Study of Cellulase Activity Produced by *Penicillium* sp., *Aspergillus niger* and *Trichoderma viride* on *Imperata cylindrica* (L.) Beauv. Enrichment Media

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Abstract. The aim of this study was to determine the value of cellulase activity produced by mould toward filter paperase (FP-ase) and endoglucanase (EG). *Penicillium* sp., *Aspergillus niger* and *Trichoderma viride* utilized as mould isolate. Cogon grass substrates pre-treated with 2% NaOH. It determined the reducing sugar. The respective cellulase activity of each isolate was measured using filter paperase (FP-ase) and endoglucanase (EG). Pre-treatment successfully damaged lignin structure. The most favourable incubation period produced in 7th days. Moreover, it increased surface area of cellulose with a cellulose level of 48.32%, hemicellulose level of 9.88% and lignin level of 1.14%. *Aspergillus niger* exhibit the highest cellulase activity with enzymes value of 3.97 U/ml for filter paperase (FP-ase) and 4.53 U/ml for endoglucanase (EG). *Penicillium* sp. reached 3.71 U/ml for filter paperase (FP-ase) and 4.11 U/ml for endoglucanase (EG) while *Trichoderma viride* got 3.37 U/ml for filter paperase (FP-ase) and 3.38 U/ml for endoglucanase (EG).

1. Introduction

Global trends demonstrate the rapid development of the use of lignocellulosic materials for the production of fuels, chemicals, and other commercial products. This is understandable considering that lignocellulosic material is a renewable natural resource and its availability is unlimited. The bioconversion process certainly provides a positive impact on the economy, community welfare, and the environment. It has been estimated that the use of lignocellulosic materials reached more than one billion tonnes per year and has replaced about 30% of total gasoline consumption in the USA [1,2].

The main component of lignocellulosic material is cellulose. Cellulose is a carbohydrate consisting of a linear glucose polymer with a length of about 1,200-15,000 degrees of polymerization (DP). This compound can be found in almost all plant cell walls, in the form of microfibrils with lignin and hemicellulose, containing 15% amorphous and 85% crystalline fractions [3]. In general, cellulose is difficult to decipher because it has a high level of crystal structure and a lignin layer that envelops cellulose tissue which acts as a barrier.

To initiate the production of fuel from lignocellulosic materials, the decomposition of cellulose into simple sugars is a very important process. Cellulose decomposition can be carried out through
enzymatic and/or acidic hydrolysis. Enzymatic hydrolysis of cellulose has been shown to be more effective because it has a high degree of conversion, minimal formation of by-products, low energy requirements, and easy operating conditions [4]. The enzyme complex involved in cellulose hydrolysis is the cellulase enzyme.

Cellulase catalyses cellulosic processes, changing compounds that are insoluble in water into simpler, water-soluble compounds [5]. Cellulase is a multi-enzyme system composed of enzymes and some isozymes, both of which can be synergized [6]. Such multi-enzyme systems include: endoglucanase (EG) which breaks cellulose randomly into oligosaccharides, cellobiohydrolase (CBH) which further degrades cellulose by releasing the cellobiose, and β-glucosidase (BG) units which break down the glucose unit from the non-reducing end of cello-oligosaccharides and cellobiose [7]. Producing fuel from lignocellulosic material on a large scale also demands larger quantities of cellulase. The high cost of cellulase is a major obstacle for some industries. It is therefore necessary to significantly reduce the cost of cellulase by focusing on the identification of new sources of cellulase with high enzyme activity, the creation of a multi-component cellulase system optimized for the selected substrate at the time of cellulose hydrolysis, and minimization of costs for cellulase production [2,8].

Based on the importance and the usefulness of cellulase enzymes, this study aims to determine the activity of cellulase enzymes produced by Penicillium sp., Aspergillus niger and Trichoderma viride through the use of paperase (FP-ase) and endoglucanase (EG) filters. Cellulase was produced by the liquid media fermentation method on cogon grass (Imperata cylindrica (L.) Beauv.) substrate. Cogon grass has a chemical content which includes: α-cellulose, 40.22%; holocellulose, 59.62%; hemicellulose, 18.40%; and lignin, 31.29% [9].

2. Methods

2.1. Pre-treatment
Before being utilized as an enzyme substrate, a cogon grass pre-treatment process was used to reduce lignin and hemicellulose levels. The grass was washed, and then dried in the sun for 12 hours. The dried grass was then cut into pieces ± 2 cm long, milled with a grinder, sifted through a 40-mesh, then stored in a dry place. The pre-treated cogon grass was then immersed in 2% NaOH at a ratio of 1:10 (w/v) at room temperature for 24 hours, before being heated in an autoclave for 1 hour. This was followed by washing with tap water to neutralise pH and drying in an oven at 65°C to obtain constant (dry) weight [10,11]. The basal medium used consisted of a solution of mineral salts and organic nitrogen to increase the production of enzymes. A total of 1.4 g of yeast extract, 2.0 g of KH$_2$PO$_4$, 0.34 g CaCl$_2$.2H$_2$O, 0.30 g MgSO$_4$.7H$_2$O, 5 mg FeSO$_4$.7H$_2$O, 1.6 mg MnSO$_4$.H$_2$O, 1.4 mg ZnSO$_4$.7H$_2$O, and 2.0 mg CoCl$_2$.6H$_2$O were added up to a volume of 1L and boiled with a magnetic stirrer, followed by sterilization using an autoclave for 15 minutes. The basal medium was set at pH 6 [12].

2.2. Mould culture
Isolates of Penicillium sp., Aspergillus niger, and Trichoderma viride were split into two subcultures, namely a stock culture and a working culture. The subcultures were grown on Potato Dextrose Agar (PDA) medium in Petri dishes which had been sterilized in an autoclave for 15 minutes. Thereafter, they were incubated at room temperature for 7 days [13]. The stock cultures were stored in the refrigerator and re-cultured every month.

2.3. Enzyme production
On the 7th day of Petri dish culture as described above, the working culture was suspended in a 50 ml centrifuge tube containing 10 ml of 0.9% Tween 80 solution. The centrifuge tube was then shaken to re-suspend the spores up to a concentration of 10$^7$ spores/ml. Cellulase production was initiated by inoculating a 5 ml starter solution into a 250 ml Erlenmeyer glass containing 50 ml of basal medium and 2.5 grams of sterilized cogon grass substrate. The culture was incubated for 8 days at room
temperature, using a rotary shaker at 200 rpm. Crude enzyme extraction was performed at incubation times of 1, 2, 3, 4, 5, 6, 7, and 8 days. Crude enzyme extraction was performed by adding 100 ml of Tween 80 0.1% (v/v) pH 6 extract solution into the incubated culture. This was then centrifuged at 4000 rpm for 15 minutes to separate the mycelium of fungi and enzymes [13,14].

2.4. Reducing sugar analysis
The Somogyi-Nelson reduction sugar test can be divided into the preparation of the reducing sugar standard curve and the determination of the reducing sugar content in each sample. The standard sugar curve is based on glucose, using absorbance measurements of a glucose standard solution with varied concentrations. Each test sample consisted of a specific substrate, acetate buffer pH 5, and an enzyme filtrate with a particular composition. The samples were incubated in a waterbath equipped with a thermostat for 30 min at a temperature of 45°C, after which 0.1 ml TCA was added and the mix was homogenised for one minute. Thereafter, 0.5 ml of Nelson C reagent was added to the appropriately diluted sample solution, followed by heating for 20 minutes in a boiling water bath. The samples were then cooled, after which 0.5 ml of arsenomolybdate reagent and 3.5 ml of distilled water were added and the mixture homogenised. Absorbance values were measured using a spectrophotometer at a wavelength of 520 nm.

2.5. Measurement of enzyme activity
From the reducing sugar standard curve, we can obtain the concentration of reducing sugar which is used to calculate cellulase enzyme activity. In this study the calculated enzyme activity was expressed in U/ml, referring to [15]. One unit of cellulase enzyme activity is the amount of enzyme needed to release 1μmol of reducing sugar per minute. Cellulase enzyme activity is expressed by the formula:

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\text{Enzyme activity} = \frac{[\text{reducing sugar - control}] \times 1000 \times \text{FP} \times \text{mol molecule mass of reducing sugar \times enzyme volume}}{\text{t (minutes)} \times \text{enzyme volume}}
\]

where: \text{FP} = \text{dilution factor} \\
1 \text{U} = \text{sugar produced (in μmol)/minute}

3. Results and Discussion

3.1. Pre-treatment
The cellulose, hemicellulose and lignin content of Imperata cylindrica (L.) Beauv. before and after pre-treatment using 2% NaOH with a ratio of 1:10 (w/v) are shown in Table 1. These data show that there was a decrease in lignin content, while cellulose and hemicellulose content increased, suggesting that the pre-treatment process successfully damaged the lignin structure.

| Treatment stage        | Cellulose | Hemicellulose | Lignin  |
|------------------------|-----------|---------------|---------|
| Before pre-treatment   | 46.92%    | 8.62%         | 4.11%   |
| After pre-treatment    | 48.32%    | 9.88%         | 1.14%   |

The process of pre-treatment was very influential in promoting delignification, due to the increased surface of cellulose and hemicellulose. These results are consistent with findings that the use of 2% NaOH pre-treatment on rice straw substrate produced a significant increase in cellulose levels after pre-treatment [16].
3.2. The combined effect of mould type and incubation time on filter paperase (FP-ase) enzyme activity value

As mentioned above, the calculation of cellulase enzyme activity is expressed in U/ml where one unit of cellulase enzyme activity is the amount of enzyme needed to release 1 μmol of reducing sugar (glucose) per minute [15]. Cellulase enzyme activity is expressed as the difference in sugar concentration between the sample and control (mg/ml), multiplied by the enzyme dilution factor, multiplied by 1000 (conversion factor), then divided by the incubation time and the molecular weight of the reducing sugar. In this study a dilution factor of 10x was used. Measurements of filter paperase (FP-ase) enzyme activity (Fig. 2) were performed after the reducing sugar produced had been determined using the glucose standard curve (Fig.1).

![Graph showing reducing sugar production of three moulds on cogon grass substrate](image1)

Figure 1. Average FP-ase reducing sugar production of three moulds on cogon grass substrate

![Graph showing enzyme activity of three moulds on cogon grass substrate](image2)

Figure 2. FP-ase enzyme activity of three moulds on cogon grass substrate

Figure 1 gives the average value of optimum filter paperase (FP-ase) reducing sugar content achieved on the 7th day of incubation. The *Aspergillus niger* isolate had an optimum reducing sugar value of 1.08 (mg/ml), while the value for *Penicillium* sp. was 1.01 (mg/ml), and for *Trichoderma viride* it was 0.92 (mg/ml). Meanwhile, Figure 2 shows the average values of the optimum filter paperase (FP-ase) enzyme activity achieved on the 7th day of incubation time. The *Aspergillus niger* isolate had an optimum filter paperase (FP-ase) enzyme activity of 3.97 (U/ml), followed by *Penicillium* sp. with 3.71 (U/ml), and *Trichoderma viride* with 3.37 (U/ml). These results are related to the average value of reducing sugar obtained (Figure 1). The value of reducing sugar and the value of filter paperase (FP-ase) enzyme activity reached the same optimum condition on the 7th day of incubation time. This is similar to the results reported for the production of FP-ase enzymes using *Aspergillus niger* on rice husks, with an activity of 19 IU/g obtained on the 7th day of hydrolysis [16].
In addition, [17] also reported a FP-ase enzyme activity value of 2.7 IU/g from *Penicillium capsulatum* on bagasse substrate. In Figure 2 it appears that on the 8th day of incubation time there is a decrease in FP-ase enzyme activity, this may be due to the limited amount of minerals and the lack of aeration so that the resulting enzymatic activity decreases.

In this research submerged fermentation was used; this means that the substrate use is in a liquid medium. The products of fermentation will be secreted by the moulds to the liquid medium. Nutrients added to the substrate can be used rapidly by microbes. In addition, the benefits of submerged fermentation include easier product purification techniques [18]. Meanwhile, [19] report that the production of cellulases enzymes by *Aspergillus niger* through submerged fermentation on coconut husk waste had an enzymatic activity value of 2.3 U/g for FP-ase and 3.9 U/g for EG on the 4th day of incubation.

3.3. The combined effect of mould type and incubation time on endoglucanase (EG) enzyme activity value

The measurement of endoglucanase (EG) enzyme activity is intended to determine cellulase enzyme activity in randomly breaking cellulose in the amorphous fraction to form oligosaccharides. The endo-1,4-β-glucanase enzyme acts on cellulose chains with low crystallinity, such as CMC, and produces shorter oligosaccharides or cellulose chains. Measurement of endoglucanase (EG) enzyme activity in this study used CMC (Carboxymethyl cellulose) substrate. Prior to the measurement of endoglucanase (EG) enzyme activity, it was necessary to know the value of reducing sugar. Figure 3 shows the average value of the optimum endoglucanase (EG) reducing sugar content achieved on the 7th day of incubation. The optimum values of reducing enzyme fluorine-enzyme sugar (FP-ase) were 0.24 (mg/ml) for *Penicillium* sp., 0.26 (mg/ml) for *Aspergillus niger* and 0.20 (mg/ml) for *Trichoderma viride*.

![Figure 3. Reducing sugar curves for endoglucanase (EG) for 3 moulds on cogon grass substrate](image)

![Figure 4. Enzymatic activity of endoglucanase (EG) for 3 moulds on cogon grass substrate](image)
As shown in Figure 4, the optimum endoglucanase (EG) enzyme activity was reached on the 7th day of incubation time. The *Aspergillus niger* isolate had an optimum endoglucanase (EG) enzyme activity value of 4.53 (U/ml), while the values were 4.11 (U/ml) for *Penicillium* sp., and 3.38 (U/ml) for *Trichoderma viride*. An EG enzyme activity value of 5.48 IU/ml has been reported from a study of cellulase enzyme production by *Trichoderma reesei* ZU-02 on corn cob substrates [20]. Meanwhile *Aspergillus niger* has been used to produce EG enzymes on rice husk substrate with an activity value of 130 IU/g [21].

The differences between the results obtained from this study with those from other similar studies may be due to a number of influencing factors, such as the type of mould used, the incubation time, and the substrate used as the mould medium. One of the factors that influenced enzymatic hydrolysis of cellulose was the length of incubation. The length of the incubation time will affect the product resulting from the hydrolysis process, because the time in the hydrolysis reaction determines the effectiveness of the enzymatic processes. The pre-treatment, by removing lignin from the substrate, was also very influential in the production of cellulase enzymes. In addition, the size of substrate particles can also have an effect [22]. The smaller the particle size of the substrate, the larger the substrate surface area for contact with other microorganisms and nutrients.

In this study, the mean value of endoglucanase (EG) enzyme activity was greater than the mean value of filter paperase (FP-ase) enzyme activity. This is because the cellulase enzyme is an enzyme complex composed of several proteins, such as endoglucanase (CMC-ase), exoglucanase, and β-glucosidase [23]. CMC-ase is one part of the cellulase complex, resulting in enzyme activity testing using a simpler CMC (Carboxymethyl Cellulose) substrate. This related to the specific enzyme work between the enzyme and its substrate, while the filter paperase (FP-ase) enzyme activity presents the total cellulase activity [24], so in the enzyme activity test using the filter paper substrate Whatmann no.1 which was more complex, because it involved three components of cellulase enzymes that require the synergism of the three enzyme complexes in breaking down cellulose.

4. Conclusion
Pre-treatment using NaOH 2% is proven to be capable of simplifying lignocellulosic structure, where cellulose content can be increased and lignin content reduced. The mould *Aspergillus niger* was more favourable than *Penicillium* sp. and *Trichoderma viride* in terms of enzyme activity. There was no difference in optimum incubation period among the three isolates utilized in this research. It is hoped that these results will contribute to fuel and energy research, especially green energy, in particular the production of ethanol from renewable sources.

References
[1] Lin H, Wanga B, Zhuang R, Zhou Q C and Zhao Y 2011 Artificial construction and characterization of a fungal consortium that produces cellulolytic enzyme system with strong wheat straw saccharification *Bioresource. Technol.* 102 10569–10576
[2] Merino S T and Cherry J 2007 Progress and challenges in enzyme development for biomass utilization *Adv. Biochem. Eng. Biotechnol.* 108 95–120
[3] Lynd L R, Weimer P J, Van Z and Pretorius I S 2002 Microbial cellulose utilization: fundamentals and biotechnology *Microbiol. Mol. Biol. R.* 66 506-577
[4] Sjostrom E 1995 *Wood Chemistry, Fundamentals and Applications* (2nd Ed.) (New York: Academic Press)
[5] Alexander M 1961 Microbiology of cellulose. In: Introduction to Soil Microbiology (2nd Ed.) (New York: Johnwiley and Son Inc.)
[6] Grassin C and Fauquembergue P 1996 *Wine and Fruit juices* (Industrial Enzymology 2nd Ed.) eds T Godfrey and S West (Macmillan Press Ltd.)
[7] Himmel M E, Baker J O and Overend R P 1994 *Approaches to cellulase purification* (Enzymatic Conversion of Biomass for Fuel Production, ACS symposium series 566)
[8] Kumar R, Singh S and Singh O V 2008 Bioconversion of lignocellulosic biomass: biochemical
and molecular perspectives. *J. Ind. Microbiol. Biotechnol.* **35** 377-391

[9] Sutiya B, Istikowati T W, Rahmadi A and Sunardi 2012 Chemical content and cogongrass fiber properties (*Imperata cylindrica*) as an illustration of pulp and paper raw materials *Bioscienciae* **9** 8-9

[10] Lin, Y S and Lee W 2011 Simultaneous saccharification and fermentation of Alkali-Pretreated cogongrass for bioethanol production *Bioresources* **6** 2744-2756

[11] Pasha, Chand B C, Sekhar B, Srinivas K, Balakrishna and Hanumalal N 2012 Sequential cellulase production, saccharification and ethanol fermentation using rice straw *J. Sci. Industrial Res.* **71** 616-620

[12] I-Son N G, Chen W, Shuang P, Jiun L, Potingchen, Chii-Gongtong, Su-Mayyu and Tuan-Hua, DH 2010 High level production of a thermoacidophilic β-glucosidase from *Penicillium citrinum* YS40-5 by solid–state fermentation with rice bran *Bioresource Technol.* **101** 1310-1317

[13] Huang Y, Qin X, Luo X M, Nong Q, Yang Q, Zhang Z, Gao Y, Lu F, Chen Y, Yu Z, Liu J and Feng J 2015 Efficient enzymatic hydrolysis and simultaneous saccharification and fermentation of sugarcane bagasse pulp for ethanol production by cellulase from *Pencillium oxalicum* EU2106 and thermotolerant *Saccharomyces cerevisiae* ZM1-5 *Biomass and Bioenergy* **77** 53-63

[14] Widjaja A, Hendy F and Yusra F 2010 Pretreatment of rice straw to produce xylose sugar by using xylanase crude enzyme *Proceedings of the National Seminar on Chemistry Unesa* B45

[15] Dybkhaer R 2001 Unit katal for catalytic activity *Pure Applied Chemistry* **73** 927-931

[16] Zhang Q and Cai W 2008 Enzymatic hydrolysis of Alkali-pretreated rice straw by *Trichoderma reesei* ZM4-F3 *Biomass and Bioenergy* **32** 1130-1135

[17] Considine P J, O’Rorke A, Hackett T J and Coughlan M P 1988 Hydrolysis of beet pulp polysaccharides by extracts if solid state cultures of *Penicillum capsulatum* *Biotechnol. Bioeng.* **31** 433–438

[18] Subramaniam R and Vimala R 2012 Solid state and submerged fermentation for the production of bioactive substance: A comparative study *Int. J. Sci. Nat.* **3** 480-486

[19] Mrudula S and Murugummal L 2011 Production of cellulose by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate *Brazilian J. Biotechnol.* **43** 1119-1127

[20] Xia L and Xueliang S 2004 High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue *Bioresource Technol.* **91** 259-262

[21] Kang S W, Park Y S, Lee J S, Hong S I, Gagdil N J, Daginawala T and Chakakrabarti S W 2004 Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass *Bioresource Technol.* **91** 153-156

[22] Maurya D P, Singh D, Pratap D, Maurya J P 2012 Optimization of solid state fermentation conditions for the production of cellulase by *Trichoderma reesei* NCIM 992 *J. Environ. Biol.* **33** 3-8

[23] Iqbal H M N, Ahmed I, Zia M A and Irfan M 2011 Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viridae* under SSF and its detergent compatibility *Adv. Biosci. Biotechnol.* **2** 149-156

[24] Oberoi S H, Chavan Y, Bansal S and Dhillon G S 2008 Production of cellulases through solid state fermentation using kinnow pulp as a major substrate *Food Bioprocess Technol.* **3** 528–536 (doi: 10.1007/s11947-008-0092-8)