

Preparation of Cellulase Enzyme and Bio-ethanol Production from Agricultural Waste Product

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Abstract

Screening for cellulase-producing fungi was routinely done on cellulose yeast extract agar plates with 1% Congo red after 4 to 7 day old culture. After 20-30 minutes it could be seen a clear zone and then a little flooded with Gram's iodine to get a sharp and distinct clear zone around the cellulase producing fungal colonies. Grams iodine is the best plate assay method for determining cellulase activity and gives the best results with prominent and distinct clear zones within 3-5 minutes. Total (19) fungal isolates were screened from two selected areas such as (12) isolates from near the heap of straw soil, Mawbi Township and (7) isolates from the litter soil, Botanical garden of Yangon University, Kamayut Township. In this study, cellulolytic fungi belonged to *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Spicaria* sp., *Rhizoctonia* sp., *Trichoderma* sp. and *Paecilomyces* sp..

The effect of experimental factors is important for the production of reducing sugar by enzyme activity. In the present study, the effect of various fermentation periods (1, 2, 3, 4, 5, 6, 7 and 8 days), the effect of different temperature (20, 30, 40 and 50° C) and the effect of different pH (pH - 3, 4, 5, 6, 7 and 8) on the appearance of reducing sugar by the crude cellulase enzymes were studied and which were extracted from *Aspergillus*, *penicillium* and *Fusarium*, *Spicaria* sp. In this study, optimal fermentation period was 3 days in *Aspergillus* and *Penicillium* sp. and 4 days in *Fusarium* and *Spicaria* sp., optimal temperature 30° C of pH 6 in *Aspergillus*, *Penicillium*, *Fusarium* and *Spicaria* sp., respectively. The optimal experimental condition was carried out by crude cellulase enzymes using filter paper cellulose, rice straw and water hyacinth, and end products were analyzed by TLC method.

Keywords: Fungi, Cellulase Enzymes, Cellulose Materials

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1. Introduction

Cellulose is the most abundant constituent of living things ranging from 15-60 % of organic matter dry weight. It provides easily available energy source to soil microflora and makes complex plant constituents available to other microorganisms for decomposition.

A wide variety of microbes including bacteria, fungi, actinomycetes and protozoa are involved in the decomposition of cellulose. Fungi have generally been considered to be the main organism responsible (Cowling, 1958; Gascoigne and Gascoigne, 1958; Siu, 1951).

Probably the most abundant energy source available to soil fungi is cellulose. All fungi do not have the same capability of breaking down this material because the enzyme cellulase is not produced by all fungi. Schubert and Nord (1950) have shown that certain fungi can preferentially decompose cellulose in wood, leaving the lignin. Mandels and Reese (1960) found that the synthesis of the enzyme cellulase could be induced in some fungi when grown upon cellobiose.

Plant biomass contains cellulose as the major component. Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes. Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid hydrolysis and enzymatic reaction. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere.

Like sugar materials, starchy materials are also in the human food chain and are thus indispensable. Third alternative exists-cellulosic materials are paper, cardboard, wood, and other fibrous plant material.

Cellulosic materials are comprised of lignin, hemicellulose, and cellulose and are thus sometimes called lignocellulosic materials. One of the primary functions of lignin is to provide structural support for the plants.

Cellulose feedstocks, which include a wide variety of material from corn stalks, wood, straw, and cotton, to old newspapers (paper) and trash, are potentially good sources of alcohol. If fully converted, for example, a ton of old newspapers would yield up to 70 gallons of alcohol. Moreover, cellulose materials are also extremely cheap and easily available.

Cellulase enzyme is used extensively in various industries, especially in textile, food and in the bioconversion of lignocellulosic wastes to alcohol.

Kumar and Singh (2001) reported that *Eichhomia* biomass could be used as

lignocellulosic biopolymer for cellulase and β -glucosidase production by co-cultivation of *A.niger* and *T. reesi*, under semi solid state fermentation. Villena *et al.*, (2001) reported cellulase production by fungal biofilms on polyester cloth.

A cellulosic enzyme system consists of three major components: endo- β -glucanase (EG) (EC 3.2.1.4), exo- β -glucanase or cellobiohydrolase (CBH) (EC 3.2.1.91) and β -glucosidase (BG) (EC 3.2.1.21). The mode of action of each of these being:

- (1) Endo- β -glucanase, 1,4- β -D-glucan glucanohydrolase, CMCase, Cx: "random" scission of cellulose chains yielding glucose and cello-oligo saccharides.
- (2) Exo- β -glucanase, 1,4- β -D-glucan cellobiohydrolase, Avicelase, C1: exo-attack on the non-reducing end of cellulase with cellobiose as the primary structure.
- (3) β -glucosidase, cellobiase: hydrolysis of cellobiose to glucose.

Thus enzyme is a complex composed of three distinct enzymes to convert cellulose to glucose. One component serves to weaken the structure of native cellulose by weakening the hydrogen bonds. A second component consists of exo- and endo- β -1,4-glucanases. The exo-glucanase removes single glucose units from the non-reducing end of the cellulose chain, while the endo-glucanase hydrolyzes the interior glucosidic bonds of cellulose to liberate oligomers of lower molecular weight. A third component consists of the beta-glucosidases, including cellobiase, which are active on the dimers and oligomers of cellulose to glucose molecules.

The complete **cellulase** system-comprising CBH, EG and BG components synergistically act to convert crystalline cellulose to glucose. The exo-cellobiohydrolases and the endoglucanases act together to hydrolyze cellulose to small cello-oligosaccharides. The oligosaccharides (mainly cellobioses) are subsequently hydrolyzed to glucose by a major beta-glucosidase.

Currently, there are two major ways of converting cellulose to glucose: chemical and enzymatic. The research on both methods has for decades occupied the attention of many investigators worldwide. Because each cellulose molecule is an unbranched polymer of 1000 to 1 million D-glucose units, linked together with beta-1,4 glycosidic bonds, cellulose from various sources are all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemicals. It is this difference that makes possible a persistent research on cellulose. The model chemical compounds most commonly used in today's research are carboxymethyl cellulose (CMC), which has a generally amorphous structure, and Avicel, which has a highly crystalline structure. In this experiment, cellulose

from a variety of sources will be subjected to depolymerization conditions.

So the rate of utilization of plant materials and its transformation into humus is largely depended on active soil mycoflora. This is the reason why scientists are studying in the at molecular level (Nevalainen and Panttila, 2003; Shimosaka *et al.*, 1996), trying to discover more cellulolytic fungi (Ariunna and Temuulen, 2001), developing new techniques to take advantage of the effect of celluloses (Norenko *et al.*, 1994; Steiner *et al.*, 1994; Szakacs-Dobozi *et al.*, 1985) and are developing mutant strains to enhance the production of cellulases (Chand *et al.*, 2005; Gupta *et al.*, 1981).

The potential of cellulose as an alternative energy source has stimulated research into bioconversion process which hydrolyzes cellulose to soluble sugars for feedstock, in alcoholic fermentations and other industrial processes (Bakare *et al.*, 2005). In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol, organic acids, detergents and other chemicals. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al.*, 2005).

Cellulases produced by the filamentous fungi, yeasts and bacteria are implicated in the degradation of cellulose fibers to soluble sugars such as glucose, cellobiose and other oligomers. Fungi are the most important microorganisms for the decomposition of organic matter due to their degradation abilities. Microorganisms excreting cellulases play an important role in the nature, due to their ability to decompose lignocellulosic residues, establishing a key link in the carbon cycle (Zhang *et al.* 2006). 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 this research work, total (19) cellulolytic fungi were isolated and screened with Congo red and Gram's iodine method from two selected areas; (12) isolates from near the heap of straw soil, Mawbi Township and (7) isolates from the litter soil, Botanical garden of Yangon University, Kamayut Township. The resulting cellulolytic fungal species were identified into genus level according to the morphological and microscopically characteristics. Some of the best cellulolytic producing activity of fungi such as *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp. will be used for producing reducing sugar using filter paper cellulose, rice straw and water hyacinth, and end products were analyzed by TLC method.

2. Materials and Methods

Glasses ware used for all the experimental purposes were first cleaned with chromosulphuric acid and rinsed several times with tap water and then with distilled water to make them free from the acid. Then they were dried and sterilized at 121°C in 15 lbs/sq inch for 15 minutes.

Sampling of soil

The soil samples near the decomposed leaves of soil (litter soil) from the Botanical garden of Yangon University, Kamayut Township and the heap of rice straw, Mawbi Township were collected at a depth of 1 inch. The samples were taken with sterile corer and put it in plastic bag. They were brought to the lab for isolation of cellulolytic fungi and carried out by serial dilution method.

Preparation of cellulose substrate

In the present investigation, the substrates cellulose was prepared from Whatman filter paper No.1, the preparation of cellulose substrate was done by the method of Amerine and Ough (1979) with slight modification. About 30 gm of cellulosic raw material was suspended in 1 liter of water and added 50 ml of concentrate HCl. It was allowed to stay over-night on the magnetic stirrer at room temperature to get complete disintegration of cellulose fibers. The resultant fibers was filtered and washed several times with Distilled water until pH 7.0. Then the fine cellulose powder was dried and kept in clean bottle or autoclaved in wet condition and used in the preparation of enzyme producing medium. It was used for preliminary determination of cellulase enzyme activity.

Other cellulose substrates were prepared from rice straw and water hyacinth. And they were air dried in the shaded place. The dried samples were grinded into small sized powder with blender. Then the fine cellulose powder of rice straw and water hyacinth were dried until constant weight and individually kept in clean bottle. It was used as substrates for the preparation of enzyme production medium and enzyme activity assay in the determination of enzyme activity and reducing sugar.



Semi-dried cellulose substrate (Whatman paper No. 1)



Dried powder of rice straw



Dried powder of water hyacinth

Figure 1. Cellulose substrates

Soil Dilution Method

Soil dilution method was used according to the method of Johnson (1957).

1. One gm sample of soil was placed in a graduated cylinder. Water was added to the soil so that a total volume of 100 ml was reached, the suspension was stirred and poured into a 300 ml Erlenmeyer flask. The flask containing the suspension was shaken on a mechanical shaker (Gallenkamp) for 30 minutes.
2. One ml sample was then transferred immediately through successive 9 ml sterile water in test tubes until the desired final dilution is reached. Each suspension was shaken by hand for a few seconds, and was in motion while being drawn into the pipette.

3. Use of the preceding method for making soil dilutions yield dilutions of soil in water $10, 10^2, 10^3, 10^4, 10^5$, etc.
4. One ml of the desired dilution is transferred to a specially into each of several petridishes and 12 to 15 ml of Cellulose Yeast Extract Medium, cooled to just above the solidifying temperature, are added to each dish.

The dishes are rotated by hand in a broad swirling motion so that the diluted soil is dispersed in the agar medium. These petridishes were incubated at $25^{\circ}\text{--}35^{\circ}\text{C}$ for 7-14 days.

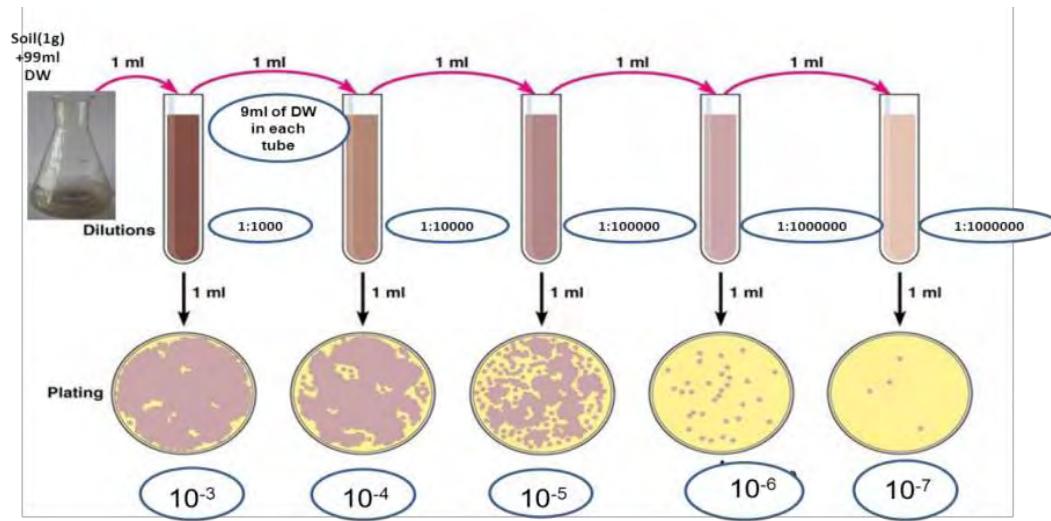


Figure 2. Soil Dilution Method (Johnson, 1957)

Table 1. Numbers of isolates from soil samples

Collected Place	Depth of soil	Soil pH	Soil concentration	No. of Isolated fungi	No. of cellulolytic fungi
Soil near the heap of straw soil (SS) (Mhawbi)	1 inch	6.0	10^{-3}	6	6
			10^{-5}	4	4
			10^{-7}	2	2
Litter soil (LS) (Botanical garden (YU), Kamayut	1 inch	6.5	10^{-3}	4	4
			10^{-5}	2	2
			10^{-7}	1	1
Total				19	19

Composition of enzyme production medium

Cellulose Yeast Extract Medium (Enzyme activity preliminary test medium) was prepared according to Mandels and Sternberg, 1976. It was included cellulose powder 10.0 g, peptone 1.0 g, yeast extract 0.5 g, malt extract 0.5 g, KH₂PO₄ 1.0 g, KNO₃ 0.1 g, MgSO₄ 0.1 g, ZnSO₄ 0.1 g, FeSO₄ 0.1 g, and agar 15.0 g in 1000 ml of demineralized water, pH 5.5.

Czapek dox agar medium

Czapek dox agar medium was used as stock culture medium or sub-culture medium for maintenance the fungus according to Raper and Thom, 1945. This medium contained sucrose 30.0 g, NaNO₃ 2.0 g, K₂HPO₄ .01 g, MgSO₄ 0.5 g, KCl 0.5 g, FeSO₄ 0.1 g, agar 15.0 g in 1000 ml of demineralized water, pH 7.0.

Preliminary test for cellulase secretion

Congo-red Reagent

Preliminary test for the excretion of cellulolytic enzyme from isolated fungi was performed according to the Congo-red method (Sharma *et al.*, 1986) with some modifications. A seven days old culture of isolated soil fungi were inoculated in the agar medium and incubated at 35°C. After 7 days small volume of Congo-red reagent (ethanol 90.0 ml, Congo-red 1.0 g in 10.0 ml of distilled water) was poured onto the surface of agar plate and kept at room temperature for 30 minutes. The development of a clear zone around the fungal colony was recorded as a positive result for the hydrolysis of cellulose by the enzyme secreted by soil fungi.

Grams Iodine Stain

Grams Iodine Stain (potassium iodide (KI) 2.0 g and iodine 1.0 g in 300 ml distilled water) was prepared according to Kasana, *et al.*, 2008.

Identification of fungi:

Fungal isolates were identified on the basis of routine cultural, morphological and microscopically characteristics (Barnett, 1969 and Watanabe, 1937).

Determination of reducing sugar by Dinitrosalicylic Acid Method

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

Dinitrosalicylic acid reagent

1gm of Dinitrosalicylic acid is dissolved in 1% NaOH solution which contains 20 gm of sodium potassium tartarate and 0.2 gm of phenol.

Glucose Standards solution

0.2 -1.0 mg of glucose concentration per ml were used for glucose standard solution.

Procedure

Into the precleaned test tubes 1ml each of sample containing 0.2 to 1 mg per ml glucose standard solution was placed and added 1ml each of DNS 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 6 ml of distilled water and then determined the absorbance by UV spectrophotometer at 570 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 was prepared prior to the actual determination of samples and shown in figure 3.

Enzyme Assay

The activity of cellulose hydrolytic enzyme was determined by detecting the amount of reducing sugar liberated by the action of crude cellulase enzymes with filter paper substrate as stated by (Mandels and Sternberg, 1976; Ghose, 1987).

$$\text{Enzyme Unit} = \frac{\mu \text{ mole of reducing sugar} \times \text{Total reaction volume}}{\text{Reaction time} \times \text{Enzyme volume in reaction}}$$
$$= \text{U/ml}$$

Determination of Filter Paper Degrading Activity (F. P. D)

Substrate : Whatman No.1 Filter Paper strip, $1.0 \times 6.0 \text{ cm}$ (50 mg)

Filter Paper Assay Procedure

Whatman No. 1 Filter Paper was cut into $1.0 \times 6.0 \text{ cm}$ 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 coiled filter paper was added to the solution and incubated for 1

hour at 50°C. And 2 ml DNS reagent was added to stop reaction and test tubes were placed in boiling water bath for 10 minutes and determined reducing sugar as glucose (Mandels and Weber, 1968; Gallo 1981; Ghose, 1987). All samples, controls and glucose standards were used as references. In the present work, the activity of cellulase enzyme extracted from isolated soil fungi was calculated and presented as FPD.

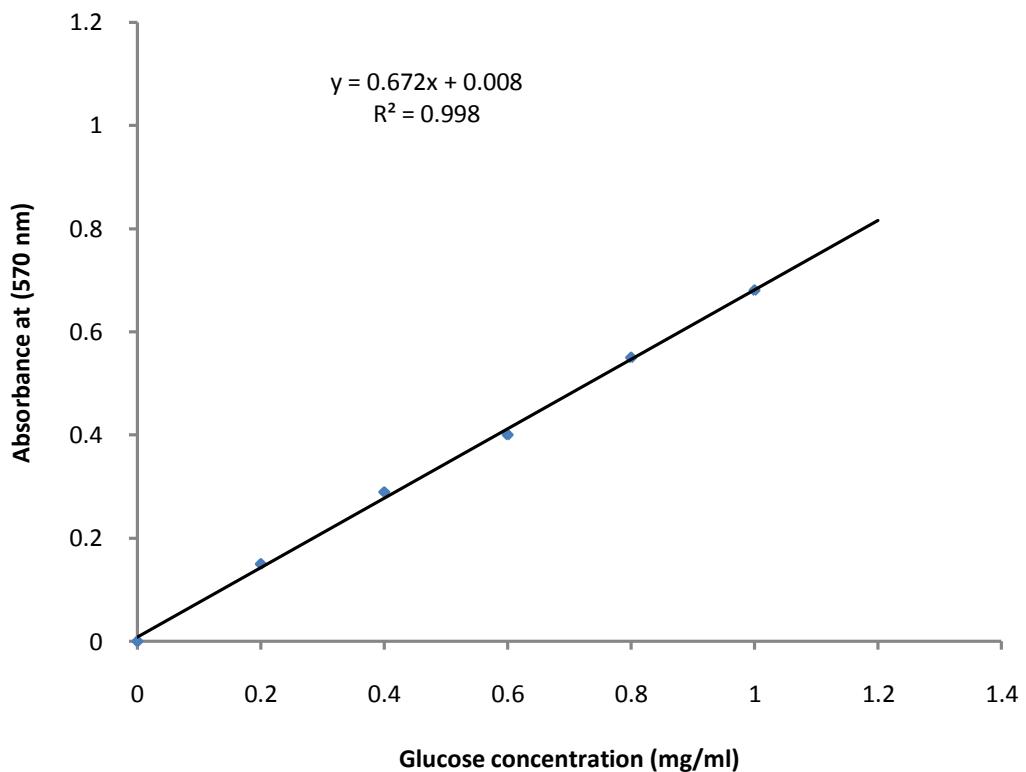


Figure - 3. Glucose standard curve

Composition of liquid medium

Composition of enzyme production liquid medium (cellulose substrate 10 g, $(\text{NH}_4)_2\text{SO}_4$ 14 g, KH_2PO_4 2 g, CaCl_2 0.3 g, MgSO_4 0.3 g, FeSO_4 0.5 g, peptone 1 g, Tween-80 1 g in 1000 ml distilled water, pH 5.5) was prepared according to Mandels and Sternberg, 1976.

Identification of hydrolysis action pattern of cellulases by Thin Layer Chromatography (TLC) method

The hydrolysis action pattern of cellulases was identified by Thin layer chromatography (TLC) method according to Stahl and Kaltenbach, 1961.

The initial hydrolysis products from the substrates by action of crude cellulase enzymes from *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. were identified by TLC as follow: one to two ml amount of samples were taken out at optimal experimental condition and the reaction was terminated by placing the sample tubes in boiling water for 5 minutes. They were centrifuged to remove the higher polysaccharides. An aliquot of 3 drops of each samples was subjected to precoated silica gel (with sodium bisulfite buffer) TLC plate and n-propanol : water (85:15 v/v) was used as the solvent system. After developing the solvent system, sugar was detected by iodine tank.

3. Results

Screening and identification of fungi:

Samples were collected from two different sources such as soil from near the heap of straw (Mhawbi) (SS) and litter soil (Kamayut) (LS). There are 12 strains were isolated from straw soil and 7 strains from litter soil. From this screening, a total number of (19) species of fungi were isolated. All of them showed cellulolytic activity. They were belonged to genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Paecilomyces*, *Fusarium*, *Rhizoctonia* and *Spicaria* sp. as shown in Table 2.

Table 2. Identification of isolates fungi that showed cellulolytic activity isolated from two different locations

No.	Sources	pH	No. of Isolated fungi	Total No. of cellulolytic fungi
1	Straw soil (SS) (Mhawbi) SS- 10^{-3}	6.0	<i>Penicillium</i> sp. (2), <i>Aspergillus</i> sp. (1), <i>Paecilomyces</i> sp. (1), <i>Fusarium</i> sp. (1), <i>Spicaria</i> sp. (1)	6
2	SS- 10^{-5}		<i>Aspergillus</i> sp. (2), <i>Penicillium</i> sp. (1), <i>Fusarium</i> sp. (1)	4
3	SS- 10^{-7}		<i>Rhizoctonia</i> sp. (1), <i>Paecilomyces</i> sp. (1)	2
4	Litter soil (LS) Botanical garden (YU) (Kamayut), LS- 10^{-3}	6.5	<i>Aspergillus</i> sp. (2), <i>Penicillium</i> sp. (1), <i>Fusarium</i> sp. (1)	4
5	LS- 10^{-5}		<i>Trichoderma</i> sp. (1), <i>Paecilomyces</i> sp. (1)	2
6	LS- 10^{-7}		<i>Trichoderma</i> sp. (1)	1
Total				19

Isolation of fungi from straw soil

Total (12) fungal strains were isolated from soil dilution 1^{-3} to 10^{-7} of straw soil (Mhawbi). All of them showed cellulolytic activity. The most outstanding clear zone was selected among them which were *Penicillium* sp. and *Spicaria* sp. for determining cellulolytic enzyme activity.



Figure 4. Various fungal colonies grown on cellulose agar medium from straw soil (Mhawbi) dilution 10^{-3}

Characters of mycelium and spore formation of *Penicillium* sp.

Conidiophores hyaline, erect, branched penicillately at the apexes with verticillate metula, terminal phialides and catenulate conidia on each phialide, forming rather divergent conidial heads: phialides pen-pointed with abruptly tapered tips. Conidia phialosporous, pale green, dark in mass, ellipsoidal or subglobose, 1-celled, smooth, apiculate at one end.

Scientific Classification

Kingdom	- Mycota
Division	- Eumycota
Sub-division	- Deuteromycotina
Class	- Deuteromycetes
Order	- Moniliales
Family	- Moniliaceae
Genus	- <i>Penicillium</i>
Species	- <i>Penicillium</i> sp.



Pure fungal colony



Micrograph of *Penicillium* sp. (2) X400



Preliminary test of enzyme secretion
on cellulose agar medium stained with
1% congo red



Effect of gram's iodine in
cellulose agar plate

Figure 5. Fungal isolate *Penicillium* sp. from straw soil

Characters of mycelium and spore formation of fungal *Spicaria* sp.

Conidiophore arrising singly, frequently branch, terminating in a group of widely divergent, phialides; conidia hyaline, 1-celled globose, catenulate, produced basipetally. The genus is much like *Penicillium*, but the spore bearing apparatus less compact and the phialides more spreading.

Scientific classification

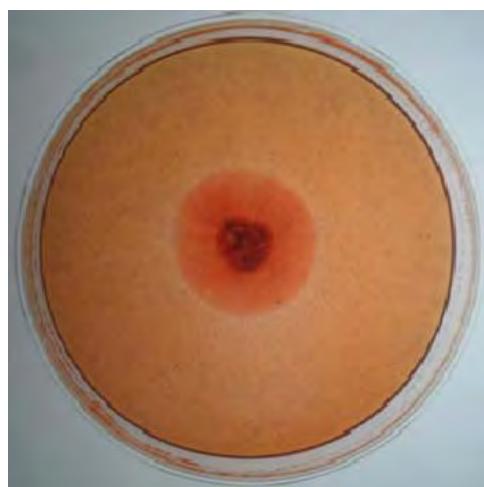
Division	- Amastigomycota
Sub-Division	- Deuteromycotina
Form-Class	- Deuteromycetes (Inperfect fungi)
Form-Subclass	- Coelomycetidae
Form-Order	- Moniliales
Genus	- <i>Spicaria</i>
Species	- <i>Spicaria</i> sp.



Pure fungal colony
(Light brown color inside with white periphery)



Micrograph of *Spicaria* sp. (x 40)



Preliminary test of enzyme secretion
on cellulose agar medium stained with
1% congo red



Effect of gram's iodine in
cellulose agar plate

Figure 6. Fungal isolate *Spicaria* sp. from straw soil

Isolation of fungi from litter soil

Total (7) fungal strains were isolated from litter soil which was diluted from 10^{-3} to 10^{-7} . All of them showed cellulolytic activity. Some fungi showed a large clear zone compared with others. *Aspergillus* sp. and *Fusarium* sp. were selected for determining cellulolytic enzyme activity.



Figure 7. Various fungal colonies grown on cellulose agar medium
from litter soil dilution 10^{-5}

Characters of mycelium and spore formation of *Aspergillus* sp.

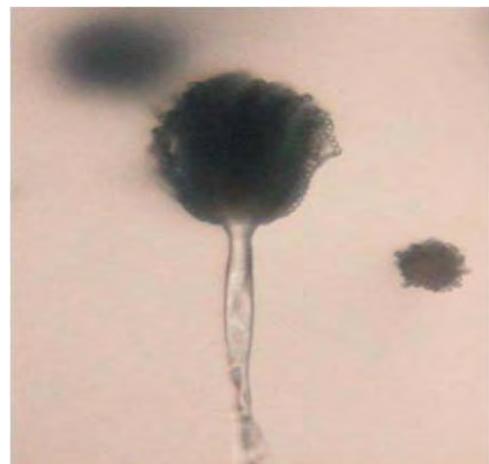
Conidiophores upright, simple, terminating in a globose or clavate swelling, bearing phialides at the apex or radiating from the apex or the entire surface; conidia 1-celled, globose, often variously colored in mass.

Scientific Classification

Kingdom	- Mycota
Division	- Eumycota
Sub-division	- Deuteromycotina
Class	- Deuteromycetes
Order	- Moniliales
Family	- Moniliaceae (Dematiaceae)
Genus	- <i>Aspergillus</i>
Species	- <i>Aspergillus</i> sp.



Pure fungal colony
(Dark green color inside with white periphery)



Micrograph of *Aspergillus* sp. X400



Preliminary test of enzyme secretion
on cellulose agar medium stained with
1% congo red



Effect of gram's iodine in
cellulose agar plate

Figure 8. Fungal isolate *Aspergillus* sp. from litter soil

Characters of mycelium and spore formation of *Fusarium* sp.

Conidiophores hyaline, simple or branched, bearing conidia in chains and/or spore masses at the apexes of branches. Conidia phialosporous, hyaline, of two kinds: macroconidia borne in spore masses, boat-shaped, with slightly curved apical cells, hooked foot cells, and 2 central cylindrical cells, mainly 4- to 5-celled, and microconidia hyaline, ellipsoidal or ovate, apiculate at one end. Chlamydospores not formed.

Scientific Classification

Kingdom	- Mycota
Division	- Eumycota
Sub-division	- Deuteromycotina
Class	- Deuteromycetes (Hyphomycetes)
Order	- Moniliales
Family	- Moniliaceae (Tuberculariaceae)
Genus	- <i>Fusarium</i>
Species	- <i>Fusarium</i> sp.



Pure fungal colony (White color)



Micrograph of *Fusarium* sp. (1) X400



Preliminary test of enzyme secretion
on cellulose agar medium stained with
1% congo red



Effect of gram's iodine in
cellulose agar plate

Figure 9. Fungal isolate *Fusarium* sp. from litter soil

Table 3. Characters of potential cellulolytic fungi from two selected sites

Sr. No.	Soil Sample	Characters of Fungi	Name of Species
1	Litter soil (LS) Botanical garden (YU) (Kamayut)	Mycelium dark green color, conidiophores upright, simple, terminating in a globose, radiating from the entire surface; conidia 1-celled, globose, catenulate.	<i>Aspergillus</i> sp.
2	Straw soil (SS) (Mhawbi)	Conidiophores arising from the mycelium singly, branched near the apex to form a brush-like, conidia-bearing apparatus; conidia 1-celled, mostly globose or ovoid, produced basipetally.	<i>Penicillium</i> sp.
3	Litter soil (LS) Botanical garden (YU) (Kamayut)	Mycelium extensive and cottony in culture, white color, conidiophores variable, slender and simple, branched irregularly, conidia hyaline, variable, principally of two kinds – macroconidia and microconidia, microconidia 1-celled, oblong or slightly curve, borne singly.	<i>Fusarium</i> sp.
4	Straw soil (SS) (Mhawbi)	Mycelium white color, conidiophore arising singly, frequently branch, terminating in a group of widely divergent, phialides; conidia hyaline, 1-celled globose, catenulate, produced basipetally.	<i>Spicaria</i> sp.

Effect of various fermentation period on the activity of cellulase enzyme

Effect of various fermentation period on the activity of cellulase enzyme by *Aspergillus*, *Penicillium*, *Fusarium*, and *Spicaria* sp. were studied in the range of 1, 2, 3, 4, 5, 6 and 7 days fermentation using Whatman No.1 paper as a substrate.

It was observed that the maximum amount of reducing sugar 0.440 mg/ml and 0.570 mg/ml by *Aspergillus* sp. and *Penicillium* sp. were studied at 3 days fermentation and 0.570 mg/ml and 0.440 mg/ml by *Fusarium* sp. and *Spicaria* sp. were observed at 4 days fermentation period, respectively, as shown in Table 4 and Figure 10.

Table 4. Determination of various fermentation period on the activity of cellulase enzyme by isolated *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp.

Fermentation period (days)	Initial pH				Amount of reducing Sugar (mg/ml)				FPD Enzyme activity (U/ml)			
	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.
1	5.5	5.5	5.5	5.5	0.280	0.270	0.295	0.385	1.036	0.999	1.091	1.424
2	6.0	6.0	6.0	6.0	0.300	0.400	0.420	0.270	1.110	1.480	1.554	0.999
3	6.5	6.0	6.0	6.5	0.440	0.570	0.440	0.385	1.628	2.109	1.628	1.424
4	6.5	6.5	6.5	6.5	0.430	0.450	0.570	0.440	1.591	1.665	2.109	1.628
5	7.0	7.0	7.0	7.0	0.410	0.440	0.450	0.385	1.517	1.628	1.665	1.424
6	8.0	8.0	8.0	8.0	0.400	0.400	0.410	0.300	1.480	1.480	1.517	1.110
7	8.0	8.0	8.0	8.0	0.370	0.358	0.400	0.280	1.369	1.424	1.480	1.036

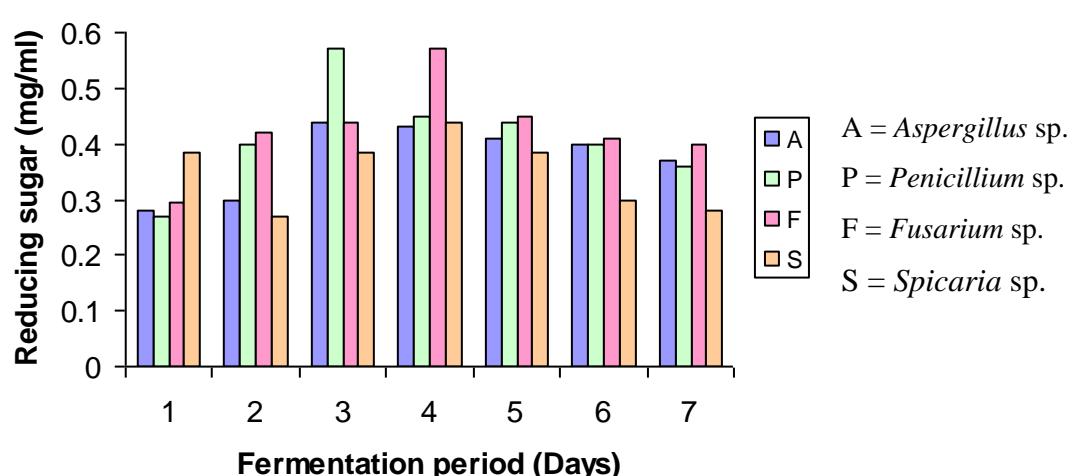


Figure 10. Comparison of the effect of various fermentation period on the activity of cellulase enzyme by *Aspergillus*, *Penicillium*, *Fusaria* and *Spicaria* sp.

Effect of different temperature on the activity of cellulase enzyme

Effect of different temperature on the activity of cellulase enzyme by *Aspergillus*, *Penicillium*, *Fusarium*, and *Spicaria* sp. were studied in the range of 20°C , 30°C, 40°C and 50°C 3 days and 4 days fermentation periods, respectively.

It was observed that the maximum amount of reducing sugar 0.440 mg/ml and 0.660 mg/ml by *Aspergillus* sp. and *Penicillium* sp. were studied at 30°C and for 3 days fermentation period and 0.440 mg/ml and 0.430 mg/ml by *Fusarium* sp. and *Spicaria* sp. were observed at 30°C and for 4 days fermentation period, respectively, as shown in Table 5 and Figure 11.

Table 5. Effect of different temperature on the activity of cellulase enzyme by isolated *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp.

Temperature (°C)	Fermentation period (days)				Amount of reducing Sugar (mg/ml)				FPD Enzyme activity (U/ml)			
	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.
20	3	3	4	4	0.150	0.200	0.170	0.111	0.555	0.740	0.625	0.407
30	3	3	4	4	0.440	0.660	0.440	0.430	1.628	2.442	1.628	1.591
40	3	3	4	4	0.130	0.110	0.130	0.090	0.481	0.407	0.481	0.333
50	3	3	4	4	0.430	0.440	0.390	0.410	1.591	1.628	1.443	1.517

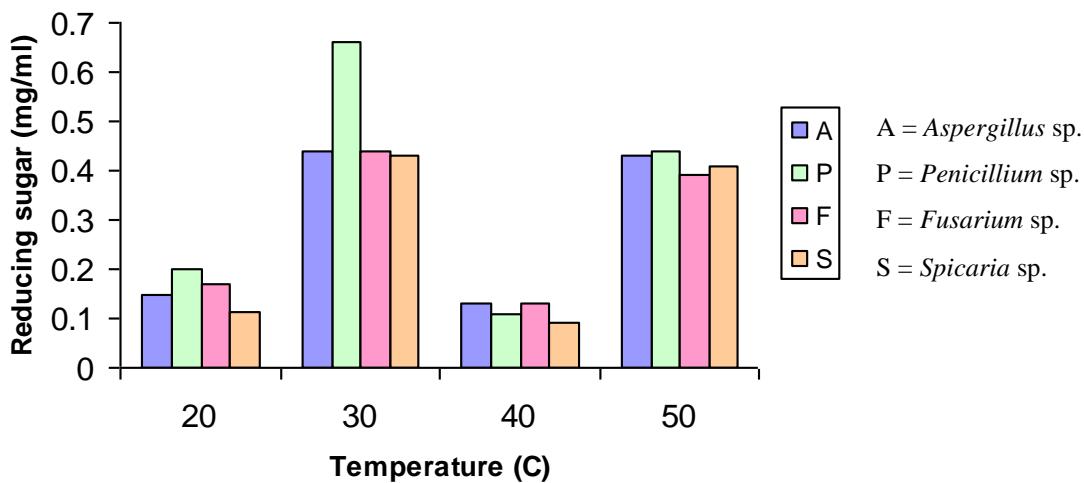


Figure 11. Comparison of the effect of different temperature on the activity of cellulase enzyme by *Aspergillus*, *Penicillium*, *Fusaria* and *Spicaria* sp.

Effect of different pH on activity of cellulase enzyme

Effect of different pH on the activity of cellulase enzyme by *Aspergillus*, *Penicillium*, *Fusarium*, and *Spicaria* sp. were studied in the range of pH 3, 4, 5, 6, 7, and 8 using Whatman No.1 paper as a substrate.

It was observed that the maximum amount of reducing sugar 0.140 mg/ml and 0.090 mg/ml by *Aspergillus* sp. and *Penicillium* sp. were studied at pH-6 for 3 days fermentation and 0.050 mg/ml and 0.090 mg/ml by *Fusarium* sp. and *Spicaria* sp. were observed at pH-6 for 4 days fermentation period at 30°C, respectively, as shown in Table 6 and Figure 12.

Table 6. Effect of different pH on the activity of cellulase enzyme by isolated *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp.

pH	Fermentation period (days)				Amount of reducing Sugar (mg/ml)				FPD Enzyme activity (U/ml)			
	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.
3	3	3	4	4	0.030	0.050	0.030	0.040	0.111	0.185	0.111	0.148
4	3	3	4	4	0.060	0.060	0.040	0.050	0.222	0.222	0.148	0.185
5	3	3	4	4	0.090	0.062	0.033	0.060	0.333	0.229	0.122	0.222

6	3	3	4	4	0.140	0.090	0.060	0.090	0.518	0.333	0.222	0.333
7	3	3	4	4	0.121	0.080	0.040	0.070	0.448	0.296	0.148	0.259
8	3	3	4	4	0.060	0.060	0.040	0.062	0.222	0.222	0.148	0.229

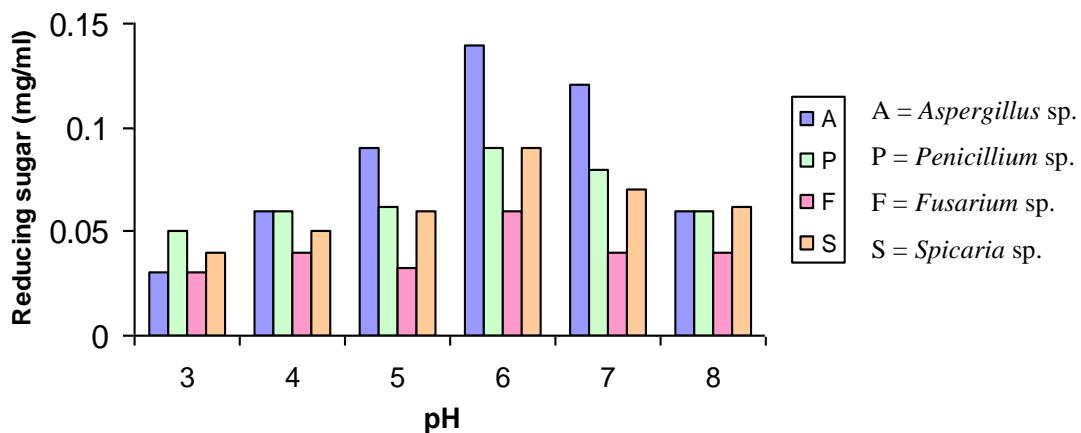


Figure 12. Comparison of the effect of different pH on the activity of cellulase enzyme by *Aspergillus*, *Penicillium*, *Fusaria* and *Spicaria* sp.

Comparison of different enzymes activities on the production of reducing sugar from filter paper cellulose, rice straw and water hyacinth at optimal experimental conditions

Four different enzymes activities on the production of reducing sugar from filter paper cellulose, rice straw and water hyacinth were studied at optimal experimental conditions.

It was observed that the maximum amount of reducing sugar 0.420 mg/ml, 0.480 mg/ml, 0.420 mg/ml and 0.440 mg/ml for filter paper cellulose, 0.430 mg/ml and 0.442 mg/ml, 0.400 mg/ml and 0.430 mg/ml for rice straw and 0.400 mg/ml, 0.410 mg/ml, 0.378 mg/ml and 0.400 mg/ml for water hyacinth (Table 7 and Figures 13, 14 and 15) were estimated at 30°C, pH 6 and 3 days fermentation period for *Aspergillus* sp. and *Penicillium* sp., 4 days for *Spicaria* sp. and *Fusarium* sp., respectively, using the substrates (filter paper cellulose, rice straw and water hyacinth) as basal carbon source. And then 0.495 mg/ml and 0.485 mg/ml were observed when the filter paper cellulose was used as a substrate at 30°C and 3 days fermentation for TG (pH-4) and FG (pH- 4.5), respectively.

Table 7. Determination of reducing sugar from substrates (filter paper cellulose, rice straw and water hyacinth) by crude cellulase enzyme from *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp. at the optimal experimental conditions

Substrates	Optimal experimental condition												Reducing sugar (mg/ml)				FPD Enzyme activity (U/ml)			
	Temperature (°C)				pH				Reaction Time (days)											
	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.	A. sp.	P. sp.	F. sp.	S. sp.
Filter paper cellulose	30	30	30	30	6.0	6.0	6.0	6.0	3	3	4	4	0.442	0.480	0.440	0.420	1.635	1.776	1.628	1.554
Rice straw	30	30	30	30	6.0	6.0	6.0	6.0	3	3	4	4	0.430	0.442	0.430	0.400	1.591	1.635	1.591	1.480
Water hyacinth	30	30	30	30	6.0	6.0	6.0	6.0	3	3	4	4	0.400	0.41	0.400	0.378	1.480	1.036	1.480	1.399

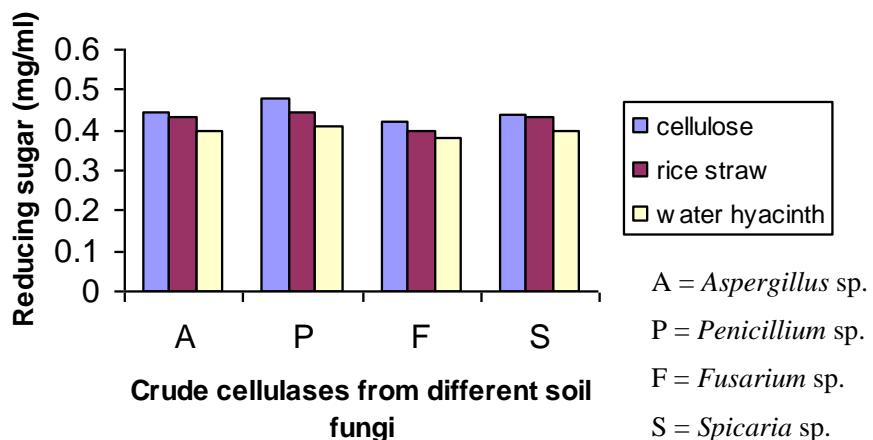


Figure 13. Comparative study on the effect of crude cellulase enzymes activity on the production of glucose from raw materials (filter paper cellulose, rice straw and water hyacinth) at optimal experimental conditions

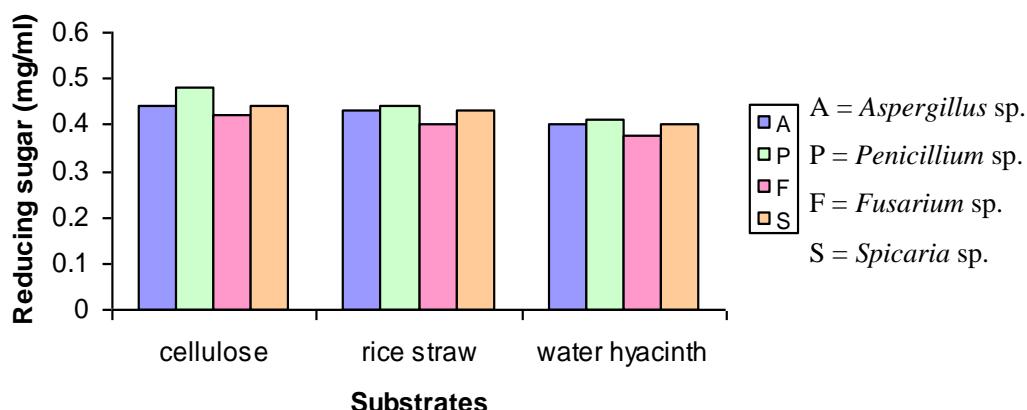


Figure 14. Comparative study on cellulose hydrolytic activity by crude cellulase enzymes from *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp. using the substrates (filter paper cellulose, rice straw and water hyacinth) at optimal experimental conditions

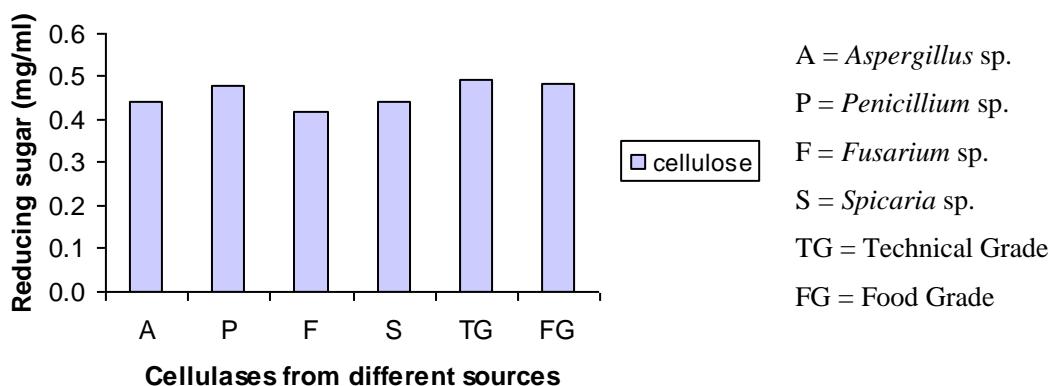
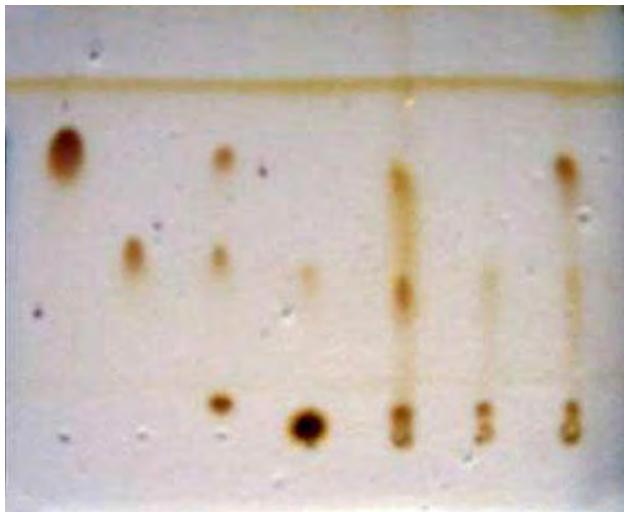


Figure 15. Comparative study on the production of glucose by crude cellulase enzymes from *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp. and two commercial cellulase enzymes (TG and FG) activities using filter paper cellulose substrate

Determination of action pattern of cellulases by TLC method (Stahl and Kaltenbach, 1961)

The hydrolysis pattern of crude cellulase enzymes on different cellulosic raw materials such as filter paper cellulose, rice straw and water hyacinth were comparatively studied and shown in Figures 16 - 19.



G = Glucose (Rf value = 0.50)
 C = Cellobiose (Rf value = 0.29)
 r = rice straw
 w = water hyacinth
 c = filter paper cellulose
 c (TG) = filter paper cellulose
 was degraded by TG
 c (FG) = filter paper cellulose
 was degraded by FG

Figure 16. Hydrolysis pattern of cellulase enzyme from *Aspergillus* sp. on different raw materials (rice straw, water hyacinth and filter paper cellulose) and two commercial cellulase enzymes Technical Grade (TG) and Food Grade (FG) activities on the production of glucose from filter paper cellulose by TLC



G = Glucose
 (Rf value = 0.53)
 C = Cellobiose
 (Rf value = 0.31)
 r = rice straw
 w = water hyacinth
 c = filter paper cellulose
 c (TG) = filter paper cellulose
 was degraded by TG
 c (FG) = filter paper cellulose
 was degraded by FG

G C r w c c c
(TG) (FG)

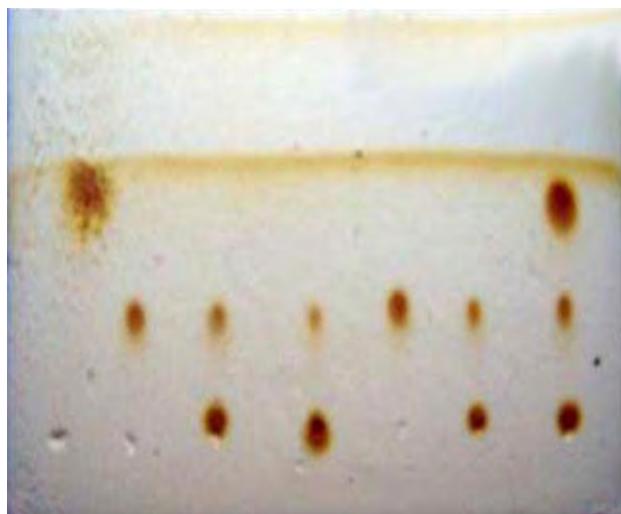
Figure 17. Hydrolysis pattern of cellulase enzyme from *Penicillium* sp. on different raw materials (rice straw, water hyacinth and filter paper cellulose) and two commercial cellulase enzymes Technical Grade (TG) and Food Grade (FG) activities on the production of glucose from cellulose by TLC



G = Glucose (Rf value = 0.48)
 C = Cellobiose (Rf value = 0.23)
 r = rice straw
 w = water hyacinth
 c = filter paper cellulose
 c (TG) = filter paper cellulose
 was degraded by TG
 c (FG) = filter paper cellulose
 was degraded by FG

c w r c c G C
TG FG

Figure 18. Hydrolysis pattern of cellulase enzyme from *Fusarium* sp. on different raw materials (filter paper cellulose, water hyacinth and rice straw) and two commercial cellulase enzymes Technical Grade (TG) and Food Grade (FG) activities on the production of glucose from cellulose by TLC



G = Glucose (Rf value = 0.48)
 C = Cellobiose (Rf value = 0.23)
 r = rice straw
 w = water hyacinth
 c = filter paper cellulose
 c (TG) = filter paper cellulose
 was degraded by TG
 c (FG) = filter paper cellulose
 was degraded by FG

G C r w c c c
(TG) (FG)

Figure 19. Hydrolysis pattern of cellulase enzyme from *Spicaria* sp. on different raw materials (rice straw, water hyacinth and filter paper cellulose) and two commercial cellulase enzymes Technical Grade (TG) and Food Grade (FG) activities on the production of glucose from cellulose by TLC

Discussion and Conclusion

Naturally, plants play a vital and significant role in environmental sustainability of weather and climate condition and the control of soil erosion and disastrous flood.

Moreover, renewable organic matter derived mainly from plant has risen to a highlight spot in solving problems and issues evolved in energy crisis and agricultural industry.

Nowadays people turn to bioproducts converted from biomass – a material from any biological materials for the save and security of their living and health.

In plants, renewable organic matters are composed of lignin, hemicellulose and cellulose. Cellulose and its derivatives provide a major source for renewable energy and raw materials.

These are widely used in the bioconversion of renewable cellulosic biomass. Glucose resulted from appropriate hydrolysis of cellulosic biomass through the treatment of advanced biotechnology can be used in different application such as a production of fuel, ethanol, and single cell protein feed stock and important chemical in agriculture industry.

There are number of cellulase enzyme secreted by microorganisms for the hydrolysis of cellulosic materials in nature. Numbers of fungi and bacteria capable of utilizing cellulose as a carbon source have existed on earth since immemorial time.

Cellulases are the enzymes responsible for the cleavage of the β (1-4) glycosidic linkages in cellulose. Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where these substrates present are the best sources for isolation of cellulolytic microorganisms can be occurred. In this study, two different soil samples were collected from near the heap of straw in Mawbi and litter soil from Botanical garden of Yangon University, Kamayut Township.

Fungi are well known agents of decomposition of organic matter in common and of cellulosic substrate in particular (Lynd *et al.*, 2002). Cellulose is world's most abundant organic substance (Ruttloff, 1987) and comprises a major storage form of organic compound and major component of biomass energy. Because a large proportion of vegetation cellulose added to soil, decomposition of cellulose has a special significance in the biological cycle of carbon (Lederberg 1992). In industry, these enzymes have found novel application in production and processing of chemicals, food and manufactured goods such as paper, rayon etc., and extraction of valuable components from plants and improvement of nutritional values of animal feed (Wiseman, 1995).

The present research work, the preliminary test of cellulase activity of soil fungi were detected by clear zone on agar plate using congo-red and iodine reagent as an indicators. Total nineteen strains of potential cellulolytic fungi had been isolated from two selected areas and identified up to genus level. They were five kinds of *Aspergillus* sp., four *Penicillium* sp., three *Fusarium* sp., one *Spicaria* sp., three *Paecilomyces* sp. (1), one *Rhizoctonia* sp. and two *Trichodama* sp..

The appearance of the clear zone around the colony can be occurred when the Congo red solution was poured. This is the evidence that the fungi showed cellulolytic activity in order to degrade cellulose. Congo red pouring plates showed low intensity of clearance zone compared with staining Gram's iodine. In the present study, using Gram's iodine remarkably enhances the sharpness of the clear zone for screening cellulase producing fungi (Kasana, *et al.*, 2008).

According to the results, Grams iodine is the best plate assay method for determining cellulolytic activity and gives the best result with prominent and distinct clear zones within 3-5 minutes. Cellulolytic fungi in the plate breaks down the polysaccharide which surrounded by fungal colony were exhausted with polysaccharides so monosaccharides and disaccharides were remained. Florencio *et al.* (2012) reported that mono and disaccharide cannot bind with dyes efficiently, so clear zone around the colony can be seen.

Congo red stain inactivates the microbe hence they cannot be used for further study. Kasana, *et al.* (2008) stated that in Gram's iodine, a distinct zone can be seen and it is not toxic to the cells which could be reused for other study.

Efficient cellulase producing isolates were finally selected based on the clear zone around the fungi colony, which was then used in the determination and analysis of reducing sugar. Among the nineteen kinds of isolated *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Spicaria* sp. were selected for extracting crude cellulase enzymes by means of fermentation process.

The crude cellulolytic enzymes were applied in the determination and analysis of reducing sugar. Two commercial cellulase enzymes (TG and FG) were also used. Different cellulosic raw material filter paper cellulose, rice straw and water hyacinth were used in reducing sugar production.

In the present work, cellulose degradation potential from isolated fungi *Aspergillus*, *Penicillium*, *Fusarium* and *Spicaria* sp. were comparatively studied. The activity of cellulose degrading potential was detected by DNS methods (Miller, 1959) using Whatman No.1 paper strips (1.0 cm x 6.0 cm) and their respective enzyme activity were expressed as F.P.D.

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), Khin Mi Mi Win (1994) and Bay Dar (1994) at the Department of Botany, University of Yangon.

Bay Dar (1994) reported that the reducing sugar was produced by the cellulase enzyme from *Chaetomium* species was found to be the best at 30°C, pH 6 and for 3 days fermentation period using filter paper cellulose as a substrate.

In 1994, Khin Mi Mi Win discovered that highest cellulose degradation enzyme activity was detected in the culture of pH 5, temperature 40°C and 3 days fermentation period by *Aspergillus* sp..

In 1994, Aye Aye Khine reported that maximum cellulase enzyme activity was detected by pH 6, temperature 40°C and 3 days fermentation period by *Trichoderma* sp..

In the present study, the best result of reducing sugar was found at 30°C and pH 6.0 and for 3 days fermentation period by *Aspergillus* sp., *Penicillium* sp. and for 4 days by *Spicaria* sp. and *Fusarium* sp. using filter paper cellulose, rice straw and water hyacinth as the substrates.

According to the results of comparative hydrolysis activity, commercial cellulase enzyme TG was found to be better hydrolysis than 4 fungal cellulases. Among them *Penicillium* sp. provided nearly the same hydrolysis of TG, moreover, a little bit better than FG using filter paper cellulose (Whatman No. 1 paper) as a substrate.

In the present work, the activities of cellulase from *Penicillium* sp. was the highest among the crude cellulase enzymes from *Aspergillus* sp., *Spicaria* sp. and *Fusarium* sp..

The activities of cellulase from *Spicaria* sp. was the lowest among them.

Comparative hydrolysis pattern of cellulase enzyme action in Figure 16 and Figure 18 it was found that glucose and cellobiose could be produced by crude cellulase enzymes from *Aspergillus* sp. and *Fusarium* sp., using rice straw and filter paper cellulose and only cellobiose was produced using water hyacinth.

In the case of filter paper cellulose was degraded by TG and FG, glucose and cellobiose were hydrolyzed by FG and only cellobiose was hydrolyzed by TG.

It was observed in Figure 17 that glucose and cellobiose could be hydrolysed from all raw materials by the crude cellulase enzyme obtained from *Penicillium* sp.. In this hydrolysis pattern TG can hydrolyse glucose and cellobiose, but only cellobiose was produced by FG using filter paper cellulose substrate.

However, according to the results of Figure 19, only cellobiose was produced by crude cellulase enzyme from *Spicaria* sp. using all raw materials. In this hydrolysis pattern glucose and cellobiose were hydrolysed by FG and only cellobiose was hydrolysed by TG using filter paper cellulose substrate.

According to the results of FPD and TLC method, cellulases were fermented by *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Spicaria* sp. may be exo- and endo-cellulase types, because the end results of reducing sugar were glucose and also cellobiose.

So, it was assume that most of the crude cellulase enzymes from *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. can hydrolyze to glucose and cellobiose, except the enzyme from *Spicaria* sp. can hydrolyze only cellobiose.

All the enzymes used in the present study seem to possess the much more cellobiohydrolase and cellobiase enzyme, except the enzyme from *Spicaria* sp. seem to possess only cellobiohydrolase enzyme.

It has been found that cellulase produced from *Penicillium* sp. showed the highest active followed by *Aspergillus* sp., *Fusarium* sp., and *Spicaria* sp. in sequence.

Although the research work is only a preliminary study it could be assumed as an initiation for the investigation on the cellulase enzyme production by the local fungal species which has not been studied, yet.

Based on the finding results the best potential of cellulolytic enzyme from *Penicillium* sp. will be selected for further investigation, bio-ethanol production.

For the commercial used, the production of cellulase enzyme should be investigated until technical grade or commercial grade is obtained for using in industries.

REFERENCES

- Amerine, M.A. and C.S. Ough (1979) **“Preparation of Culture Powder”, Methods for Analysis of Murts and Wines.** A Wiley Interscience Publication. John Wiley and Sons.
- Ariunaa, J., G. Temuulen (2001) **Distribution of Cellulolytic Fungi in Soil of Mongolia.** No. 10. Ulaanbaatar: Scientific Journal “Biology” Mongolian National University; pp. 71–77. (in Russian).
- Aye Aye Khine (1994) **“The study on the production of cellulose enzyme from *Trichoderma* species”.** M.Sc thesis, University of Yangon, department of Botany.
- Bay Dar (1994) **“The study on the production of cellulose enzyme from *Chaetomium* species”.** M.Sc thesis, University of Yangon, department of Botany.
- Bakare, M.K., I.O. Adewale, A. Ajayi and O.O. Shonukan (2005) **Purification and characterization of cellulase from the wild type and two improved mutants of *Pseudomonas fluorescens*,** African J. Biotechnol, 4(9): 898-904.
- Barnett, H.L. (1969) **Illustrated Genera of Imperfect fungi.** Printed in the United States of America. Ph.D Thesis.
- Chand, P., A. Aruna, A.M. Maqsood, and L.V. Rao (2005) **Novel mutation method for increased cellulase production.** Journal of Applied Microbiology. 98(2):318
- Cowling, E.B. (1958) **A Review Literature on the Enzymatic Degradation of Wood and Cellulose.** USDA Forest Service Report, No. 2116.
- Florencio, C., S. Couri and C.S. Farinas (2012) **Correlation between Agar Plate Screening and Solid-State Fermentation for the Prediction of Cellulase Production by *Trichoderma* Strains.** Enzyme research, pp.1 7.
- Gallo, B. J. (1981). U. S. Patent, 4: 163-275.
- Gascoigne, J.A. and M.M. Gascoigne (1958) **Biological Degradation of Cellulose.** London: Butter Worths Publ. Co. p. 231
- Ghose, T.K. (1987) **Masurement of cellulase activities.** Pure & Appl. Chem., 59 (2): 257-268
- Gupta, J.K. C.K. Shirkot, and S. Dhawan (1981) **Isolation and mutation of cellulolytic fungi.** Acta Microbiol Acad Sci Hung. 28(1):31–36.

- Johnson, D.F., E.A. Curl, and J.H. Bond (1957) “**Formulase of Selected Culture Media**”, Methods for Studying Soil Microflora, 145.
- Kasana, R.C. (2008) **A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine**. Current Microbiology, 57(5), pp.503-507.
- Khin Kyi (1994) “**The study on the production of cellulose enzyme from *Emericella* species**”. M.Sc thesis, University of Yangon, department of Botany.
- Khin Mi Mi Win (1994) “**The study on the production of cellulose enzyme from *Aspergillus* species**”. M.Sc thesis, University of Yangon, department of Botany.
- Kumar R. and R.P. Singh (2001) **Semi-solid-state fermentation of *Eichhornia crassipes* biomass as lignocellulosic biopolymer for cellulose and β-glucosidase production by co-cultivation of *Aspergillus niger* RK3 and *Trichoderma reesei* MTCC 164**. Applied Biochem. And Biotech. 96(1-3): 71-82
- Lynd, L.R., P.J. Weimer, W.H. van Zyl, and I.S. Pretorius (2002) **Microbial cellulose utilization: 'undamentals and biotechnology**. Microbiol. Mol. Biol. Rev. 66(3): 506-577.
- Mandel, M. and E. T. Reese (1960) **Induction of Cellulose in fungi by cellobiose**. J. Bact. 79: 81.
- Mandels, M. and D. Sternberg (1976) **Recent Advances in cellulase Technology**. J. Ferment. Technol., 54, 4:267-286
- Mandels, M. and J. Weber (1968) **The production of cellulase**. 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
- Nevalainen, H. and M. Penttilä (2003) **Molecular Biology of Cellulolytic Fungi**. In: Kück U., editor. The Mycota, Vol. II, Genetics and Biotechnology. 2nd Ed. Berlin, Heidelberg: Springer Verlag.
- Norenko, L.N.N., J. Ariunaa and E.S. Morozova (1994) **8th E.C. on Biomass for Energy, Environment, Agriculture and Industry**. Vienna, Austria. Cyclic Utilization of Lignocellulosics; pp. 1337–1342.
- Raper, K.B. and C. Thom (1949) **A General purpose Media, Methods for studying soil microflora**. 141.

- Ruttloff, H. (1987) **Impact of biotechnology on food and nutrition.** In: Food Biotechnology. (Eds): D. Knorr. Marcel Dekker, Inc.
- Schubert , W.J. and F.F. Nord (1950) **Investigation on lignin and lignification. I Studies on softwood lignin.** J. Am. Chem. Soc. 72:977-981
- Sharma, P., S. Pajin, N. Dhillon, D.V. VAdehra, and D.K. Dube (1986) “**The staining solution of Lactophenol cotton-blue**”, The Practice of Medical Microbiology, 12th ed., 2.
- Shimosaka, M., M. Kumehara, X.Y. Zhang, M. Nogawa and M. Okazaki (1996) **Cloning and characterization of a chitosanase gene from the plant pathogenic fungus *Fusarium solani*.** J Ferment Bioeng. 82(5):426–431
- Siu, R.G.H. (1951) **Microbial Decomposition of Cellulose.** New York: Reihd. Publ. Corp. p. 531.
- Stahl, E, u. U. Kaltenbach, (1961) **J. Chromatog.** 5, 351
- Steiner, J., C. Socha and J. Eyzaguirre (1994) **Culture conditions for enhanced cellulase production by a native strain of *Penicillium purpurogenum*.** World J Microbiol and Biotech. 10(3):280–284.
- Szakács-Dobozi, M., G. Szakács, D. Meyer and G. Klappach (1985) **Enhancement of enzymatic degradation of cellulose by application of mixed enzyme systems of different fungal origin.** Acta Biotechnologica. 5(1):27–33
- Villena, G.K., P. Moreno and M. Gutierrez-Correa (2001) **Cellulose production by fungal biofilms on polyester cloth.** Agro-food-industry Hi-Tech. pp. 32-35.
- Watanabe, T. (1937) **Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species.** Second Edition. CRC Press.
- Wen, Z., W. Liao and S. Chen (2005) **Production of cellulose by *Trichoderma reesei* from dairy manure.** Bioresour. Technol. 96(4): 491-499.
- Wiseman, A. (1995) **The application of enzymes in industry.** In: Handbook of Enzyme Biotechnology (3rd Ed.). Ellis Hardwood.
- Zhang, Y.H. and L.R. Lynd (2004) **Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulose systems.** Biotechnol. Biocng. 88(7): 797-824.