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# Xylanase Production by the Thermophilic Mold *Humicola lanuginosa* in Solid-State Fermentation

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## Abstract

Among the lignocellulosic substrates tested, wheat bran supported a high xylanase (EC 3.2.1.8) secretion by *Humicola lanuginosa* in solid-state fermentation (SSF). Enzyme production reached a peak in 72 h followed by a decline thereafter. Enzyme production was very high (7832 U/g of dry moldy bran) when wheat bran was moistened with tap water at a substrate–to–moistening agent ratio of 1:2.5 (w/v) and an inoculum level of $3 \times 10^6$ spores/10 g of wheat bran at a water activity ($a_w$) of 0.95. Cultivation of the mold in large enamel trays yielded a xylanase titer comparable with that in flasks. Parametric optimization resulted in a 31% increase in enzyme production in SSF. Xylanase production was approx 23-fold higher in SSF than in submerged fermentation (SmF). A threshold constitutive level of xylanase was secreted by *H. lanuginosa* in a medium containing glucose as the sole carbon source. The enzyme was induced by xylose and xylan. Enzyme synthesis was repressed beyond 1.0% (w/v) xylose in SmF, whereas it was unaffected up to 3.0% (w/w) in SSF, suggesting a minimization of catabolite repression in SSF.

**Index Entries:** *Humicola lanuginosa*; solid-state fermentation; submerged fermentation; wheat bran; xylanase.

## Introduction

Plant cell wall contains hemicelluloses with xylan as a major constituent, and it is composed of a backbone of 1,4-β-linked D-xylose units, which may be substituted with acetic acid, arabinose, and 4-*O*-methyl-D-glucuronic

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acid. Xylanolytic enzymes, a repertoire of hydrolytic enzymes, facilitate the complete hydrolysis of xylan. Xylanases have attracted considerable attention because of their potential industrial applications including hydrolysis of lignocellulose to fermentable sugars, clarification of juices, and improvement of the nutritive quality of animal feed *(1)*. Alkaline, thermostable, and cellulase-free xylanases find application in the production of dissolving pulps; enzymatic beating; and prebleaching of kraft pulps to reduce chlorine consumption, thus reducing the emission of toxic compounds from the bleaching process *(1)*. The application of thermophilic microorganisms in the production of thermostable enzymes has technical and economical advantages. Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications, including xylanases, whose levels in fungi are generally much higher than those in yeast and bacteria *(2)*. The thermophilic fungus *Humicola lanuginosa* is one of the best xylanase producers reported to date *(3,4)*.

The technique of solid-state fermentation (SSF) describes the microbial growth on and/or within the particles of solid substrate where the liquid content, bound with them, is at the level corresponding to the water activity ($a_w$) ensuring growth and metabolism of cells, but not exceeding the maximal water-holding capacity of the solid matrix *(5)*. Such a system, which is closer to the natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites. SSF technique offers a number of economically and industrially important advantages, including simplicity in preparation of media; less fermentation space required; less water for fermentation; reduced energy demand; simpler machinery, equipment, and control systems; minimized catabolite repression; easier scale-up of the process; and superior yields. These advantages justify the reasons for the resurgence of SSF as a method for production of microbial products. However, SSF has some limitations too, such as a limited choice of microorganisms that can grow at a low moisture level, difficulty in monitoring parameters such as pH and moisture level, the need for pretreatment of the substrate, and a complex method of determining cell biomass *(6,7)*.

The synthesis of xylanolytic enzymes is regulated by induction and repression systems in microorganisms *(3,8)*. Information on the regulatory mechanism of enzyme production in SSF is, however, limited. There are a few reports on the inhibition of enzyme production in SSF in the presence of easily fermentable sugars *(2,9)*. Relatively high sugar concentrations did not affect enzyme production in SSF, whereas the production was lowered in submerged fermentation (SmF), probably owing to catabolite repression *(6)*.

The present investigation dealt with the optimization of cultivation parameters for maximum enzyme production by *H. lanuginosa* in SSF, and the effect of the end product of xylan hydrolysis (xylose) on xylanase synthesis.

## Materials and Methods

### *Thermophilic Fungal Strain*

The thermophilic mold *H. lanuginosa* (syn. *Thermomyces lanuginosus*) (TMD-3) was obtained from the culture collection of the Department of Microbiology, University of Delhi South Campus. The mold was maintained on Emerson's YpSs agar *(10)* slants at 4°C.

### *Xylanase Production in SSF*

Various lignocellulosic substrates were washed twice in boiling water to remove the dirt and reducing sugars present in them. Erlenmeyer flasks (250 mL) containing 10 g of wheat bran were moistened with 25 mL of mineral solution *(11)* and autoclaved at 15 psi for 45 min, cooled, and inoculated with freshly prepared spore suspension ($7.5 \times 10^5$). The spore suspension was prepared from agar slopes of *H. lanuginosa* grown for 4 d at 45°C using 20 mL of sterile 0.1 *M* saline containing 0.1% Tween-80 followed by shaking and filtration. The inoculum was mixed thoroughly and incubated in a humidified chamber (relative humidity: 65%) at 45°C.

### *Enzyme Extraction*

The enzyme from the moldy bran of each flask was extracted with 100 mL of 0.1 *M* sodium phosphate buffer (pH 7.0). The flasks were kept on a rotary shaker (200 rpm) for 90 min, and the enzyme extracted from the moldy bran was centrifuged at 10,000*g* for 15 min. The clear supernatants were used in enzyme assays. The dry weight of wet moldy bran was determined gravimetrically by drying it at 80°C to a constant weight.

### *Enzyme Assays*

Birchwood xylan (Sigma, St. Louis, MO) and carboxymethylcellulose were used for assaying xylanase *(12)* and cellulase *(13)*, respectively, at pH 7.0 and 55°C. One unit of xylanase/cellulase is defined as the amount of enzyme required for liberation of 1 µmol of reducing sugars as xylose/glucose per min/mL under the assay conditions. Enzyme production is expressed as units per gram of dry moldy bran (DMB). Total protein was determined according to Lowry et al. *(14)* using bovine serum albumin as the standard. Specific enzyme activity is defined as the xylanase activity per milligram of protein.

### *Optimization of Cultivation Parameters*

The mold was cultivated in 250-mL flasks containing 10 g of various substrates and moistened with 25 mL of mineral solution. The flasks containing wheat bran were moistened with tap water and buffers of various pH values (sodium citrate buffer [0.1 *M*, pH 3.0–5.0], sodium phosphate buffer [0.1 *M*, pH 6.0–7.0], and glycine-NaOH buffer [0.1 *M*, pH 8.0–11.0]).

The flasks were inoculated with different levels of 4-d-old inoculum ($7.5 \times 10^5$ to $4.5 \times 10^6$ spores/10 g) and incubated at different temperatures for 72 h. Six different mineral solutions, tap water, and distilled water were used for moistening the wheat bran. The effect of moisture level on enzyme production was assessed by varying the solid:liquid (w/v) ratios. Xylanase production as a function of $a_w$ was determined using glycerol as a depressant of water activity *(15)*.

### *Enzyme Production in Trays*

Wheat bran (40 g) was moistened with 100 mL of tap water and evenly spread on enamel-coated metallic trays ($36 \times 22.5 \times 5.1$ cm), covered with aluminum foil, autoclaved, cooled, inoculated, and incubated in a humidified chamber (relative humidity: 65%) at 45°C. After 72 h, the moldy bran was extracted and assayed.

### *Repression of Xylanase by Catabolite Xylose*

Increasing concentrations of xylose were included in the xylanase production medium *(3)*. The flasks were incubated in an incubator shaker (Innova 4330; New Brunswick Scientific) at 45°C and 200 rpm for 72 h. Medium containing wheat bran was supplemented with various levels of xylose in submerged as well as solid-state cultivations to determine their effect on xylanase secretion.

### *Saccharification of Lignocellulosics*

Lignocellulosic substrates were saccharified according to Okeke and Obi *(16)* in an incubator shaker at 100 rpm at 70°C for 24 and 48 h. Reducing sugars were determined using dinitrosalicylic acid (DNSA) reagent *(12)* in the supernatants after centrifugation.

All experiments were carried out in triplicate and the mean values were recorded.

## Results and Discussion

The use of pure xylan as a substrate for the production of xylanases increases the cost of enzyme production. Consequently, there have been attempts to develop bioprocesses to produce such enzymes using different lignocellulosic residues *(17)*. Of the various lignocellulosic substrates used, wheat bran supported a high xylanase secretion with minimum levels of cellulase (Table 1) and, therefore, was selected for further experiments. Similar results were reported for *H. lanuginosa (18)*, *Bacillus* sp. *(19)*, and *Bacillus licheniformis (6)*, where wheat bran supported good enzyme production. Cellulase-free xylanases from *T. lanuginosus* have been reported *(3,20)*. The higher xylanase titer in wheat bran could be attributed to its rich content of hemicellulose and protein, and also its suitable physicochemical structure *(21)*. Low enzyme titer in bagasse could be attributed to its com-

Table 1
Production of Xylanases and Cellulases
by *H. lanuginosa* in SSF on Different Agroresidues at 45°C

| Serial no. | Carbon source | Xylanase (U/g of DMB) | Cellulase (U/g of DMB) |
|---|---|---|---|
| 1 | Wheat bran | 5977.8 | 7.4 |
| 2 | Mustard oilseed cake | 3.4 | 0.5 |
| 3 | Rice husk | 5.9 | 0.2 |
| 4 | Rice straw | 91.6 | — |
| 5 | Wheat straw | 210.9 | — |
| 6 | Sugarcane bagasse | 1.9 | — |
| 7 | Citrus pulp | 1.4 | 1.3 |
| 8 | Citrus peel | — | 2.8 |
| 9 | Gram bran | 63.7 | 5.1 |

plex structure. Rice husk is an unsuitable substrate owing to its high silica content *(22)*. High extracellular xylanolytic enzyme levels were observed on cultivation of *Aspergillus awamori* on milled sugarcane bagasse and organic nitrogen sources in SSF *(17)*.

Xylanase production in SSF by *H. lanuginosa* reached a peak in 72 h followed by a decline thereafter (data not shown). This observation is consistent with that of *Aspergillus niger* A3, which yielded maximum xylanase in 72 h in SSF using wheat bran and bagasse *(23)*. The time interval needed for incubation is generally dictated by the composition of the substrate and properties of the strain, such as its growth rate, enzyme production profile, and initial inoculum *(24)*.

An extrinsic parameter such as pH acts synergistically with other environmental parameters in addition to being a regulatory parameter in biotechnological processes. A high enzyme production by *H. lanuginosa* was observed at pH 7.0 (Fig. 1A). Highest xylanase was reported previously for *T. lanuginosus* at pH 6.5 on coarse corncob medium *(4)*.

The production of xylanase was higher at 45°C with high specific activity, followed by at 50°C (Fig. 1B). This behavior is typical of a thermophile wherein the metabolic activities get slowed down above and below the optimum range. A slightly higher temperature of 50°C was reported for maximal xylanase production by *T. lanuginosus (3,4)*.

Enzyme production steadily increased with an increase in inoculum size, and a high xylanase secretion was attained when inoculated with $3 \times 10^6$ spores of *H. lanuginosa*/10 g of wheat bran (Fig. 2). Any further increase in inoculum size lowered the enzyme production. In *Trichoderma harzianum*, an inoculum size of $3.0 \times 10^6$ spores/50 g supported maximum xylanase production *(25)*.

A high enzyme production was recorded using tap water as the moistening agent (Fig. 3A). A number of moistening agents have been employed in SSF processes such as tap water *(6,7)*, distilled water *(26)*, and various mineral solutions *(3,4)*.

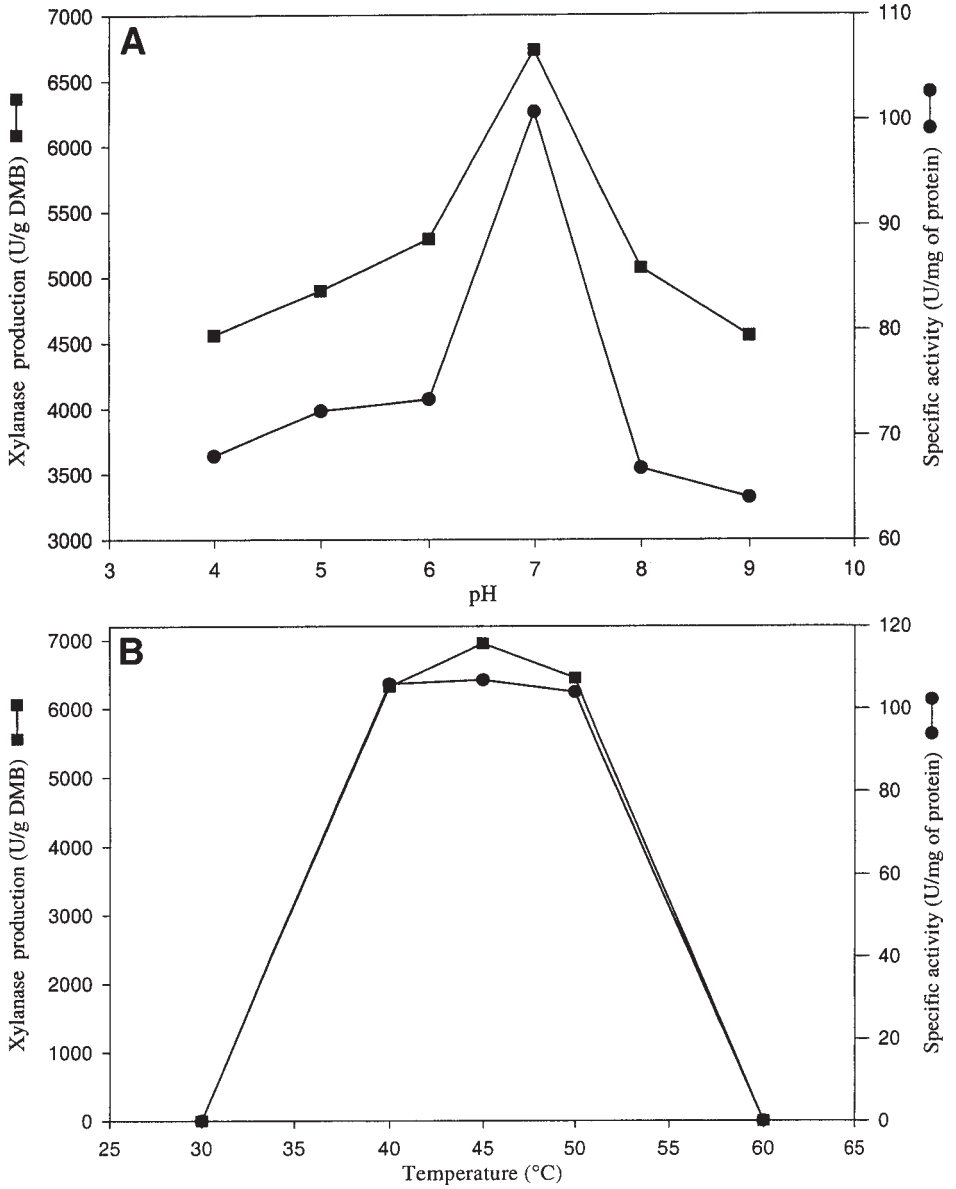


Fig. 1. **(A)** Effect of different pH values on xylanase production; **(B)** effect of different temperatures on xylanase production.

An increase in enzyme production was achieved when the ratio of wheat bran to moisture level was raised from 1:1 to 1:2.5, with production decreasing thereafter (Fig. 3B). Similar observations were recorded with *B. licheniformis (6)*. By contrast, a slightly higher ratio of 1:4 to 1:5 was optimal for *Thermoascus auranticus (1)*, and slightly lower ratios of 1:0.5 to 1:1.5 were suitable for *Bacillus* sp. *(19)*. A higher-than-optimum moisture level causes decreased porosity, alteration in wheat bran particle structure,

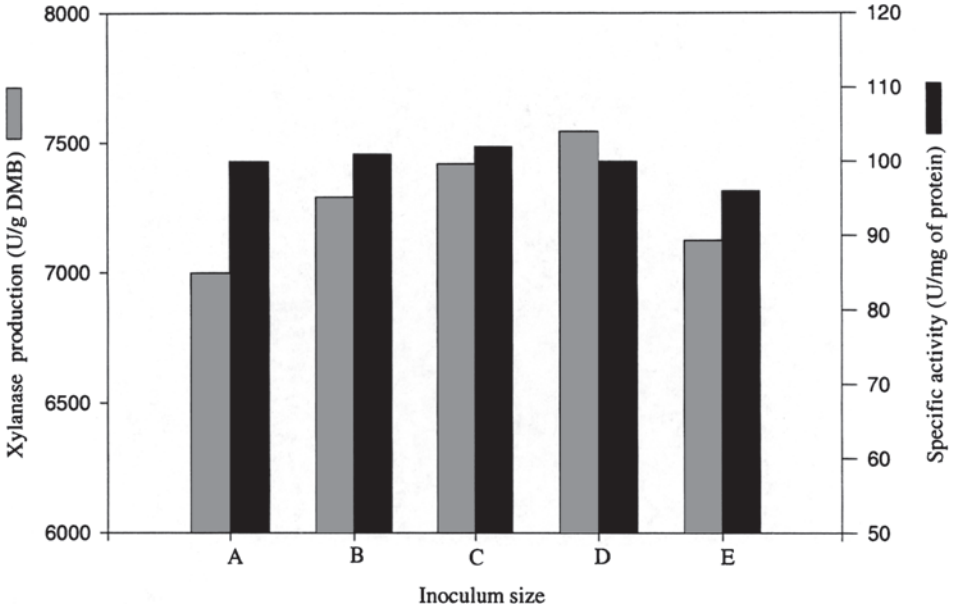


Fig. 2. Effect of inoculum size on xylanase production in wheat bran: A, $7.5 \times 10^5$; B, $1.5 \times 10^6$; C, $2.25 \times 10^6$; D, $3.0 \times 10^6$; and E, $4.5 \times 10^6$ spores/10 g.

gummy texture, lower oxygen transfer, and enhanced formation of aerial mycelia. Lower moisture levels are known to reduce solubility of the nutrients of the solid substrate, lower the degree of swelling, and increase water tension *(21,27)*.

The $a_w$ is an estimate of the proportion of free water that is available for biologic and physiologic activity. The enzyme titers of *H. lanuginosa* were consistently high at $a_w$ 0.95, with a sharp decline at 0.92 (Fig. 4). Enzyme production was not observed at $a_w$ values of 0.80 and 0.75. This is consistent with the observation that at relatively low moisture contents, growth and metabolism of the microorganism could be limited. Values of $a_w$ in the range of 0.982–0.986 were found to be suitable for the production of hydrolases *(28)*.

Xylanase production in enamel-coated and aseptically maintained trays (6892 U/g of DMB) was comparable with that obtained in flasks, indicating that the system is amenable for scale-up. A possible reason for a decrease in enzyme production during scale-up could be the reduction in effective aeration and heat transfer. Similar observations have been recorded in the production of fungal rennet by *Mucor miehei (29)* and xylanases by *B. licheniformis (6)* in trays.

Attempts have been made to understand the mechanisms that regulate the synthesis of xylanases *(3,8)*. The xylanase synthesis level in wheat bran (xylan-containing carbon source) suggested that xylan/xylose was necessary for the effective induction of xylanase in *H. lanuginosa* (Fig. 5A). This is consistent with xylanase production in *T. lanuginosus* strain RT9 *(20)*.

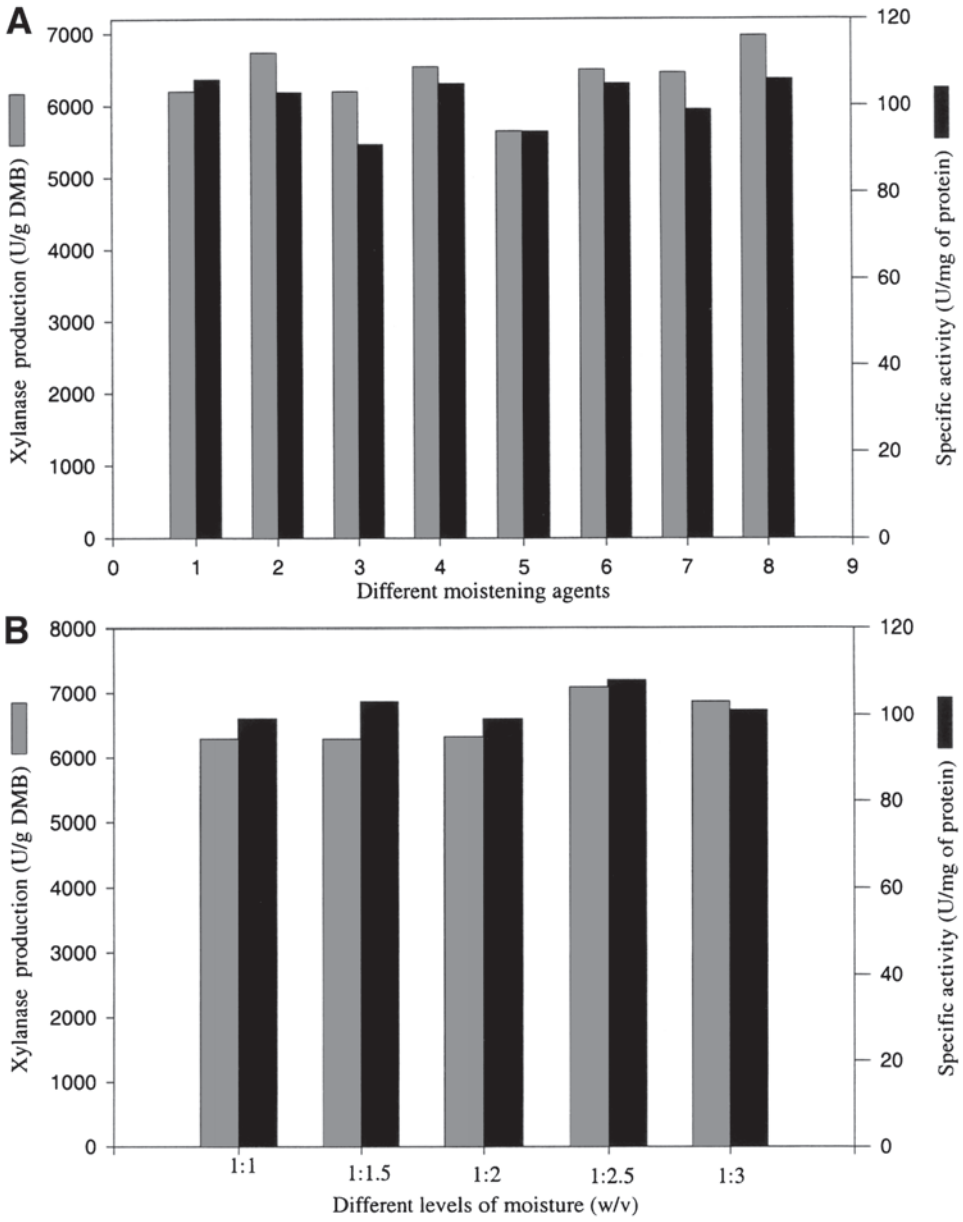


Fig. 3. **(A)** Effect of different moistening agents (MAs) on xylanase production: MA 1: *(3)*; MA 2: 0.4 g/L of $(NH_4)SO_4$, 2.1 g/L of $KH_2PO_4$, 0.3 g/L of $CaCl_2{\cdot}2H_2O$, 0.11 g/L of $FeSO_4$, 0.3 g/L of $MgSO_4$; MA 3: *(11)*; MA 4: 2.0 g/L of $NH_4NO_3$, 6.0 g/L of $KH_2PO_4$, 0.5 g/L of KCl, 0.5 g/L of $MgSO_4{\cdot}7H_2O$; MA 5: 0.1 g/L of $KH_2PO_4$, 1.0 g/L of $NH_4H_2PO_4$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 0.1 g/L of $CaCl_2{\cdot}2H_2O$, 0.1 g/L of $FeSO_4$, 0.1 g/L of $MnSO_4$; MA 6: 11.0 g/L of $Na_2HPO_4$, 6.0 g/L of $NaH_2PO_4$, 3.0 g/L of KCl, 0.1 g/L of $MgSO_4{\cdot}7H_2O$; MA 7: distilled water; MA 8: tap water. **(B)** Effect of different moisture levels on xylanase production.

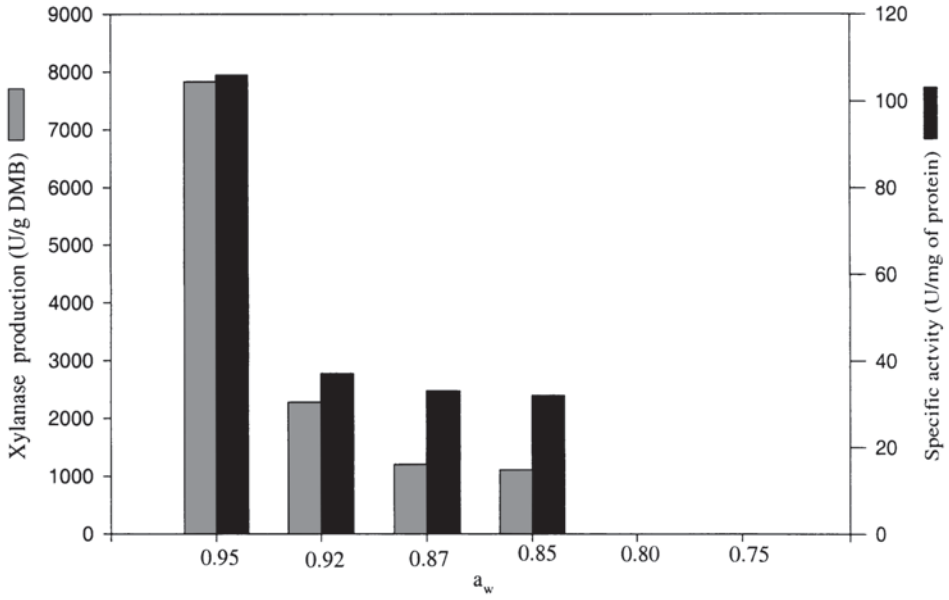


Fig. 4. Effect of $a_w$ on xylanase production.

A threshold constitutive level of xylanase was observed when the strain was grown in a medium containing glucose as the sole carbon source. Xylan, being a high-molecular-mass polymer, cannot penetrate the cell wall. Therefore, low-molecular-mass fragments of xylan are produced by the action of low basal levels of extracellular enzymes that are constitutively expressed *(8)*. Low constitutive levels of xylanase were widely reported previously *(3,20)*. The induction of enzyme synthesis by xylose was concentration dependent (Fig. 5B). The role of xylose in various microorganisms is not consistent. It acts either as an inducer or as a repressor of enzyme synthesis. Purkarthofer et al. *(3)* reported induction of xylanase in *T. lanuginosus* by xylose. In the present investigation, xylose induced xylanase up to 1.5% (w/v) and repressed thereafter.

When wheat bran was supplemented with xylose in SmF, a xylose concentration up to 1% (w/v) did not affect enzyme secretion. At higher concentrations of xylose, xylanase synthesis was repressed. This suggested that xylose repressed xylanase synthesis in a concentration-dependent manner (Fig. 6A). Kyu et al. *(8)* reported a decrease in xylanase secretion in *Bacillus circulans* in xylan medium supplemented with xylose.

In SSF, xylose did not affect xylanase synthesis to any significant extent up to 3% (w/w), and thereafter, it repressed xylanase production (Fig. 6B). These results suggested that xylose affected enzyme production in both fermentation systems, but the sensitivity of enzyme synthesis to xylose was different. The productivities of pectinesterase and polygalacturonase in *A. niger* were affected by glucose in both SSF and SmF, but the inhibition was very high in SmF compared with that in SSF *(9)*. Similarly, in *Penicil-*

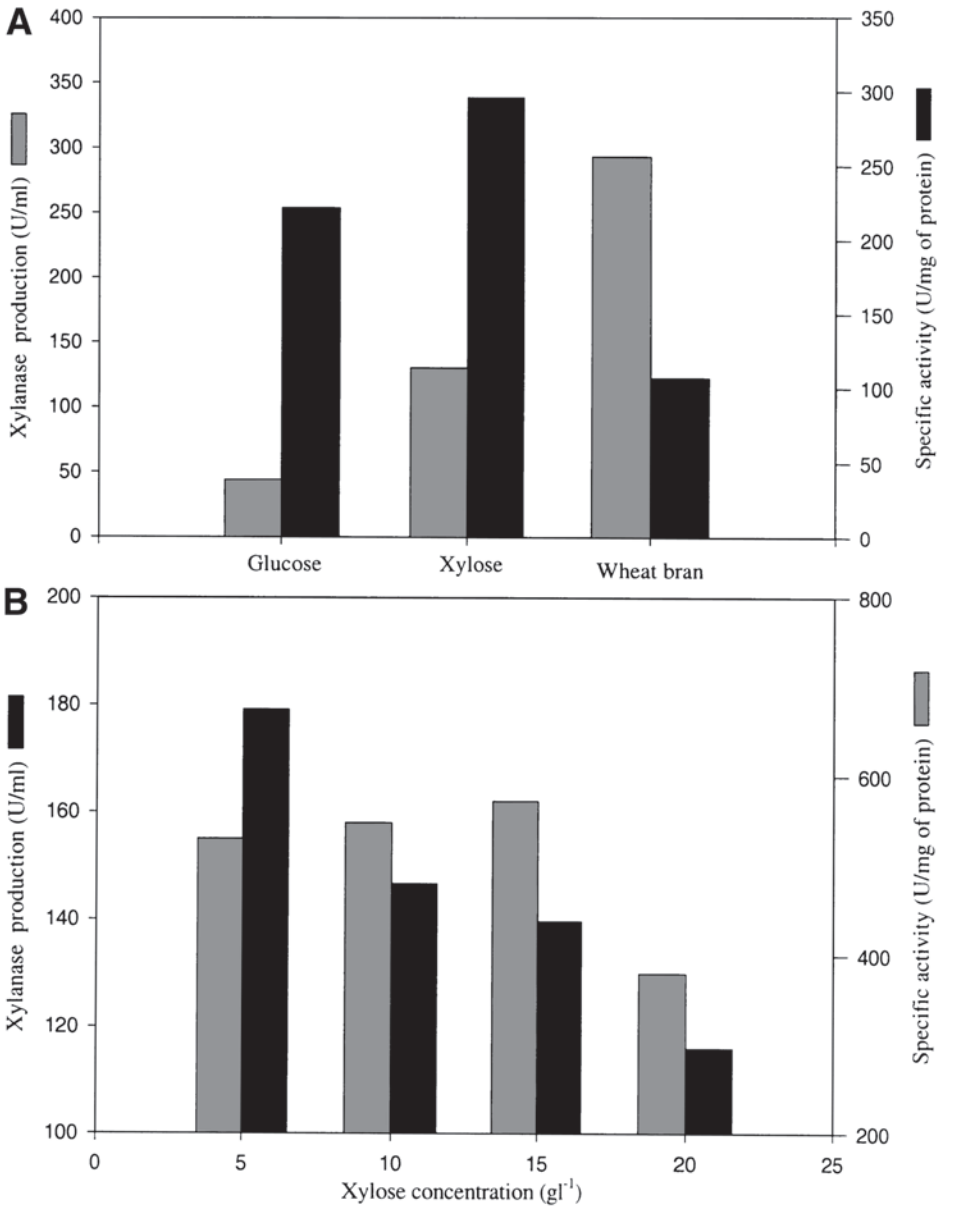


Fig. 5. **(A)** Effect of carbon sources on xylanase production; **(B)** effect of xylose concentration on xylanase production in SmF.

*lium canescens* 10-10c, minimization of catabolite repression was reported in SSF *(2)*. The existence of physiological differences between SSF and SmF is possible and may be related to different patterns of xylanase production. Although the production of xylanases by various microbes has been reported earlier in SSF, the enzyme titer was much lower (with wheat bran as substrate) than that attained in our investigation (Table 2).

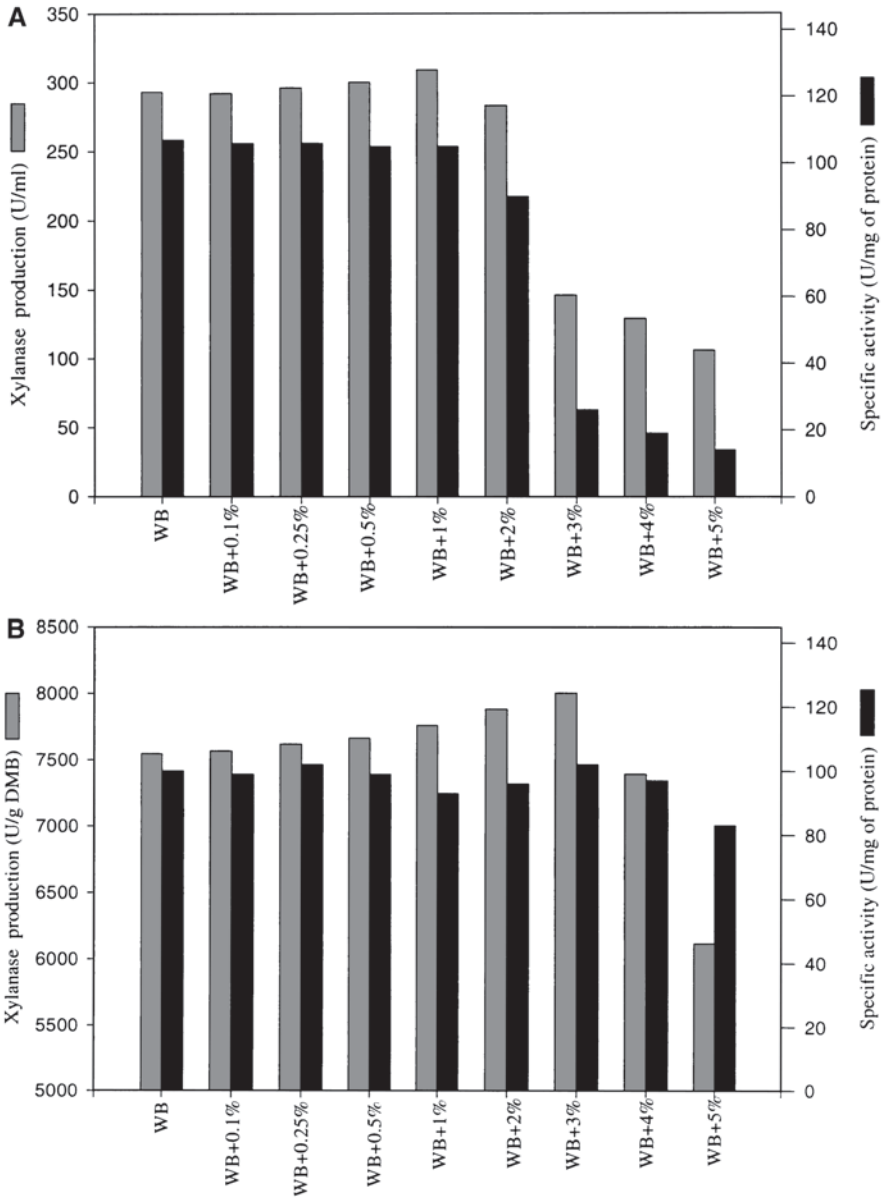


Fig. 6. **(A)** Xylanase production in different levels of xylose along with wheat bran (WB) in SmF; **(B)** xylanase production in different levels of xylose along with wheat bran in SSF (WB + xylose % [w/w]).

Solis-Pereira et al. *(30)* used a method of comparison of pectinase production from *A. niger* by dividing the yield obtained from SSF (U/g of DMB) by the yield in SmF (U/mL) of spent broth. According to this method, the xylanase yield in SSF was 23-fold higher than that in SmF. Archana and Satyanarayana *(6)* reported a 22-fold higher xylanase production in SSF than in SmF. *T. lanuginosus* also produced higher enzyme titers in SSF than

Table 2
Xylanase Production by Different Microorganisms in SSF

| Organism | Units (U/g) | Reference |
|---|---|---|
| *Bacillus licheniformis* | 19 | *6* |
| *Thermoascus aurantiacus* | 500 | *38* |
| *Bacillus* sp. | 720 | *19* |
| *Humicola lanuginosa* | 2050 | *18* |
| *Thermoascus aurantiacus* | 2700 | *33* |
| *Trichoderma reesei* and *Aspergillus niger* | 2842 | *34* |
| *Aspergillus niger* mutant | 5071 | *37* |
| *Aspergillus niger* | 5147 | *23* |
| *Thermoascus aurantiacus* | 6193 | *1* |
| *Melanocarpus albomyces* | 7000 | *35* |
| *Trichoderma hamatum* | 7600 | *36* |
| *Humicola lanuginosa* | 7832 | Present study |

Table 3
Saccharification of Lignocellulosic Substrates Using Xylanase of *H. lanuginosa*

| | Reducing sugar (mg/g) liberated after reaction times | |
|---|---|---|
| Substrate | 24 h | 48 h |
| Wheat bran | 60.4 | 76.4 |
| Wheat straw | 46 | 52.8 |
| Rice straw | 39.2 | 39.6 |
| Rice husk | 36.4 | 36 |

in SmF *(3)*. Parametric optimization resulted in a 31% increase in enzyme production in SSF.

The xylanolytic enzymes of *H. lanuginosa* released reducing sugars from lignocellulosic substrates. Since the strain produced a high titer of xylanase with negligible amounts of cellulase, the reducing sugars were released from the hydrolysis of hemicelluloses. Wheat bran was rapidly saccharified by xylanase, followed by wheat straw and rice husk (Table 3). Ball and McCarthy *(31)* had also reported a significant difference in enzymatic profiles of more than 200 strains and their ability to release reducing sugars from milled wheat straw. Saccharification of lignocellulosic substrates (wheat bran, soybean hull, rice straw, sugarcane bagasse) by *A. niger* and *Aspergillus terreus* also suggested wheat bran to be more susceptible to enzymatic hydrolysis, while soybean hulls were more resistant to enzymatic saccharification *(32)*.

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