OPTIMIZATION OF XYLANASE PRODUCTION FROM FREE AND IMMOBILIZED CELLS OF *FUSARIUM SOLANI* F7

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The aim of the present investigation was to characterize a xylanase-producing *Fusarium solani* isolate and to optimize cultural conditions for xylanase enzyme production from free and immobilized cells. Screening of *Fusarium solani* isolate was based on the diameter of the clear zone formation in oat spelt xylan agar plates. *Fusarium solani* isolate F7 was selected and optimized for xylanase enzyme production using cheaper substrates such as wheat straw, rice straw, rice bran, and wood husk. Maximum enzyme activity was observed in wheat straw (78.32 U ml-1 for free cells and 94.68 U ml-1 for immobilized cells). Optimum pH and temperature for xylanase activity were found to be 5.5 and 30°C at 3% substrate concentration for free cells and 5.0 and 30°C at 3% substrate concentration for immobilized cells. In the purification step, 75% ammonium sulphate saturation was found to be suitable, giving maximum xylanase activity. Production of xylanase was greater from immobilized cells than from free cells. Purified xylanase from free cells yielded a single band with a molecular weight of 89kDa, while it was 92.8kDa for immobilized cells. The use of wheat straw as a major carbon source is particularly valuable, because oat spelt xylan is very expensive. The *Fusarium solani* F7 isolate proved to be a promising microorganism for xylanase production.

Keywords: Xylanase; Fusarium solani; Free cells; Immobilized cells

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INTRODUCTION

Xylans are the major hemicelluloses in wood from angiosperms, where they account for 15-30% of the total dry weight. In gymnosperms, however, xylans contribute only 7-12% of the total dry weight. The structure of xylans is complex, and their complete biodegradation requires the concerted action of xylanase enzymes. Xylans are heterogeneous polysaccharides with a backbone consisting of beta-1,4 linked D-xylosyl residues. Endo-beta-1,4 xylanases are the main enzymes responsible for cleavage of the linkages within the xylan backbone (Amani et al. 2007). To date xylanases have gained increasing attention because of their various biotechnological applications, and they have great potential for industrial applications, mainly for the bioconversion of lignocelluloses to sugar, ethanol, and other useful substances (Biely 1985; Viikari et al. 1994; Beg et al. 2001). They are extensively used in pre-treatment of forage crops and other

lignocellulosic biomass, added to swine and poultry cereal-based diets to improve nutrient utilization, flour modification for bakery products, and saccharification of agricultural, industrial and municipal wastes (Sá-Pereira et al. 2002). Moreover, it is reported that xylanases have been widely used for clarifying fruit juices and wine (Hang and Woodams 1997), and in the food processing industries, for the production of several valuable products like xylitol and ethanol (Salles et al. 2005), and for improving the nutritional properties of agricultural silage and grain feed (Kuhad et al. 1993).

To reach commercial feasibility, enzyme production must be increased by introducing a more potent strain and by optimizing culture conditions. Xylanases are produced by numerous microorganisms, among which the fungi are the most potent producers (Pham et al. 1997). Haltrich et al. (1996) gave an overview of fungal xylanases and showed that the enzyme can be produced by a number of microorganisms, including bacteria, yeasts, and filamentous fungi such as *Trichoderma, Aspergillus, Penicillium, Fusarium, Chaetomium, Humicola, Talaromyces*, and many others. *Fusarium oxysporum* and other *Fusarium* sp. have been shown to be promising organisms for enhanced production of xylanases (Chirstakopoullos et al. 1999). Extracellular enzymes are considered important from an industrial viewpoint, as they ease the extraction procedure. Immobilized enzymes are also used in food technology, biotechnology, biomedicine, and analytical chemistry. They have various advantages over free enzymes, including easy separation of the reactants, products, and reaction media, easy recovery of the enzyme, and repeated or continuous reuse. Initially, most work was carried out on the immobilization of bacterial cells. Eventually, yeasts and filamentous fungi received increasing attention (Anderson 1975). Recently, there has been an appreciation of the potential of immobilized fungal cells for the production of enzymes (Fiedurek and Ilczuk 1991).

The use of purified xylan as a substrate to induce xylanase synthesis increases the cost of enzyme production. Therefore, for commercial applications, there have been attempts to develop a bioprocess to produce xylanase in high quantities from simple and inexpensive substrates. Wheat straw, wheat bran, and corncob have been shown to be efficient substrates in the production of xylanase, and the enzyme production is related to the type and concentrations of nutrients and growth conditions (Alam et al. 1994; Hoq and Deckwer 1995; Haltrich et al. 1996; Gawande and Kamat 1999; Kang et al. 2004; Sonia et al. 2005). Filamentous fungi are interesting producers of this enzyme from an industrial point of view due to extracellular release of xylanases, higher yield compared to bacteria and yeast, and production of several auxiliary enzymes that are necessary for debranching of the substituted xylanase (Haltrith et al. 1996).

Thus, since there have been fewer reports on efficient xylanase production by the fungus *Fusarium solani*, therefore an attempt has been made in this study to provide prospective information on optimization process for xylanase production from free and immobilized cells of Indian isolates of *Fusarium solani*.

EXPERIMENTAL

Isolation of *Fusarium solani* Isolate

The soil samples for the isolation of *Fusarium solani* were collected from the different nearby areas of Faizabad region, U.P., India (Lat. 26° 47' N ; Long. 82° 12' E). The *Fusarium solani* cultures were isolated from soil samples by the serial dilution method (Clark et al. 1958). The study was carried out at Microbiology Laboratory, Dr. R. M. L. Awadh University, Faizabad-224001, U.P., India during 2007-2008.

Identification of *Fusarium solani* isolate was based on cell and colony morphology characteristics as per the method described by Booth (1971). The young colonies of *Fusarium solani* were aseptically picked up and transferred to PDA slants and incubated at 28±2ºC for 4-5 days for maximum growth.

Screening for Xylanolytic Activities

The fungal isolates formed were subcultured to purity and examined for xylanolytic activities. Screening for xylanolytic activities was performed on malt extract agar (MEA) containing 0.1% (w/v) of xylan from oat spelt. Positive xylanolytic isolates were detected based on the clear zones of hydrolysis on the xylan. The potential isolates were subcultured and maintained on MEA slants. The slants were stored at 4ºC prior to use.

Immobilization of *Fusarium solani* Cells

Fusarium solani cells were immobilized and enzyme was isolated as per the method described by Gonçalves et al. (1996).

Xylanase Production Medium

Erlenmeyer flasks (250 ml) containing 10 g of wheat straw were added with the Mandels and Sternburg's basal medium (Mandels and Sternburg 1976) just to wet the wheat straw. The Mandel's medium was prepared with the following composition ($g.L^{-1}$) 10.0g; urea, 0.3; peptone, 0.75; yeast extract, 0.25; $(NH4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3 and trace elements (mg. L^{-1}): $FeSO_4.7H_2O$, 5; $MnSO_4$. $4H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4 and $CoCl_2.6H_2O$, 20.0 and Tween 80 0.1% (v/v) (pH 5.0). The flasks were inoculated with 2 ml of spore suspension prepared from a week old PDA slants of the culture grown at 30°C. Sterile distilled water containing 0.1% (v/v) Tween 80 was used to prepare fungal spore suspension. Inoculated flasks were incubated at 30°C under static conditions for 10 days.

Inoculum and Fermentation

An inoculum size of conidia (Each ml of cells suspension contained 2.0 X 10^6 cells) was transferred from a stock culture in a 250 ml flask containing 50 mL of growth medium. The flasks were incubated for 72 hrs at $28^oC \pm 2^oC$ on a rotatory shaker at 150 rpm.

Preparation of Enzyme

The enzyme from each flask was extracted using 50 ml of 0.05M citrate buffer (pH 5.3) and filtered through a wet muslin cloth by squeezing. The extract was centrifuged at 5000 rpm for 20 min. The clear supernatant was partially purified by ammonium sulphate fractionation at 4^0C to achieve 75% saturation and dialysed using the same buffer for 24 h with three intermittent changes.

Xylanase Assay

The supernatant was used as a source for enzyme sample. Xylanase activity was measured with the optimized method described by Bailey et al. (1992), using 1% oat spelt xylan, (Himedia, Mumbai, India) as the substrate. The solution of xylan and the enzyme at appropriate dilution were incubated at 55 °C for 7 min, and the reducing sugars were determined by the dinitrosalicylic acid method described by Miller (1959), with xylose as standard. The released xylose was measured spectrophotometrically at 540 nm. One unit of xylanase was defined as the amount of enzyme required to release 1 µmol of reducing sugar as xylose equivalent per min under the above assay conditions. All experiments were repeated thrice.

SDS-PAGE of Xylanase Enzyme

The cell free supernatant of crude enzyme was essayed by means of sodium dodecyl sulphate-poly acrylamide gel electrophoresis for protein profiling to confirm the molecular weight of xylanase enzyme.

Optimization of Process Parameters

The optimization of composition of medium and cultural conditions was carried out based on stepwise modification of the governing parameters for xylanase production. The effects of various substrates, consisting of rice straw, wheat straw, rice bran, and wood husk were examined by adding 10 g of each substrate in a 250 ml Erlenmeyer flask with 10 ml of sterile distilled water, which was added to moisten the substrates. Cultivation was carried out at ambient temperature (28 ± 2^{o}C) for 7 days. By changing the pH from 3 to 8 in the production medium, the effect of pH was observed. The effect of cultivation temperature on the enzyme production was examined at different temperatures starting from 25 to 50^{o}C with 5^{o}C intervals. The effects of incubation period were evaluated at 24 h intervals by checking the enzyme activity. The optimization was carried out by adding standardized concentration wheat straw using supplemental carbon sources such as glucose, sucrose, galactose, starch, dextrin, xylose, sorbitol, corboxymethyl cellulose, fructose, as well as nitrogen sources such as peptone, urea, yeast extract, sodium nitrate, ammonium sulphate, ammonium nitrate, and beef extract.

RESULTS AND DISCUSSION

Applications of xylanases are widely dispersed in the paper and pulp industries as well as in the food and pharmaceutical industries. In the present investigation, purification of xylanase was carried out. The molecular weight of the single protein band

was calculated to be about 89 kDa for xylanse enzyme isolated from free cells and 92.8kDa for enzyme isolated from immobilized cells of *Fusarium solani* (Fig. 1). Microbial xylanases are single subunit proteins with molecular masses within the range of 8 - 145 kDa (Kulkarni et al. 1999). In a previous study, a purified xylanase enzyme preparation also showed a single protein band on sodium dodecyl sulphate-poly acrylamide gel electrophoresis, and the molecular weight of this enzyme was found to be 24 kDa (Sardar et al. 2000).

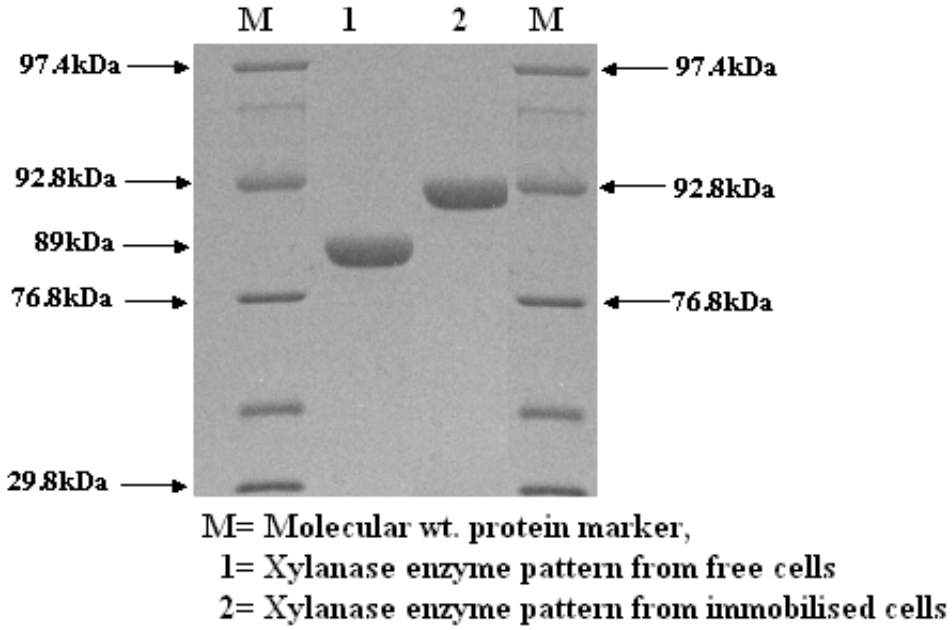

Fig. 1. Purification of xylanase from *Fusarium solani* F7

Selection and Identification of Isolate *Fusarium solani*

Based on the screening programme, a total of 16 isolates were capable of exhibiting xylanolytic activities on MEA-xylan agar, with the diameters of the clear zones ranging from 34 mm to 48 mm. However, 6 isolates identified as F2, F5, F7, F11, F13, and F15 were selected for further confirmation using the MEA-xylan agar plates. The isolates F7 demonstrated reproducible zones of hydrolysis of 48 mm diameter, and this isolate was selected as the potential producer of xylanase. Isolate F7 was identified based on the structural morphologies as observed under the light and scanning electron microscopes. It was observed that the isolate possessed distinct macro and micro conidia formation. Macro-conidia were of 3-5 celled slightly curved at the pointed ends, typically canoe shaped, whereas micro-conidia were 1-celled and ovoid. Based on these characteristics, isolate *Fusarium solani* isolates were identified to be *Fusarium solani* (Booth 1971), and isolate F7 was used for further investigation for optimization of xylanase producing property.

Effect of Incubation Period

The xylanase activity was determined after every 24 hours of incubation in order to determine the optimum incubation period for maximum production of xylanase. The enzyme production however, started after 24 hour of inoculation and showed maximum production (56.31 U ml^{-1}) on the 6^{th} day of the incubation period at $28^oC \pm 2^oC$ from free cells and 72.42 U ml^{-1} on 5^{th} day from immobilized cells of *F. solani* (Fig. 2). High xylanase production has been shown to be linked strictly due to time course or incubation period (Haltrich et al. 1996; Chirstakopoullos et al. 1999; Kang et al. 2004).

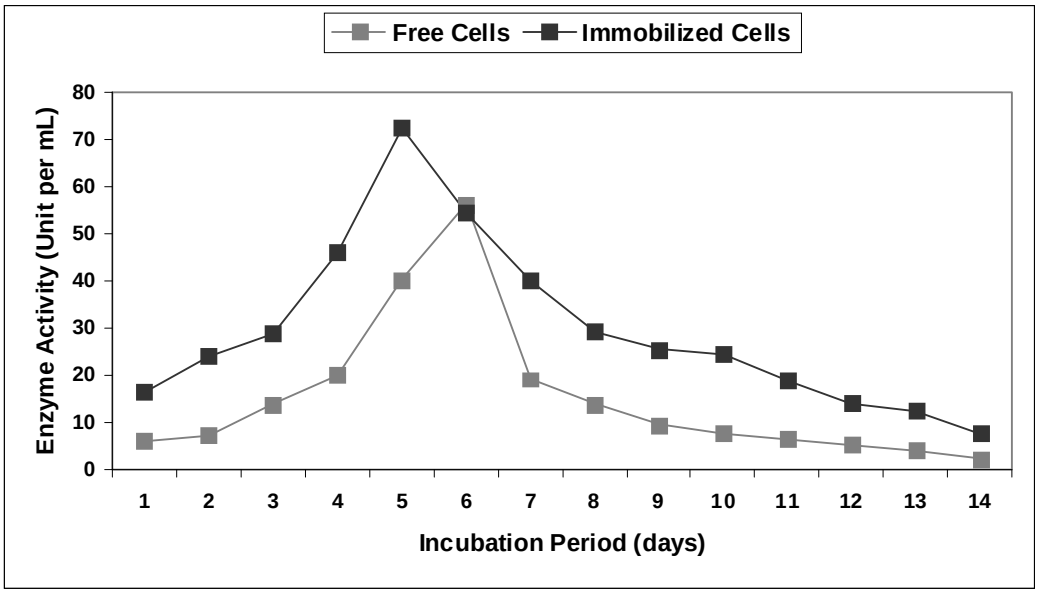

Fig. 2. Effect of incubation period on xylanase production by free and immobilized cells

Effect of Temperature

Temperature is one of the important parameters that determine the success of an optimization system. Therefore, the effect of temperature on xylanase production by *Fusarium solani* F7 was examined within the temperature range 20 to 50^oC for 72 hrs, and the results obtained are shown in Fig. 3. The production of xylanase was maximum at the temperature near to ambient temperature (30^oC) with an activity of 58.8 U ml^{-1} and 69.88 U ml^{-1} from free and immobilized cells, respectively. A lower activity was recorded with cultivation temperatures lower or above the ambient temperature. The results obtained indicated that the enzyme production corresponded closely to temperature optima close to room temperature, i.e. 28^oC. This observation was in agreement with those reported by Chirstakopoullos et al. (1999) and Biswas et al. (1988, 1990), who showed that the highest xylanase activities were obtained at temperatures that were optimum for the growth of the *Fusarium solani* F7 fungi at room temperature. Thus, ambient temperature, which was the optimum temperature for xylanase production, was similar to the temperature of the natural habitat of the fungus where it was initially isolated.

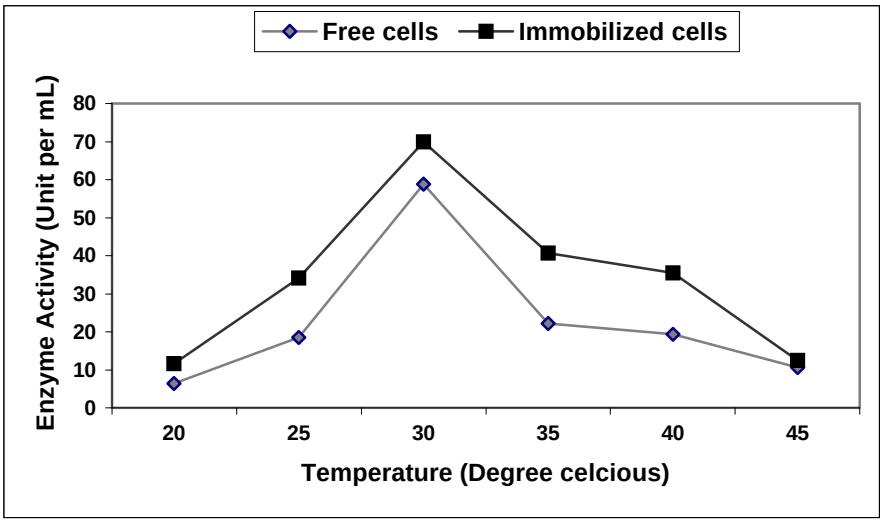

Fig. 3. Effect of temperature on xylanase production by free and immobilized cells

Effect of pH

The initial pH of the medium was adjusted to variable pH values within the range by adding 0.1N HCl. Purified enzyme was tested in the pH range between 3 and 8. The production of xylanase was found to be the best at pH 5.0 for immobilized cells and pH 5.5 for free cells. Below and above this pH production of xylanase was significantly lower (Fig. 4).

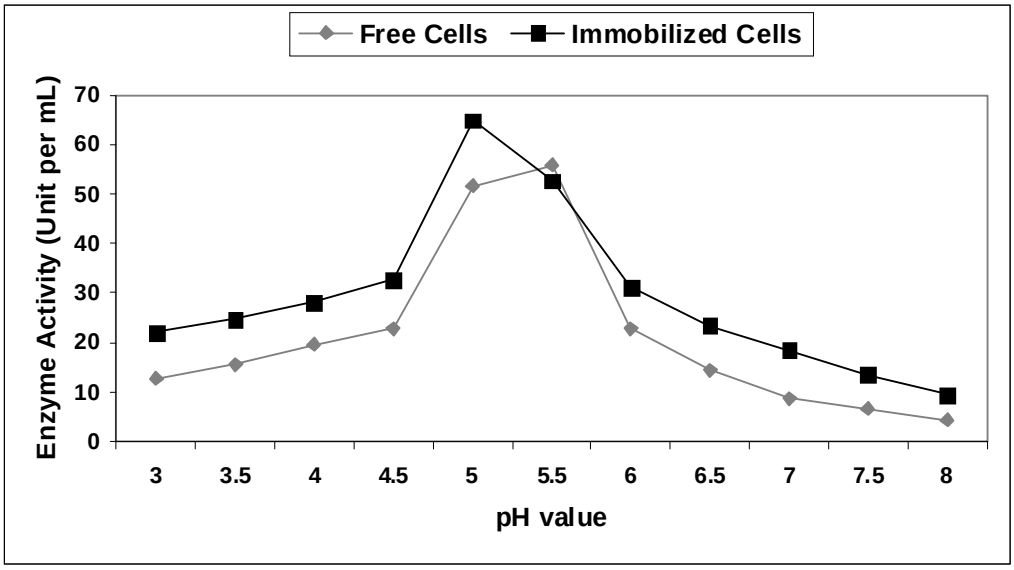

Fig. 4. Effect of pH on xylanase production by free and immobilized cells

The results showed that xylanase production by *Fusarium solani* F7 was highly dependent on pH, and the optimum initial pH was between pH=5.0 and 5.5. However, when the pH was increased or decreased to values other than 5.0 and 5.5, the production of xylanase gradually decreased. This might be due to the fact that alkaline pH has an

inhibitory effect on the growth of *Fusarium solani* F7 (an acidophilic fungi) and enzyme production. Xylanases from different organisms show an optimum pH within a range of 4.0-7.0. However, certain xylanases from *Aspergillus kawachii, Penicillium herque*, and some other fungi including *Fusarium oxysporum* exhibit an optimum pH more on the acidic side (pH 2.0 - 6.0) (Funaguma et al. 1991; Ito et al. 1992; Kulkarni et al. 1999). Endoxylanase I and II from *Aspergillus awamori* show an optimum pH at 5.5 – 6.0 and 5.0, respectively (Kormelink et al. 1992).

Effect of Substrates and their Concentration

Standard xylanase activities (52.81 U ml^{-1} and 65.64 U ml^{-1} for free and immobilized cells, respectively) were obtained in the medium containing 1% wheat straw after 144 h of incubation in comparison with rice straw, rice bran, and wood husk (Figs. 5 and 6). In order to determine the best amount of wheat straw for xylanase production, different concentrations (1–6 % by mass per volume) were tested.

The results showed that the highest yield of xylanase was 78.32 U ml^{-1} for free cells, while it was 94.68 U ml^{-1} for immobilized cell with 3% wheat straw (Figs. 5 and 6). Increasing the concentration by more than 3% resulted in a significant decrease in xylanase activity. This might be attributed to the fact that high concentration of substrate led to the increase in medium viscosity, which influenced the mixture medium components and oxygen transfer. Similar results were obtained by other researchers by using high concentrations of lignocellulosic materials as substrates for enzyme production (Cao et al. 2008). Wheat straw and wheat bran have been known for being ideally suitable for xylanase production in *T. aurantiacus* and *Penicillium citrinum* cultures (Kalogeris et al. 1998; Nair et al. 2008).

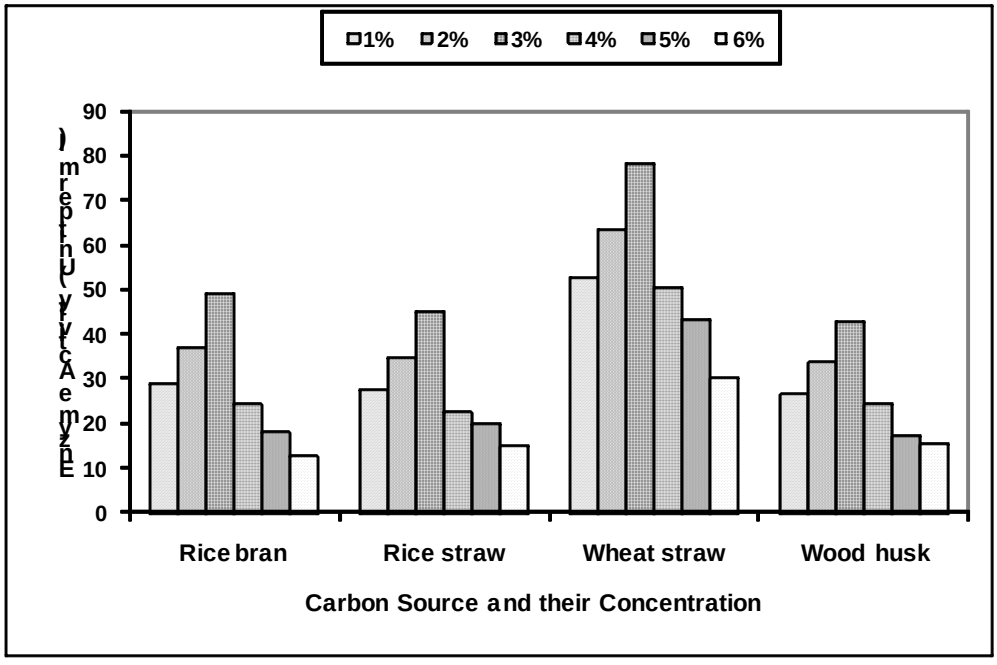

Fig. 5. Production of xylanase from free cells as influenced by substrates and their concentration

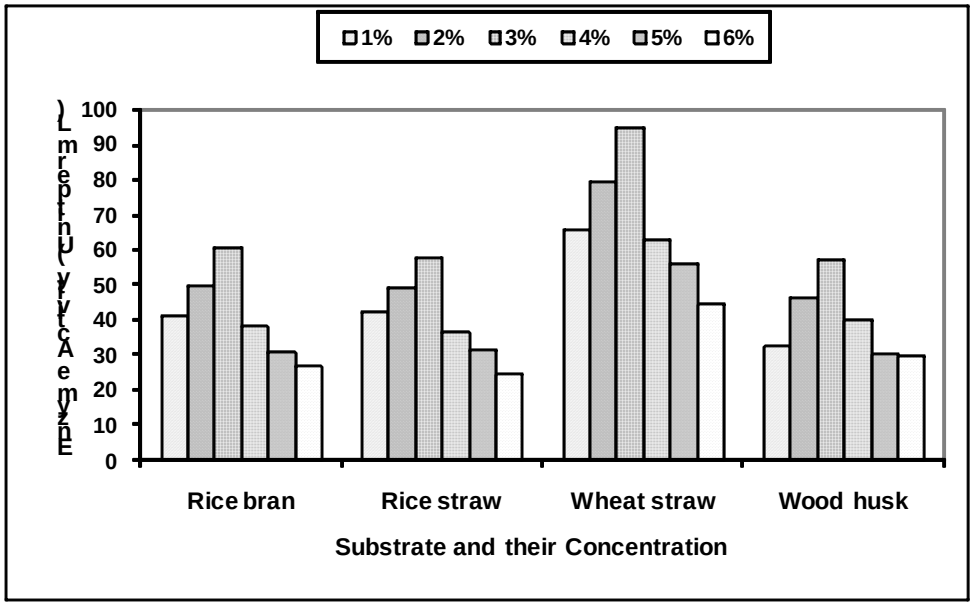

Fig. 6. Production of xylanase from immobilized cells as influenced by substrates and their concentration

Effect of Supplemented Carbon Source

The production of primary metabolites by microorganisms is highly influenced by their growth, which is determined by the availability of the nutrients in the substrates. Therefore, it is expected that the improvement of the nutritional value of wheat straw by the supplementation of carbon will also improve the growth of *Fusarium solani* F7 and subsequently the enzyme production. Flasks containing production media supplemented with carbon sources (glucose, sucrose, starch, carboxymethyl cellulose, fructose, sorbitol, xylose, galactose, and dextrin) were tested for the influence of carbon sources at 1% concentration. Figures 7 and 8 show the supplementation of sugars, which may act either as carbon sources or inducers. Starch, sucrose, dextrin, fructose, sorbitol, and galactose were moderate carbon source for xylanase production. Carboxymethyl cellulose was a poor source of xylanase (Figs. 7 and 8). As shown in the figures, the addition of xylose resulted in an increment (42% and 51% from free cells and immobilized cells respectively) in xylanase production from other supplemented carbon sources. These findings confirmed that the size of carbon source was an important factor in xylanase production (Kalogeris et al. 1998).

Xylose has been described as an effective inducer and carbon source for xylanase production in several microorganisms for xylanase production, including *A. pullulans* (Priem et al. 1991), *Fusarium oxysporum* (Chirstakopoullos et al. 1999), and *T. lanuginosus* (Purkarthofer et al. 1993). Xylan is costly for large-scale production of xylanases, whereas lignocellulosic materials can be used as cost-effective substrates for xylanase production (Haltrich et al. 1996; Beg et al. 2000). Various lignocellulosic materials and microbial cultures have been used successfully in solid-state fermentation for xylanase production (Topakas et al. 2003; Sonia et al. 2005). The significant difference in xylanase titers, when wheat straw was used as the carbon source, may be

attributed to its hemicellulose nature and favorable degradability, due to the presence of some nutrients in the carbon source (Sonia et al. 2005).

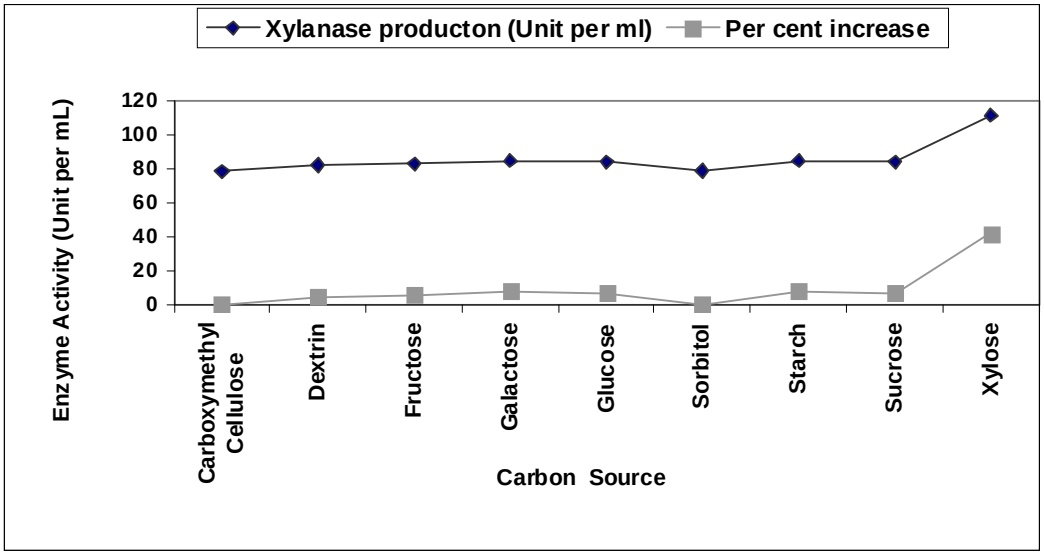

Fig. 7. Production of Xylanase from free cells as influenced by carbon sources with 3% wheat straw as substrate

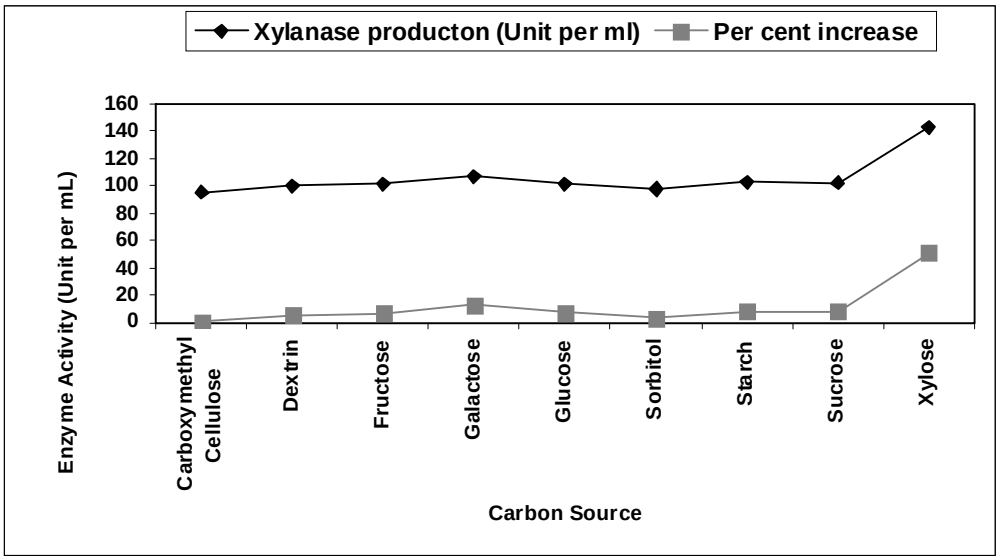

Fig. 8. Production of Xylanase from immobilized cells as influenced by carbon sources with 3% wheat straw as substrate

Effect of Supplemental Nitrogen Source

Effects of different nitrogen sources viz. peptone, urea, yeast extract, sodium nitrate, ammonium sulphate, ammonium nitrate, and beef extract on the production of xylanase were studied. It was observed that ammonium sulphate, yeast extract, and beef extract caused poor enzyme production. Peptone and sodium nitrate supported maximum production of enzyme, whereas ammonium nitrate and urea produced considerable

amounts of xylanase. The optimum concentration of peptone was 0.3% (Fig. 9). The results obtained also did not exhibit any significant difference between the organic or inorganic nitrogen sources on the production of xylanase by *Fusarium solani F7*. These results are in agreement with those reported in the literature where fungi were found to produce higher xylanase activities on organic nitrogen sources (Purkarthofer et al. 1993; Lemos et al. 2001).

The mineral salt solution, distilled water, organic nitrogen source solution, and mineral salt solution plus organic nitrogen source solution used as MA supported xylanase production and extracellular protein synthesis to varying degrees. The effects of several MAs have been reported on xylanases production in SSF (Grajek 1986, 1987; Shamla and Sreekantiah 1987; Alam *et al*. 1994; Gutierrez-Correa and Tengerdy 1998; Chirstakopoullos et al. 1999).

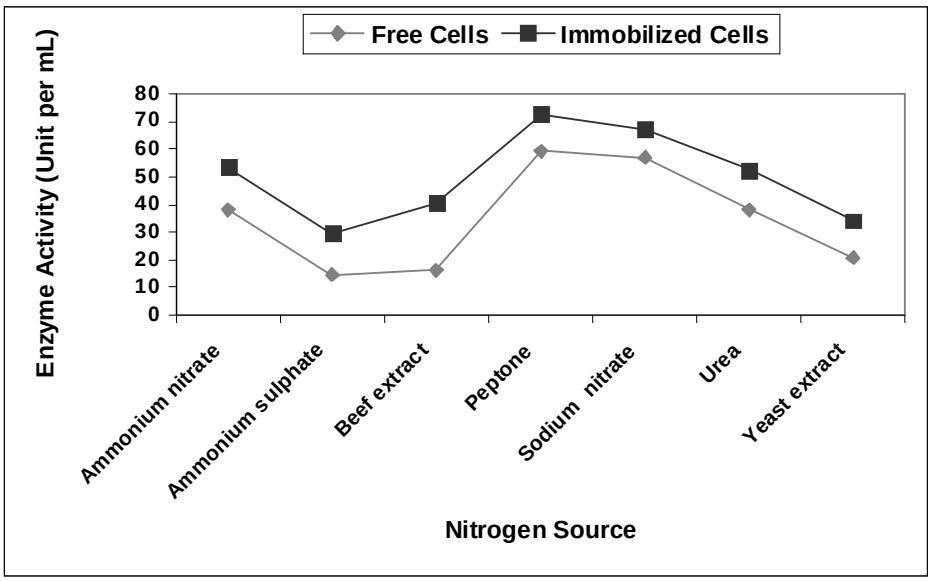

Fig. 9. Effect of different nitrogen sources at 0.3% concentrations on xylanase production by free and immobilized cells

CONCLUSION

1. The results obtained from the present study indicate that significant improvement of xylanase production by *Fusarium solani* F7 isolate could be obtained by selective use of nutrients and growth conditions, especially when using an immobilized cell system, rather than a free cell system.
2. Since oat spelt xylan is an expensive substrate for commercial scale xylanase production, the possibility of using wheat straw for xylanase production was investigated. Wheat straw (3% by mass per volume) could be used as a less expensive substrate for efficient xylanase production.

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