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# Solid-State Fermentation of Cellulases by Locally Isolated *Trichoderma harzianum* for the Exploitation of Agricultural Byproducts

Ikram-ul-Haq, Kiran Shahzadi, Uzma Hameed, Muhammad Mohsin Javed and M.A. Qadeer
Institute of Industrial Biotechnology, Government College University Lahore, Pakistan

**Abstract:** The present study deals with isolation, screening and optimization of cultural conditions for biosynthesis of cellulases by *Trichoderma harzianum* using agricultural byproduct. Twenty different strains of *Trichoderma harzianum* were isolated from different soil samples by serial dilution method. Of all the strains tested, KM07 gave maximum production of cellulases by solid-state fermentation. Different agricultural byproducts such as wheat bran, wheat straw, rice bran, rice husk and soybean meal were tested for the production of cellulases and wheat bran was found to be the best substrate. The production of enzyme was significantly improved as the wheat bran was moistened with mineral salts CMC solution (ratio 1:1). The cultural conditions such as temperature 28°C and pH 6.5 were also optimized. The production of cellulases was maximum 72 h after the inoculation.

**Key words:** Cellulases, *Trichoderma*, solid fermentation

## INTRODUCTION

Pakistan being an agricultural country has a lot of recyclable residues, obtained from wheat, rice, sugarcane and cotton. These crops yield wheat straw, wheat bran, rice husk, rice bran and sugarcane bagasse as by-products and are abundantly available for utilization as substrates for the production of bio-chemicals such as cellulases. Utilization of these environment friendly biocatalysts in animal feed, textile wet processing, pulp and paper production, production of plant protoplast for genetic manipulation, wine production, pharmaceutical and biomass conversion has greatly increased the prospects of enzymatic hydrolysis (Boisset *et al*., 2001; Miettinen-Oinonen and Suominen, 2002).

Fungi can be cultivated in a relatively short time by establishing the methods of fermentation to produce a regular supply of the desired enzyme (Thiry and Cingolani, 2003). More than 14,000 species of fungi have been found to be active in degrading cellulose. The developments of microbial strains, media composition and process control all contribute in the accumulation of high level of extracellular cellulases (Szengyel *et al*., 2000). Solid-state fermentation (SSF) involves the growth of microorganism on moist substrate. It offers some advantages over liquid fermentation, as there is higher productivity, reduced energy requirements, lower capital investment, low waste water out put and low downstream processing cost (Kumaran *et al*., 1997).

The present study is concerned with exploitation of agricultural by-products for production of industrially important enzyme cellulases by the SSF using locally isolated *Trichoderma harzianum.*

## MATERIALS AND METHODS

**Organism:** *Trichoderma harzianum* strains were isolated from soil samples, collected from different areas of District Lahore by serial dilution method (Clark *et al*., 1958). Modified mineral-salt-cellulose-agar medium containing (g $L^{-1}$, w/v): $(NH_4)_2SO_4$, 1.4; $KH_2PO_4$, 2.0; Urea, 0.3; $MgSO_4.7H_2O$, 0.3; $ZnSO_4.7H_2O$, 0.0014; $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.0016; $COCl_2$, 0.002; $CaCl_2$, 0.002; Tween 80, 2.0 mL; Poly-peptone, 1.0; Cellulose powder, 10.00; Agar, 20.00 (pH 5.6) was used as isolation medium (Ikram-ul-Haq *et al*., 2005). The young colonies of *Trichoderma harzianum* were identified after Gilman (1957) and maintained on potato dextrose agar slants.

**Fermentation:** Diluents: Mineral salt solution containing (g $L^{-1}$, w/v): $NaNO_3$, 3.0; $KH_2PO_4$, 1.0; Tween 80, 1.0 mL; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$ $7H_2O$, 0.01

**Inoculum:** The conidia inoculum from 5 days old slant culture was prepared by adding 10 mL of sterilized 0.005% Monoxal O.T (Diactyl ester of sodium sulpho succinic acid). Inoculum size was measured with Haemacytometer, according to Sharma (1989).

**Corresponding Author:** Uzma Hameed, Institute of Industrial Biotechnology, Government College University Lahore, Pakistan

**Batch culture:** Ten gram of wheat bran was transferred to the individual 250 mL conical flasks and moistened by adding 10 mL of diluents. The flasks were cotton plugged, sterilized in an autoclave and cooled at room temperature. The flasks were then inoculated with 1.0 mL of conidial suspension and incubated at 30±1°C for 72 h. The flasks were shaken twice a day. All the experiments were run parallel in triplicate.

**Enzyme harvestation:** The enzyme was extracted by adding 100 mL of 0.05 M citrate buffer (pH 4.8) to the fermented substrate in each flask. The flasks were rotated on a rotary shaker at 200 rpm for 1 h at 40±1°C. The fermented broth was filtered by using filter paper (Whattman No. 1). Filtrate was analyzed for cellulolytic activity.

**Bioassays:** Different enzyme assays for cellulases were performed using various substrates such as carboxymethyl cellulose (CMC) and Filter Paper (FP). The released reducing sugar (glucose) was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity in each case was defined as the amount of enzyme required to liberate one μmol of reducing sugars per minute. The results of the analysis were expressed as units per gram of initial dry substrate.

**CM-cellulase activity (CMC-ase):** The CMC-ase activity was determined by the method of Wood and Bhat (1988).

**Filter paper-cellulase activity (FP-ase):** FP-ase activity was determined according to Mandels and Sternberg, (1976).

**Statistical analysis:** Treatment effects were compared by the method of Snedecor and Cochran (1980) using a computer software CoStat 3.03 CoHort Software, Berkeley, CA 94701. Significance has been presented as Duncan's multiple range tests in the form of probability ($p<0.05$) values.

## RESULTS AND DISCUSSION

Agricultural byproducts rich in cellulosic biomass can be exploited as cheap raw material for the production of industrially important enzymes and chemicals (Bigelow and Wyman, 2004). In present investigation twenty different strains of *Trichoderma harzianum* were isolated from different soils samples of Lahore. Isolate, *T. harzianum* KM07 gave maximum production (Table 1), therefore, was selected for the further studies to investigate the exploitation of different agricultural byproducts by SSF for cellulases. Different agricultural byproducts such as wheat bran, wheat straw, rice bran, rice straw and soybean meal were tested for the production of enzymes (Fig. 1). Of all the substrates tested, wheat bran was found to be the best substrate for the production of cellulases. The other substrates gave comparatively less production of cellulases. It might be due to the fact that wheat bran contains adequate amount of nutrients like proteins 1.32%, carbohydrates 69.0%, fats 1.9%, fiber 2.6%, ash 1.8%, Ca 0.05%, Mg 0.17%, P 0.35%, K 0.45%, S 0.12%, various amino acids and porosity for oxygen supply. All these nutrients are necessary for the production of enzymes as well as cell mass formation (Bakri *et al*., 2003). Roussos *et al.* (1991) also reported the wheat bran as best source of carbon and nitrogen for cellulases biosynthesis. Cellulases production in fermentation medium was found to be maximal when 10 g of wheat bran was used (Fig. 2). Further increase in amount of wheat bran resulted decrease in the production of enzyme. It might be due to the fact that increase in the level of substrates decreases the aeration and porosity of the medium, which were very essential for the proper growth of the organism. As the growth of organism was not proper therefore the production of cellulases was significantly decreased (Bigelow and Wyman, 2004).

Time course of enzyme production plays a very critical role in enzyme synthesis. The *Trichoderma harzianum* KM07 was incubated for 0-96 h (Fig. 3). The production of cellulases was increased with increase in the incubation period and found maximum at 72 h after inoculation. It might be due to the fact, that organism entered in the stationary phase of growth. Further increase in the incubation period led to a decrease in the production of cellulases by *Trichoderma harzianum*

Table 1: Screening of *Trichoderma harzianum* isolates for the production of cellulases

| | Enzyme activity (U $g^{-1}$ $min^{-1}$) | |
|---|---|---|
| No. of strains | CMCase | Fpase |
| KM01 | 1.3±0.208$^{k}$ | 0.9±0.152$^{ij}$ |
| KM02 | 5.9±0.808$^{f}$ | 3.3±0.700$^{e}$ |
| KM03 | 7.7±0.404$^{cd}$ | 3.3±0.470$^{e}$ |
| KM04 | 3.9±0.251$^{hi}$ | 1.8±0.300$^{h}$ |
| KM05 | 4.8±0.350$^{g}$ | 2.5±0.250$^{fg}$ |
| KM06 | 6.6±0.200$^{ef}$ | 3.7±0.250$^{c-e}$ |
| KM07 | 10.4±0.320$^{a}$ | 5.7±0.550$^{a}$ |
| KM08 | 9.1±0.521$^{b}$ | 5.4±0.470$^{ab}$ |
| KM09 | 8.4±0.560$^{bc}$ | 4.6±0.500$^{b}$ |
| KM10 | 7.9±0.680$^{cd}$ | 4.7±0.400$^{b}$ |
| KM11 | 7.8±0.380$^{cd}$ | 4.4±0.450$^{bc}$ |
| KM12 | 6.9±0.50$^{e}$ | 4.1±0.660$^{b-d}$ |
| KM13 | 7.1±0.260$^{de}$ | 3.5±0.260$^{de}$ |
| KM14 | 5.9±0.200$^{f}$ | 3.1±0.570$^{ef}$ |
| KM15 | 4.4±1.150$^{gh}$ | 3.0±0.200$^{ef}$ |
| KM16 | 3.1±0.330$^{ij}$ | 1.8±0.300$^{h}$ |
| KM17 | 2.9±0.180$^{ij}$ | 1.4±0.200$^{hi}$ |
| KM18 | 2.8±0.300$^{ij}$ | 1.1±0.300$^{h-j}$ |
| KM19 | 1.2±0.410$^{jk}$ | 0.56±0.300$^{ij}$ |
| KM20 | 1.16±0.150$^{k}$ | 0.83±0.200$^{ij}$ |
| LSD | 0.790 | 0.691 |

Each value is an average of three parallel replicates±denotes standard deviation among replicates. Number(s) followed by different letter(s) differ significantly at $p \leq 0.05$

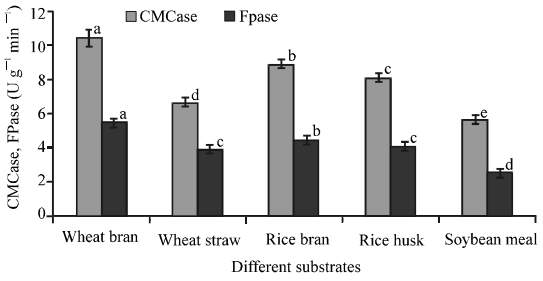


Fig. 1: Effect of different substrates on the production of enzyme cellulases by *Trichoderma harzianum* KM07. Each value is an average of three parallel replicates. Y error bars indicate standard deviations among replicates. Bars followed by different letters differ significantly at $p \leq 0.05$. Incubation time 72 h, Temperature 30°C

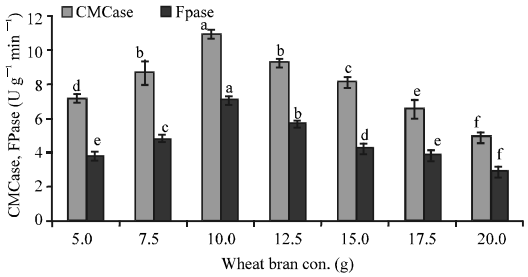


Fig. 2: Effect of different concentrations of wheat bran on the production of cellulases by *Trichoderma harzianum* KM07. Each value is an average of three parallel replicates. Y error bars indicate standard deviations among replicates. Bars followed by different letters differ significantly at $p \leq 0.05$. Incubation time 72 h, Temperature 30°C

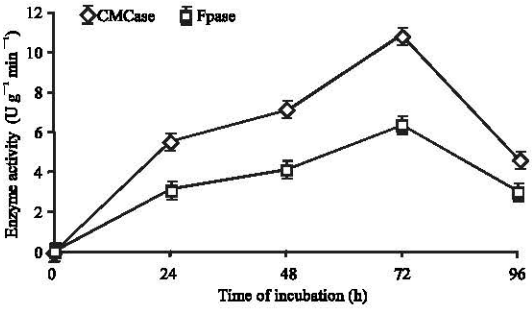


Fig. 3: Rate of enzyme cellulase biosynthesis by *Trichoderma harzianum* KM07 Y error bars indicate the standard deviation among the three parallel replicates. The value differs significantly at $p \leq 0.05$

KM07. It might be due to the depletion of the nutrients and production of other by products in the

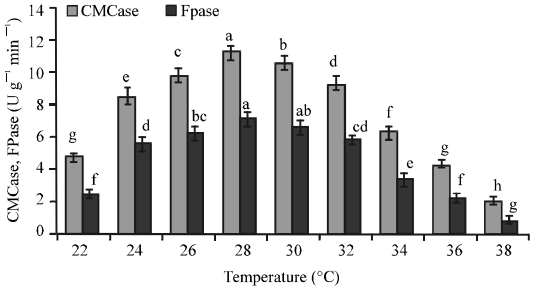


Fig. 4: Effect of incubation temperature on the production of cellulases by *Trichoderma harzianum* KM07. Each value is an average of three parallel replicates. Y error bars indicate standard deviations among replicates. Bars followed by different letters differ significantly at $p \leq 0.05$. Incubation time 72 h

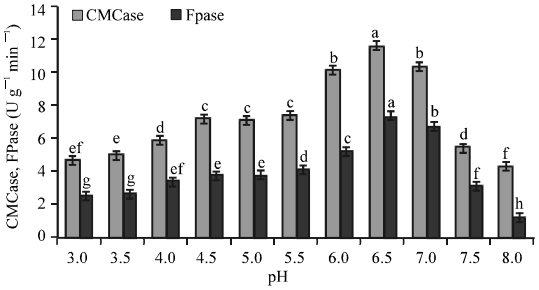


Fig. 5: Effect of different initial pH on the production of cellulases by *Trichoderma harzianum* KM07. Each value is an average of three parallel replicates. Y error bars indicate standard deviations among replicates. Numbers followed by different letters differ significantly at $p \leq 0.05$. Incubation time 72 h, Temperature 28°C

fermentation medium (Haq *et al*., 2006). The accumulation of these by-products resulted decrease in the production of cellulases. In contrast, Zhang *et al*. (1999) reported the highest cellulases production by *Trichoderma* in solid-state fermentation after 144 h fermentation.

The production of enzyme is very sensitive to the incubation temperature and pH (Smits *et al*., 2003). In the present study fermentation was carried out at different temperature (22-38°C). The production of enzyme was maximal in flasks incubated at 28°C (Fig. 4). As the temperature was increased, there was a gradual decrease in the enzyme production. It might be due fact that high temperature can change membrane composition and can cause the protein catabolism and inhibition of fungal growth. Similar kind of findings has also been reported by Haq *et al*. (2006). In contrast, Margaritis and Merchant (1986) reported 44-55°C as the optimal temperature for cellulase production. Therefore, our findings are more encouraging involving the less utilization of energy. The effect of pH (3.0-8.0) of moistening agent on

the production of cellulases by *Trichoderma harzianum* KM07 is depicted in Fig. 5. The production of cellulases was found to be optimal when the initial pH of the moistening agent was kept 6.5. Further, increase or decrease in pH of medium resulted decrease in the production of cellulases. However, Margaritis and Merchant (1986) reported the initial pH 4.5-5.0 for maximum volumetric productivity of cellulases. The optimal production at initial pH 6.5 in present investigation might be due to the organism require slightly acidic pH for the growth of organism and cellulases production as the productivity of enzyme by mould culture is very specific to pH (Puntambekar, 1995).

## CONCLUSIONS

Cellulosic biomass such as agricultural byproducts provides a low-cost feedstock for biological production of cellulases. In present study, a wild strain *T. harzianum* KM07 was isolated from soil. Wheat bran was selected as the best agricultural byproduct for the cellulases production at optimal temperature and pH 28°C and 6.5, respectively 72 h after the inoculation. However, further study is needed to optimize the nutritional requirements of this newly isolate strain of *T. harzianum* for the better exploitation of wheat bran for the biosythesis of cellulases.

## ACKNOWLEDGMENTS

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