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Extracellular chitinase production by *Trichoderma harzianum* in submerged fermentation

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Extra-cellular chitinase production by a chitinolytic fungus *Trichoderma harzianum* TUBF 966 using submerged fermentation was studied. Colloidal chitin (1.5% w/v) was used as sole carbon source. Maximum chitinase production (14.7 U/ml) was obtained when fermentation was carried out at 30 °C for 96 h using 72 h old mycelium in a medium containing colloidal chitin 1.5% (w/v) as carbon source and 0.42 (% w/v) peptone as nitrogen source (pH 5.5). Supplementation of additional carbon sources (0.75% w/v) showed no further enhancement in chitinase production while supplementation of nitrogen sources (0.42% w/v) such as peptone and tryptone in the fermentation medium showed a marked increase in production. The process parameters that controlled chitinase production by the fungus were studied and presented here.

Chitin, a naturally abundant mucopolysaccharide and the supporting material of crustaceans, insects etc consist of 2-acetamido 2-deoxy-β-D-glucose (N-acetyl glucosamine) through a $\beta(1-4)$ linkage (MAJETI 2000). The complete enzymatic hydrolysis of chitin to free N-acetyl glucosamine is performed by a chitinolytic system consisting of two fractions, endo-chitinase and chitobiase. The physiological function of chitinases (EC 3.2.1.14) depends on their source. In bacteria, chitinases play roles in nutrition and parasitism whereas in fungi, protozoa and invertebrates they are involved in morphogenesis. Chitinases are involved in the defense mechanism of plants and invertebrates (GOODAY 1995). The possible role suggested for chitinase activity in human serum is a defense against fungal pathogens (ESCOTT *et al.* 1996, AERTS *et al.* 1996).

During the last decade, chitinases have received increased attention because of their wide range of applications. The major applications include use of chitinases for the biocontrol of plant pathogens (LORITO *et al.* 1993, MATHIVANAN *et al.* 1998) and for developing transgenic plants (LORITO and SCALA 1999, BOLAR *et al.* 2000). They are used extensively in biological research for the generation of fungal protoplasts (VYAS and DESHPANDE 1989, KUMARI and PANDA 1992) due to its ability to degrade fungal cell wall and also be employed in human health care such as making ophthalmic preparations with chitinases and microbicides (PATIL 2000). Unfortunately, large-scale production of chitinases is too expensive and uneconomical to make this enzyme available in sufficient quantities. Hence there is a wide market for potent, economically viable source of chitinase.

Trichoderma spp. are known to produce chitinases, β-1,3-glucanases, proteases and volatile and non-volatile antibiotics (ELAD *et al.* 1982). It has been pointed out that the growth inhibition of fungal mycelium by *Trichoderma* is not mediated by hyphal penetration, indicating that extra cellular metabolites could be responsible for the fungal degradation (CHERIFF *et al.* 1990). Mass production of *Trichoderma* spp. spores for biocontrol pur-

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poses is mainly confined to submerged fermentation technology (JIN *et al.* 1991). The chitinolytic enzymes of *T. harzianum* was demonstrated to have higher activity and wider spectrum antifungal nature than those of higher plants or bacteria (LORITO *et al.* 1993a) and hence *T. harzianum* is being studied both as a part of a complex and fascinating microbial system significant today for commercial agriculture, as well as molecular tools for enhancing protection against fungal diseases (WOO *et al.* 2001).

Chitinase was produced by *T. harzianum* when it was grown in presence of chitin or isolated fungal cell walls (ELAD *et al.* 1983, RIDOUT *et al.* 1988, TOKIMOTO 1982). It has been found that optimization of fermentation parameters very often led to markedly increased enzyme production (PETRUCCIOLLI *et al.* 1995, PARK *et al.* 1997). In this respect understanding factors governing synthesis and activity of chitinase is critical and worth investigation.

The present study aimed to optimize culture conditions that influenced chitinase production by *T. harzianum* TUBF 966 in submerged fermentation.

Materials and methods

Microorganism and maintenance: Six strains of *Trichoderma harzianum* (TUBF 966, 968, 964, 789, 791, 688) obtained from Technical University of Budapest, Hungary, were used in the study. The microorganisms were maintained on potato-dextrose-agar (PDA) slants, subcultured regularly at every two weeks and stored at 4 °C.

Inoculum preparation: The spore inoculum was prepared by dispersing spores from fully sporulated culture on PDA slants in 0.1% Tween 80 under aseptic conditions. The concentration of spores was adjusted to 4×10^5 colony forming units (cfu) ml^{-1} suspension. Vegetative mycelial inocula was prepared in a seed culture medium of pH 5.0, having the following composition (in kg/m^3) dextrose 10.1 $(NH_4)_2SO_4$ 4.2, NaH_2PO_4 6.9, KH_2PO_4 2.0, $MgSO_4 \cdot 7\,H_2O$ 0.3, peptone 1.0, citric acid monohydrate 10.5 and urea 0.3 (FELSE and PANDA 2000). Forty milliliters of medium were taken in 250 ml ERLENMEYER flasks, sterilized at 121 °C for 15 min, cooled to 30 °C), inoculated with 1 ml of spore suspension and incubated at 30 °C on a rotary shaker at 180 rpm. The mycelium was collected aseptically after a desired period of fermentation by filtration and washed three times with sterile distilled water.

Colloidal chitin preparation: Colloidal chitin was prepared by the method of ROBERTS and SELITRENIKOFF (1988) with some modification. One hundred grams of chitin flakes were added slowly to 1.75 liter concentrated HCl and agitated gently for 3 hours on a magnetic stirrer. This solution was then filtered to 20 liter of prechilled distilled water with constant mixing and allowed to settle. A dense white precipitate formed was then centrifuged at 10,000 rpm for 10 min at 4 °C. The precipitate was then washed in cold distilled water repeatedly until the pH of the wash reached near to 5.5. The supernatant was discarded and colloidal chitin was then kept in refrigerator for future use.

Submerged fermentation: A known quantity of inoculum was transferred to 40 ml of the sterilised production medium of pH 5.0 having composition (in kg/m^3): $(NH_4)_2SO_4$ 4.2, NaH_2PO_4 6.9, KH_2PO_4 2.0, $MgSO_4 \cdot 7\,H_2O$ 0.3, Tween 80 0.2, $FeSO_4 \cdot 7\,H_2O$ 0.005, $MnSO_4$ 0.0016, $ZnSO_4$ 0.0014, $CaCl_2 \cdot 2\,H_2O$ 0.002 (FELSE and PANDA 2000) and colloidal chitin 5.0 under strict aseptic condition. The culture was incubated at 30 °C for desired period on a rotary shaker at 180 rpm and was harvested by centrifuging. The clear supernatant was used for enzyme assay.

Optimization of culture conditions: To determine the optimum conditions for chitinase production, the strategy employed was to vary only one parameter at a time and monitoring the enzyme yield and in the process selecting the conditions that provided maximum yield. The effects of various parameters influencing chitinase production were tested.

Comparison of chitinase production using 1 ml of spore inoculum incubated for different intervals of time (0–120 h) and 0.075% dry weight equivalent of vegetative mycelium of different ages (0–120 h) incubated for 96 h; effect of inoculum size was studied using inoculum discs (1–5 discs, where 1 disc

corresponded to 5 loops of mycelial inoculum) incubated for 96 h; incubation time was evaluated for 0–144 h; different levels of pH was noted over a wide range (4.0–6.5); concentration of colloidal chitin was optimized (0.125–2.0%, w/v); influence of supplementary carbon sources was studied by replacing 0.75%(w/v) of colloidal chitin with 0.75%(w/v) of glycerol, glucose, lactose, maltose, sucrose and soluble starch; supplementation of organic and inorganic nitrogen sources was evaluated using 0.42(% w/v) of $NaNO_3$, $(NH_4)_2HPO_4$, NH_4NO_3, urea, peptone, tryptone, yeast extract, beef extract and malt extract.

Analytical procedure: Chitinase assay is based on the estimation of reducing sugars released during the hydrolysis of colloidal chitin. The reaction mixture contained 0.5 ml of enzyme, 0.5 ml of 0.5% colloidal chitin and 1.0 ml of citrate phosphate buffer pH 5.6. The mixture was kept in a water bath at 50 °C for 1 h. The amount of reducing sugar liberated was estimated by MILLER's (1959) method. One unit (U) of activity was defined as the amount of enzyme which catalyzed the release of one µg of reducing sugar per ml per minute under the reaction conditions. All the experiments were conducted in triplicate and data were presented as means of three replicates ± SD (MS Excel 2000).

Chitinase binding assay was carried out to by the method of ESCOTT *et al.* (1998) with slight modification. This assay helps to determine whether there was any absorption of chitinase by high concentrations of colloidal chitin in the medium. The culture medium containing high concentrations of colloidal chitin (1.5% and 2%) were filtered through a cheese cloth and centrifuged at 1000 rpm for 15 min at 4 °C to obtain the culture supernatant. The residual colloidal chitin obtained was then washed with 10 ml of 0.1 M citrate phosphate buffer pH 5.6 and centrifuged to remove any unbound protein. The residue was then washed after resuspension in 10 ml of 80 mM acetic acid and 50% ethylene glycol in saturated NaCl. Both the supernatants were collected together, dialysed and assayed for chitinase activity.

Results and discussion

Results on enzyme production during initial screening of six strains belonging to *Trichoderma harzianum* for chitinase production in submerged fermentation showed that *T. harzianum* TUBF 966 was best strain (data not shown). A single parameter optimization study was then conducted to evaluate the influence of various culture conditions on enzyme production.

Comparison of chitinase production using different ages of mycelial inoculum incubated for 96 h and spore inoculum incubated for different time intervals

The efficiency of using spore inoculum and mycelial inoculum for chitinase production by *T. harzianum* TUBF 966 is presented in Fig. 1. In the case of spore inoculum, maximum enzyme production was obtained at 96 h (4.8 U/ml), while with mycelial (vegetative) inoculum, the yield was maximum (6.2 U/ml) by using 72 h old inoculum incubated for 96 h. 72 h old mycelial inoculum showed 29% increase in yield compared to spore inoculum. The variation in chitinase production could be attributed to difference in the physiological condition of the inoculum, which would affect the growth and product formation.

Thus, it became evident that the nature of the inoculum had a strong influence on enzyme production and mycelial (vegetative) inoculum was found to be better than the spore inoculum. Higher enzyme yield with mycelial inoculum could be due to rapid growth initiation of the cultures and early entry of the organisms into the production phase.

Effect of inoculum size

Inoculum concentration is considered critical for any bioprocess and it has to be added at optimal level. Effect of inoculum size on chitinase production was carried out with disc inoculum method. Fermentation was carried out using 5 different inoculum sizes (1–5 disc) for 96 h. Maximum yield (5.8 U/ml) was obtained with an inoculum size of 3 discs. From

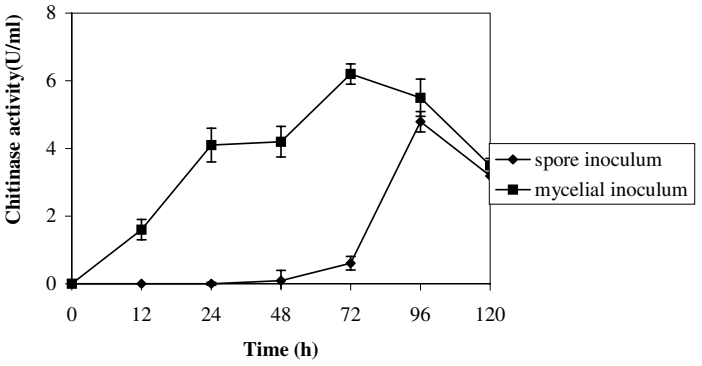

Fig. 1
Comparison of chitinase production by *T. harzianum* using mycelial inoculum of different ages incubated for 96 h and spore inoculum incubated for different time intervals (values are means of three replicates ± SD)

the data presented in Fig. 2, it was clear that enzyme yield increased to 97% with 3-disc inoculum when compared with the yield obtained with 1-disc inoculum. Lower inoculum concentration may not be sufficient to effect maximal transformation of biological materials in the natural state whereas the marginal decrease seen with larger inocula could be due to the shortage of nutrients available for the larger biomass and faster growth of the culture.

Impact of incubation time

During the time course study with 72 h old mycelial inoculum (0–144 h), maximum chitinase yield (6.6 U/ml) was recorded at 96 h of incubation. Beyond this period, the enzyme yield was found to be decreasing as shown in Fig. 3. At 144 h, the yield was only 33% of the yield obtained at 96 h. This decrease of chitinase yield after an optimum period of time was probably due to the reduced nutrient level of the medium affecting the enzyme synthesis and also enzyme denaturation by protease secreted by the fungus. SMITH and GRULA (1983) reported a sharp drop of chitinolytic activity after a maximal peak in *B. bassiana* in liquid culture.

Effect of pH of the medium

Most microbial extra-cellular enzyme yield were maximum at a growth pH, which is optimal for enzyme activity (MCTIGUE *et al.* 1994). The results presented in Fig. 4 indicated a strong influence of initial pH of the medium on the rate of chitinase production. For the study,

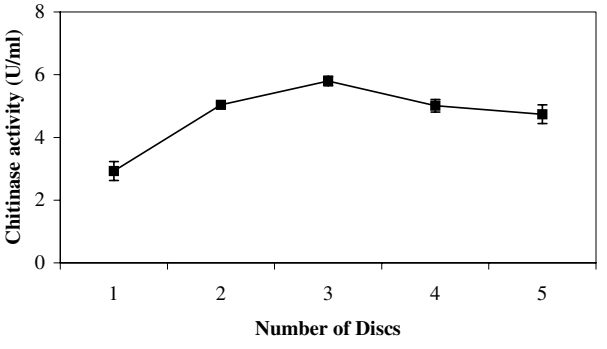

Fig. 2
Effect of inoculum size on chitinase production by *T. harzianum* TUBF 966 (values are means of three replicates ± SD)

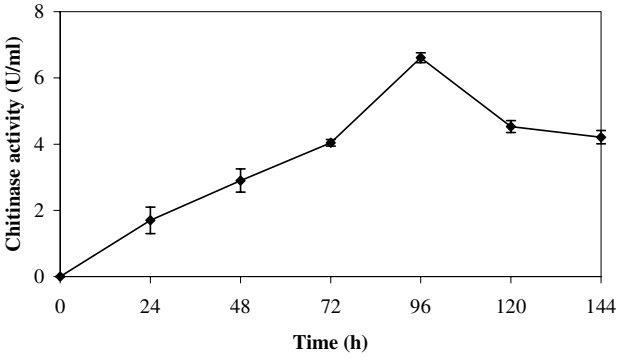

Fig. 3
Impact of incubation time on chitinase production by 72 h old mycelial inoculum of *T. harzianum* (values are means of three replicates ± SD)

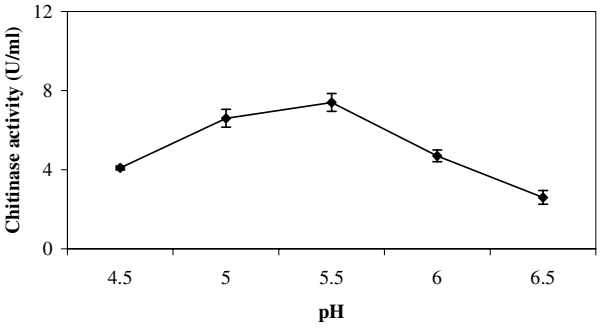

Fig. 4
Effect of initial pH on chitinase production by *T. harzianum* TUBF 966 (values are means of three replicates ± SD)

study, enzyme yield was compared in a broad range of initial pH (4–6.5) in shake flask cultures. It was found that medium of initial pH 5.5 produced maximum enzyme yield (7.4 U/ml). Beyond this pH, chitinase production was found to be decreasing.

Chitinase production was found to be stimulated by acidic pH and this result was in accordance with KATATNY *et al.* (2000), who reported that chitinase production by *Trichoderma harzianum* RIFAI was stimulated by acidic pH from 5.5 to 6. Another study by ULHOA *et al.* (1991) also showed that production of chitinase by *T. harzianum* 39.1 was markedly affected by pH with the optimum at 6.0.

Optimization of colloidal chitin concentration

It is known that an ideal substrate concentration in any fermentation process results in higher conversion efficiencies and optimum substrate utilization. The study determined the variation in chitinase production with different concentrations of colloidal chitin in the medium and the results are presented in Fig. 5. Maximum enzyme yield was recorded when 1.5% (w/v) of colloidal chitin was present in the medium (9.2 U/ml). Chitinase production was substrate concentration dependent but above 1.5% (w/v) colloidal chitin, there was no further enhancement in enzyme synthesis. Hence chitinase binding assay was carried out to determine whether the produced enzyme was absorbed to colloidal chitin (1.5% and 2% w/v) in the medium. The result showed that no residual chitinase activity was bound to these higher concentrations of colloidal chitin.

Chitin is produced as an inducible enzyme with chitin or its degradation products acting as inducers. In most cases chitin concentration in the range of 1–2% was found to be suitable for chitinase production. Chitinase production by *Talaromyces emersonii* studied in submerged cultures showed that optimum chitin concentration was 1–2% w/v at a pH of 5 and 45 °C (MCCORMACK *et al.* 1991). According to GUPTA *et al.* (1995) *Streptomyces viridificans* produced maximum levels of chitinase at 1.5% colloidal chitin after six days of

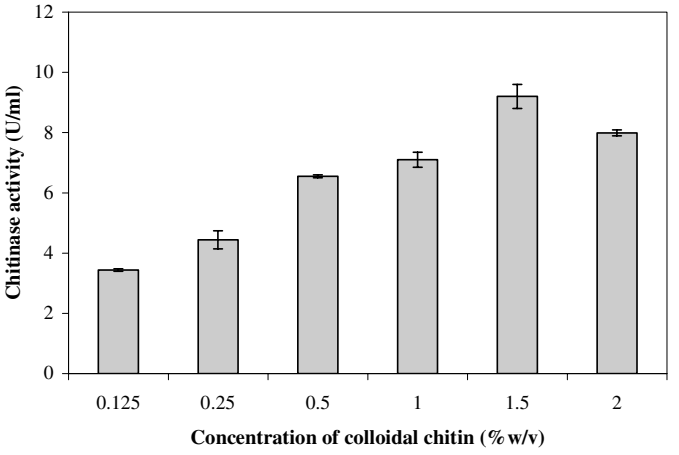

Fig. 5
Optimization of colloidal chitin concentration for chitinase production by *T. harzianum* TUBF 966 (values are means of three replicates ± SD)

fermentation at 30 °C and 200 rev/min. The possible reason, which has been so far unsubstantiated could be that the signal for induction of synthesis of chitinase could be resulted from the physical contact between the cell surface and the insoluble substrate, as has been proposed for cellulose biosynthesis (BERG and PETTERSON 1977).

Influence of supplementary carbon sources

The type and nature of carbon source is one of the most important factors for any type of fermentation process (PANDEY *et al.* 1999). The carbon source represents the energetic source that will be available for the growth of the microorganism.

The optimized colloidal chitin concentration for chitinase yield was found to be 1.5 (%w/v) (Fig. 5). The influence of additional carbon sources on enzyme production was evaluated by supplementing 50% of colloidal chitin (CC) with various carbon sources such as glycerol, glucose, lactose, maltose, sucrose and starch in the medium at 0.75% (w/v). The medium containing 0.75 (%w/v) colloidal chitin as sole carbon source was taken as control. Fig. 6, clearly shows that supplementation of glycerol and lactose reduced chitinase production considerably, of which glycerol showed 30% and lactose 8.7% of reduction respectively. Similar repression in chitinase yield by addition of glycerol was observed by FRANDBERG *et al.* (1994). However, supplementation of glucose and maltose showed 30% increase in chitinase yield. MATHIVANNAN *et al.* (1997) observed maximum production of extra cellular chitinase by *Fusarium chlamydosporum* in a medium containing a combination of colloidal chitin (0.5%) and sucrose 10 mM as carbon source. The above result also showed an increase in chitinase yield by supplementation of disaccharides. On the contrary, all the hexoses and pentoses added along with 1% colloidal chitin, repressed chitinase production by *Streptomyces lydicus* WYEC 108 (MAHADEVAN and CRAWFORD 1997).

The medium containing optimized colloidal chitin concentration (1.5% w/v) as sole carbon source showed better yield than any other medium containing additional carbon sources. The medium showed a dose dependent increase (42%) in chitinase production when compared to the control medium and the enzyme produced was not found to be bound to colloidal chitin.Thus the above results confirmed that significant chitinase activity was present when the fungus was incubated in 1.5 (%w/v) colloidal chitin containing medium, in the absence of any other carbon sources. Most of the chitinolytic systems reported in the literature are inducible (SMITH and GRULA 1983).

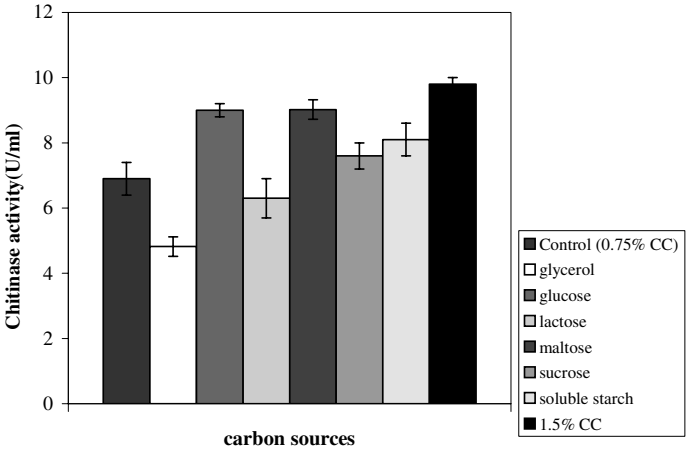

Fig. 6
Influence of supplementary carbon sources on chitinase production by *T. harzianum* TUBF 966 (values are means of three replicates ± SD)

Effect of inorganic and organic nitrogen sources

Fig. 7 shows the effect of incorporation of inorganic and organic nitrogen sources at 0.42% (w/v) on the enzyme yield. Nitrogen enrichment of fermentation medium with chemically defined nitrogen sources such as $NaNO_3$, $(NH_4)_2HPO_4$, NH_4NO_3 showed marginal reduction in the enzyme yield in comparison to control having $(NH)_2SO_4$ as nitrogen source. Addition of urea into the medium was found to reduce chitinase yield by 39.7%. This result was in agreement with KAPAT *et al.* (1996) who observed that exclusion of urea form the medium increased chitinase production. However, this report was contrary to the observation made by ULHOA and PEBERDY (1991) for chitinase production by *T. harzianum* and VYAS and DESPANDE (1989) for *Myrothecium verrucaria* where addition of urea increased chitinase production. In several fermentations, it has been found that rapidly utilizable nitrogen sources act as inhibitors.

Organic nitrogen sources such as peptone and tryptone in the medium increased chitinase production by 50% and 42%, respectively. This result was similar to that obtained by HO-SEONG *et al.* (1994) and VAIDYA *et al.* (2001) where addition of organic nitrogen sources to

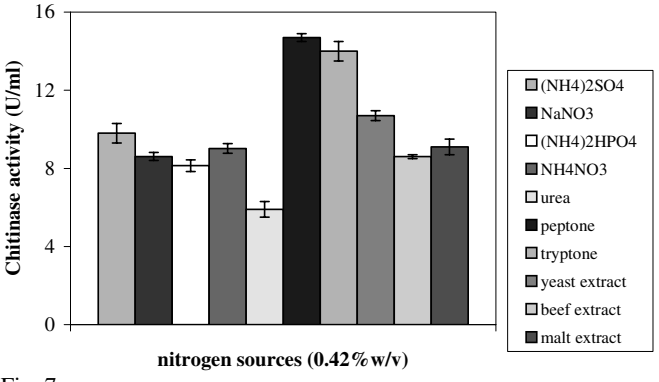

Fig. 7
Effect of supplementary nitrogen sources on chitinase production by *T. harzianum* TUBF 966 (values are means of three replicates ± SD

the culture medium increased chitinase production by *Pseudomonas stutzeri* YPL-1 and *Alcaligenes xylosoxydans* IMI 385022, respectively, but inclusion of peptone was also reported to reduce chitinase yield (KAPAT *et al.* 1996). In a novel chitinolytic strain of *Aeromonas* sp., inclusion of tryptone increased chitinase production (HUANG *et al.* 1996). Yeast extract in the medium also marginally enhanced chitinase yield whereas supplementation of beef extract and malt extract reduced enzyme synthesis. The results of the study indicated that organic nitrogen sources such as peptone and tryptone in the medium enhanced the enzyme production.

From the results it was clear that culture conditions have a strong influence on chitinase production by the fungus. By varying the process parameters a two-fold increase in chitinase yield was obtained. These observations, thus, confirmed that *T. harzianum* TUBF 966 could be a potential mesophilic fungus for production of extra-cellular chitinase by submerged fermentation.

Acknowledgements

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