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Studying the optimum conditions for cellulase production from wheat straw and papyrus straw using *Trichoderma viride*

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Abstract

The cellulase production in solid state fermentation (SSF) by *Trichoderma viride* was investigated using wheat straw and papyrus straw as the substrate. Present study described the optimization of process parameters for the production of cellulases. The fermentation experiments were carried out in shake flasks using pretreated wheat straw and papyrus straw. Maximum production of cellulases from wheat straw (CMCase 1.62 U/ml/min) and from papyrus straw (PAPase 0.81 U/ml/min) was observed after a fermentation period of 70hrs at an incubation temperature of 30ºC. Initial pH of the culture medium was also optimized and a pH of 5.5 was found to support maximum growth and enzyme production. Different inorganic nitrogen sources were evaluated for the production of cellulases and ammonium sulphate was found to be the best. The enzyme production was further enhanced by carrying out fermentation experiments using 25 ml of culture medium in 250 ml flask inoculated with 4% inoculum.

Key words: *Trichoderma viride*, Solid state fermentation, Cellulose, Wheat straw, Papyrus straw.

Introduction

Cellulases are being widely used in industrial fields, such as in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry (Ogel *et al*., 2001). Cellulases have now becoming more and more important in hydrolyzing biomass into fermentable sugars which are then converted to bio-fuel like ethanol (Sun and Cheng, 2003; Sukumaran *et al*., 2009) and hydrogen (Kumar and Das, 2001; Kapden and Kargi, 2006) by microbial fermentation.

Cellulose, a polymer of β-D-glucopyranose with 1, 4 β-glycosidic bonds, is the most abundant amongst all the naturally occurring organic compounds (Chandra *et al*., 2009). Thousands of million tons are being produced by photosynthesis annually and accumulate in large quantity in the form of agricultural forest and municipal residue which deteriorate the environment. Cellulases are the hydrolytic enzymes which are responsible for the decomposition of the natural cellulose polymer (cotton, filter paper or lignocellulosic biomass) by acting at 1,4 β-D-glucosidic linkages thus finally converting into glucose monomer (Sternberg *et al*., 2000). Cellulases are composed of three major components, endo β-glucanase (EC.3.2.1.4.), exo β-glucanase (EC.3.2.1.91) and β-glucosidase (EC.3.2.1.21). These enzymes act together to convert native crystalline cellulose to oligosaccharides and glucose (Mekala *et al*., 2008). Endo β-glucanase (1, 4 β-Dglucanhydrolase or CMCase) attacks randomly on internal glycosidic bonds of cellulose chain resulting in a rapid scission to yield oligosaccharides and glucose (Wood and Bat, 1988). Exo β-glucanase (1,4 β-D-glucanocellobiohydrolyase or cellobiohydrolase) hydrolyzeshighly crystalline cellulose attaching on newly generated ends (Sun *et al*., 2008). The enzyme β-glucosidase hydrolyzes the aryl- and alkyl-glucoside as well as cellobiose and cello-dextrin to glucose (Chapple *et al*., 2007).

The objective of the present study was to optimize various parameters for the enhanced cellulases production by *Trichoderma viride* through the use of pretreated wheat straw and papyrus straw in solid state fermentation.

Materials and Methods

Microorganism: *Trichoderma viride* GCBT-11 was obtained from the Biotechnology Laboratory, School of Bioscience and Biotechnology, University Kebangsaan Malaysia (UKM). It was maintained on potato dextrose agar (PDA) slants, stored at 4 °C and sub-cultured every two weeks. For inoculum preparation, the cultures were incubated on PDA at 30 °C for 16 or 18 h with 150 rpm agitation on rotary shaker and then transferred into solid-state fermentation medium according to ten percent inoculation quantity (10^6 spores /ml) (Mandels , 1982).

Substrate: Two substrates were used in this study. The first one was untreated wheat straw and the second was papyrus straw. Each substrate was washed with water to remove all residual, dried and milled to 40 mesh powders.

Inoculum preparation: The spore suspension was used as inoculum in the present studies. It was prepared from a 5 days old slant by adding 10 ml of sterilized 0.005 % Monoxal O.T (Diacetyl ester of Sodium sulphosuccinic acid) to it. The spores were scratched with the help of a sterilized wire loop to make a homogeneous suspension of spores. Spore count was measured using Haemocytometer (Hawary and Mostafa, 2001).

Fermentation technique: Twenty five milliliters of the fermentation medium consisting of (%, w/v); (NH4)2SO4, 0.14: KH2PO4, 0.20; Urea, 0.03; MgSO4,.7H20, 0.03; ZnSO4.7H2O, 0.00014; FeSO4.7H2O, 0.0005; MnSO4 0.00016; CoCl2 ,0.0002 ; CaCl2 , 0.0002; Tween-80, 2.0 ml; Polypeptide, 0.10; and sugarcane bagasse, 1.0 (pH 6.0), was transferred to the individual 250 ml cotton wool plugged conical flasks and autoclaved at 15 psig for 15 min. The flasks were inoculated with 1 ml of this inoculum containing 1.2×10^6 spores after cooling at room temperature and incubated at 30°C at 200 rpm in an orbital shaker incubator. After 70 hrs, the fermented broth was centrifuged at 6000 rpm for 10 min and the supernatant was assayed for enzyme activity.

Enzyme assay: The cellulases were assayed for CMCase and PAPase using wheat straw (carboxymethyl cellulose) and papyrus straw as substrates respectively. The released reducing sugar was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity is defined as " the amount of enzyme required to liberate one μmol of reducing sugars per minute under the assay conditions".

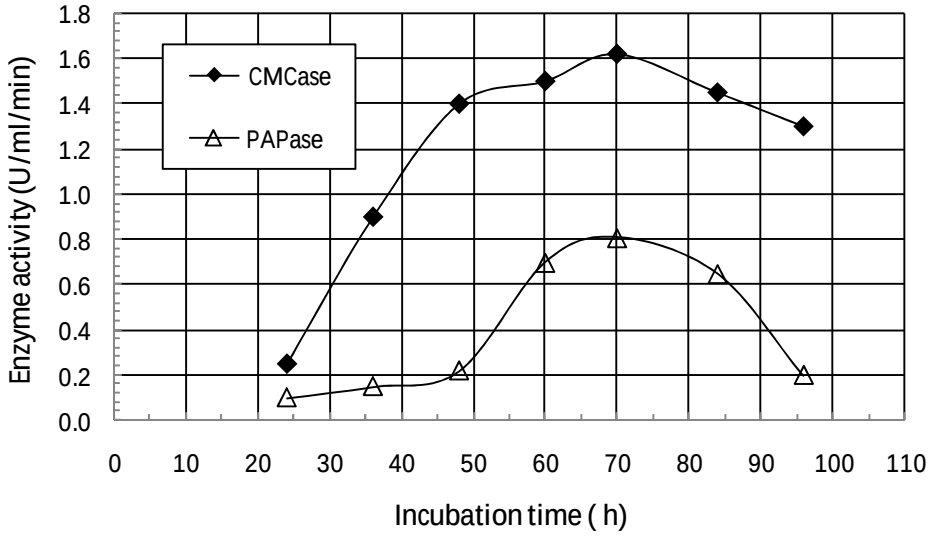
CM-Cellulases activity (CMCase): CMCase activity was determined according to Wood and Bhat (1988), one milliliter of appropriately diluted enzyme was incubated with 1.0 ml of 0.05 M citrate buffer (pH 5.0) and 1.0 ml of 1.0 % carboxymethyl cellulose for 30 min at50°C followed by determination of reducing sugar.

Papyrus-cellulases activity (PAPase): A 50 mg papyrus powder was suspended in a mixture containing 1.0 ml of dilutedenzyme extract and 1.0 ml of 0.05 M Sodium citrate buffer (pH 4.8). This mixture wasincubated for 1 h at 50ºC followed by the estimation of reducing sugar (Mandels, 1982).

Results and Discussion

Effect of incubation period: Figure (1) showed the rate of production of cellulases by *Trichoderma viride* in shake flasks. The production of cellulases increased with the increase in incubation period and reached maximum (CMCase 1.62 U/ml/min, PAPase 0.81 U/ml/min) after 70 hrs of incubation. Further increase in the incubation period however, resulted in the gradual decrease in the production of cellulases. Therefore, incubation period of 70 hrs was found to be optimal for cellulases production by *Trichoderma viride*. The optimization of the time course is of prime importance for cellulasesbiosynthesis by fungi (kuhad and Singh, 1993). The decrease in the production of cellulases by *Trichoderma viride* after 70 hrs of incubation period might be due to the depletion of the nutrients and accumulation of other byproducts like proteases in the fermentation medium (Hawary and Mostafa, 2001). However, Mekala *et al*., (2008) got maximum cellulases yield (25.6 CMCase units per gram dry substrate) at an incubation period of 67 hrs by *Trichoderma ressei* RUT C30 using an inducer in the culture medium.

Effect of incubation temperature: The effect of incubation temperature (25-35ºC) on the cellulase biosynthesis by *Trichoderma viride* is shown in Figure (2). There was a gradual increase in the production of PAPase as the temperature was increased. But it showed maximum yield at 30ºC i.e., PAPase 0.92 U/ml/min. The production of CMCase was also found to be maximum at 30ºC. As the temperature was further increased, there was a gradual reduction in the enzyme production. This may be due to the fact that higher temperatu-



Figure(1): Effect of incubation period on cellulases biosynthesis by *Trichoderma viride* GCBT-11 (Incubation Temperature, 30°C; Initial pH = 5.5)

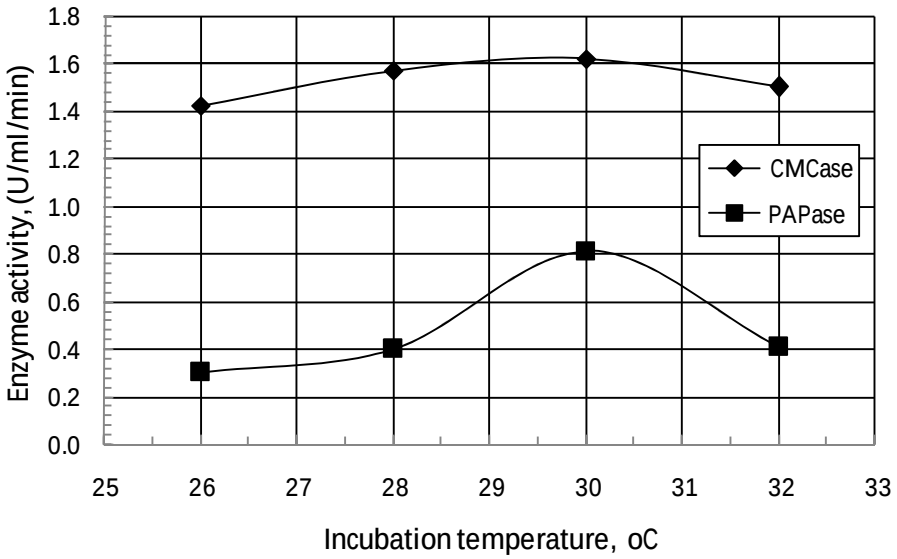


Figure (2): Effect of different incubation temperatures on cellulases production by *Trichoderma viride* GCBT-11 in shake flasks (Incubation period, 70 hrs; Initial pH, 5.5).

re denatures the enzymes. Mekala *et al*. (2008) showed that cellulases production was maximum in flasks incubated at 33^{o}C and decreased with high temperature. High temperature may also lead to inhibition of microbial growth.

Effect of initial pH: The effect of initial pH (4.5-6.5) of the culture medium on the biosynthesis of cellulases by *Trichoderma viride* GCBT 11 was studied (Figure 3). At the pH value of 4.5, there was very little production of enzyme (CMCase 0.2 U/ml/min and PAPase 0.5 U/ml/min), however, it started to increase as the initial pH of the growth medium was increased and reached maximum (CMCase 1.51 U/ml/min and PAPase 0.9U/ml/min) at pH 5.5. Further increase in pH resulted in a gradual reduction of cellulases biosynthesis by the organism. Hence, pH of (5.5) was optimized for the maximum cellulases biosynthesis by *Trichoderma viride.* Enzyme production is greatly influenced by initial pH of the culture medium. After pH value of (5.5), the production of cellulases decreased which might be due to the fact that cellulases are acidic proteins and are greatly affected by the neutral pH values (Juhasz *et al*., 2004 ; Chandra *et al*., 2009).

Effect of different nitrogen sources: The different inorganic nitrogen sources such as NH_4Cl, KNO_3, $(NH_4)_2SO_4$, (NH_4) CH_3COO, $NH_4H_2PO_4$, $NaNO_3$ and NH_4NO_3 were evaluated for the production of cellulases by *Trichoderma viride* GCBT 11 (Figure 4). The fermentation medium was supplemented with each of these nitrogen sources at a level of 1%. Among all the nitrogen sources tested, $(NH_4)_2SO_4$ gave the maximum production of cellulases (CMCase 1.62, PAPase 0.91 U/ml/min). Thus $(NH4)_2SO_4$ was selected as the best inorganic nitrogen source for the production of cellulases by *Trichoderma viride*. It was due to the fact that $(NH_4)_2SO_4$ provided both the ammonium as well as sulfate ions for the cell growth and enzyme production (Chen *et al*., 1998; Mekala *et al*., 2008). Mangat and Mandhar (1998) showed that nitrogen sources greatly influence cellulases biosynthesis hence they should be used with proper concentration. High concentration of nitrogen sources may lead to verification (the medium appears to be yellow and glassy) that is usually unstable for the micro-organisms.

Volume of culture medium: Figure (5) showed the effect of different volumes (10-70ml) of fermentation medium contained in 250 ml Erlenmeyer flasks on the production of cellulases by *Trichoderma viride* GCBT 11. The production of cellulases increased with the increase in the volume and was found to be maximum (CMCase 1.6 U/ml/min, PAPase 0.95 U/ml/min) with 25 ml of medium. Further increase in the volume, however, resulted in decreased amount of cellulases yield by *Trichoderma viride*. Thus 25 ml of fermentation medium was selected for the production of cellulases by *Trichoderma viride* GCBT 11. Substrate quantity is very important in the growth of microbial culture because cells show proper growth in the proper volume of substrate (Haq *et al*., 2003). Low volume of diluent may result into restricted utilization of the fermentation medium by *Trichoderma viride* GCBT 11, hence less production of cellulases. Increase in volume decreased air supply to the cells germination and growth of the micro-organism in the medium, resulting into anaerobic conditions in the fermentation flasks. Yang *et al*. (2004) showed that anaerobic conditions may lead to less growth as well as repressed biosynthesis of celluloses.

Effect of inoculum size: Figure (6) showed the effect of inoculum sizes (2.0-8.0%) on cellulases biosynthesis by Trichoderma viride GCBT-11 in shake flasks. The production of enzyme was minimum at 2% inoculum and reached maximum (CMCase 1.6 and PAPase 0.81 U/ml/min) with 4% inoculum containing 2.1 × 10^7cells/ml. Further, increase in inoculum size resulted in the gradual decrease in production of cellulases by *Trichoderma viride* GCBT-11. At low inoculum size i.e., 2%, conidial cells were not enough to utilize the fermentation medium in a better way hence, resulted in less growth and cellulases biosynthesis. On the other hand, at high concentration of conidial cells, anaerobic condition of fermentation medium, due to the tremendous growth of microorganism may lead to nutritional imbalance in medium, which resulted into gradual reduction of cellulases yield (Haq *et al*., 2003).

Conclusions

The optimal conditions for production cellulase by *Trichoderma viride* using Wheat straw and Papyrus straw as substrate by solid fermentation

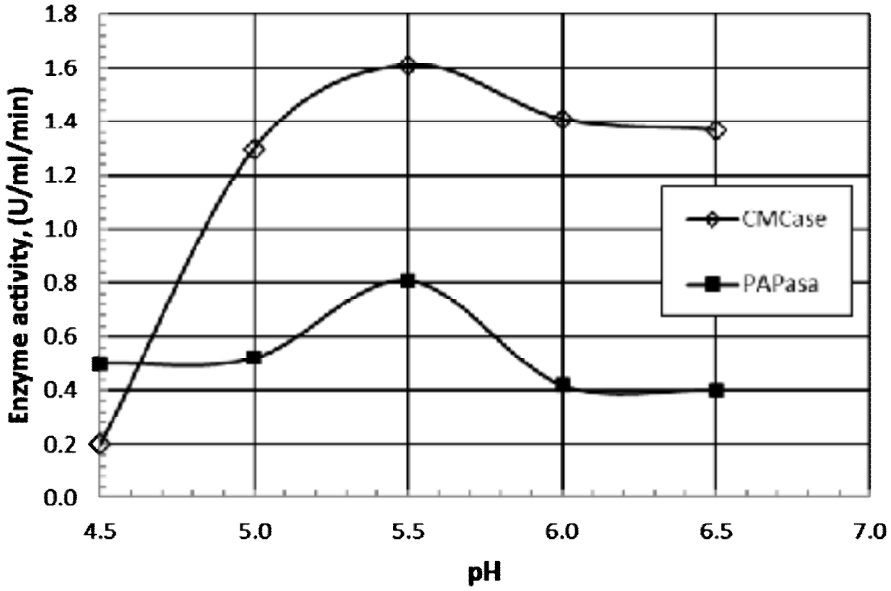


Figure (3): Effect of different initial pH on cellulases biosynthesis by *Trichoderma viride* GCBT-11 in shake flasks (Incubation period, 70 h; Incubation Temperature, 30°C).

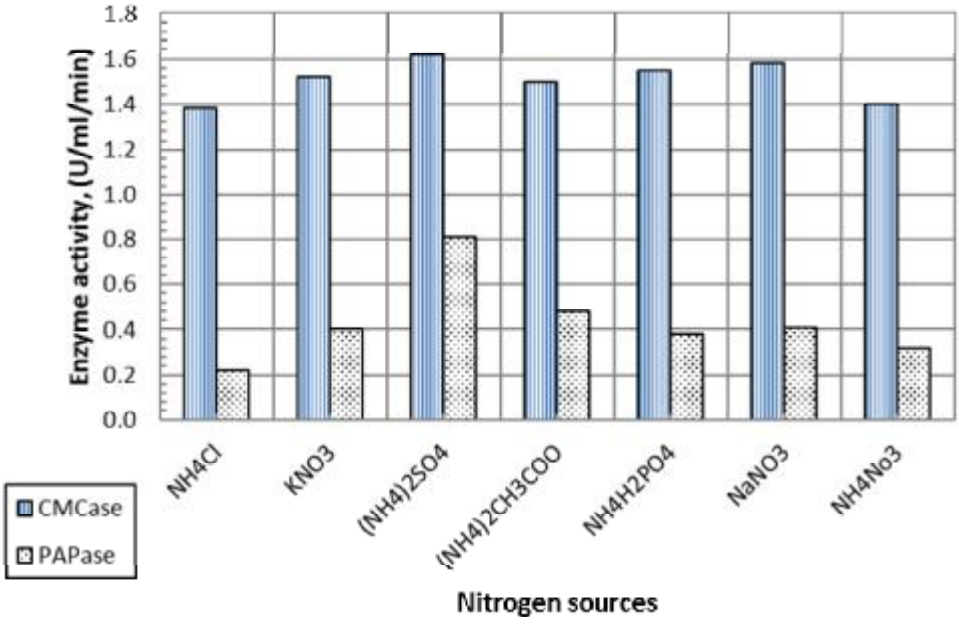


Figure (4): Effect of different inorganic nitrogen sources on the cellulases biosynthesis by *Trichoderma viride* GCBT-11 in shake flasks (Incubation period, 70 hrs; Incubation Temperature, 30°C; Initial pH, 5.5)

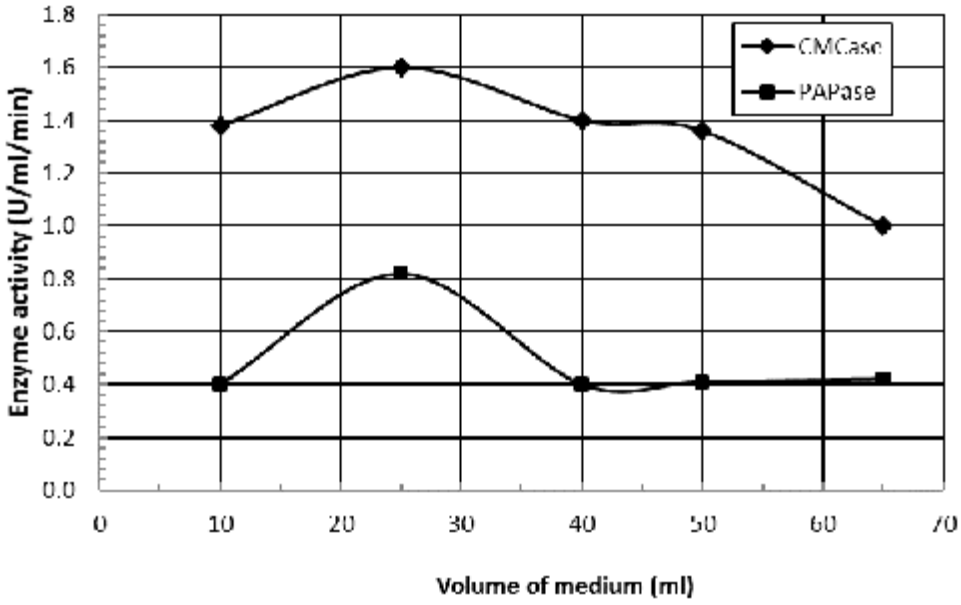


Figure (5): Effect of different volumes of medium on cellulases biosynthesis by *Trichoderma viride*GCBT-11 in shake flasks.(Incubation period, 70 hrs; Incubation Temperature, 30ºC; Initial pH, 5.5).

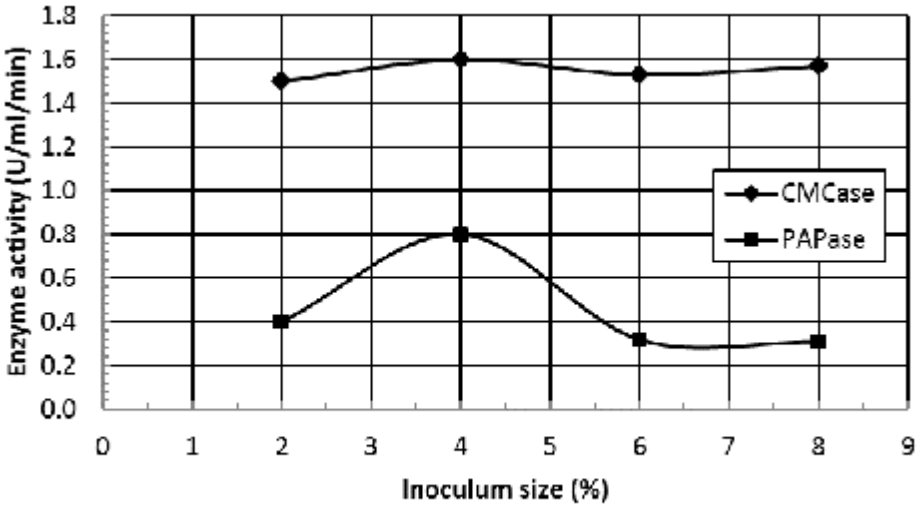


Figure (6): Effect of inoculum size on cellulases production by *Trichoderma viride* GCBT-11 in shake flasks (Incubation period, 70 hrs; Incubation Temperature, 30ºC; Initial pH, 5.5, volume of medium, 25ml)

were: incubation period of 70 hrs, incubation temperature of 30 ^{o}C, pH =5.5, $(NH_4)_2SO_4$ as nitrogen source with 25 ml of medium volume and 4% inoculum containing 2.1x10^{7} cells/ml.

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