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CELLULASE BIOSYNTHESIS BY SELECTED *TRICHODERMA* SPECIES

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Abstract

The enzyme cellulase, a multi enzyme complex made up of several proteins, catalyses the conversion of cellulose to glucose in an enzymatic hydrolysis. The indigenous fungi of Pakistan viz., *Trichoderma viride* Pers. ex Gray, *T. reesei* Rifai and *T. harzianum* Rifai were selected and analyzed on the basis of extent of hydrolyzing zones for the evaluation of their enzymatic activity in solid state fermentation on PDA and plate screening medium (PSM) at pH 4, 5 and 6. PSM at pH 4 depicted best results for all the strains tested. Strains FCBP-142 and FCBP-232 of *T. viride*, strains FCBP-271 and FCBP-364 of *T. reesei* and strains FCBP-210 and FCBP-325 of *T. harzianum* were considered best in their extent of hydrolyzing ability and were selected for evaluation of their cellulolytic activity through submerged fermentation using 3, 5-dinitrosalicylic acid (DNS) method. The maximum enzymatic activity was achieved after 72 hours of incubation at 30 ± 2^{o}C at initial pH 4.

Introduction

Cellulose is the most abundant and renewable biopolymer on earth. Cellulose has been used by man for centuries, however, its enormous potential as a renewable source of energy was recognized only after cellulose degrading enzymes or "cellulases" had been identified (Bhat & Bhat, 1997).

A cellulosic enzyme system consists 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) (Knowles *et al*., 1987). The mode of action of each of these is as follows:

1. Endo-β-glucanase, 1,4–β–D–glucan glucanohydrolase, CMCase, Cx: "random" scission of cellulose chains yielding glucose and cell-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.

These components act synergistically in the conversion of cellulose to glucose (Eveleigh, 1987).

Ali & Akhand (1992) worked on cellulase production by mesophilic *Trichoderma* isolate during growth on water hyacinth under optimized conditions. The cellulase complex of *Trichoderma reesei* has been most thoroughly studied. It is complete in that it can convert native cellulose as well as derived celluloses to glucose (King & Nessal 1969). Ahmad *et al*., 2003 worked on *Trichoderma harzianum* for cellulase enzyme production by using different carbon sources and reported that CMC is the best for substantial amount of enzyme production.

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Since growth of fungi as well as the enzyme production depends on the composition of the growth media, water activity (a_w), pH, temperature, light and the surrounding atmospheric gas mixture. The effect of environmental factors on the growth of fungi is generally less specific and restricted than the effect on secondary metabolite production. For example, the ranges of water activity, growth medium and pH within which formation of certain secondary metabolites occur, is narrower, than the range of conidial growth (Northolt & Bullerman, 1982).

The work on cellulase enzyme production by *Trichoderma* species has been conducted all over the world. But the physiological responses of same organism or species may vary with ecological variations. Therefore, the present research work was aimed to evaluate the cellulase production potential by native *Trichoderma* strains, in particular by strains of *Trichoderma viride, Trichoderma reesei* and *Trichoderma harzianum* that were available in First Fungal Culture Bank of Pakistan (FCBP). Thus, the novelty of this work is that the work is concerned with commercialization of mycoflora of FCBP to meet industrial sector demands.

Materials and Methods

Experimental design: For *Trichoderma reesei* and *Trichoderma viride* a 3×3×2 factorial and for *Trichoderma harzianum* 6×3×2 factorial with 3 and 6 fungal strains respectively, 3 pH levels viz. 4, 5 and 6, were assessed on two growth media viz., Potato Dextrose Agar medium (PDA) and Plate Screening Medium (PSM), with 6 replicates each in a completely randomized design.

Microorganism: The strains of *Trichoderma viride* (isolates FCBP-142, FCBP-167, FCBP-232), *T. reesei* (isolates FCBP-84, FCBP-271, FCBP-364) and *T. harzianum* (isolates FCBP-125, FCBP-139, FCBP-140, FCBP-193, FCBP-210, FCBP-325) were obtained from stock cultures of First Fungal Culture Bank of Pakistan, Institute of Mycology and Plant Pathology, University of the Punjab, Lahore. The cultures were maintained as direct stock cultures on Potato Dextrose Agar (PDA) plates at 30 ± 2°C and stored at 4°C with regular sub culturing.

Inoculum/spore suspension: The inoculum was prepared according to the method of Noomrio & Dahot, (1992). The stock suspension was serially diluted to prepare a conidial suspension of 5 x 10^5 conidia m L^{-1} with the help of haemacytometer (Neubauer Precidor HBG, Germany). This appropriate dilution was used as inoculum.

Plate screening assay: The experiment was carried in two sets. In one set, the plate screening medium (PSM) contained Mendel's mineral salt solution that is: Urea 0.3 g L^{-1}, $(NH_4)_2SO_4$ 1.4 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, $CaCl_2$ 0.3 g L^{-1}, $MgSO_4$ 0.3 g L^{-1}, yeast extract 0.25 g L^{-1} and proteose peptone 0.75 g L^{-1} with 10 g L^{-1} of cellulose and 17.5 g L^{-1} agar (Mandels, 1974) while the other set was composed of PDA. Three levels of pH: 4, 5 and 6 were adjusted for each set (pH was adjusted by either 0.5M NaOH or 0.5M HCl) before autoclaving. The suspensions were plated on both sets and were incubated at 30 ± 2°C for five to seven days. After 7 days, the colonies with clear zones of cellulose digestion were selected and top two strains among each species were further assayed for cellulase activity.

Fermentation conditions: Fermentation experiment was run according to Mendel's method (Mandels, 1975). The mass concentration of nutrients in Mendel's salt solution is: Urea 0.3 g L^{-1}, $(NH_4)_2SO_4$ 1.4 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, $CaCl_2$ 0.3 g L^{-1}, $MgSO_4$ 0.3 g L^{-1}, yeast extract 0.25 g L^{-1} and proteose peptone 0.75 g L^{-1} with 10 g L^{-1} of Carboxymethylcellulose (CMC) in 0.05M citrate buffer (pH 4). In 250 mL conical flasks, 50 mL of sterilized culture medium was taken and inoculated with 1.0 mL of conidial suspension (5 x 10^5 conidia mL^{-1}) of each fungal species in triplicates. The flasks were incubated in orbital shaker incubator (100rpm) at 30 ± 2^0C for 96 hours. After every 12 hours interval, the fermented broth was centrifuged at 3,000 rpm for 15 minutes and the supernatant (crude enzyme) was used for further analysis.

Enzyme estimation assays: The overall cellulolytic activity in broth culture was determined by 3-5, dinitrosalicylic acid (DNS) method of Miller (1959). The reaction mixture contained 0.5 mL of 1% CMC in 0.05 M citrate buffer (pH 4) and 0.5 mL of enzyme solution. It was incubated at 50°C for 15 min. Then 3 mL of DNS reagent was added and the mixture was boiled for 5 min. After cooling, the absorbance was measured at 600 nm using spectrophotometer. Sugar content was measured using a calibration curve of glucose. Enzymatic activity of cellulase complex was expressed as International Units/mL defined as the amount of enzyme which releases one micro mole of reducing sugar expressed as glucose per minute.

Statistical analysis: Data regarding the hydrolyzing zones formed by different strains of three test species of *Trichoderma* as affected by pH and growth media were statistically analyzed using three way analysis of variance (ANOVA) followed by LSD method to demarcate mean differences. Data regarding cellulase activity of the selected strains of *Trichoderma* spp., were subjected to one way ANOVA followed by Duncan's Multiple Range Test to separate the means (Steel & Torrie, 1980).

Results

The test strains of each *Trichoderma* species viz., *Trichoderma viride*, *T. reesei* and *T. harzianum* were analyzed on the basis of extent of hydrolyzing ability. Cellulose hydrolysis of all three strains of *T. viride* was immensely affected by varying pH and medium. The results indicated statistically significant interaction in all correlating factors of strain, growth medium and pH level in case of *T. viride* (Table 1). Cellulose digestion occurred in all three isolates between pH 4–6 but the maximum extension rate of approximately 53 ± 1 mm was exhibited at pH 4 on PSM. The rate of hydrolysis was reduced tremendously as pH was increased from 4–6 (Fig. 1A-C).

The three strains of *T. reesei* followed similar trends in response to changes in pH and medium (Fig. 2). Highest cellulose digestion, in terms of hydrolyzing zone, was observed at low pH (4) in PSM (Fig. 2A-C). There appeared reduction in hydrolyzing activity followed by further increase in pH. This effect was more pronounced and significant in strain FCBP-364 (Fig. 2C).

Analysis of variance for *Trichoderma harzianum* (Table 3) depicted that increasing pH and substrates variation had a highly significant influence ($p \leq 0.001$) on hydrolyzing capabilities of different strains. The interaction between strain x growth medium had significant effect ($p \leq 0.05$) whereas the interactive effect between strain x pH x growth medium was highly significant ($p \leq 0.001$). All test strains showed the best hydrolyzing zone formation potential at pH 4, PSM further improved their activity significantly ($p \leq 0.05$) (Fig. 3). Cellulose digestion was inhibited by further increase in pH level.

Table 1. ANOVA for hydrolyzing zones of different strains of *Trichoderma viride* under three pH levels and on two growth media.

Sources of variation	df	SS	MS	F-values
Treatments	17	2266	133	70***
Strains (S)	2	878	439	231***
pH	2	904	452	238***
Growth media (G)	1	334	334	176***
S × pH	4	65	16	8.5***
S × G	2	12	6	3.2*
pH × G	2	48	24	12.7***
S × pH × G	4	24	6	3.2*
Error	90	171	1.9	
Total	**107**	**2437**		

Note: numbers represent F-values *** = p≤ 0.001, * = p≤ 0.05.

Table 2. ANOVA for hydrolyzing zones of different strains of *Trichoderma reesei* under three pH levels and on two growth media.

Sources of variation	df	SS	MS	F-values
Treatments	17	825	49	78***
Strains (S)	2	171	86	138***
pH	2	520	260	418***
Growth media (G)	1	82	82	131***
S × pH	4	30	7.6	12.2***
S × G	2	8	4	6.5**
pH × G	2	6.3	3.2	5.1**
S × pH × G	4	6.4	1.6	2.5*
Error	90	56	0.62	
Total	**107**	**881**		

Note: Numbers represent F-values *** = p≤ 0.001, * = p≤ 0.05.

Table 3. ANOVA for hydrolyzing zones of different strains of *Trichoderma harzianum* under three pH levels and on two growth media.

Sources of variation	df	SS	MS	F-values
Treatments	35	6659	190	149***
Strains (S)	5	4693	939	733***
pH	2	1317	658	514***
Growth media (G)	1	81	81	63***
S × pH	10	452	45	35***
S × G	5	15	3	2.3*
pH × G	2	50	25	19***
S × pH × G	10	51	5.1	4***
Error	180	230	1.3	
Total	**215**	**6889**		

Note: Numbers represent F-values *** = p≤ 0.001, * = p≤ 0.05.

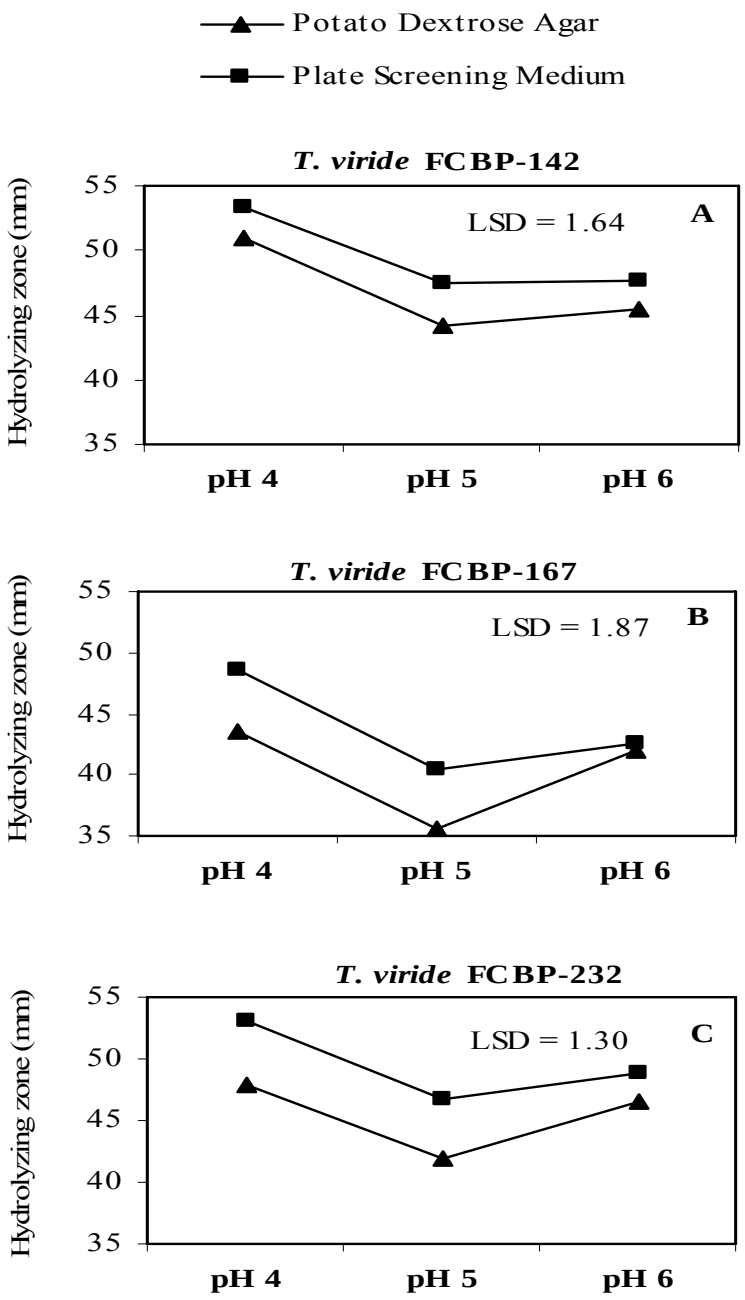

Fig. 1. Effect of pH and growth media on hydrolyzing zones of different strains of *Trichoderma viride*. LSD at $p \leq 0.05$

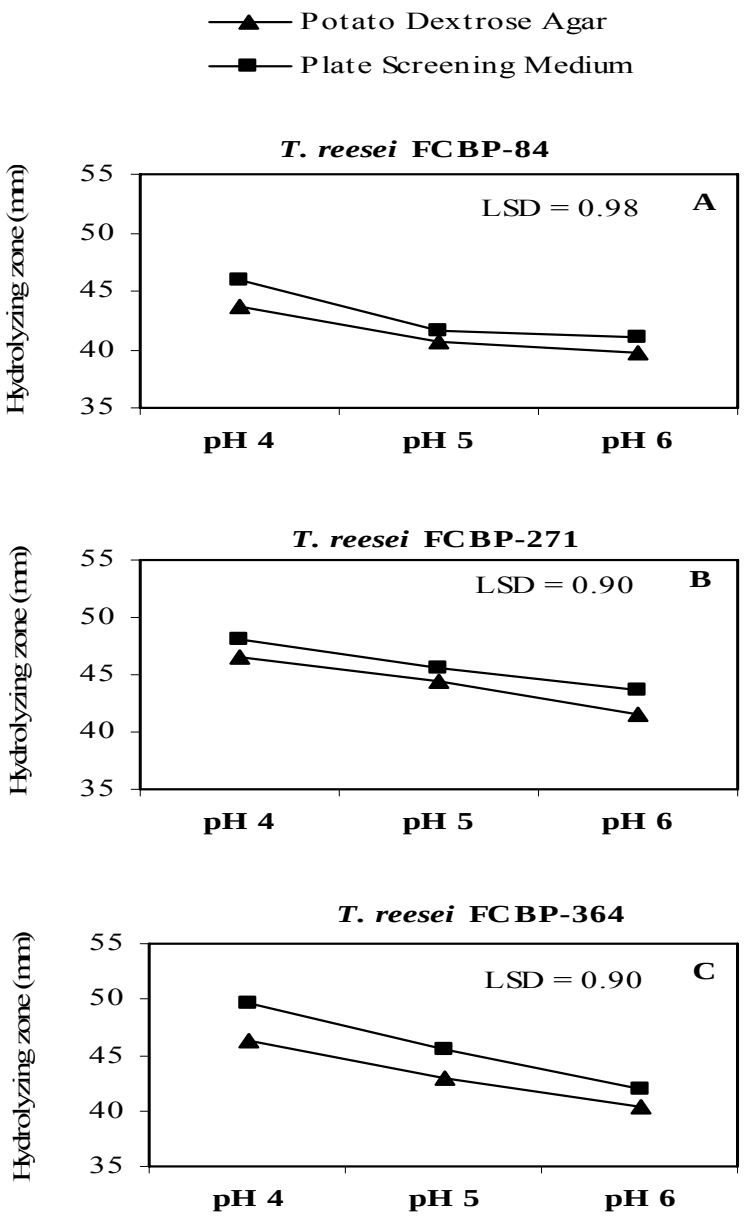

Fig. 2. Effect of pH and growth media on hydrolyzing zones of different strains of *Trichoderma reesei*. LSD at $p \leq 0.05$

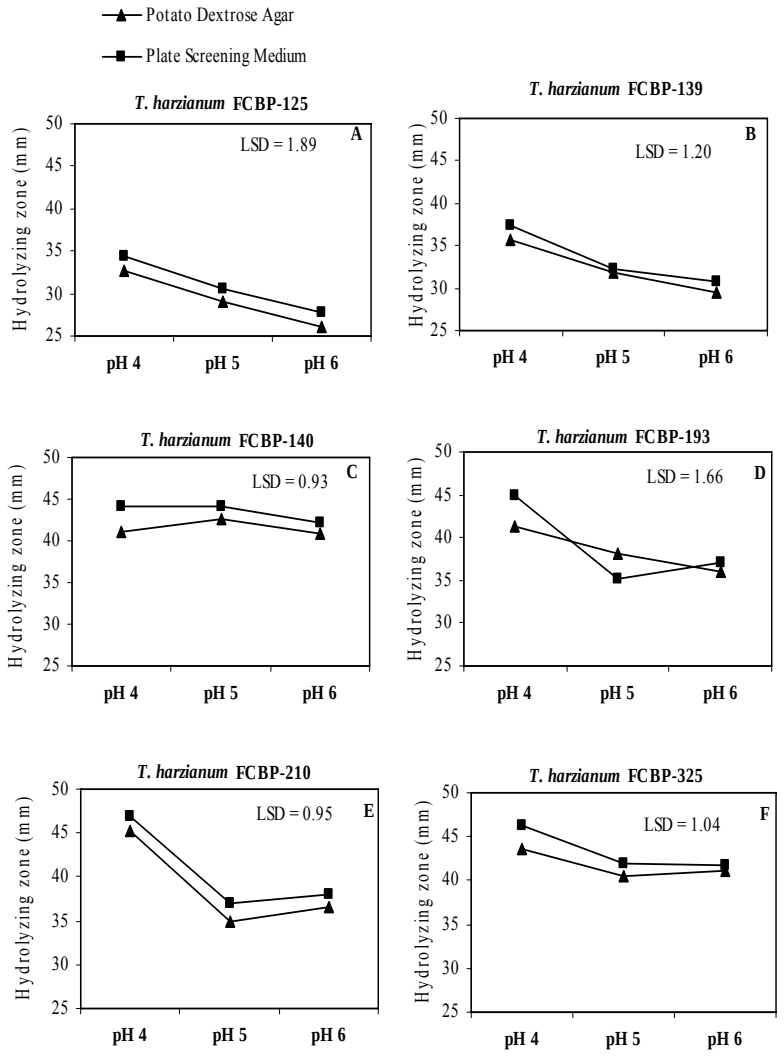

Fig. 3. Effect of pH and growth media on hydrolyzing zones of different strains of *Trichoderma harzianum*.
LSD at $p \leq 0.05$

The selected fungal isolates were tested and compared for their cellulase activity by submerged fermentation. According to results obtained, it became obvious that enzyme activities of these fungi varied considerably even though they belong to the same genus. The comparison of *Trichoderma* species involving in the quantitative assay of cellulase activity has been presented in Fig. 4. The rate of saccharification of cellulases produced by *Trichoderma* species was carried out up to 96 hours. The maximum enzyme production was recorded up to 53.42 units mL^{-1} after incubation period of 72 hours at 30 ± 2°C. All the strains exhibited over all good cellulolytic activity after 72 hours of incubation (Fig. 4A-C).

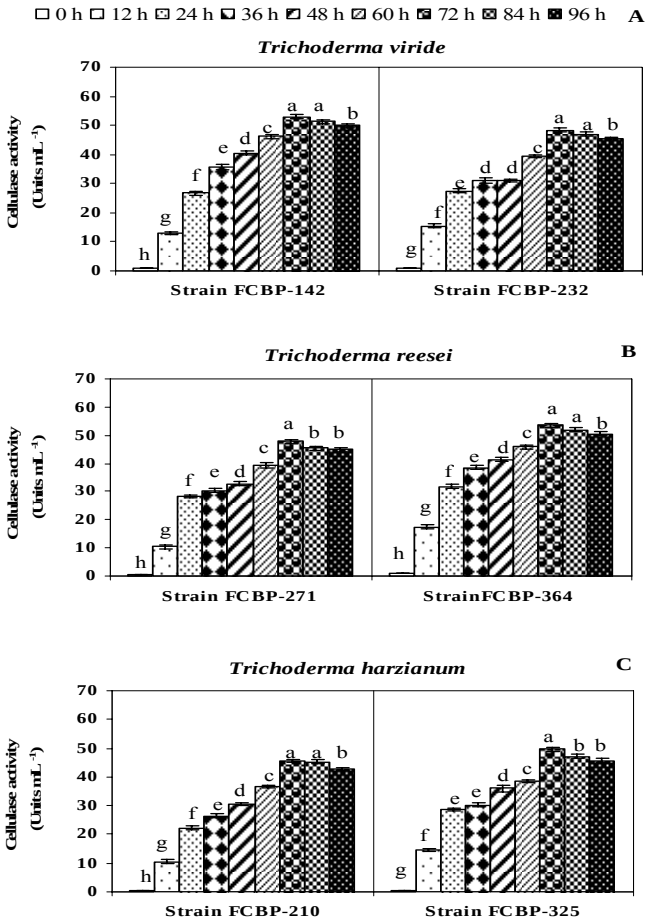

Fig. 4. Cellulase activity of selected *Trichoderma viride*, *T. reesei* and *T. harzianum* strains. Each value is a mean average of three parallel replicates and Y-error bars indicate standard error among the replicates.

Of all the cultures tested, *Trichoderma reesei* strain FCBP-364 and *Trichoderma viride* strain FCBP-142 showed promising results by exhibiting 53.42 and 52.97 units mL^{-1} respectively. The strain FCBP-271 of *T. reesei* and strain FCBP-232 of *T. viride* though showed significant increase in enzymatic activity up to 72 hours but the level of their enzyme production was significantly lowered (47.755 and 48.333 units mL^{-1}, respectively) than the other strains of same species.

In case of *T. harzianum* strains FCBP-210 and FCBP-325 almost similar patterns of enzyme activity prevailed. However, the cellulase activity was found to be lowest i.e., 45.366 units mL^{-1} of strain FCBP-210 and 49.422 units mL^{-1} of strain FCBP-325, as compared to other two species of *Trichderma*. While among both strains of *T. harzianum,* the strain FCBP-325 was found to be more efficient as compared to other one.

Discussion

The pH of the medium has a direct influence on the growth of microorganisms carrying out fermentation process (Figs. 1-3). The rate of enzyme synthesis was favoured most at pH 4. Similar results were reported earlier by Suhr *et al*., (2002) while working on *Penicillium caseifulvum* who stated that low pH induces the production of secondary metabolites.

Efficient colonization and utilization of lignocellulosic growth substrate depends upon the capacity of the organisms to produce the extracellular enzymes required to degrade the main polymers of the substrate, cellulose, hemicellulose and lignin. The physiology and extracellular lignocellulolytic enzyme production have been studied for some fungi from genus *Pleurotus* in submerged cultivation on synthetic media (Lee *et al.,* 2000) and in solid-state fermentation of lignocellulosic substrates (Giardina *et al*., 2000).

The results showed that all the selected isolates produced detectable quantities of cellulase on plate screening medium. Thus solid-state fermentation process was found to give fairly reliable indication of elevated cellulolytic activities. These findings are in line with the work conducted by Zaldivar *et al*., 2001. However, the selection of more efficient cellulolytic strains was made on biochemical basis (Elander, 1982).

The ability of crude enzyme of *Trichoderma* species to hydrolyze cellulose presented remarkable variation in activity. Elisashvili *et al*., 2003, reported similar variations in enzymatic activity by different species of *Pleurotus*. Considerable variations in results have been observed among different strains of same species analyzed. These findings are correlated with the work on saccharifying activity of cellulases by *T. harzianum* (Haq *et al*., 2005).

The temperature of the medium was kept at 30 ± 2°C with shaking at 100 rpm, which is in accordance with the previous work conducted by Esterbaur *et al*., (1991) who found that optimum temperature for cellulase production from *Trichoderma reesei* was 15-28°C and optimal temperature for its growth was 30°C. Similarly, Smith & Wood (1991) have reported the optimum temperature of 30°C and 35°C for the production of extra cellular xylanases and xylosidase by *Aspergillus awamori*.

The maximum activity was achieved after incubation period of 72 hours at 30 ± 2°C. This is due to the fact that during this phase the microbes were in stationary phase. The results are in accordance with those of earlier work conducted on cellulolytic fungi by Aurangzeb *et al*., 1997. Further increase in the incubation period did not show any enhancement (Haq *et al*., 2005). Similarly glucose produced during fermentation may also be responsible for the feed back inhibition of the enzyme production as reported by Podukhe & Soman (1993).

The data recommend that *Trichoderma reesei* strain FCBP-364 and *Trichoderma viride* strain FCBP-142 illustrated promising result by exhibiting 53.422 and 52.977units mL^{-1} respectively. The cellulolytic activities of these strains can be further enhanced by the mutagenic treatment (UV and chemical) of fungus. Further studies on these lines are in progress in our laboratory.

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