds of easily available cellulosic raw materials such as rice hull, timber, straw, bagasse, waste paper, woody pulp, coconut fiber and peanut hull etc. In native cellulose up to 10,000 β-D glucose residues are linked in long chains by oxygen bridges with 1,4-β-linkages.

A cellulose hydrolyzing enzyme system consists of three major components (1) Endo-β-glucanase or carboxymethylcellulase (Cx or CMCase) (2) Exo-β-glucanase or cellobiohydrolase (C1) and (3) β-glucosidase or cellobiase (CB) (Whitaker, 1971; Emert *et al.,* 1974 and Miyamoto, 1997). The combination of these three types of enzyme components isolated from crude culture filtrates, it is

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necessary for the complete hydrolysis of crystalline cellulose (King and Vessal, 1969). Crude cellulose is a very resistant material and thus its conversion to fermentable sugar such as glucose is somewhat difficult. The end product of cellulose by cellulase is β–D glucose. This is the main carbon source for the production of alcohol by a fermentation process. Several microorganisms can produce the cellulose hydrolysis enzyme (the cellulase). It is produced by a number of bacteria and fungi though species of *Trichoderma* and *Aspergillus* are most reported (Zaldivar *et al.*, 2001).

The subject of ethyl alcohol production by fermentation has assumed new interest on account of attempts to find substitution for gasoline. In Brazil, over the last four years only about 30% of the total ethanol production was blended with gasoline (Heitland *et al.*, 1977). The remainder was used for industrial purposes, exports and other uses. Recently, the Brazilian Government had sponsored research in the use of fermentation alcohol in diesel engines and in gas turbines (Carvalho *et al*., 1977).

Rice straw is one of the abundant lignocellulosic waste materials in the world. Yeast based fermentation, for example, has yielded ethanol from sugar or crops. Ethyl alcohol is also used widely as an industrial solvents as raw materials for synthesis (Stark, 1954). Ethanol from renewable resources has been of interest in recent decades as an alternative fuel or oxygenate additive to the current fossil fuels. Production of ethanol (ethyl alcohol) from biomass is one way to reduce both the consumption of crude oil and environmental pollution (Lang *et al*., 2001).

Ethyl alcohol, grains alcohol has been produced for hundreds of years by the fermentation of carbohydrates by yeast. This method is still a major source of alcohol. The raw materials for the fermentation can be divided into two classes: materials that contain sugar such as potatoes, rice, barley and other cereals (or) cellulose containing substances such as cotton linters, wood (sawdust), straw and grasses (Henry and Rose, 1966). Ethanol is made by converting starch crops into sugars, the sugars are fermented into ethanol which is then distilled into its final form.

Cellulase is used in the fermentation of biomass into biofuels, although this process is relatively experimental at present. So it is necessary to understand the knowledge and the advance technology of alcoholic fermentation in Myanmar.

The aims of this research are to determine the reducing sugar (β-D glucose), to study the optimal parameter of pH, temperature, and reaction time on rice straw, grass, water hyacinth, bagasse and corn cob by two commercial cellulase enzymes (TG and FG) and to study the percentage of alcohol production from rice straw by TG and FG with optimal fermentation parameters.

2. Materials and Methods

Cellulase enzymes

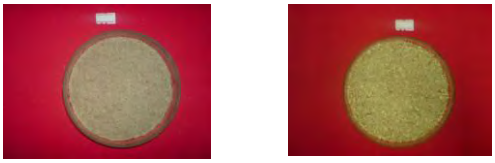
Technical Grade (TG) and Food Grade (FG) cellulase enzymes were produced by controlled fermentation of *Trichoderma reesei*.

Preparation of cellulose substrates

In the present study, the cellulose substrates were prepared from rice straw, grass, water hyacinth, bagasse and corn cob. And they were air dried in the shaded place. The dried samples were ground into powder with blender. Then the fine cellulose powder of rice straw, grass, water hyacinth, bagasse and corn cob were dried and separately kept in clean bottles. It was used for the determination of enzyme activity and preparation of ethanol production.

Dried powder cellulose substrates

Rice straw Grass Water hyacinth



Bagasse Corn cob

Determination of reducing sugar by Dinitrosalicylic Acid Method

The reducing sugar appeared from the hydrolytic action of cellulase was determined by DNS Method (Miller, 1959).

Dinitrosalicylic acid reagent

1gm of Dinitrosalicylic acid was dissolved in 1% NaOH solution which contains 20 gm of sodiumpotassiumtartrate and 0.2 gm of phenol.

Glucose Standard solution

A series of glucose concentration (0.2-1.0 mg per ml) was used for glucose standard solution.

Procedure

1ml each of samples containing 0.2-1.0 mg per ml glucose standard solution was placed into the precleaned test tubes and added 1ml each of reagent. They were well capped by glass balls and heated in a vigorously boiling water bath for 15 minutes.

Then they were cooled for 10 minutes in running tap water. After cooling, they were diluted with 5ml of distilled water and then the absorbance was determined by UV spectrophotometer at 575 nm. A standard curve with exact amount of glucose containing 0.2 mg, 0.4 mg, 0.6 mg, 0.8 mg and 1.0 mg were prepared prior to the actual determination of samples (Figure 1).

Enzyme Assay

The activity of cellulase hydrolysis enzyme was determined by detecting the amount of reducing sugar liberated by the action of TG and FG cellulase enzymes from filter paper substrate (Mandels and Sternberg, 1976).

Technical Grade (TG) = 10815 Unit
Food Grade (FG) = 94.3 Unit

One unit of cellulase activity is defined as the amount of enzyme which catalyzed the liberation of 1 micromole (µ mole) reducing sugar as glucose per minute from the cellulose substrate (filter paper) under the above experimental conditions.

Determination of Filter Paper Degrading Activity (FPD)

***Substrate* :** Whatman No. 1 Filter Paper strips, 1.0 × 6.0 cm (50 mg).

Filter Paper Assay Procedure

Whatman No. 1 Filter Paper was cut into 1.0 × 6.0 cm strips (50 mg). Each enzyme solution 0.5 ml and 1.0 ml of 0.05 M sodium citrate buffer solution (pH - 4.8) were placed in 18 mm test tube.

A filter paper was added and mixed in vortex mixer to coil the paper in the solution and incubated for 1 hour at 50°C. DNS reagent 3.0 ml was added to stop reaction and test tubes were placed in boiling water bath for 10 minutes and reducing sugar was determined as glucose (Mandels and Weber, 1968 and Gallo, 1981). All samples, controls and glucose standards were used as reference.

Microorganisms

The yeast *Saccharomyces boulardii* was used in producing alcoholic fermentation. Microorganism was cultured on Yeast extract Malt extract agar medium and maintained under refrigeration at 5°C to prevent the contamination of mold and other microorganisms. The yeast was then transferred to new medium at monthly intervals.

Composition of maintaining culture medium

Yeast extract Malt extract agar medium (malt extract 0.3 %, yeast extract 0.3 %, peptone 0.5 %, glucose 1 %, agar 2 % in distilled water, pH 3.0 - 3.5) developing by Collins (1964) was used for maintaining the microorganisms.

Determination of packed cell volume (PCV)

Packed cell volume (PCV) was determined according to Onions (1971). About 5ml of broth was taken from the culture flask and poured into graduated centrifuge tube and rotated in model KOKUSAN H-107 Type centrifuge at 2000 r.p.m for 20 minutes. After centrifugation, the upper portion of the suspension was removed and the remaining portion of the residue was measured by using graduated centrifuge tube.

Determination of the isolated yeast cell number

Total cell count method was used according to Cook (1958). The method that requires the least experiment consists in counting the total number of cells in a known volume of liquid. For this purpose, the yeast cell is thoroughly dispersed in the culture medium, diluted to a convenient cell density and a small volume of this suspension is transferred to a hemocytometer chamber. The total number of cells in a known volume, as revealed by microscopical examination was determined. When a "Thoma" type chamber was used, the following formula can be employed:

$$\text{Total yeast cells number/ml} = \frac{\text{No. of cells counted x 4 x } 10^6 \text{ x dilution}}{\text{No. of small squares examined}}$$

In this experiment it was used 1% of 24 hours age of *Saccharomyces boulardii* culture (1×10^7 cells⁄ml).

Composition of inoculums medium and main fermentation medium

Inoculums medium (glucose 3%, potassium dihydrogen phosphate 0.5%, ammonium sulphate 0.2%, magnesium sulphate 0.1% and yeast extract 0.1 % in distilled water, pH 6.0) was developed by Raherimandimby (1986) using for inoculating the microorganisms. Basal fermentation medium contains glucose 20% instead of glucose 3% of inoculums medium and other ingredient of the same as those medium.

Determination of alcohol concentration from fermented broth

Determination of alcohol concentration was carried out according to the method prescribed by (Rebelein, 1973). The procedure was as follows:

Reagent

(1) 67.445 g of potassium dichromate was dissolved in 1 litre of distilled water in 1 litre volumetric flask at 20°C.

(2) 770 ml of nitric acid (65%) was diluted to 1 litre of distilled water in 1 litre volumetric flask at 20°C.

(3) 300 g of potassium iodide was dissolved in 100 ml of 1N sodium hydroxide and brought to 1 litre volumetric flask at 20°C with distilled water.

(4) 86.194 g of potassium thiosulfate was dissolved in 100 ml of 1N sodium hydroxide and 500 ml of distilled water in a 1 litre volumetric flask at 20°C.

(5) 10 g of soluble starch was added into 500 ml of distilled water in which 20 g of potassium iodide and 10 ml of 1N sodium hydroxide have been dissolved at 20°C.

Procedure

(1) 10 ml of dichromate solution (Reagent - 1) and 25 ml of the nitric acid solution (Reagent - 2) were added into the 500 ml Erelenmeyer receiving flask.

(2) 12 ml of distilled water and 1ml of sample were added and rapidly heated at boiling for 3 minutes.

(3) After boiling, the concentrated alcohol sample was added and then added 300 ml of distilled water into the flask.

(4) Then 10 ml of potassium iodide solution (Reagent - 3) and 10 ml of starch solution (Reagent - 5) were added as indicator.

(5) A magnetic stirrer bar was added and titrate the solution with thiosulfate solution (Reagent - 4) to a blue starch end point.

Calculation Formula

30 ml of standard thiosulfate = 120 g/L of alcohol solution

alcohol 1g/ 100ml = 12 - 0.4A

Where, A = thiosulfate standard solution (ml)

To convert volume % the answer was divided by 0.7933.

3. Results

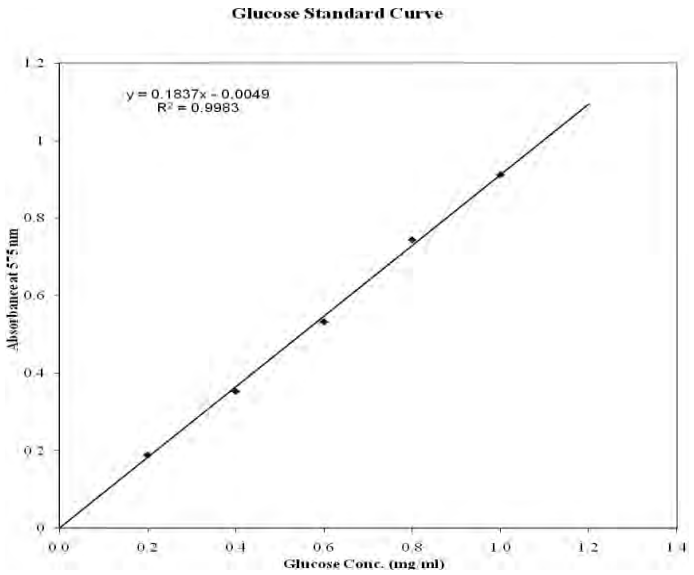


Figure 1- Standard Curve for standard glucose solution

Determination of reducing sugar for TG and FG by FPD method

The amount of reducing sugar liberated was measured according to the D.N.S method as formerly described. The maximum amount of reducing sugar 2.982 mg/ml was estimated by TG at 0.6 ml and 2.183 mg/ml was observed by FG at 0.4 ml (Table 1 and Figure 2).

Table 1 - Determination of reducing sugar for Technical Grade (TG) and Food Grade (FG) by Filter Paper Degrading Activity (FPD) method

Enzyme (Technical Grade) (ml)	Reducing Sugar (mg/ml)	Enzyme (Food Grade) (ml)	Reducing sugar (mg/ml)
0.2	0.520	0.2	0.325
0.4	1.245	0.4	2.183
0.6	2.982	0.6	1.079
0.8	2.088	0.8	1.829
1.0	1.579	1.0	1.082

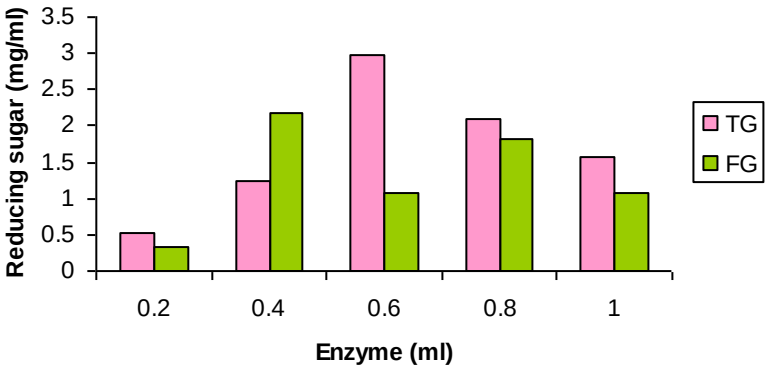


Figure 2 – Effect of different concentration of enzymes (TG and FG) on determination of reducing sugar by FPD method.

Effect of different weight of Rice straw, Grass, Water hyacinth, Bagasse and Corn cob on the glucose production by TG and FG

Effect of different weight of substrates on the glucose production by TG and FG were studied in the range of 100, 200, 400, 500 and 1000 mg, the results were shown in the Table - 2 and Figure - 3 and 4. It was noted that the maximum amount of reducing sugar 3.850 mg/ml, 3.660 mg/ml, 3.602 mg/ml, 2.981 mg/ml and 2.327 mg/ml were estimated by TG and 3.243 mg/ml, 2.296 mg/ml, 2.243 mg/ml, 2.579 mg/ml and 2.819 mg/ml were observed by FG at 400 mg when the rice straw, grass, water hyacinth, bagasse and corn cob, respectively, were used as basal carbon source (Table - 2and Figure - 3 and 4).

Table 2 - Determination of reducing sugar on different weight of rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG

Straw (mg)	Enzyme (ml)		Reducing Sugar (mg/ml)									
			Rice straw		Grass		Water Hyacinth		Bagasse		Corn cob	
	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG
100	0.6	0.4	1.092	1.348	1.248	1.948	1.676	1.024	1.699	0.783	1.402	0.550
200	0.6	0.4	2.066	1.142	2.065	1.643	2.516	1.528	2.956	1.805	1.531	1.946
400	0.6	0.4	3.850	3.243	3.660	2.296	3.602	2.243	2.981	2.579	2.327	2.819
500	0.6	0.4	2.468	2.588	2.379	2.271	2.328	2.127	2.257	1.330	1.416	1.868
1000	0.6	0.4	1.676	1.656	1.499	1.789	1.271	1.578	1.278	1.532	1.550	1.790

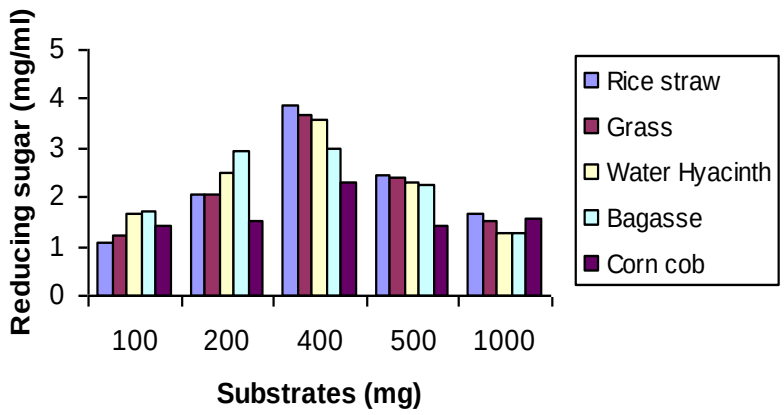


Figure 3 – Effect of different weight of substrates on determination of reducing sugar by Technical Grade (TG)

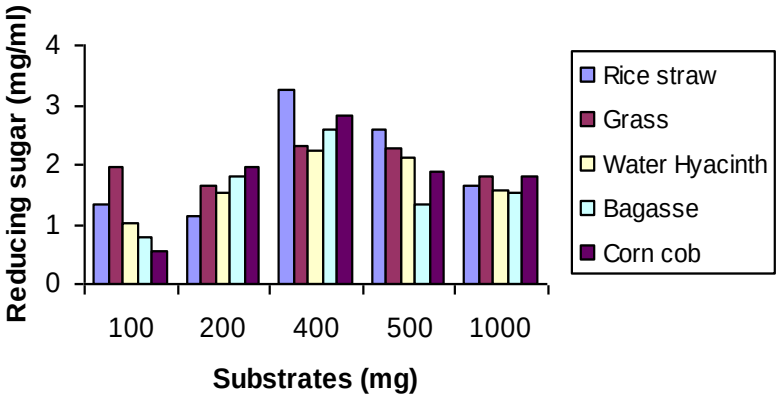


Figure 4 - Effect of different weight of substrates on determination of reducing sugar by Food Grade (FG)

Effect of different temperature on the production of glucose from rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG

Effect of different temperature on the amount of reducing sugar from rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG were studied in the temperature range of 30, 40, 50, 60 and 70 °C.

It was observed that the maximum amount of reducing sugar 3.850 mg/ml, 3.66 mg/ml, 3.602 mg/ml, 2.981 mg/ml and 2.327 mg/ml were estimated by TG and 3.243 mg/ml, 2.271 mg/ml, 2.243 mg/ml, 2.579 mg/ml and 2.819 mg/ml were observed by FG using rice straw, grass, water hyacinth, bagasse and corn cob, respectively, at 50°C when 400 mg substrate was used as basal carbon source (Table -3 and Figure - 5 and 6).

Table 3 - Determination of reducing sugar on different temperature effect on rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG

Temperature (°C)	Enzyme (ml)		Reducing Sugar (mg/ml)									
			Rice straw		Grass		Water Hyacinth		Bagasse		Corn cob	
	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG
30	0.6	0.4	1.927	1.135	2.159	2.142	2.322	1.700	1.631	1.214	1.179	1.080
40	0.6	0.4	2.704	2.725	3.117	2.269	2.535	2.147	2.313	1.412	2.242	1.179
50	0.6	0.4	3.85	3.243	3.66	2.271	3.602	2.243	2.981	2.579	2.327	2.819
60	0.6	0.4	3.608	2.747	3.00	2.268	3.307	2.124	2.327	2.221	2.294	1.346
70	0.6	0.4	2.697	2.485	2.98	2.188	3.199	1.467	1.024	1.172	1.913	0.798

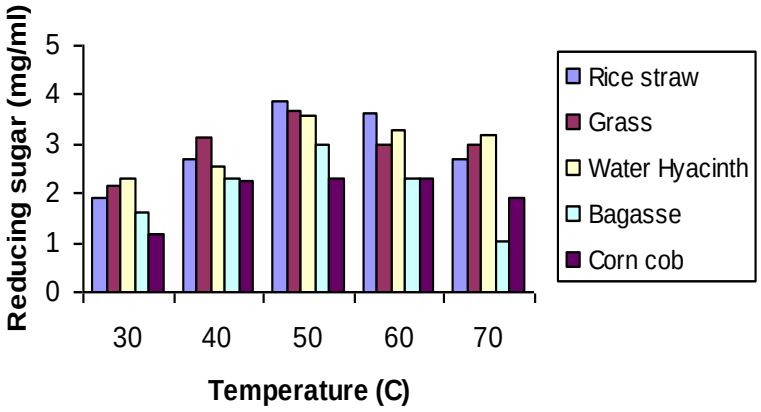


Figure 5 - Effect of different temperature on the glucose production from raw materials (Rice straw, Grass, Water hyacinth, Bagasse and Corn cob) by TG

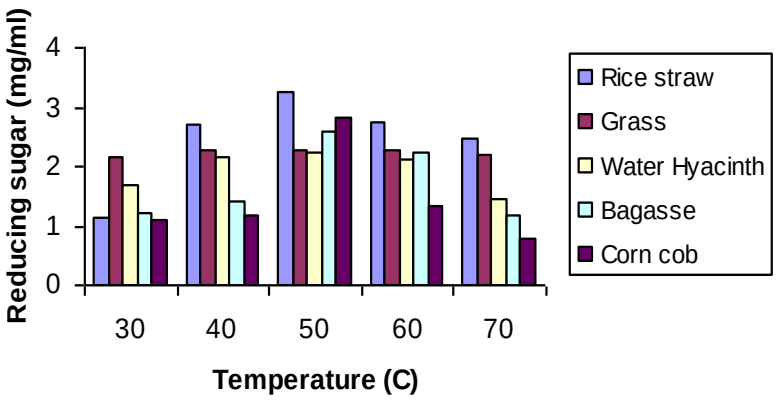


Figure 6 - Effect of different temperature on the glucose production from raw materials (Rice straw, Grass, Water hyacinth, Bagasse and Corn cob) by FG

Effect of different pH on the production of glucose from rice straw, grass, water hyacinth bagasse and corn cob by TG and FG

Effect of different pH on the production of glucose from rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG were studied in the range of pH - 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5.

It was observed that the maximum amount of reducing sugar 1.221 mg/ml, 1.651 mg/ml, 1.776 mg/ml, 1.218 mg/ml and 1.179 mg/ml were observed by TG at pH 4 and 1.327 mg/ml, 1.546 mg/ml, 1.271 mg/ml, 1.891 mg/ml and 1.880 mg/ml were estimated by FG using rice straw, grass, water hyacinth, bagasse and corn cob at pH 4.5, respectively, when 400 mg substrate was used as basal carbon source (Table - 4 and Figure - 7 and 8).

Table 4 - Determination of reducing sugar on different pH effect on rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG

pH	Enzyme (ml)		Reducing Sugar (mg/ml)									
			Rice straw		Grass		Water Hyacinth		Bagasse		Corn cob	
	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG
3.0	0.6	0.4	0.903	0.621	1.271	0.437	0.932	0.381	0.451	0.550	0.459	0.593
3.5	0.6	0.4	1.059	0.829	1.405	0.767	1.108	0.896	0.501	0.981	0.536	0.727
4.0	0.6	0.4	1.221	1.128	1.651	1.326	1.776	1.179	1.218	1.437	1.179	1.402
4.5	0.6	0.4	1.024	1.327	1.521	1.546	1.543	1.271	1.146	1.891	0.918	1.880
5.0	0.6	0.4	1.011	1.066	1.158	1.327	1.327	1.197	0.980	1.550	0.374	1.402
5.5	0.6	0.4	0.536	0.790	0.656	0.875	0.579	1.052	0.642	0.923	0.233	0.883

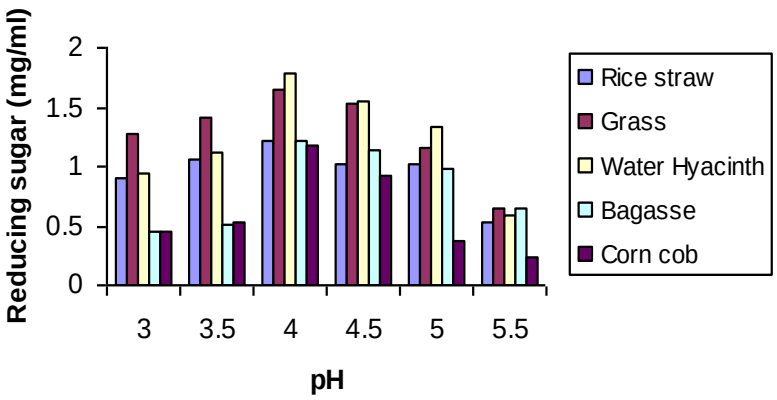


Figure 7 - Effect of different pH on the glucose production from raw materials (rice straw, grass, water hyacinth, bagasse and corn cob) by TG

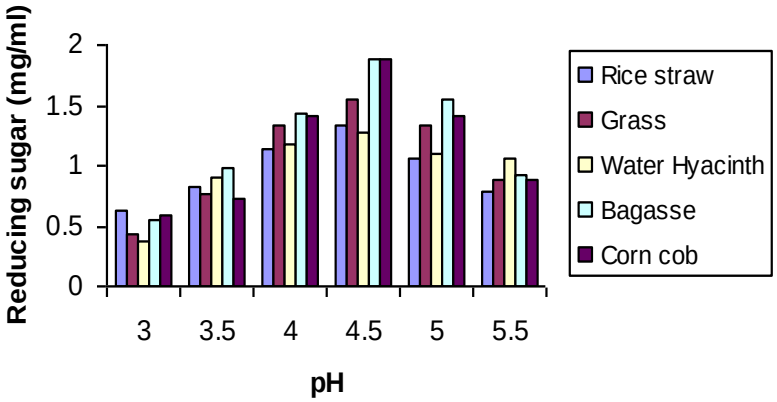


Figure 8 - Effect of different pH on the glucose production from raw materials (rice straw, grass, water hyacinth, bagasse and corn cob) by FG

Effect of different reaction time on the production of glucose from rice straw, grass, water hyacinth, bagasse and corn cob on the glucose production by TG and FG

Effect of different reaction time on the production of glucose from rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG were studied in the range of 1, 2, 4, 8, 16 and 24 hours.

It was observed that the maximum amount of reducing sugar 3.233 mg/ml, 2.336 mg/ml, 3.072 mg/ml, 1.678 mg/ml and 1.122mg/ml were observed by TG and 3.567 mg/ml, 3.699 mg/ml, 2.358 mg/ml, 2.768 mg/ml and 2.810 mg/ml were estimated by FG using rice straw, grass, water hyacinth, bagasse and corn cob at 4 hr reaction time, respectively, when 400 mg substrate was used as basal carbon source (Table - 5 and Figure - 9 and 10).

Table 5 - Determination of reducing sugar on different reaction time effect on rice straw, grass, water hyacinth, bagasse and corn cob by TG and FG

Reaction time (Hours)	Enzyme (ml)		Reducing Sugar (mg/ml)									
			Rice straw		Grass		Water Hyacinth		Bagasse		Corn cob	
	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG	TG	FG
1	0.6	0.4	2.053	2.182	1.367	1.193	1.511	1.278	1.066	1.518	0.769	1.122
2	0.6	0.4	2.817	3.072	1.885	2.372	2.237	1.666	1.596	1.942	0.829	1.398
4	0.6	0.4	3.233	3.567	2.336	3.699	3.072	2.358	1.678	2.768	1.122	2.810
8	0.6	0.4	1.596	2.040	1.800	2.103	2.196	1.468	1.073	1.158	1.016	1.857
16	0.6	0.4	1.426	1.822	1.285	1.652	1.772	1.278	0.868	0.946	0.670	1.312
24	0.6	0.4	1.271	1.059	1.143	0.083	1.539	0.184	0.530	0.868	0.435	0.826

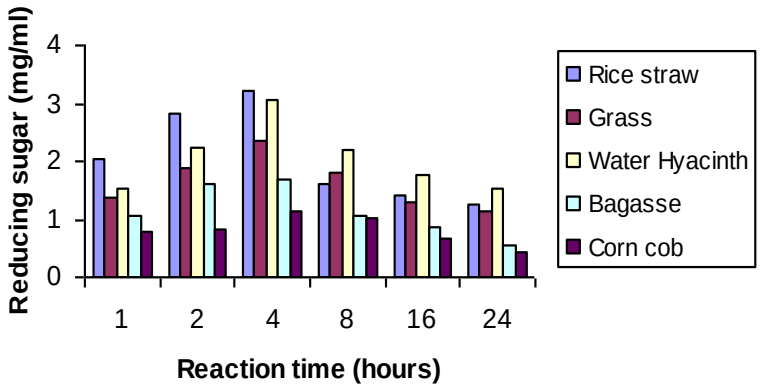


Figure 9 - Effect of different reaction time on the glucose production from raw materials (Rice straw, Grass, Water hyacinth, Bagasse and Corn cob) by TG

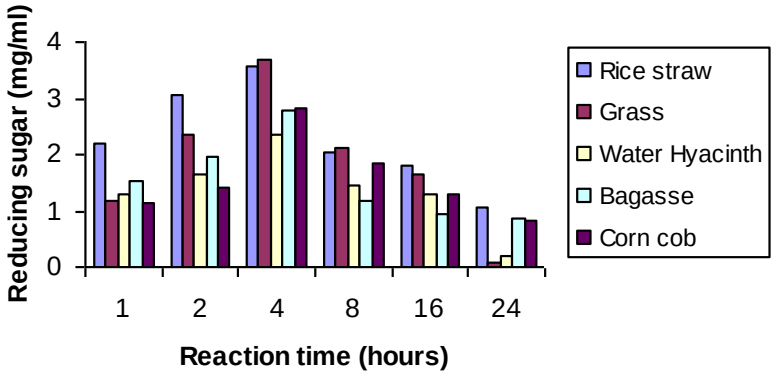


Figure 10 - Effect of different reaction time on the glucose production from raw materials (Rice straw, Grass, Water hyacinth, Bagasse and Corn cob) by FG

Comparison of different enzymes (TG and FG) activity on the production of reducing sugar from rice straw, grass, water hyacinth, bagasse and corn cob at the optimal experimental conditions

Two different enzymes (TG and FG) activity on the production of reducing sugar from rice straw, grass, water hyacinth, bagasse and corn cob were comparatively studied at the optimal experimental conditions.

It was observed that the maximum amount of reducing sugar 4.642 mg/ml, 3.390 mg/ml, 4.653 mg/ml, 3.632 mg/ml and 3.459 mg/ml were observed by TG at pH 4 and 4.547 mg/ml, 3.086 mg/ml, 3.145 mg/ml, 3.871 mg/ml and 3.744 mg/ml were estimated by FG at pH 4.5 using rice straw, grass, water hyacinth, bagasse and corn cob at 50°C for 4 hours, respectively, when 400 mg substrate was used as basal carbon source (Table - 6 and Figure - 11 and 12).

Table 6 - Determination of reducing sugar from rice straw, grass, water hyacinth, bagasse and corn cob at optimal experimental conditions by cellulase enzymes (TG and FG)

Straw (mg)	Cellulase Enzyme	Enzyme (ml)	Optimal experimental condition			Reducingsugar (mg/ml)				
			Temperature (°C)	pH	Reaction Time (hours)	Rice straw	Grass	Water hyacinth	Bagasse	Corn cob
400	TG	0.6	50	4	4	4.642	3.39	4.653	3.632	3.459
400	FG	0.4	50	4.5	4	4.547	3.086	3.145	3.871	3.744

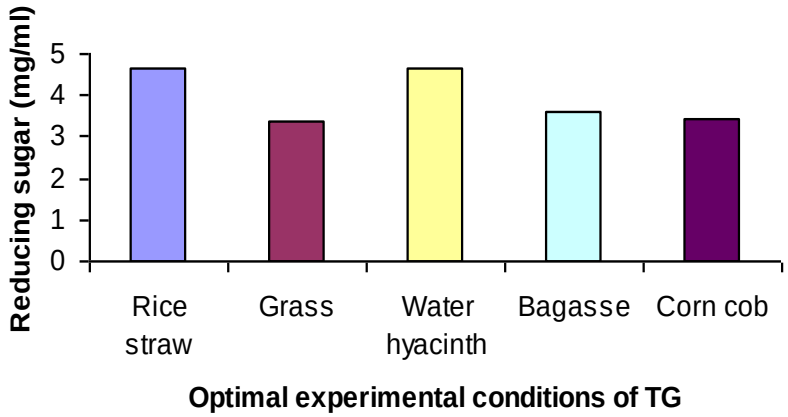


Figure 11- Comparative study on the effect of enzyme activity (TG) on the production of reducing sugar using raw materials (rice straw, grass, water hyacinth, bagasse and corn cob) at optimal experimental conditions

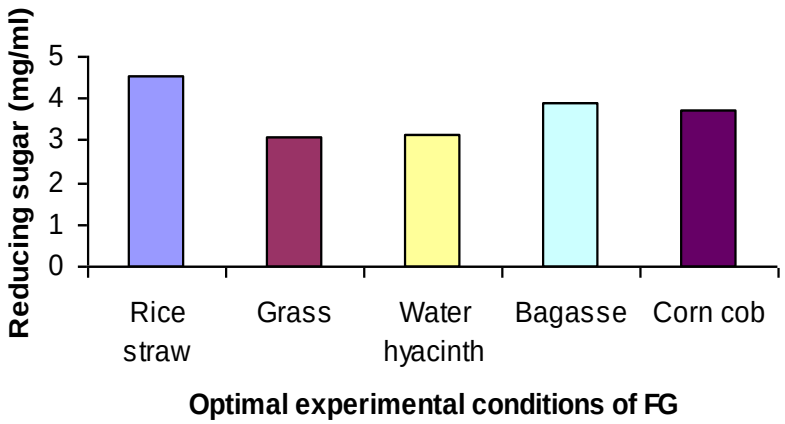


Figure 12 - Comparative study on the effect of enzyme activity (FG) on the production of reducing sugar using raw materials (rice straw, grass, water hyacinth, bagasse and corn cob) at optimal experimental conditions

The effect of different fermentation period on the production of alcohol by TG and FG

Effect of different fermentation period on the production of alcohol by TG using rice straw and water hyacinth and by FG using rice straw and bagasse were studied in the range of 0, 24, 48 and 72 hrs, respectively.

It was observed that the maximum alcohol concentration 4.36 % for rice straw and 4.22 % for water hyacinth by TG (Table - 7 and Figure - 13) and 4.31 % for rice straw and 4.29 % for bagasse by FG (Table - 8 and Figure - 14), respectively, when fermentation period was for 48 hrs

Table 7 - Determination of different fermentation period on the production of alcohol by TG using rice straw and water hyacinth

Fermentation period (hours)	Initial sugar (g/ml)	Reducing sugar (g/ml)	Consume sugar (g/ml)	Packed cell volume (PCV) ml	Alcohol concentration on Consume sugar (%)	
					Rice straw	Water hyacinth
0	0.20	0.20	0	0.01	-	-
24	-	0.08	0.12	0.02	3.59	3.85
48	-	0.07	0.13	0.04	4.36	4.22
72	-	0.07	0.13	0.03	4.11	3.93

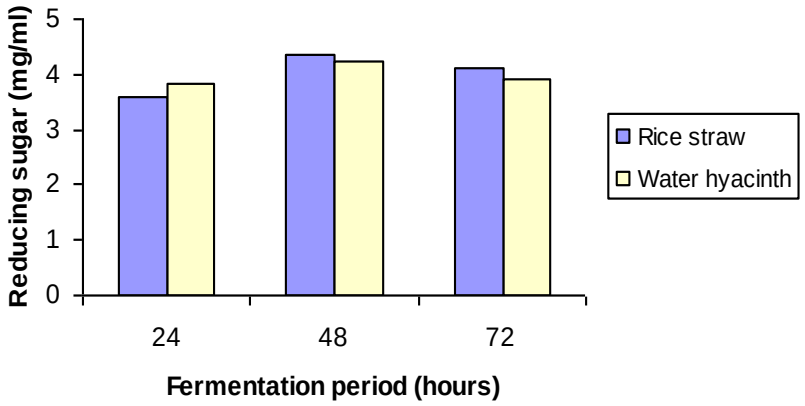


Figure 13 - Comparison of different fermentation period of rice straw and water hyacinth on the production of alcohol by TG

Table 8 – Determination of different fermentation period on the production of alcohol by FG using rice straw and bagasse

Fermentation period (hours)	Initial sugar (g/ml)	Reducing sugar (g/ml)		Consume sugar (g/ml)		Packed cell volume (PCV) ml		Alcohol concentration on Consume sugar (%)	
		Rice straw	Bagasse	Rice straw	Bagasse	Rice straw	Bagasse	Rice straw	Bagasse
0	0.20	0.20	0.20	0	0	0.02	0.02	-	-
24	-	0.07	0.08	0.13	0.12	0.03	0.03	3.52	3.56
48	-	0.06	0.07	0.14	0.13	0.04	0.05	4.31	4.29
72	-	0.06	0.07	0.14	0.13	0.03	0.04	3.93	3.42

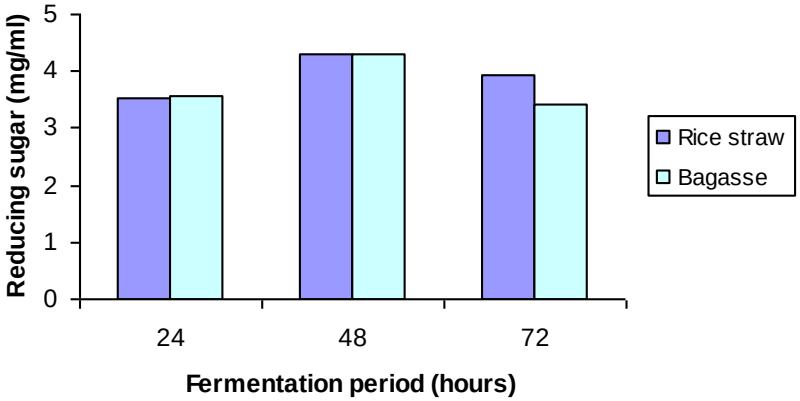


Figure 14 - Comparison of different fermentation period of rice straw and bagasse on the production of alcohol by FG

The effect of different temperature on the production of alcohol by TG and FG using rice straw for 48 hrs fermentation

Effect of different temperature (28, 30, 32, 34 and 37°C) on the production of alcohol by TG and FG using rice straw for 48 hours fermentation was studied.

It was observed that the maximum alcohol concentration was 6.35 % and 6.22% by TG and FG, respectively, at 30°C for 48 hours fermentation using rice straw (Table - 9 and Figure - 15).

Table 9 – Determination of different temperature on the production of alcohol by TG and FG using rice straw for 48 hours fermentation

Temperature (Ċ)	Initial sugar (g/ml)	Reducing sugar (g/ml)		Consume sugar (g/ml)		Packed cell volume (PCV) ml		Alcohol concentration on Consume sugar (%)	
		TG	FG	TG	FG	TG	FG	TG	FG
28	0.20	0.06	0.07	0.14	0.13	0.03	0.03	6.21	6.13
30	-	0.06	0.06	0.14	0.14	0.04	0.04	6.35	6.22
32	-	0.06	0.06	0.14	0.14	0.04	0.04	6.11	6.09
34	-	0.06	0.06	0.14	0.14	0.03	0.04	5.69	5.03
37	-	0.06	0.07	0.14	0.13	0.03	0.03	5.28	4.75

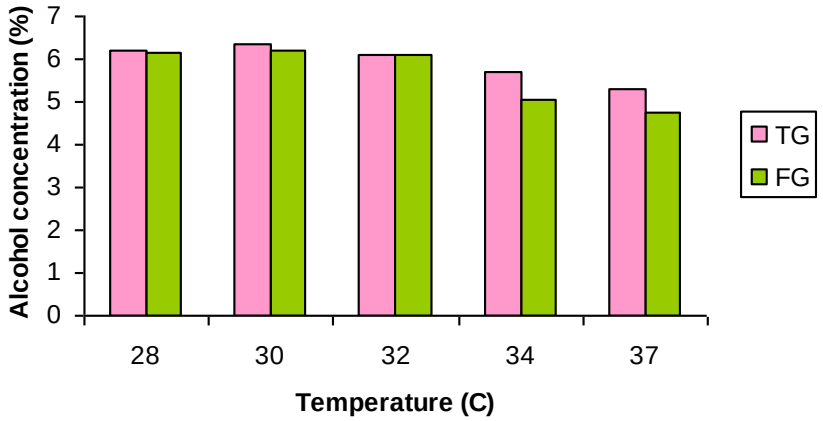


Figure 15 - Comparison of different temperature on the production of alcohol by TG and FG using rice straw for 48 hours fermentation

The effect of different pH on the production of alcohol by TG and FG using rice straw at 30°C for 48 hrs fermentation.

Effect of different pH (pH - 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) on the production of alcohol by TG and FG using rice straw were studied at 30° C for 48 hours fermentation.

It was observed that the maximum alcohol concentration was 6.61 % by TG at pH 5 and 6.55% by FG at pH 5.5 using rice straw at 30°C for 48 hours fermentation (Table-10 and Figure-16).

Table 10 - pH of fermentation broth on the production of alcohol by TG and FG using rice straw at 30°C for 48 hours fermentation

pH	Reducing Sugar (g/ml)		Consume Sugar (g/ml)		Packed cell volume (PCV) (ml)		Alcohol Concentration on consume sugar (%)	
	TG	FG	TG	FG	TG	FG	TG	FG
3.5	0.04	0.03	0.16	0.17	0.02	0.01	5.76	5.42
4	0.05	0.04	0.15	0.16	0.03	0.02	5.89	5.67
4.5	0.06	0.05	0.14	0.15	0.04	0.03	6.22	5.93
5	0.07	0.06	0.13	0.14	0.05	0.04	6.61	6.19
5.5	0.06	0.07	0.14	0.13	0.04	0.05	6.39	6.55
6	0.05	0.06	0.15	0.14	0.03	0.04	5.98	6.23

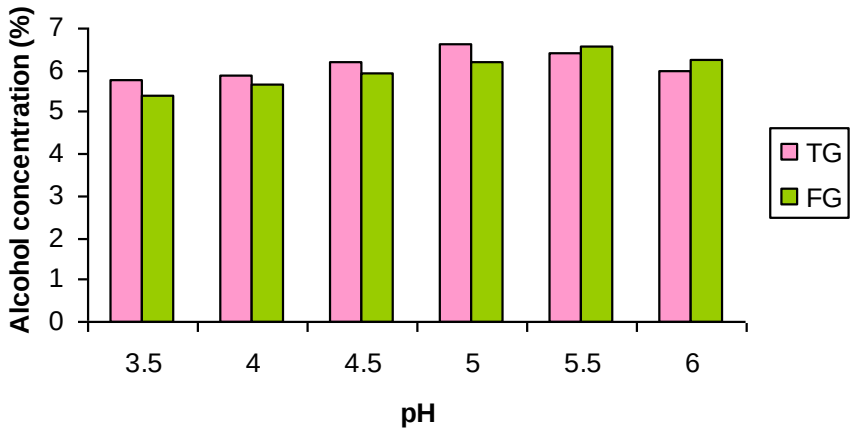


Figure 16 - Comparison of different pH on the production of alcohol by TG and FG using rice straw at 30°C for 48 hours fermentation

4. Discussion and Conclusion

In Myanmar, preliminary studies on the production of cellulase enzyme from *Trichoderma* species, *Emericella* species, *Aspergillus* species and *Chaetomium* species were carried out by Aye Aye Khine (1994), Khin Kyi (1994) and Khin Mi Mi Win (1994) and Bay Dar (1994) at the Department of Botany, University of Yangon.

In the present work, it was determined the amount of cellulase enzyme in the production of reducing sugar by FPD method reported by Mandels and Weber (1968). It was found that the maximum amount of reducing sugar was liberated at 0.6 ml by TG and at 0.4 ml by FG, respectively.

One of the main objectives was to find out the easy and effective use of very cheap, easily available cellulosic plant wastes such as rice straw, grass, water hyacinth, bagasse and corn. The maximum amount of reducing sugar was obtained in the reaction mixture containing 400 mg each of cellulosic substrate, pH 4.0 for TG and 4.5 for FG, respectively. The optimal temperature was found to be 50°C and the optimum reaction time was 4 hours for both TG and FG using rice straw, grass, water hyacinth, bagasse and corn cob as the substrates.

Bay Dar (1994) reported that the reducing sugar produced by the cellulase enzyme from *Chaetomium* species was found to be the best at the pH 6 and the temperature 30°C using filter paper as a substrate.

The basal fermentation medium was Raherimandimby medium in which different types of plant biomass were used as substrates such as rice straw, water hyacinth and bagasse. The microorganism used in the alcohol production was yeast, *Saccharomyces boulardii*. The age of culture was 24 hours old culture of yeast, grown in yeast extract malt extract medium. The basal fermentation medium (Raherimandimby medium) was inoculated with 1% size of inoculum in which 1×10^7 cells/ml was contained. The cellulosic biomass was subjected in the hydrolysis reactions of cellulase enzymes (TG and FG) and the final products were appeared as the liquid containing reducing sugars (glucose) as hydrolyzates. This glucose containing liquid served as basal raw material in the fermentation of ethanol by yeast.

In the present result, the production of alcohol was found more in the medium containing rice straw than water hyacinth and bagasse by TG and FG, respectively. The optimal fermentation period, temperature and pH on the production of alcohol were 48 hours, 30°C and pH 5 for TG (alcohol concentration 6.56%) and pH 5.5 for FG (alcohol concentration 6.45%), respectively, using rice straw with *S. boulardii*. But alcohol concentration in the fermentation medium by TG is a little more concentrated than by FG. Thein Thein Nwe (2007) reported that the optimum temperature on the production of ethanol was at 30°C for 2 days fermentation period by *S. fructuum* (alcohol concentration 6.20%) and *S. cerevisiae* (alcohol concentration 6.28%). Shwe Wah Sein (1994) stated that alcohol yield 6.84% was produced by the fermentation process containing *S. cerevisiae*. Prescott and Dunn (1949) stated that the best sugar concentration of the production of alcohol was between 10 and 18% in the fermentation medium. Raherimandim (1986) also found that the best alcohol production was in 14% sugar concentration. In this study, 20% sugar concentration was used in the fermentation medium.

Although there may be awareness in the biotechnological utilization of biomass in the manufacture of renewable energy, the present research work is a very important step in the discovery of practical application of different agro-waste materials in alcohol production. Recent global concerns due to the resource depletion and environmental pollution bring new aspect to present work of applied microbiology. Plentiful amount of agro-waste biomass must be unavoidably utilized in many possible ways for the benefits of local people of Myanmar. Not only the characteristics of social infrastructure in the locality but also biological interrelations, especially microbial ecosystem, regarding the biomass must be taken into account for the feasible utilization of the different types of biomass.

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References

Aye Aye Khine (1994). **The Study on the Production of Cellulase enzyme from *Trichoderma* Species**. MSc Thesis, Department of Botany, University of Yangon.

Bay Dar (1994). **The Study on the Production of Cellulase enzyme from *Chaetomium* Species**. MSc Thesis, Department of Botany, University of Yangon.

Bhat, M.K. (2000). **Cellulases and related enzymes in biotechnology**. Biotech. Adv. 18: 355-383.

Carvalho, Jr. A.V.W.N. Milfont, Jr and V. Yang. (1977). **Ethyl alcohol and use in Brazil. In, Ethyl alcohol production and use as a motor fuel**. edited by J. K. Paul. Noyeo Data Corporation Park Ridge, New Jersey. U.S.A,. 274- 289.

Collins, C.H. (1964). **Microbiological Methods**. 2nd edition. Butterworth. London. 91-93.

Cook, A.H. (1958). "**The chemistry and Biology of yeasts**" Academic Press Inc. New York.

Ferrer, A., C. Aiello, O. Bravo, A. Ledesma, I. Zabala, & M. Davila (1994**). Utilizacion de residuos de cosechay subproductos agricolas en la produccion de alimentos para animals ruminates.** Revi. Fac. Agron. (LUZ). 11:207 – 223.

Gallo, B.J. (1981). U. S Patent, 4, 275, 163.

Heitland, H., H. W. Czaschke, and N. Pinto (1977). **The use of ethanol from biomass as an alternative fuel in Brazil. In, Proceeding as International Simposium and Alcohol Fuel Technology**. Published by the U. S. Department of energy. June 1978.

Henry, B. and N.C. Rose (1966). **Organic Chemistry**, Macmillan Company, New York. London.

Hodge and Hildebrandt. (1954). **Industrial Microbiology**. Edited by A.H Rose. Butterworth and Co (publishers) Ltd., London.

Khin Kyi (1994). **The Study on the Production of Cellulase enzyme from *Emericella* Species**. MSc Thesis, Department of Botany, University of Yangon.

Khin Mi Mi Win (1994). **The Study on the Production of Cellulase enzyme from *Aspergillus* Species**. MSc Thesis, Department of Botany, University of Yangon.

King, K.W. and M.I. Vessal (1969). **Enzymes of the cellulase complex.Advances in Chemistry**, Series No.95.R.Gould,Amer.Chem. Soc., Washington, D.C, 2:7-24.

Lang, X., D.G. Macdonald, and G.A. Hill (2001a). **Recycle bioreactor for bioethanol production from wheat starchII.** Fermentation and economics. Energy sources 23:427-436.

Lynd, L.R., P.J. Weimer, W.H. Van Zyl, and I.S. Pretorius (2002). **Microbial cellulose utilization: Fundamentals and Biotechnology.** Microbial. Mol. Biol. Rev. 66: 506-577.

Mandels, M. and J. Weber (1968). **The Production of Cellulases Food Microbiology Division** , 136th Meeting of the ACS, Atlantic City, New Jersey, 391 – 403,

Miller, G.L. (1959). "**Use of Dinitrosalicylic Acid Reagent From Determination of Reducing Sugar** ", Analytical Chemistry, 31(3):426.

Onions, A.H.S. (1971). Preservation of Fungi. In "**Methodsin Microbiology**" edited by C. Booth. Vol.4. Academic Press. New York, London.

Prescott, S.C. and C.G. Dunn (1949). **Industrial Microbiology**. 2nd edition. McGraw, Hill Book company inc., New York.

Raherimandimby, M. (1986**). Contribution d'etude de la Fermentation alcoholiqueper levules floculees surmelasses *et* hydrolysate d'amidon**. These de INSA, Toulouse.

Schlesinger, W.H. (1991**). Biogeo chemistry: an analysis of global change.** Academic, San Diego. p. 443.

Shwe Wah Sein (1994) Production of Alcohol From Indigenous Raw Materials by *Saccharomyces cerevisiae.* M.Sc. Thesis, Departyment of Botany, University of Yangon.

Singh, A. and K. Hayashi (1995). **Microbial cellulase, protein architecture, molecular properties and biosynthesis**. Adv. Appl. Microbiol. 40: 144.

Stark, W.H. (1954). Alcoholic **Fermentation of Grain. In, Industrial Fermentation.** edited by Lelanda. Underkofler and Richard J. Hickey. Chemical Publishing Co. Inc. New York. 1: 17-72.

Thein Thein New (2007) **Effects of Starchy Substrates and Factors Effecting Fermentation of Ethanol by Yeast Isolated from Toddy and Dani.** Ph.D Thesis, Departyment of Botany, University of Yangon.

Van Wyk, J.P.H. (2001). **Biotechnology and the Utilization of Biowaste as a Resource for Bioproduct Development.** T I B. 19(5):172 – 177.

Zaldivar, M., J.C. Velasquez, I. Contreras and L.M. Perez (2001). ***Trichoderma aureoviridae* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol.** EJB Electronic J. Biotechnology ISSN: 0717-3458, 4, 10 pages.