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Process optimization for antifungal chitinase production by *Trichoderma harzianum*

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Abstract

Single-parameter optimization was carried out for the production of chitinase using a soil isolate belonging to *Trichoderma harzianum* in solid-state fermentation. Maximum chitinase activity (3.18 U/gds) was obtained after 96 h of incubation at 30 °C when wheat bran moistened (65.7%) with salt solution was supplemented with colloidal chitin (1%, w/w) and yeast extract (2%, w/w) used as the substrate. The inoculum contains 4×10^7 spores of *T. harzianum* (TUBF 781). The growth of the fungus on wheat bran particle was visualized by a scanning electron microscope. Chitinase activity was measured as the amount of *N*-acetyl glucosamine (NAG) liberated in μmol/min under reaction conditions. The crude extract showed antifungal activity against a wide range of fungal strains belonging to *Aspergillus*, *Rhizopus* and *Mucor* sp., and was found significant against *Aspergillus niger* (NCIM 563).

Keywords: Chitinase; *Trichoderma harzianum*; Solid-state fermentation; Antifungal activity

1. Introduction

Microbial production of chitinase has captured the worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method. Chitinases (EC.3.2.1.14) are hydrolytic enzymes responsible for the degradation of chitin, a high molecular weight linear polymer of *N*-acetyl-D-glucosamine (*N*-acetyl-2-amino-2-deoxy-D-glucopyranose) units linked by β(1– 4)-glucosidic bond [1]. Chitinolytic enzymes play a key role in biological pest control, in food/feed industry, for producing growth factors, sweeteners, single-cell protein, etc. and finally for degrading the chitin rich waste materials [2]. The complete enzymic hydrolysis of chitin to free *N*-acetyl-D-glucosamine is performed by a chitinolytic system comprising exochitinase, endochitinase and chitobiase [3].

Solid-state fermentation (SSF) has emerged as an appropriate technology for the management of agro-industrial residues and for their value addition. SSF is a promising technology for the development of several bioprocesses and products including production of industrial enzymes on large-scale [4,5]. Different types of substrates, which contain chitin, have been tried for the production of chitinase, which included fungal cell walls, crab and shrimp shells [6–8]. The use of *Trichoderma* sp. in SSF for the production of lytic enzymes such as cellulose and chitinase has tremendous impact for an industrial scale production [9–11].

In the present study, the most vital fermentation parameters were optimized to monitor the production of extracellular chitinase by *Trichoderma harzianum* TUBF 781 under solid-state fermentation using wheat bran as the substrate. The antifungal activity of the crude chitinase was also tested.

2. Materials and methods

2.1. Micro-organism and inoculum preparation

A fungal isolate, *T. harzianum* TUBF 781 obtained from the Technical University of Budapest, Hungary was used in the present study. The culture was maintained on

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potato-dextrose agar (PDA) medium and subcultured every fortnightly. Slants were incubated for 8 days at 30 °C and stored at 4 °C. The spores of a fully sporulated slant were dispersed in 10 ml of 0.1% Tween 80 solution by dislodging them with a sterile loop under aseptic conditions. The spore suspension obtained was used as the inoculum. Viable spores present in the suspension were determined by serial dilution followed by plate count.

2.2. Substrate preparation for SSF

Five grams of dry wheat bran having both coarse and fine particles in 1:1 ratio (w/w) contained in a 250 ml Erlenmeyer flask and was supplemented with 2 ml salt solution containing 0.5% NH_4NO_3, 0.2% KH_2PO_4, 0.1% NaCl, 0.1% $MgSO_4{\cdot}7H_2O$. The initial moisture level in the substrate was adjusted by adding adequate amount of distilled water. The thoroughly mixed substrate was autoclaved for 20 min at 121 °C (15 lb) and cooled to room temperature before inoculation.

2.3. Solid-state fermentation

Under aseptic conditions, the sterilized solid substrate medium was inoculated with 1 ml fungal spore inoculum. Unless otherwise mentioned, the spore suspension contained 2×10^7 spores/ml. The contents were mixed thoroughly and the flasks were placed in an incubator at 30 °C for desired time intervals. All the sets were prepared in duplicate.

2.4. Enzyme extraction

To the fermented substrate an adequate amount of distilled water containing 0.1% Tween 80 was added to get a total extraction volume of 100 ml. The contents were thoroughly mixed by keeping the flasks on a rotary shaker at 150 rotations per minute for 30 min. The mixture was centrifuged at 9000 rotations per minute for 10 min at 4 °C. The supernatant was collected and used for enzyme assay.

2.5. Chitinase assay

Chitinase activity was determined by a dinitrosalicylic acid (DNS) method [12]. This method works on the concentration of *N*-acetyl glucosamine (NAG), which is released as a result of enzymic action [13,14]. The 2 ml reaction mixture contained 0.5 ml of 0.5% colloidal chitin in phosphate buffer (pH 5.5), 0.5 ml crude enzyme extract and 1 ml distilled water. The well vortexed mixture was incubated in a water bath shaker at 50 °C for 1 h. The reaction was arrested by the addition of 3 ml DNS reagent followed by heating at 100 °C for 10 min with 40% Rochelle's salt solution. The coloured solution was centrifuged at 10,000 rotations per minute for 5 min and the absorption of the appropriately diluted test sample was measured at 530 nm using UV spectrophotometer (UV-160 A, Shimadzu, Japan) along with substrate and enzyme blanks. Colloidal chitin was prepared by the modified method of Roberts and Selitrenkoff [15]. One unit (U) of the chitinase activity is defined as the amount of enzyme that is required to release 1 μmol of *N*-acetyl-β-D-glucosamine per minute from 0.5% of dry colloidal chitin solution under assay conditions.

2.6. Scanning electron microscopy

Growth of *T. harzianum* on wheat bran particles was characterized using an electron microscope (JEOL JSM 5600LV, Japan). The fermented sample (72 h) was adequately dried and mounted on a brass stud followed by a mild gold coating (100 Å) and was subjected to electron microscopy at an accelerating voltage of 10 kV.

2.7. Optimization of process parameters

The wheat bran based medium was used to carry out various single-parameter optimization studies for maximum chitinase yield in solid-state fermentation. It included incubation time (0–168 h), incubation temperature (25–45 °C), initial moisture content of the substrate (61–74%), inoculum size (1–6 × 10^7 spores), initial pH, effect of supplementation substrate with various chitin containing carbon sources such as chitin powder, chitin flakes, colloidal chitin (1%, w/w) and additional nitrogen sources such as peptone, yeast extract, corn steep liquor, urea, ammonium chloride and ammonium sulphate (1%, w/w). For each parameter optimization, three sets of independent experiments were carried out and the average values are reported.

2.8. Antifungal activity

A 96 h old culture of *T. harzianum* was used for the preparation of a crude extract. It was prepared as described and filtered through a 0.2 μm sterile membrane. This crude extract was tested for inhibitory activity against the growth of fungal strains: *A. niger*, *A. ficuum*, *A. oryzae*, *Rhizopus oligosporus* and *Mucor racemosus*. Antifungal activity was observed directly on Petri plates of PDA medium where the test organism was plated together with 500 μl of the prepared extract. The growth of the particular test fungi on plates with and without the crude chitinase extract was compared.

3. Results and discussion

The approach of single-parameter optimization was employed to optimize various process parameters.

3.1. Optimization of incubation time

Wheat bran (5 g) was supplemented with colloidal chitin (1% dry) and inoculated with 1 ml of spore suspension (2 × 10^7 spores/ml). SSF was carried out with the initial moisture

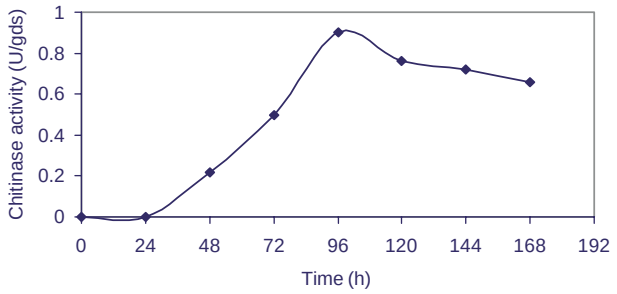

Fig. 1. Effect of incubation time on chitinase production.

content of 68% and incubation temperature of 30 °C. Flasks were removed every 24 h and the enzyme extraction was done as described earlier. The enzyme production showed growth relatedness as the incubation period progressed and maximum enzyme production (0.90 U/gds) was observed after 96 h (Fig. 1). Growth rate and enzyme production pattern were generally dependent on the duration of incubation. After 96 h, the production started to decrease as the growth of the organism might have reached a stage, from which it could no longer balance its steady growth with the availability of nutrient resources.

3.2. Optimization of inoculum size

The spore suspension made from a PDA slant contained 2×10^7 spores/ml. SSF was carried out using various volumes (0.5–3 ml) of the suspension as inoculum. The initial moisture of the substrate was 68%. Incubation was carried out for 96 h at 30 °C. As shown in Fig. 2, the maximum chitinase production (1.26 U/gds) was noted when an inoculum size of 2 ml (4×10^7 spores) was used. It was the size of the inoculum, that determines total biomass production on the solid medium [5]. An increase in the number of spores in the inoculum would ensure a rapid proliferation and biomass synthesis. However, after a certain limit, the competition for the nutrients resulted in the decreased metabolic activity of the organism. With optimum inoculum size for the enzyme production, there is a balance between proliferating biomass and availability of nutrients that supports production of enzyme.

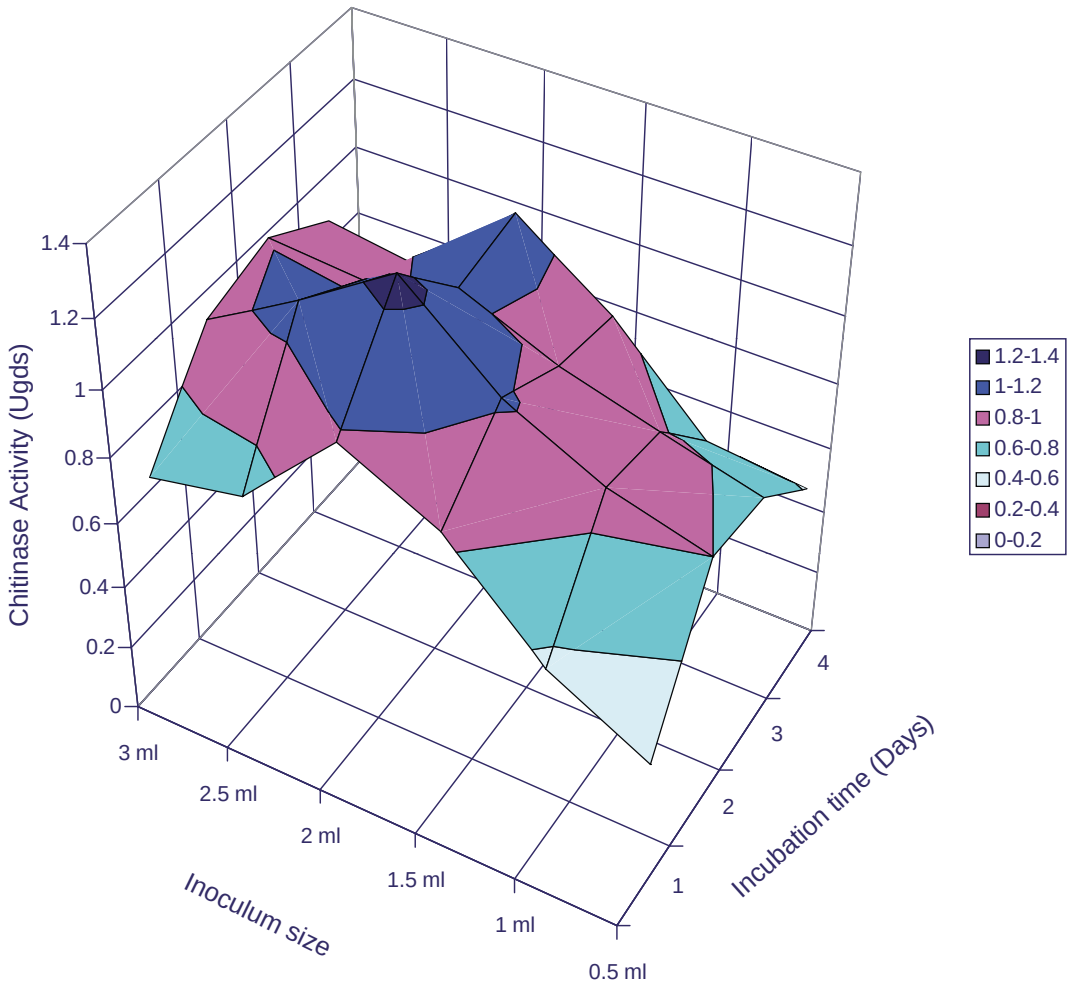

Fig. 2. Effect of inoculum size on chitinase production.

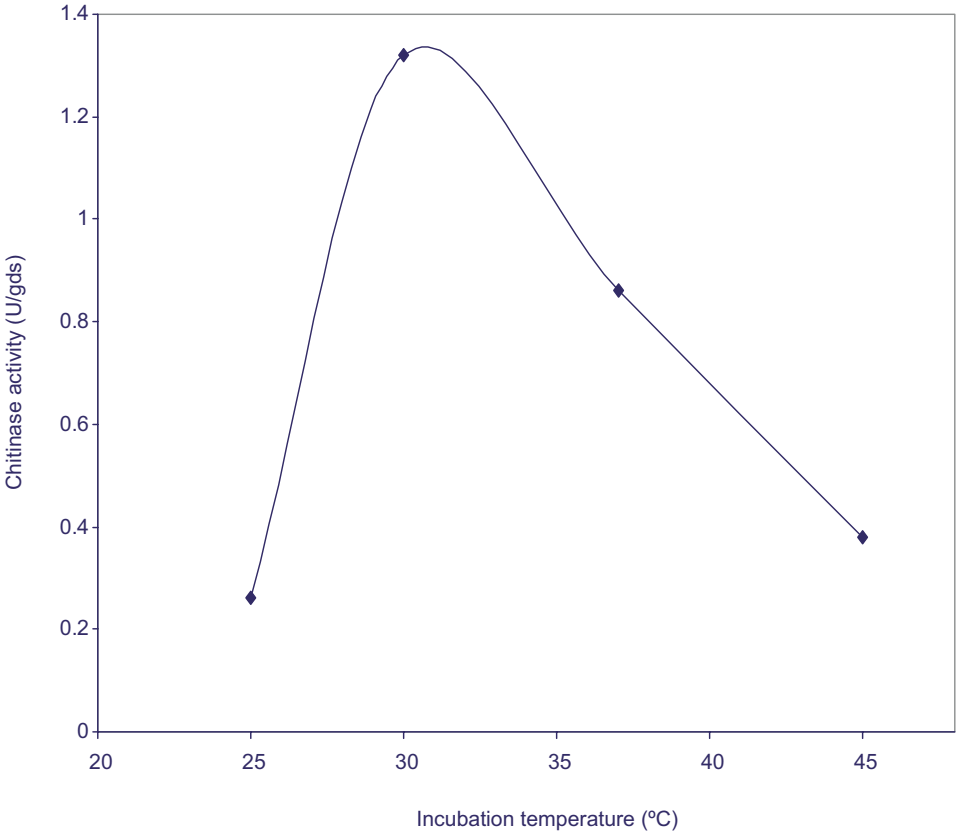

Fig. 3. Effect of temperature on chitinase production.

3.3. Optimization of incubation temperature

SSF was carried out at different incubation temperatures ranging from 25 to 45 °C. The initial moisture content was maintained at 68% and spore concentration of the inoculum was 4×10^7 spores. Samples were extracted after 96 h of fermentation. The organism exhibited a better growth as well as enzyme production at 30 °C and it was 1.32 U/gds as shown in Fig. 3. The significance of temperature in development of biological process is such that it could determine the effects of protein denaturation, enzyme inhibition, promotion or suppression of the production of a particular metabolite, cell viability and death [16]. In SSF, during fermentation there is a general increase in the temperature of the fermenting mass due to respiration [17]. Heat built-up is in fact a drawback in SSF system. However, problems of heat and mass transfers are generally severe during the scale-up of SSF. In present study using flasks, no such difficulty was noted.

3.4. Optimization of initial moisture content

SSF was carried out with substrates having different levels of initial moistures (60–75%). The samples were incubated at 30 °C and extracted after 96 h of fermentation. Maximum enzyme activity (2.2 U/gds) was obtained when initial moisture of the substrate was adjusted to 65.7% (Fig. 4). The enzyme yield was lower when the substrate moisture was higher or lower than this level. The decrease in the enzyme activity with increase in substrate moisture might be attributed to the phenomenon of flooding of interparticle space of the substrate. A higher than optimum moisture level causes decreased porosity, alteration in wheat bran particle structure, lowers oxygen transfer and enhanced formation of aerial mycelia [18,19]. Fungi would prefer unbound moisture for survival in such a way that it may not hamper with its metabolic pathways. Similarly, a moisture level lower than optimum leads to higher water tension, lower degree of swelling and reduced solubility of the nutrients of the solid substrate. A related criterion with this is the water activity of the medium, which is considered as the fundamental parameter for mass transfer of water and isolates across the cell membrane. When water is made available in a lower or higher quantity than that is optimally required, the productivity of the process is significantly affected. Moisture optimization can be used to regulate and to modify the metabolic activity of the micro-organism [20].

3.5. Optimization of initial pH of the substrate

For these experiments, the pH of the moistening solution used were set from 3 to 7 using 1N HCl/NaOH. Initial moisture content was adjusted as 65.7% and incubated in 30 °C for 96 h. However, there was no further increase in

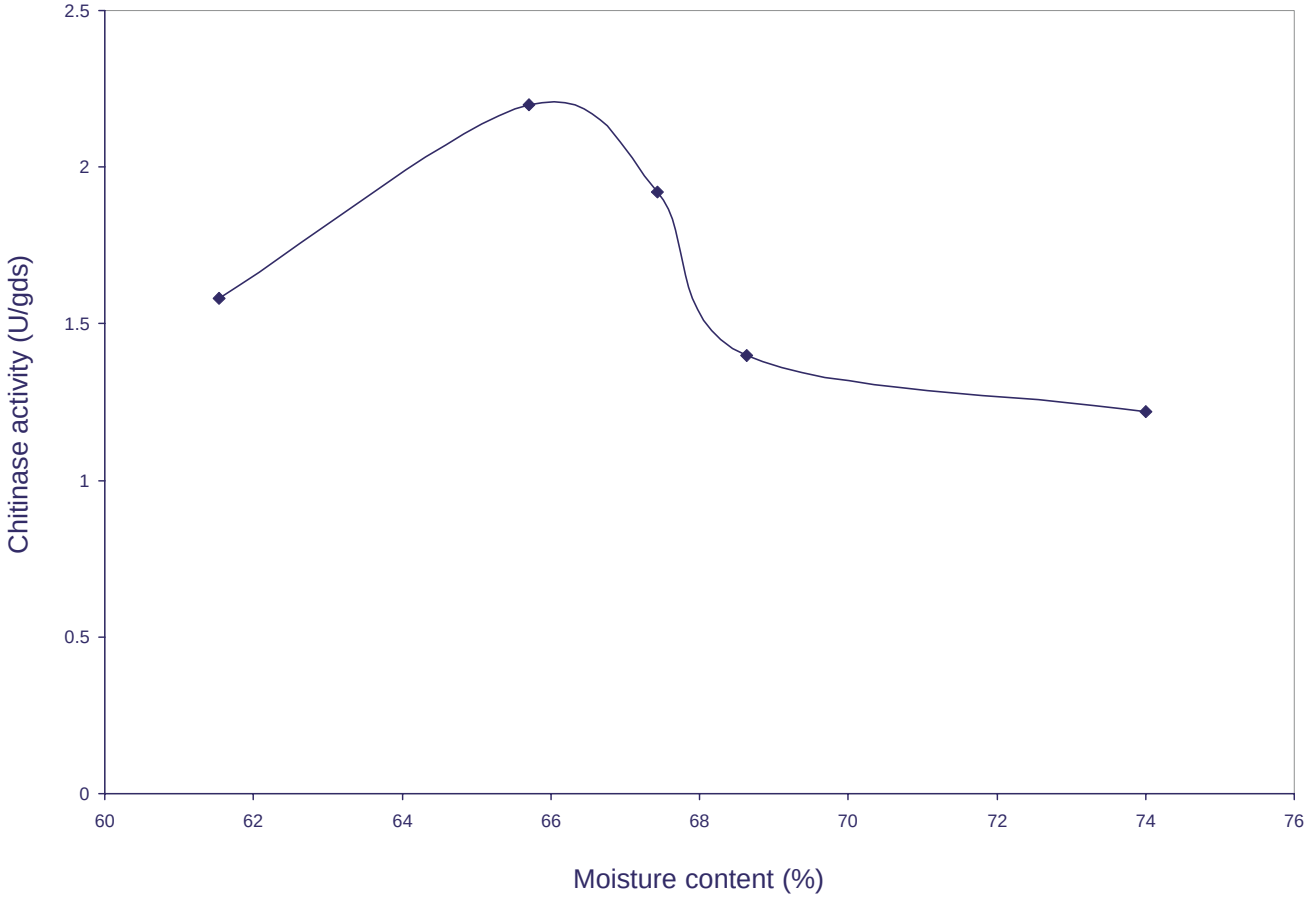

Fig. 4. Effect of initial moisture on chitinase production.

the enzyme yields when compared with the previous experiment. Maximum chitinase production was found with the substrate moistened with a solution having pH 4.5 (2.12 U/gds). However, there was little significant difference in enzyme production with the other pH (all data not shown). In view of the reports that generally the agro-industrial residues possess excellent buffering capacity [16] and that their use offers advantage for SSF processes, the pH of the substrates prepared with moistening solutions having different pH were examined. After autoclaving, the initial pH of all the substrate remained similar. This indicates that there was no need to change/adjust the pH of the wheat bran based medium. However, the pH of the medium strongly affects the growth and activity of the micro-organisms. Generally, microbial enzymes are produced in higher yield at a pH near to the maximal for enzyme activity. Fungal strains are noted for their best performance in the range of 3.5–6 and low pH avoids contamination by other microbes [16].

3.6. Effect of different sources of chitin

SSF was carried out with different sources of chitin containing carbon sources (1%, dry weight basis). Chitinase production was at best (2.24 U/gds) when colloidal chitin was used as the source material (Fig. 5). The other supplements, i.e. chitin powder and chitin flakes were not as effective as colloidal chitin. Due to the colloidal nature, probably it offered ease to the fungal culture to metabolize it. According to previous studies, SSF with 1% colloidal chitin by certain species of mould showed high chitinase activity [21] and certain bacterial species showed an increase in chitinase production on the addition of colloidal chitin [22].

3.7. Effect of supplementation of various nitrogen sources

Among the various nitrogen supplementation tried (Fig. 6), addition of yeast extract resulted in a slight increase

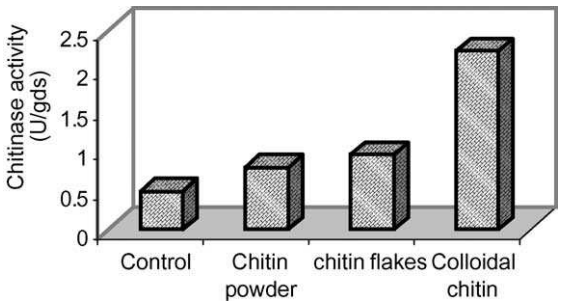

Fig. 5. Effect of various chitin sources on enzyme production.

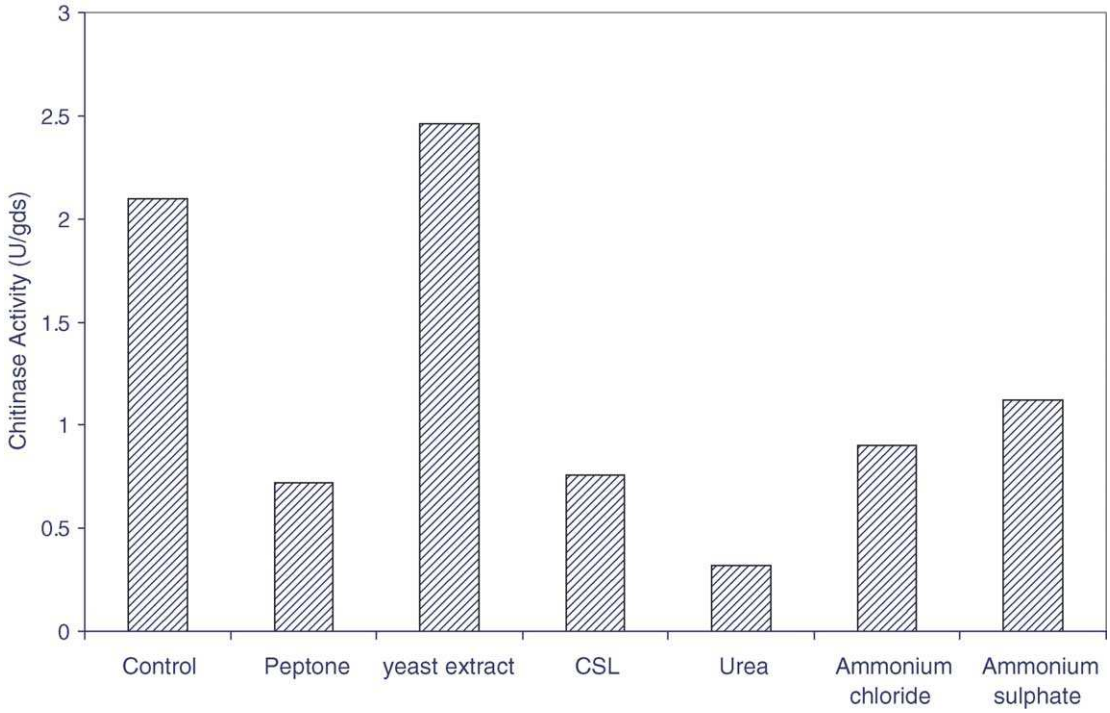

Fig. 6. Effect of various nitrogen sources on the production of chitinase by *T. harzianum* TUBF 781.

in chitinase production (2.46 U/gds) compared to the control. This could be due to the presence of chitin in it or some other growth factor, which might have stimulated enzyme production. There are reports on the favourable influence of supplementation of fermentation medium with yeast extract. For example, *S. cinereoruber* produced maximum chitinase activity on a medium containing 0.5% *A. niger* cell wall and 0.5% yeast extract [6]. Suresh and Chandrasekharan [23] also reported enhancement in the enzyme yield when the production medium was supplemented with phosphate and yeast extract.

3.8. Effect of different concentrations of yeast extract

Since supplementation of yeast extract stimulated chitinase production, attempts were made to determine the suitable concentration of yeast extract for maximum enzyme synthesis by the fungal culture. For this, SSF was carried out with different concentrations of yeast extract supplementation ranging from 0.25 to 2.5% (w/w). Enzyme activity showed a related increase with the yeast extract concentration and was maximum (3.18 U/gds) as it shown in Fig. 7 with the addition of 2.0% yeast extract.

3.9. Scanning electron microscopy (SEM)

Visual observation of the growing culture showed that *T. harzianum* grew very well on wheat bran particles and formed a thick growth in 72 h of incubation. Fig. 8 shows the SEM of this sample, which showed a mat like knitted

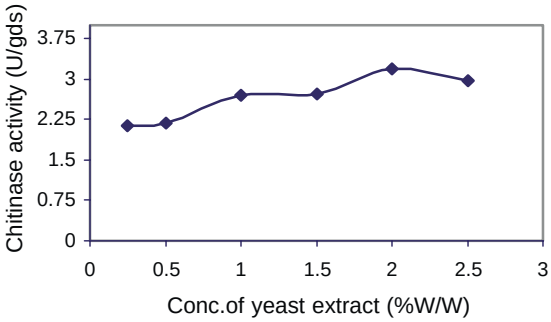

Fig. 7. Effect of yeast extract on chitinase production.

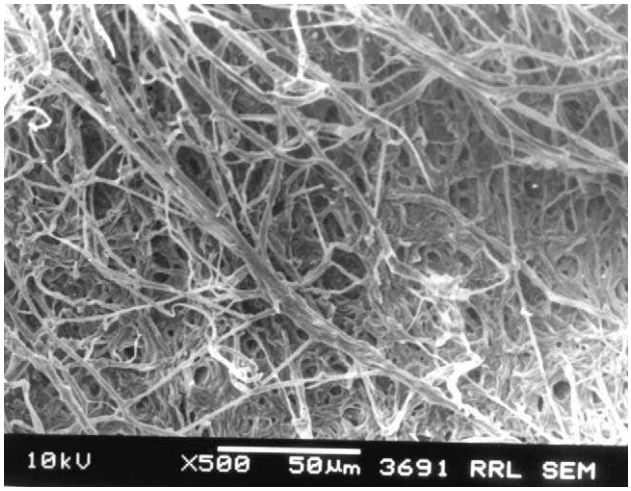

Fig. 8. SEM photograph showing the growth of *T. harzianum* TUBF 781 on wheat bran particles (72 h).

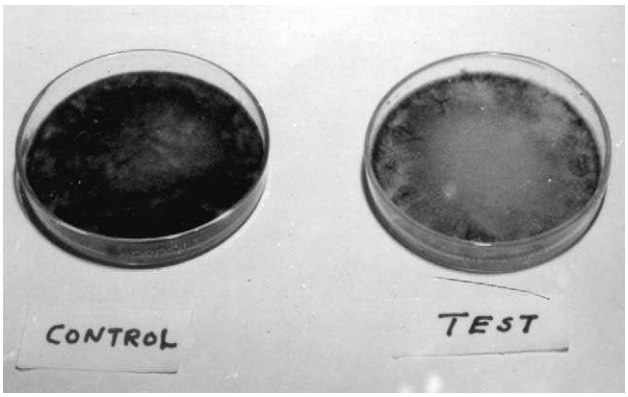

Fig. 9. Antifungal activity of crude chitinase of *T. harzianum* against *A. niger* NCIM 563.

structure in which mycelia were embedded in the wheat bran particles. The degree of substrate transformation in SSF depends upon the capability of fungal mycelia to penetrate deep in to the intracellular space.

3.10. Antifungal activity

Antifungal activity of the crude chitinase extract was observed against all the fungal cultures studied (results not shown) and it was maximal against *A. niger* NCIM 563 as shown in Fig. 9. It is known that the extracellular cell wall degrading enzymes are the main mechanism involved in the biological control of fungi by *T. harzianum* [24]. Chitinase producing organisms can inhibit fungal growth especially plant-pathogenic fungi and possible explanation is the action of chitinases and β-glucanases on chitin or glucan present in the fungal cell walls, acting as protective agents [25,26]. The present isolate also proved to be an efficient producer of chitin degrading enzyme.

The physiological function of chitinase depends on their source. In micro-organisms, chitinases are known to be involved in spore germination, branching and mycelial development, hyphal growth, cell separation, autolysis, nutrition and parasitism [27]. Chitinase activity has not yet been uniformly defined in the literature. Many variations in defining chitinase activity in terms of incubation temperature, time, and pH can easily been seen in the literature [28,29]. The success in using chitinase for different applications depends upon the supply of highly active preparations at reasonable cost.

4. Conclusion

T. harzianum chitinase is active over a wide range of operating and environmental conditions and hence it is designated as one of the best organism to study the production as well biochemical aspects of chitinase. In short, understanding more about the various chitinolytic enzymes such as the standardizations of suitable process parameters for its production, the method of estimation and biochemical characterization will make them more useful in a variety of process in near future.

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