PROCESS OPTIMIZATION OF L-GLUTAMINASE PRODUCTION BY *Trichoderma koningii* UNDER SOLID STATE FERMENTATION (SSF)

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ABSTRACT : Solid state fermentation (SSF) was carried out for the production of L-glutaminase by the fungal strain *Trichoderma koningii* using sesamum oil cake as the solid substrate. L-glutaminase has received significant attention in recent years owing to its potential applications in medicine as an anticancer agent, as an efficient anti-retroviral agent and as a biosensor. In food industries it is used as a flavor and aroma enhancing agent. The overall maximum yield of L-glutaminase (19.41 U/gds) was achieved with the optimized process parameters of initial moisture content 65%, initial pH of the medium 7.0, supplemented with D-maltose (1.0% w/v) and malt extract (1.0% w/v), inoculated with 2ml of 6 day old fungal culture and incubated at 33°C for 5 days. Both physico-chemical and nutritional parameters had played a significant role in the production of the enzyme, L-glutaminase. The enzyme production was found to be growth associated with the growth of the fungal culture.

Keywords: L-glutaminase, *Trichoderma koningii*, Sesamum oil cake, Solid state fermentation, Process parameters, Optimization

INTRODUCTION

L-glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) is a hydrolytic enzyme, that deaminates L-glutamine to L-glutamic acid and ammonia. The action of glutaminase plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes. In recent years glutaminase has attracted much attention with respect to its wide applications in pharmaceuticals as an anti-leukemic agent (Roberts et al., 1970, 1977; Pal and Maity,1992) and also as an efficient anti-retroviral agent (Roberts and McGregor, 1991) along with its use in food industry as a flavor and aroma enhancing agent (Yokosuta and Loeliger, 2000). Another important application of glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid (Sabu et al., 2000).

As far as the occurrence of glutaminase is concerned, it is widely distributed in animal tissues, plants and in microorganisms including bacteria, fungi and yeast. Different methods of fermentation technology can be applied for the production of glutaminase. Commercial production of glutaminase had been carried out using submerged fermentation (SmF) technique (Imada et al., 1973; Yamamoto and Hirooka, 1974). But nowadays, solid state fermentation (SSF) has been emerged as a promising technology for the development of several bioprocesses and products including the production of therapeutic enzymes on a large-scale (Pandey et al., 1999). The primary advantage of SSF is the fact that many metabolites are produced at higher concentration. There are a few reports on the production of extra-cellular L-glutaminase under SSF using microbial strains (Prabhu and Chandrasekharan, 1997; Sabu et al., 2000; Kashyap et al., 2002; Shindia et al., 2007).

In this paper, the optimized production of extracellular L-glutaminase enzyme by *Trichoderma koningii* using sesamum pil cake under solid-state fermentation was reported.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The fungal strain *Trichoderma koningii* MTCC 6348 used in this study was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on Malt extract Agar (MA) medium. Inoculated slants were grown in an incubator at 30°C for 7 days. The slants were stored at 4°C and sub-cultured once every 4 weeks.

Conidial suspension was prepared from a freshly raised 7 day old culture of *Trichoderma koningii* on Malt extract agar slants by suspending in 10ml of 0.85% sterile saline solution.

Fermentation medium and culture conditions

Sesamum oil cake obtained from the local market of Visakhapatnam, India was utilized as the solid substrate for the production of L-glutaminase by *Trichoderma koningii*. Five grams of dried sesamum oil cake having both coarse and fine particles in 1:1 ratio (w/w) was taken in 250 ml Erlenmeyer flasks and moistened with 10ml of salt solution containing glucose 0.6%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.05% and KCl 0.05%. The thoroughly mixed flasks were autoclaved at 121°C (15 lb) for 20 min, cooled to room temperature and inoculated with 2 ml of the fungal conidial suspension which was prepared previously. The contents were mixed thoroughly and the flasks were placed in an incubator at 30°C for desired time period.

Optimization of the culture condition for L-glutaminase production

Various process parameters that enhance the yield of L-glutaminase by *T. koningii* under solid state fermentation were investigated. The impact of incubation time (0-168 h), initial moisture content of the substrate (30-90 % v/w), inoculum concentration (0.5-4ml), initial pH (4-10, adjusted with 1N HCl or 1N NaOH), incubation temperature (25-45°C) were evaluated. Moreover, the effect of incorporation of additional carbon sources (maltose, glucose, fructose, soluble starch, xylose, lactose, and sucrose at 1% w/v), nitrogen sources (peptone, yeast extract, malt extract, beef extract, ammonium nitrate, sodium nitrate and ammonium sulphate at 1% w/v) were also studied. All the experiments were conducted in triplicate and the mean values are considered.

Crude enzyme extraction

The crude L-glutaminase was extracted from the fermented solid substrate by using 0.1M phosphate buffer (pH-8). After mixing the fermented substrate with 41 ml of buffer, the flasks were kept on a rotary shaker at 150 rpm for 30 min. The slurry was centrifuged at 10,000 rpm for about 10 min at 4°C in a cooling centrifuge. The clear supernatant was collected and used for enzyme assay. The clear supernatant was collected and used as the crude enzyme for subsequent assays.

L-glutaminase assay

L-glutaminase was assayed according to Imada et al., (1973). The enzymatic reaction mixture contains 0.5ml of L-glutamine (0.04 M), 0.5 ml of Tris-HCl buffer 0.1M (pH 8.0), 0.5 ml of distilled water and 0.5ml of enzyme solution was incubated at 37°C for 30 min. The enzymatic activity was stopped by the addition of 0.5ml of 1.5M Trichloroacetic acid. Then to 3.7ml of distilled water, 0.1 ml of the above mixture and 0.2ml of Nessler's reagent was added and colour developed was read after 10-15 min at 450 nm in a spectrophotometer. One unit (U) of L-glutaminase was defined as the amount of enzyme that liberates 1µ mol of ammonia under optimal assay conditions. Enzyme yield was expressed as the activity of L-glutaminase per grams dry substrate (U/gds).

RESULTS AND DISCUSSION

Solid substrates utilized in solid state fermentation processes are generally insoluble in water and play a dual role - supply of nutrients for the growth of microbial culture growing and anchorage for the growing cells. Unlike bacteria and yeasts that grow by surface adhering to solid substrates, filamentous fungi have the potentiality to deeply penetrate the solid particles for nutrient up taking.

In SSF, the selection of a suitable solid substrate for the fermentation process is a critical factor. In the present investigation, sesamum oil cake has been selected as the solid substrate due to its excellent particle size properties and nutritional composition required for the fungal growth and enzyme formation by the culture. The optimization studies were carried out using sesamum oil cake as the substrate for the production of L-glutaminase throughout the study.

Effect of incubation time:

SSF was carried out for a period of 168h and the flasks were taken at regular intervals of every 24h and the enzyme extraction was done as described earlier. The enzyme production showed growth relatedness as the incubation period progressed and enzyme production was maximum (10.52 U/g dry substrate) was observed after 120 h (i.e. 5days) (Fig.1). After 5 days, the enzyme 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 available nutrient resources or the enzyme might be inactivated by the presence of some kind of proteolytic activity or due to the denaturation of the enzyme protein (Kashyap et al., 2002).

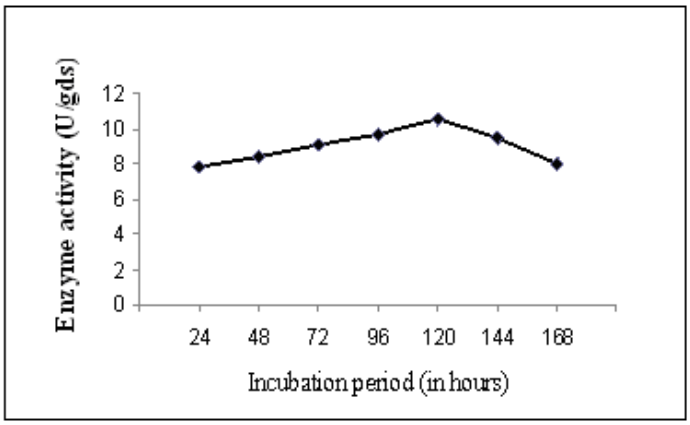

Fig.1: Effect of incubation period on L-glutaminase production by *Trichoderma koningii*

Effect of initial moisture content:

Different initial moisture levels (60-80% v/w) were established in the groundnut oil cake system and the fermentation was carried out for 5 days. Results obtained were shown in Fig.2. The maximum glutaminase yield (13.36 U/g of dry substrate) was achieved with 65% (v/w) initial moisture content. The initial moisture content of any substrate is of utmost importance in a solid-state fermentation system, which controls the growth of microorganisms, metabolite production and enzyme activity. It is reported that higher moisture levels may cause decrease in porosity, alteration in particle structure, enhancement of bacterial growth or low oxygen transfer. Similarly, the moisture level lower than the optimum might be due to the higher water tension, lower degree of swelling and reduced solubility of the nutrients of the solid substrate (Raimboult and Alazard, 1980; Pandey, 1992). Moisture optimization can be used to regulate and to modify the metabolic activity of the microorganism (Pandey et al., 1994).

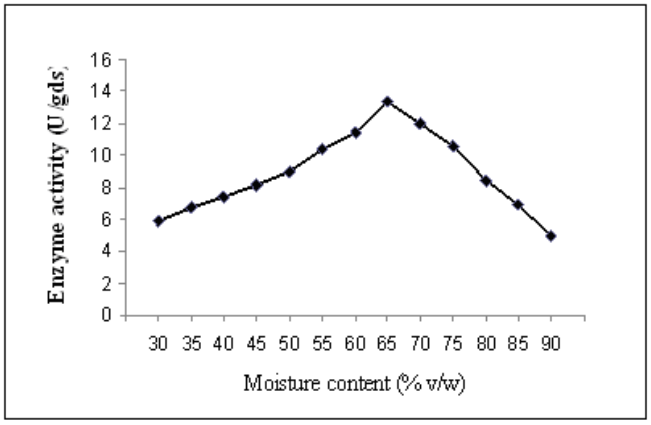

Fig.2: Effect of moisture content on L-glutaminase production by *Trichoderma koningii*

Effect of inoculum volume:

To evaluate the effect of inoculum volume on glutaminase production, different inoculum concentrations (0.5-5.0 ml of 6 day old culture) were added to different flasks. Fermentation was carried out for 5 days and results were shown in Fig. 3. The maximal glutaminase production (14.19 U/g of dry substrate) was observed when an inoculum concentration of 2ml of 6 day old fungal culture was added. At lower and higher inoculum levels, poor glutaminase production was observed. It is very important to provide an optimum inoculum level in fermentation processes. A low inoculum density may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass and deplete the substrate of nutrients or accumulation of some non-volatile self inhibiting substances inhibiting the product formation (Kashyap et al., 2002). With the optimum inoculum concentration, there is a balance between the proliferating biomass and availability of nutrients that supports maximum enzyme production.

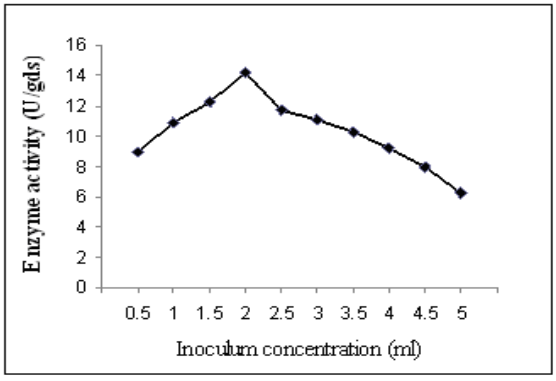

Fig.3: Effect of Inoculum volume on L-glutaminase production by *Trichoderma koningii*

Effect of initial pH:

For these experiments, the pH of the moistening solution adjusted with either 1N HCl or 1N NaOH from 4.0 to 10. Fermentation was carried out for 5 days. Maximum glutaminase production (14. 71 U/g of dry substrate) was found with the substrate moistened with a solution having 7.0 as shown in Fig. 4. This may be attributed the balance of ionic strength of the fungal plasma membrane (Bilgrami and Verma, 1981). Generally, agro-industrial residues possess excellent buffering capacity and that their use offers advantage for SSF processes. However, the pH of the medium strongly affects the growth and activity of the microorganisms. Microbial enzymes are produced in higher yield at a pH near to the maximal for enzyme production. Fungal strains are noted for their best performance in the range of 3.5-7.0 and also low pH avoids the contamination by other microbes (Pandey et al., 2001).

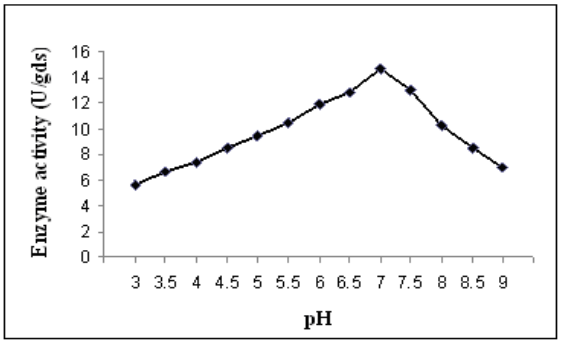

Fig.4: Effect of pH on L-glutaminase production by *Trichoderma koningii*

Effect of incubation temperature:

SSF was carried out at different temperatures ranging from 25-45°C. Samples were extracted after 5 days of fermentation. The organism exhibited a better growth as well as enzyme production at 33°C and it was 15.59 U/gds as shown in Fig. 5. The significance of incubation temperature in development of a biological process is such that it could determine the effects of protein denaturation, enzyme inhibition, cell viability and death (Pandey et al., 2001). In SSF, during fermentation there is a general increase in the temperature of the fermenting medium due to respiration (Pandey and Radhakrishnan, 1992). Heat built-up is in fact a drawback in solid state fermentation process. However, problems of heat and mass transfers are generally severe during the scale-up of SSF. In the present investigation using Erlenmeyer flasks, no such difficulty was observed.

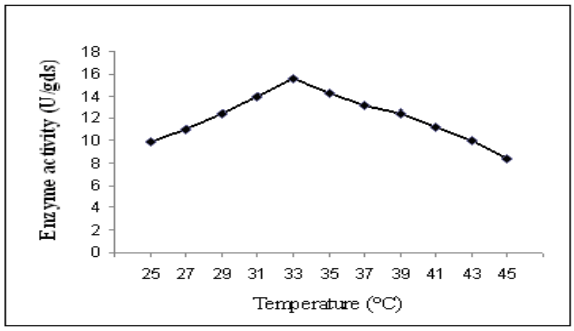

Fig. 5: Effect of Incubation temperature on L-glutaminase production by *Trichoderma koningii*

Effect of carbon sources:

Incorporation of additional carbon sources enhanced enzyme yield from 15.09 to 15.88 U/g of dry substrate (Fig. 6). Among the various carbon sources tested maltose (1% w/v) promoted maximal yield (16.38 U/g of dry substrate) compared to others. Similar results were reported for the production of L-glutaminase by the halophilic *Z.rouxii* under solid state fermentation (Kashyap et al., 2002). The enhanced production of L-glutaminase by incorporation of carbon sources may be attributed to the positive influence of additional carbon sources on enhanced biosynthesis. Optimal glucose concentration was studied by varying the glucose concentration in the medium and maximum enzyme production was observed at the same concentration of 1.0% (w/v).

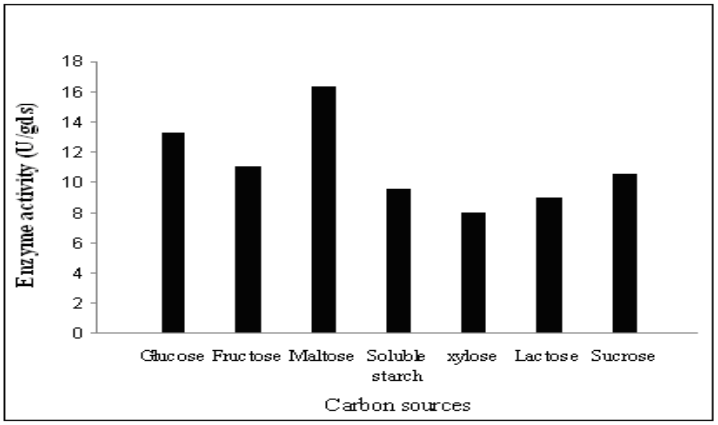

Fig. 6: Effect of different carbon sources on L-glutaminase production by *Trichoderma koningii*

Effect of nitrogen sources:

Among the different nitrogen sources tested, Malt extract (1% w/v) was the best source for maximal enzyme production (19.41 U/g of dry substrate) (Fig.7). Optimal malt extract concentration was studied by varying its concentration in the medium and maximum enzyme production was observed at the same concentration of 1.0% (w/v). Similar results were observed for the production of L-glutaminase by *B.bassiana* under solid state fermentation (Sabu et al., 2000). Probably the presence of additional nitrogen sources in the medium promoted enhanced growth and consequent enzyme production.

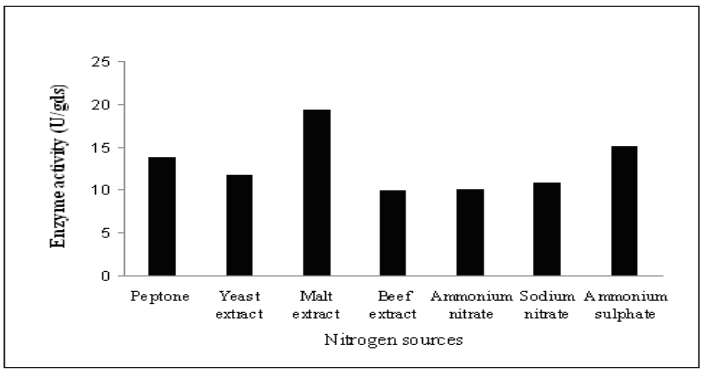

Fig.7: Effect of different nitrogen sources on L-glutaminase production by *Trichoderma koningii*

CONCLUSIONS

Sesamum oil cake , one of the major agricultural byproducts of India that is used as a feed material now finds a different use. Solid-state fermentation on sesamum oil cake is a cheaper and an economically viable bioprocess for the production of L-glutaminase, an enzyme with potent therapeutic and industrial significance. But the yields obtained in the present investigation would have to be further increased for its industrial importance, as it has proved solid state fermentation process as a prospective technique for the large-scale production of microbial metabolites of biotechnological importance.

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