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Optimization of Growth Conditions for Xylanase Production by *Aspergillus niger* in Solid State Fermentation

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Abstract

The objectives of the present study were isolation, identification and characterization of xylanase producing fungi, optimization of medium composition and cultural conditions for xylanase enzyme production using cheaper sources. The fungal strains were isolated from garden soil by serial dilution technique and *Aspergillus niger* was identified and isolated in pure form. In conformation screening by congo red test, based on the reddish zone of enzyme activity formation in oat spelt xylan agar plates, *A. niger* was selected and optimized for xylanase enzyme production in solid state fermentation using cheaper sources like wheat bran, rice bran, soya bran, ragi bran and saw dust. Maximum enzyme activity was observed in wheat bran (9.87 U/ml). The use of wheat bran as a major carbon source is particularly valuable because oat spelt xylan or birch wood xylan are more expensive. The effects of time course, incubation substrate, inoculum size, moisturizing agent, moisture content, temperature and volume of fermentation medium on the production of xylanase were studied. The maximum xylanase production (12.65 U/ml) was observed at optimized condition, incubation temperature of 28°C after 6 days of incubation period while minimum production (9.38 U/ml) at unoptimized condition. The maximum production of enzyme was found to be in wheat bran when the volume of fermentation medium was kept as 10 g/250 ml conical flasks, with mineral solution as moisturizing agent and moisture ratio 1:0.7. Thus the present study proved that the fungal strain *A. niger* used is highly potential and useful for xylanase production.

Key words: *Aspergillus* sp. optimized for production, solid state fermentation, xylanases.

Introduction

Solid state fermentation can be performed on a variety of lignocellulosic materials, such as rice bran, wheat bran, ragi bran, corn cob, soya bran *etc.*, which proved to be highly efficient technique in the production of xylanase (Hoq and Deckwer, 1995; Sonia *et al*., 2005). Solid state fermentation (SSF) is an attractive method for xylanase production, especially for fungal cultivations, because it presents many advantages, such as the higher productivity per reactor volume as well as the lower operation and capital cost (Purkarthofer *et al*., 1993a; Pandey *et al*., 1999). The cost of carbon source plays another major role in the economics of xylanase production. Hence, an approach to reduce the cost of xylanase production is the use of lignocellulosic materials as substrates rather than opting for the expensive pure xylans (Senthilkumar *et al*., 2005). For the development of suitable xylanase as a pre-bleaching agent, the stability of enzyme at higher optimum pH and temperature is desirable (Beg *et al*., 2000).

There are several applications of xylanases in industry (Subramaniyan and Prema, 2002). Xylanolytic enzymes are receiving increasing attention because of their potential applications in improving digestibility of animal feed (Wong *et al*., 1988), pulp bleaching (Viikari *et al*., 2001) and bioconversion of lignocelluloses into feed-stocks and fuels (Kim *et al*., 2000). Principal xylanolytic enzymes are endo-β-xylanases, which attack the main chain of xylans, and β-xylosidases, which hydrolyze xylooligosaccharides into D-xylose. Several studies have shown that the xylanases are co-induced in response to xylan or natural substrates containing hemicellulose or even by pure cellulose (Kadowaki *et al*., 1997). In food industry, the enzyme treatment has favorable effects on dough handing, bread volume, texture and stability (Li *et al.*, 2000; Bhat *et al*., 2001). Other potential applications include the conversion of agricultural waste and the

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production of fuel ethanol (Damaso *et al*., 2003). Xylanases have been produced by a variety of microbes on different sources, which is of great importance and application. In view of this, there is a need to search for new sources of xylanases and their characterization. The present paper reports on the isolation and characterization of xylanase from a fungal source *Aspergillus niger* in SSF.

Experimental

Materials and Methods

Collection of samples. The garden soil samples were collected using pre-sterilized sample bottles and sterile spatula from Bangalore south of India. Precautionary measures were taken to minimize the contamination. The soil was collected from 5 to 6 places in and around Bangalore and the collected soil samples were pooled. The mixed soil sample was taken for isolating xylanase producing fungi.

Isolation of fungi. The xylanase producing fungal strains was isolated by preliminary screening where the dilution plating technique was followed (Montenecourt and Veileigh, 1977).

Identification of fungi. A total of 7 fungal strains were isolated from the mixed soil sample from Bangalore south of India, they are: *Aspergillus niger*, *Trichoderma* sp., *Cladosporium* sp., *Rhizopus* sp., *Fusarium* sp., *Aspergillus* sp. end *Mucor* sp. *Aspergillus niger* being the most predominant was used for xylanase production. Fungi isolated were identified using standard reference manuals (Raper and Fennell, 1965) by wet mount preparation. *A. niger* isolates were preserved on Czapek-Dox agar plates as pure culture (Pitt and Samson, 2000) for further studies.

Secondary screening. *A, niger* isolated from the preliminary screening were cultured in Czapek-Dox broth in Erlenmeyer flasks. After incubation on a rotary shaker (28°C, 180 rpm) for 6 days, the culture broth was filtered using Whatmann filter paper 1and the supernatant was collected for enzyme assay for xylanase production. Its ability to produce xylanase enzyme was further confirmed by congo red test by growing *A. Niger* on oat spelt xylan agar plates. After incubation plate was treated with congo red and washed with 1 M NaCl. (Teater R.M. and Wood P.J., 1982). This confirmed *A. niger* xylanase producing strain were preserved on Czapek-Dox agar plates as pure culture for further studies.

Xylanase production, assay of xylanase and optimization were carrried out using the selected strain in triplicates

Xylanase production in solid state fermentation. *A. nige*r culture was grown on Czapek-Dox agar plate at 28°C for 7 days. Using sterile distilled water fungal spore suspension was prepared. Erlenmeyer flasks (250 ml) containing 10 g of wheat bran was taken to which 10 ml of distilled water was added as moisturizing agent. The flasks were then inoculated with 2 ml of spore suspension and the flasks were incubated at 28°C under static conditions for 6 days. The enzyme from each flask was extracted using 50 ml of distilled water and filtered through Whatmann filter paper 1. The clear supernatant was collected for enzyme assay for xylanase production (Deschamps and Huet, 1985).

Solid state fermentation (SSF) is currently used only to a small extent for enzyme and secondary metabolites production because of severe process engineering problems. A scale-up of solid state fermentation seems to be difficult due to the generally known problems of heat transfer, the fact that media is not homogenous and difficulties with aeration (Purkarthofer *et al.*, 1993b).

Xylanase assay. Xylanase activity was assayed by using oat spelt xylan (Fluka) as enzyme substrate. The reaction mixture contained 0.5 ml of substrate solution of 0.25 g of xylan wetted with 2 ml 95% ethanol and 22.5 ml of distilled water in a beaker and covered with alluminium foil and boiled to dissolve on hot plate at 100°C for 10 min, and make up the volume to 25 ml with distilled water. To this solution, 0.1 ml of the culture filtered supernatant crude enzyme and 1ml sodium acetate buffer was added. The mixture was incubated at 40°C in water bath with shaking for 15 min. Released reducing sugar was measured using 3, 5-dinitrosalicylic acid (DNSA) (Miller, 1959) and glucose as standard. Colour was developed by boiling in water bath for 5 min and read, using spectrophotometer at 540 nm. One unit of activity was defined as amount of enzyme required to liberate 1 µmol of glucose or xylose per minute under the assay conditions (Maria *et al.*, 2006).

Optimization. The optimization of the growth conditions was carried out based on stepwise modification of the governing parameters for xylanase production. The effect of various substrates for SSF, consisting of rice bran, wheat bran, ragi bran, corn cob, soya bran *etc.*, and sterile distilled water as moistening agent was examined. Cultivation was carried out at ambient temperature (28 ± 3°C) for 6 days (Ghosh *et al*., 1993).

The effect of cultivation temperature on the enzyme production was examined at different temperatures starting from 25 to 60°C for enzyme assay and 28°C, 32°C, 37°C, 40°C for growth (Lenartovicz *et al*., 2002). The effects of incubation period were evaluated by 24 h interval by checking the enzyme activity (Bakri *et al*., 2003). The effect of moisture level on the enzyme production was determined by

varying the ratio substrate wheat bran to moistening agent at the ratio of 1:0.5, 1:0.75, 1:1.0 and 1:1.25. (Pang Pei Kheng and Ibrahim, 2005). The moistening agent used was sterile distilled water. The effect of mineral salts solution and tap water as moistening agents on xylanase production was also determined as comparison to that of distilled water. (Pang Pei Kheng and Ibrahim, 2005).

Optimization of lodage of substrate for xylanase production was carried out using wheat bran as substrate and distilled water as moistening agent at 1:1 ratio. The different volumes of lodage used were 5 grams, 10 grams and 15 grams. (Cai-qin Liu *et al*., 2007). Optimization of inoculum size for xylanase production was carried out by adding approximately 100 to 150 conidia of *A. niger* to 9 ml of sterile water blank. This suspension was serially diluted to 10^{-1} to 10^{-6}. 1 ml of the diluted samples from each dilution was transferred to sterile media and incubated at 28 ± 2°C for 5–7 days. Optimization of growth conditions for xylanase production by *A. niger* in Czapek-Dox broth before and after optimization considering the above parameters were also carried out. (Pang Pei Kheng and Ibrahim, 2005).

All the above readings obtained at 540 nm absorbance were represented in the graphical form.

Result and Discussion

Identification of fungi was done based on colony characters and microscopic examination. Further work was carried out with the most predominant fungi being *A. niger.* (congo red test forming reddish orange halozone of hydrolysis) *A. niger* xylanase producing strain (in hydrolysin) were preserved on Czapek-Dox agar plates as pure culture by point inoculation for further studies. Standard glucose was estimated by DNSA method at 540 nm for the further enzyme activity.

The time course of xylanase production was investigated and maximum production was observed after 6 days (6.11 U/ml) while minimum was noted at 24 h (0.66 U/ml) (Figure 1). Further incubation after

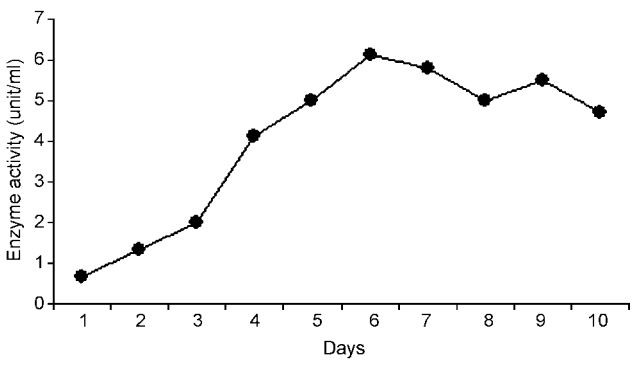

Fig. 1. Effect of incubation time on xylanase production.

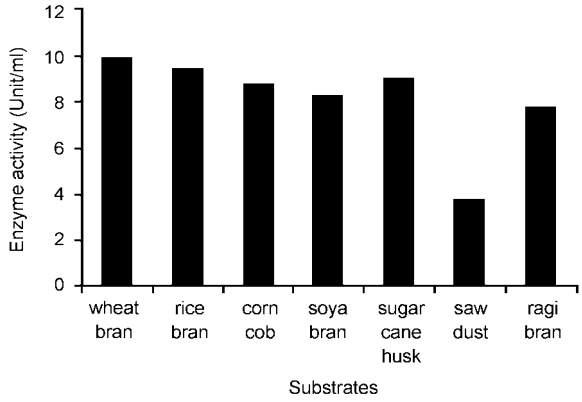

Fig. 2. Effect of substrates on xylanase production.

this did not show any increment in the level of enzyme production, probably due to increase in toxic unwanted wastes and depletion of nutrients in the media, which leads to decreased growth and enzyme.

Solid state fermentation (SSF) is a popular method to produce xylanases by fungi. SSF means that the microorganism grows on moist solid substrates in the absence of free-flowing water. When different substrates were used in the solid state fermentation medium, the highest enzyme activity was obtained in wheat bran (9.87 U/ml) and minimum at saw dust (3.75 U/ml). Wheat bran is an inexpensive by product, which contains a lot of xylan. Therefore, it is one of the most popular components of complex media for xylanase production when compared to rice bran. The other substrates did not show any significant enzyme activity when compared to wheat bran. (Figure 2) However, the wheat bran particles suspended in the cultivation medium have to be decomposed to form soluble compounds to be used by the fungus and also to protect the fungal mycelium from the shear forces. Several substances have been indicated in the literature as suitable carbon sources for xylanase producing microorganisms, oat wheat (Carmona *et al*., 1998), birchwood xylan (Duarte *et al*., 1999), oat spelt xylan (Chivero *et al*., 2001), bagasse xylan, wheat bran arabino-xylan (Batailon *et al*., 2000), wheat bran (Fujimota *et al*., 1995; Gawande and Kamat, 1999), and rice bran (Dhillon *et al*., 2000). However, *Bacillus licheniformis* shows lower xylanase production in wheat bran (Archana and Satyanarayana, 1997; Zychlinski *et al*., 1994).

The cultivation temperature does not only affect the growth rate of an organism, but it also has a marked effects on the level of xylanase production. *A. niger* grew well at 28°C (8.98 U/ml) when cultivated in Czapek-Dox media, but xylanase production was significantly decreased at higher temperature (2.64 U/ml at 40°C), (Haltrich *et al*., 1996). The net temperature is influenced not only by the environmental temperature, but also by the increase in temperature generated from

the metabolic activities of the fungi growing on the solid substrates. It might be due to that at higher or lower temperature than optimum, the growth of the fungus was inhibited and hence the xylanase production was also decreased (Yaun and Rangyu, 1999; Rahman *et al.*, 2003).

Optimization of the volume/lodage of fermentation medium is very important for air supply, nutrient supply, growth of microorganism and production of enzyme (Mimura and Shinichi, 1999). The results revealed that 10 g dry matter of medium contributed to higher enzyme activity than other dry matter weight of medium, and that the maximum reached 10.12 U/ml of dry matter. As the volume of the fermentation medium increased, the production of enzyme decreased. It might be due to the reduction in the agitation of medium, decrease in air and mineral supply and subsequent decreased growth of the organism. Similarly, at low level of fermentation medium, the production of enzyme was also decreased. It might be due to not sufficient nutrient supply in the fermentation medium for good growth of the *Aspergillus niger* and hence enzyme formation.

Solid substrates used in SSF are insoluble in water therefore water will have to be absorbed onto the substrate particles, which can be used by the microorganisms for growth and metabolic activity (Pandey, 1992). It is also expected that the rate of water absorbed by different substrates vary from one substrate to another. This is another possible explanation for the variation in the xylanase production using different substrates. Thus, it is concluded that the degree of hydration of the substrate plays an important role on the growth of the fungi and subsequently the enzyme production. Besides distilled water, other moistening agents consisting of mineral salt solution and tap water were also examined. Mineral salts solution and tap water resulted in higher xylanase production compared to distilled water. However, the enzyme production using tap water of 8.73 U/ml showed no significant difference from that of using the mineral salts solution of 8.98 U/ml. Thus, tap water was chosen as the moistening agent for economical reasons. Water causes the swelling of the substrate and facilitates good utilization of substrates by the microorganisms. Increasing moisture level is believed to have reduced the porosity of substrate, thus limiting the oxygen transfer into the substrate. Likewise, a lower moisture ratio leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling and a higher water tension (Ikasari and Mitchell, 1994). Initial moisture content is one of the key factors increasing xylanase production. The results in Figure 3 shows highest xylanase activity obtained with initial moisture content of 75% with 1:0.75 ratio (9.38 U/ml). The moisture content of the medium has critical importance to SSF. Many researchers have reported the similar examples of moisture content on xylanases production (Ferreira *et al*., 1999). This could be attributed to the faster growth of microorganism at higher moisture content and the subsequent early initiation of the enzyme production (Kalogeris *et al*., 1998). As reported elsewhere, high moisture enhanced fungal growth and xylanases production when lignocellulosic substrates were the carbon sources in SSF (Alam *et al*., 1994).

Incubation temperature for enzyme substrate reaction plays a critical role in enzyme productivity (Seyis and Aksoz, 2003). So, the enzyme activity in present studies was investigated at different temperatures ranging from 25–60°C (Figure 4). The *A. niger* gave promising results, with maximum activity of xylanase, when incubated at 45°C (16.00 U/ml) and minimum was observed at 25°C (7.33 U/ml). When the temperature increased or decreased from 45°C, the activity of xylanase gradually reduced. Probably due to enzyme denaturation, conformation change, as enzymes are proteins.

The effect of inoculum size was examined using the spore suspension of concentration from 10^{-1} to 10^{-6} dilution for a fermentation period of up to 6 days. The results of the enzyme productivity profiles at different inoculum sizes are shown in Figure 5. Higher inoculum size of 10^{-1} dilutions resulted in a higher xylanase productivity compared to other inoculum

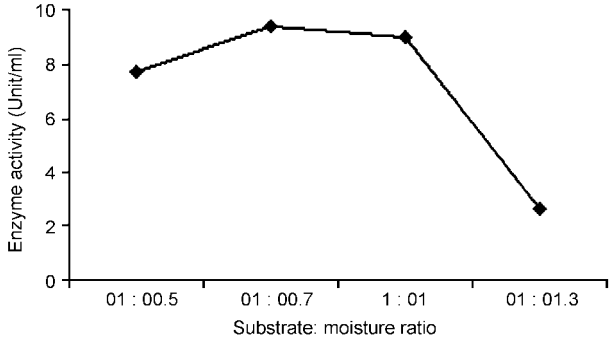

Fig. 3. Effect of moisture content on xylanase production.

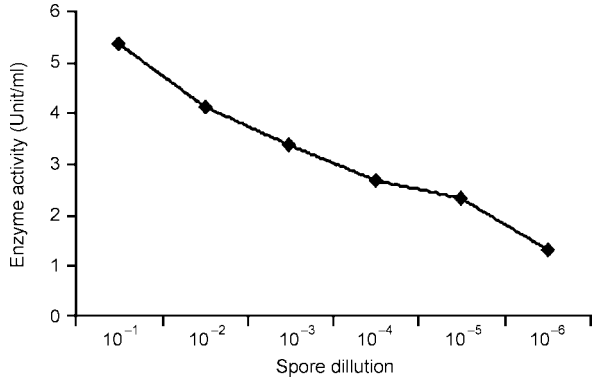

Fig. 5. Effect of inoculum size on xylanase production.

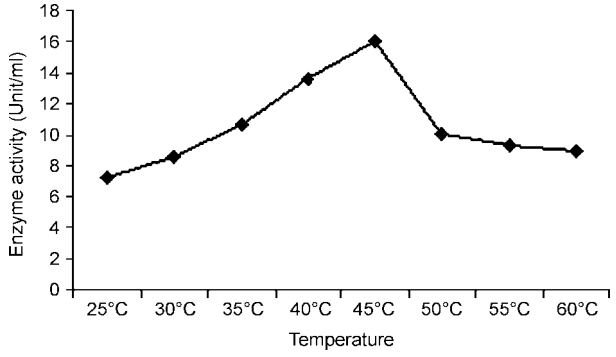

Fig. 4. Effect of temperature on xylanase enzyme activity.

sizes with the maximum productivity of 5.37 U/ml. At lower inoculum sizes, it was observed that the time taken to achieve maximum growth or enzyme productivity was much longer. This is clearly shown that with the inoculum size of 10^{-6} dilutions, the enzyme has not achieved maximum productivity even after 6 days of fermentation. Higher enzyme production at higher inoculum is related to the rapid growth of the fungus, which resulted in higher degradation of the substrates and increases availability of the nutrients.

The results show the profiles of the xylanase production by *A. niger* before and after optimization of the cultural conditions and medium composition. Xylanase production is higher (12.65 U/ml) in optimized conditions when compared to unoptimized (9.38 U/ml) conditions. This confirms that the isolated *A. niger* is a good producer of xylanase when compared to *Aspergillus casiellus*, *Aspergillus awamori, Aspergillus ochraceous, Aspergillus sydowii* and *Aspergillus fischeri* (Judith and Nei, 2002).

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