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OPTIMIZATION OF PROCESS PARAMETERS FOR THE BIOSYNTHESIS OF CELLULASES BY *TRICHODERMA VIRIDE*

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Abstract

Present studies describe the optimization of process parameters for the production of cellulases by *Trichoderma viride* using submerged fermentation technique. The fermentation experiments were carried out in shake flasks using pretreated bagasse. Maximum production of cellulases (CMCase 1.57 U/ml/min, FPase 0.921 U/ml/min) was observed after a fermentation period of 72 hrs 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 (CMCase 1.66 U/ml/min and FPase 0.932 U/ml/min) by *T. viride*. 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% conidial inoculum.

Introduction

Cellulose, a polymer of β-D-glucopyranose with 1,4 β-glycosidic bonds, is the most abundant amongst all the naturally occurring organic compounds (Emert *et el*., 1974). 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 synergistically and cooperatively to convert native crystalline cellulose to oligosaccharides and glucose (Garcia-Kirchner, 2002). Endo β-glucanase (1,4 β-D-glucanhydrolase or CMCase) attacks randomly on internal glycosidic bonds of cellulose chain resulting in a rapid scission to yield oligosaccharides and glucose (Wood 1985). Exo β-glucanase (1,4 β-D-glucanocellobiohydrolyase or cellobiohydrolase) hydrolyzes highly crystalline cellulose attaching on newly generated ends (Hoshino 1997). The enzyme β-glucosidase hydrolyzes the aryl- and alkyl-glucoside as well as cellobiose and cello-dextrin to glucose (Kubicek, 1994).

The production and applications of cellulases have central importance in bioprocess industries because it causes 30% more hydrolysis than acids (Headon & Wash, 1994). Mode of action of cellulases in animal feed, fruit processing, textile wet processing, preparation of dehydrated vegetables, food products, essential oils, flavors, starch processing, botanical extracts, pulp and paper production, jams, juice, production of plant protoplast for genetic manipulation, wine production, pharmaceutical and biomass conversion have greatly increased the prospects of enzymatic hydrolysis over chemical processes (Latif *et al*., 1998).

Although cellulases are distributed throughout the biosphere, they are manifested in fungi, bacteria and a few actinomyctes (Kim & Wimpenny, 1981; Rojaka & Malik, 1997). These microorganisms produce cellulases to release sugar for cell growth and product formation under certain environmental conditions. More than 14,000 species of fungi have been found to be active in cellulose degradation (Esterbauer, 1991). The saprophytic filamentous fungi, especially *Trichoderma* spp., have been extensively studied due to their strong cellulolytic activity against crystalline celluloses which results into saccharification (Deschamps *et al*., 1985). Among them *Trichoderma viride*, *T. harzianum*, *T. reesei* and *T. konigii* have been studied (Saddler, 1982; Deschamps, 1985; Macris, 1985; Hawary *et al*., 2001). However, *Trichoderma viride* have proven to be an efficient candidate for the biodegradation of pretreated bagasse (Hawary *et al*., 2001).

During fermentation, cultural parameters and nutritional requirements such as carbon and nitrogen sources, ionic concentration, pH, temperature, cultivation time, aeration, water content of the substrate and inoculum size have fundamental role in the growth of microorganisms and subsequent product formation (Mangat & Mandahr, 1998; Mekala *et al*., 2008). Cellulolytic cultures have been investigated with the use of carbon sources such as avicel and crystalline cellulose. Cheaper carbon and nitrogen sources are desired in order to reduce the production costs (Ryu & Mandels, 1980; Mandels 1982; Nochure 1993; Saez *et al*., 2002, Ulger & Saglam, 2001; 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 bagasse in submerged fermentation.

Materials and Methods

Microorganism: *Trichoderma viride* GCBT-11 was obtained from the available stock culture of Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan. The strain was transferred to fresh PDA slants (3.9%, w/v) and was allowed to grow in an incubator at 30ºC for 48 hrs. After sufficient growth and sporulation, the slants were placed in cool lab at 40ºC for storage.

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 with the help of Haemocytometer.

Fermentation technique: Twenty five milliliters of the fermentation medium consisting of (%, w/v); $(NH_4)_2SO_4$, 0.14: KH_2PO_4, 0.20; Urea, 0.03; $MgSO_4.7H_20$, 0.03; $ZnSO_4.7H_2O$, 0.00014; $FeSO_4.7H_2O$, 0.0005; $MnSO_4$ 0.00016; $CoCl_2$,0.0002 ; $CaCl_2$, 0.0002; Tween-80, 2.0 ml; Polypeptone, 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 lb/inch^2 for 15 min. The flasks were inoculated with 1 ml of this inoculum containing 1.2×10^6 ml^{-1} of spores after cooling at room temperature and incubated at 30ºC at 200 rpm in an orbital shaker incubator. After 72 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 FPase using carboxymethyl cellulose and filter paper 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 after Wood & 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 at 50ºC followed by determination of reducing sugar.

Filter paper-cellulases activity (FPase): A 50 mg rolled strip of Whattman filter paper No. 1, (measuring 1x6 cm) was suspended in a mixture containing 1.0 ml of diluted enzyme extract and 1.0 ml of 0.05 M Sodium citrate buffer (pH 4.8). This mixture was incubated for 1 hrs at 50ºC followed by the estimation of reducing sugar (Mandels & Sternberg, 1976).

Results and Discussion

Effect of incubation period: Fig. 1 shows 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 1.57 U/ml/min, FPase 0.921 U/ml/min) after 72 hrs of incubation. Further increase in the incubation period however, resulted in the gradual decrease in the production of cellulases. Therefore, incubation period of 72 hrs was found to be optimal for cellulases production by *Trichoderma viride*.

The optimization of the time course is of prime importance for cellulases biosynthesis by fungi (Khud & Sing, 1993). The decrease in the production of cellulases by *Trichoderma viride* after 72 hrs of incubation period might be due to the depletion of the nutrients and accumulation of other byproducts like proteases in the fermentation medium (Hsieh *et al*., 2000). However, Mekala *et al*., (2008) got maximum cellulases yield (25.6 FPase units per gram dry substrate) at an incubation period of 67 hrs by *Trichoderma ressei* RUT C30 using an inducer in the culture medium. Their results showed that crude inducer was found to be very effective in inducing cellulases and reducing the incubation period.

Effect of incubation temperature: The effect of incubation temperature (26-32ºC) on the cellulase biosynthesis by *Trichoderma viride* is shown in Fig. 2. There was a gradual increase in the production of FPase as the temperature was increased. But it showed maximum yield at 30ºC i.e., FPase 0.932 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 temperature denatures the enzymes. Mekala *et al*., (2008) showed that cellulases production was maximum in flasks incubated at 33C 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 (Fig. 3). At the pH value of 4.5, there was very little production of enzyme (CMCase 0.2 U/ml/min and FPase 0.5 U/ml/min), however, it started to increase as the initial pH of the growth medium was increased and reached maximum (CMCase 1.660 U/ml/min and FPase 0.932 U/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*.

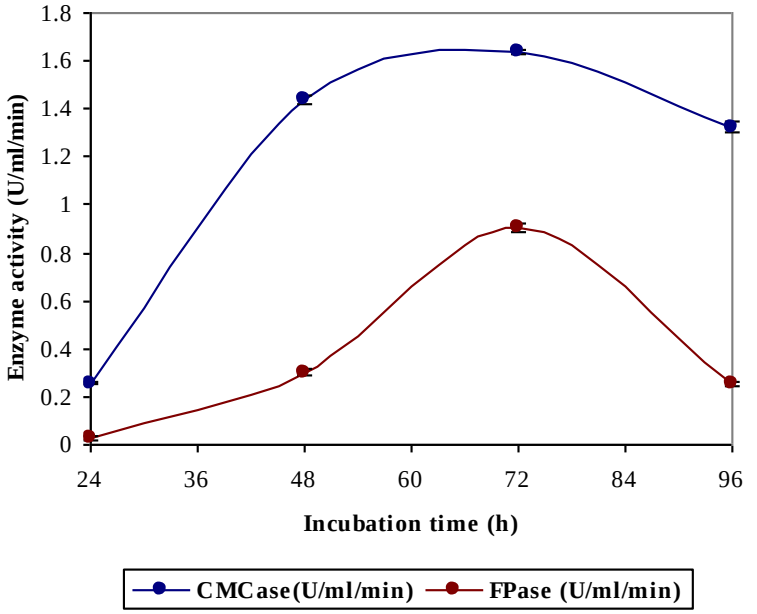

Fig. 1. Effect of incubation period on cellulases biosynthesis by *Trichoderma viride* GCBT-11
Incubation Temperature, 30°C; Initial pH, 5.5
Y error bars indicate the standard deviation among the three parallel replicates.

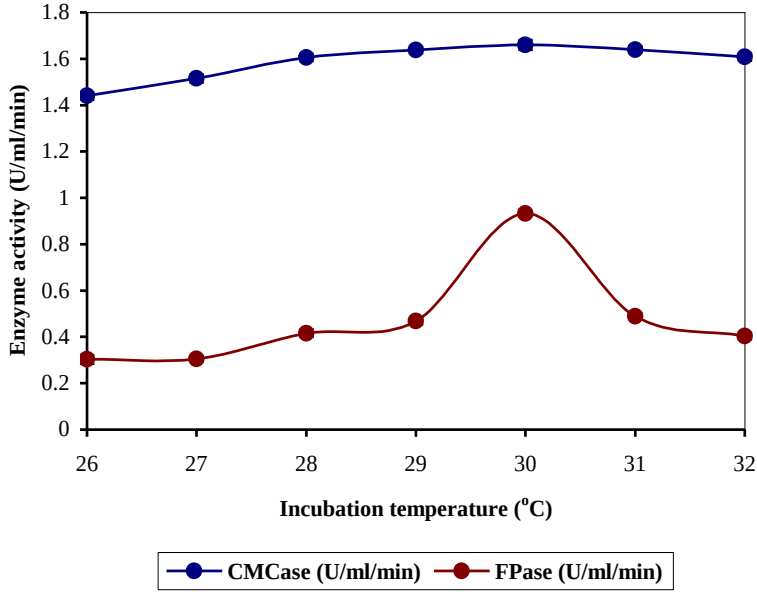

Fig. 2. Effect of different incubation temperature on cellulases production by *Trichoderma viride* GCBT-11 in shake flasks
Incubation period, 72 h; Initial pH, 5.5
Y error bars indicate the standard error of means among the three parallel replicates.

Enzyme production is greatly influenced by initial pH of the culture medium. Fungal metabolism progressed from imminent to actual exhaustation of carbon source (indicated by increase in pH). This may lead to the situation in which at least part of the biomass started to sporulate, after which a return to the productive phase no longer occurred. 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 (Fig. 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 1.68, FPase 0.926 U/ml/min). Thus $(NH_4)_2SO_4$ 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 conidial cell growth and enzyme production (Chen *et al*., 1998; Mekala *et al*., 2008). Mangat & Mandahr (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 vitrification (the medium appears to be yellow and glassy) that is usually unstable for the micro-organisms.

Volume of culture medium: Fig. 5 shows the effect of different volumes (12.5-65ml) 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 1.650 U/ml/min, FPase 0.920 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 ministering is very important in the growth of microbial culture because conidial cells show proper growth in the proper volume of substrate (Reese *et al*., 1969; Haq *et al*., 1993). 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 conidial cells germination and growth of the micro-organism in the medium, resulting into anaerobic conditions in the fermentation flasks. Durrant (1996) showed that anaerobic conditions may lead to less growth as well as repressed biosynthesis of celluloses.

Effect of inoculum size: Fig. 6 shows 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 1.640 and FPase 0.928 U/ml/min) with 4% inoculum containing 2.1×10^7 conidia/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*., 1991).

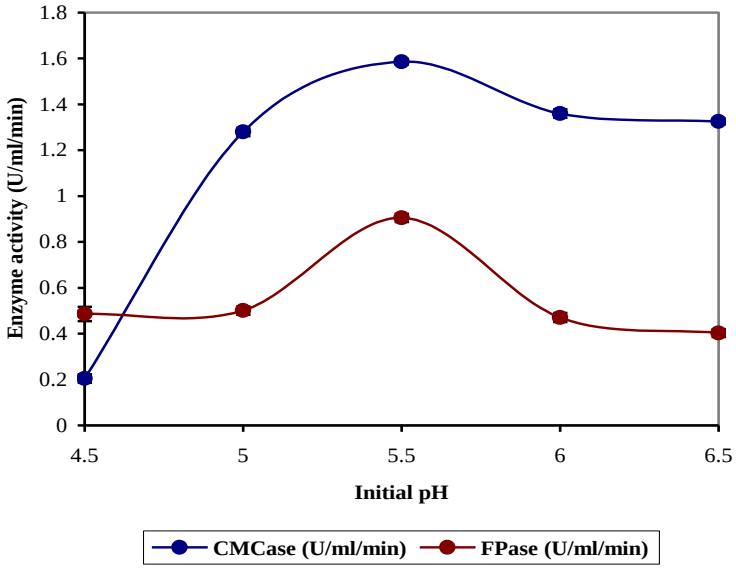

Fig. 3. Effect of different initial pH on cellulases biosynthesis by *Trichoderma viride* GCBT-11 in shake flasks.
Incubation period, 72 h; Incubation Temperature, 30ºC
Y error bars indicate the standard error of means among the three parallel replicates

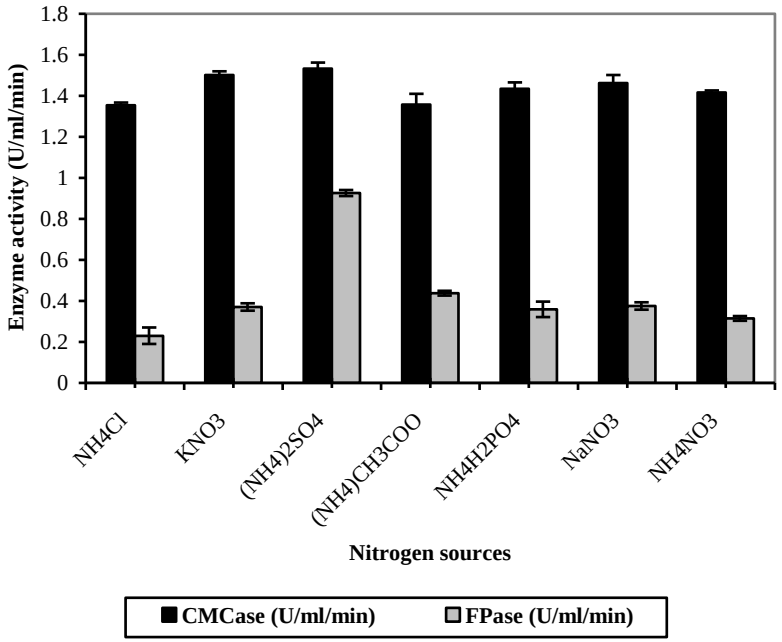

Fig. 4. Effect of different inorganic nitrogen sources on the cellulases biosynthesis by *Trichoderma viride* GCBT-11 in shake flasks.
Incubation period, 72 h; Incubation Temperature, 30ºC; Initial pH, 5.5
Y error bars indicate the standard error of means among the three parallel replicates.

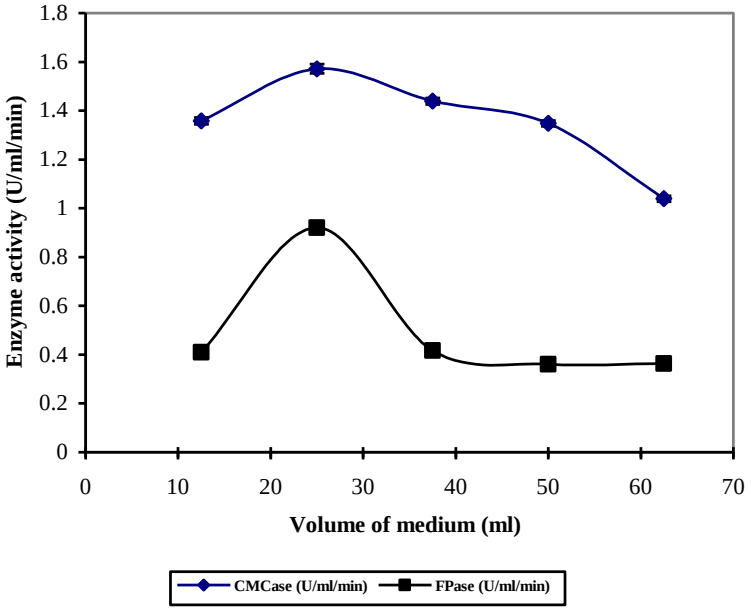

Fig. 5. Effect of different volumes of medium on cellulases biosynthesis by *Trichoderma viride* GCBT-11 in shake flasks.
Incubation period, 72 h; Incubation Temperature, 30ºC; Initial pH, 5.5
Y error bars indicate the standard error of means among the three parallel replicates.

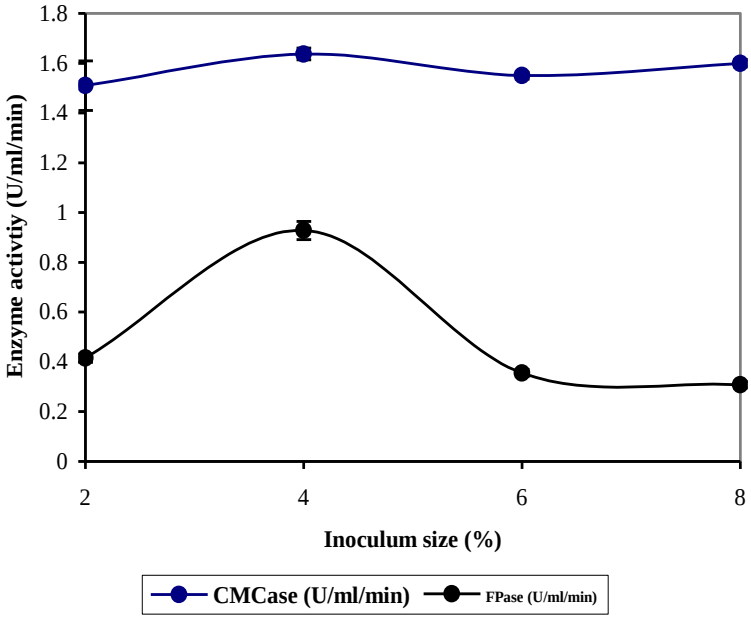

Fig. 6. Effect of inoculum size on cellulases production by *Trichoderma viride* GCBT-11 in shake flasks.
Incubation period, 72 h; Incubation Temperature, 30ºC; Initial pH, 5.5, volume of medium, 25ml
Y error bars indicate the standard error of means among the three parallel replicates.

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