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Production of Xylanases by *Humicola lanuginosa* in Solid State Culture by Screening Lignocellulosic Substrates

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Abstract: The production of xylanases by *Humicola lanuigosa* in solid state fermentation was carried out on different carbon and nitrogen sources. Optimum temperature for xylanase and β-xylosidase production was 45°C while maximum productivity was on pH 5.5 to 6 of the fermentation medium by *Humicola lanuigosa* in solid culture screening from 15 different carbon sources (inducers). These studies revealed that wheat bran and xylan were good inducers of hemicellulases. Xylanase and β-xylasidase accumulated extra cellular. Vogel's salt medium was found optimum to provide ionic strength. The productivities (IU/F/h) of xylanases from *Humicola lanuigosa* at pH 5.5 and 45°C in solid state fermentation using xylan was 35.5 IU/F/h and wheat bran 32.9 IU/F/h after 120 hours. Similarly β-xylosidase showed maximum productivity 5.49 IU/F/h from xylan in solid state fermentation and 2.97 IU/F/h on wheat bran. The hemicellulases complex including xylosidase of such organism needs further improvements to more effectively again monomeric sugars.

Key words: *Humicola lanuginosa* · Xylanases · Wheat bran · Hemicellulases · β-xylosidase

INTRODUCTION

Pakistan is an agriculture country and the waste of major crops of lignocellulosic material, its accessibility and cost effective agricultural residues, such as wheat straw, wheat bran, xylan, sugarcane bagasse, corn cobs α-cellulose, carboxy methyl cellulose and rice bran were used to achieve higher xylanase yields, less chance of contamination using solid-state fermentation. Lignocellulose consists primarily of three major polymers cellulose, hemicellulose and lignin. Among all, hemicellulose is the second largest component (20-40%) of lignocellulosic material after cellulose [1].

Hemicellulose does not have a homogenous chemical composition and microbial xyalanses enhanced dough theological properties with increase in loaf volume that improves its baking performance and so have great importance in cereal industry [2]. But fungi are the most common industrial sources of hemicellulases such as glucanases, xylanases, galactanasese, mannases, glalactomannases and pentosanases [3] investigated commercial enzymes from three fungi using crude enzyme solutions including xylanase.

Xylanase from *Trichoderma reesei* were found to increase bleachability of the pulps depending on cooking method used in pulp production [4]. The effect of xylanase treatment on brightness was highest in the conventional Kraft pulps and in pulps produced by extended cooking methods. High amounts of lignin with higher average molecular mass also could be extracted from these pulps after xylanase treatment. The enzymes were especially effective in improving the bleachability of oxygen-delignified pulps [5].

Several attempts have been made to convert xylan into useful products as the conventional methods of food production are becoming scarce with ever increasing human population. The production of cellulase free endoxyalnase by thermophilic fungus, *Thermomyces lanuginosus* was investigated in semi-solid fermentation. Simultaneous production of cellulases and xylanases has also been reported in many microorganisms including fungi [6]. *Humicola lanuginosus* is a thermophilic fungus that produces xylanase without any accompanying cellulase activity.

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MATERIALS AND METHODS

Microorganisms *Humicola lanuginosus* obtained from National Institute of Biotechnology and Genetic Engineering Faisalabad. The cultures were maintained on vogel's medium with ball mill cellulose as a carbon source. The slants were incubated at 45°C for 48 h for sporulation and subsequently stored at 4°C.

Inoculum Preparation: Inoculum medium (100 mL) was prepared in VOGEL'S MEDIUM. The flask was then incubated on an orbital shaker (120 rpm) for 96 h at 35°C.

Enzyme Production by Solid-State Fermentation: Agricultural residues, such as wheat straw, wheat bran, xylan, sugarcane bagasse, corn cobs α-cellulose and carboxy methyl cellulose and rice bran were used to achieve higher xylanase yields using solid-state fermentation. Sugars like glucose, galactose, fructose, arabinose, sucrose, maltose; lactose were also used for enzyme production.

Sample Harvesting in Solid State Fermentation: After predetermined incubation period 24, 48, 72, 96 and 120 h, experimental flasks in triplicate were harvested. In each flask 50 mL distilled water and 1mL tween-80 were added. All flasks were agitated for 30 min at 4°C. Biomass was filtered through muslin cloth and washed twice before being dried in oven 100°C for 24 h till constant weight. The filtrates were centrifuged at 15000rpm for 30 minute at 4°C. The filtrate was subjected to enzyme assay. The pellet in centrifuge tube was mixed with water poured in a pre-weighed petri plate and dried at 100°C for 24 h till constant weight.

Effect of Nitrogen Source: Different nitrogen sources were used to see their inductive effect on enzyme production. These included $(NH4)_2SO_4$ and corn steep liquor. Corn steep liquor provided macro and micro nutrients.

Optimization of pH for Enzyme Activity: Enzyme produced by solid-state fermentation at different pH (3.5, 4.0, 4.5, 5, 5.5, 6 and 6.5) of the medium was tested.

Optimum Temperature for Enzyme Activity: The culture was grown and harvested at different temperatures (40°C, 45°C, 50°C and 55°C). The optimum temperature which supported maximum activity was selected.

Enzyme Assay and Determination of Proteins: Activity of enzyme was determined by the method described by Tuncer *et al.* [7] against oat spelt xylan. β-xylosidase activity was determined by the method described by Kim *et al.* [8] against p-nitro phenyl β-xylopyranoside. Protein of the sample was estimated by the method of Bradford [9].

Determination of Growth Kinetic Parameters: For determining kinetic parameters the procedures described previously were adopted [10]. Dry cell mass (g/l) of cultures after growth on different carbon sources was determined on duplicate samples as described earlier and residual carbohydrates (g/l) were determined on dry mass after [11]. The volumetric rate of enzyme production (Qp, IU/I/h) was determined from the maximum slope in plot of produced (g/I). Product yield coefficient namely Yp/x and Yp/s were determined by using the relationships.

Yp/x = dP/dX and Yp/s = dP/dS respectively

RESULTS AND DISCUSSION

Growth and Kinetics of *Humicola lanuginosus* on Various Substrates by Solid State Fermentation: Inoculum of *Humicola lanuginosus* was used for solid state fermentation. Different substrates namely as wheat straw, rice bran, corn cobs, sugarcane bagasse, wheat bran, α cellulose, CMC, glucose, glactose, arabinose, fructose, sucrose, maltose, lactose and xylan. (2 g) in ratio of 1:4 (Solid: Liquid) were used independently as carbon sources. However, the enzyme was harvested after 5 days incubation at 45°C. Substrate utilization by inoculum of *H.lanuginosus* is shown in Fig. 3. The enzyme activities obtained from these substrates were dependent on carbon sources used Table 1. Maximal enzyme activities were obtained after 96-120 h. Xylan followed by wheat bran showed overall highest enzyme production is better extent than the rest.

Potential kinetic parameters for a batch fermentation process, namely, enzyme productivity (Qp), process product yield namely Yp/s and specific product yield Yp/x were determined (Table 1 and 2).

Table 1: Production of xylanase enzymes by *Humicola lanuginosus* using different carbon sources in solids state fermentation

Carbon Sources	Qp (IU/flask h)	Yp/s (IU/gs)	Yp/x (IU/g cell)	μ	qp (u/gh)
Wheat Bran	28.200	538.30	2568.60	0.23	0.050
Rice Bran	10.750	399.33	1815.15	0.20	0.043
Xylan	10.250	887.50	3707.77	0.22	0.055
Wheat Straw	10.950	412.73	1620.73	0.23	0.057
Corn cobs	5.300	352.30	1487.50	0.21	0.048
Sugarcan Bagass	6.550	331.43	1902.17	0.20	0.031
CMC	4.600	203.50	1389.13	0.19	0.030
α-Cellulose	0.750	263.32	2966.66	0.21	0.021
Arabinose	3.490	162.87	971.42	0.21	0.035
Fructose	2.650	91.25	1042.86	0.20	0.018
Glucose	1.350	110.35	809.25	0.21	0.029
Galactose	0.100	155.50	948.38	0.20	0.032
Sucrose	4.100	201.21	1434.78	0.19	0.028
Lactose	3.840	183.96	975.00	0.21	0.039
Maltose	0.165	146.60	786.36	0.20	0.038

Table 2: Production of β xylosidase enzymes from *Humicola lanuginosus* by using different carbon sources on different substrate in solid state fermentation

Carbon Sources	Qp (IU/flask h)	Yp/s (IU/gs)	Yp/x (IU/g cell)	Yx/s
Wheat Bran	1.03	85.95	410.14	0.21
Rice Bran	1.79	57.00	259.09	0.22
Xylan	3.30	86.41	361.10	0.23
Wheat Straw	1.42	58.69	230.48	0.25
Corn cobs	0.61	7.52	255.55	0.23
Sugarcane Bagasse	0.41	32.91	188.90	0.13
CMC	0.03	12.45	85.00	0.15
α-Cellulose	0.93	27.17	305.66	0.10
Arabinose	.01	35.77	34.46	0.17
Fructose	0.37	9.53	108.92	0.09
Glucose	.01	20.00	146.11	0.14
Galactose	0.28	9.00	55.67	0.16
Sucrose	0.42	17.50	124.70	0.15
Lactose	1.59	25.62	135.83	0.19
Maltose	1.65	16.66	89.39	0.18

***Humicola lanuginosus* Grown at Different Temperatures:** Different temperatures of (40, 45, 50 and 55°C) were used for determination of optimum temperature for xylanase and β-Xylosidase production. Table 7 and 8 show maximal xylanase of 31.0 U/mL and β-Xylosidase of 6 U/mL obtained from wheat bran at 45°C. However, near maximal activity was also attained at 50°C.

Production of Xylanase and β-Xylosidase by *Humicola lanuginosus* at Different pH: Different pH values (of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5) were used for determination of optimum pH of the medium for xylanase and protein production. Table 9 and 10 show maximal xylanase of 31U/mL and β-Xylosidase of 6 31U/mL obtained from wheat bran at pH 5.5 and 6.

DISCUSSION

The production of microbial xylanases has attracted great interest due to their potential application in chemical, pharmaceutical and food industries. Xylanases preparations free of cellulose activity are of particular interest for the pre-treatment of paper pulps to decrease the xylan content and, therefore, reduce the dependence on chlorine used for bleaching in the brightening process [12]. The production of enzyme by using xylan rich substrate mainly depends on the selection of a suitable strain from an appropriate habitat. Fungal systems have been mainly used for enzyme production [13-15]. Extensive work has been going on in many laboratories to select a proper organism for production of concentrated xylanase and efficient inducer for large-scale enzyme production particularly using biomass wastes (highly rich in carbohydrates) in solid fermentation.

Effect of Carbon Sources on Production of Xylanase in Solid State Fermentation: Solid-state fermentations have suited fungal growth because the enzyme is produced in high amounts. In these studies 1 to 4 ratio of solid to liquid medium (Vogel's medium,) was used. Different carbon sources were used to find economical and potent inducer of xylanase using wild culture of H. lanuginosus in solid fermentation. The H. lanuginosus showed significantly higher amount of xylanase production (40-42 % increase) on all substrates. However, the maximum activity was obtained from wheat bran and xylan followed by wheat straw, rice bran and corn cobs.

By conducting screening of various carbon sources it is realistic to identify highly efficient inducer of enzymes which can lead to substantial increase in yield. The time course production of xylanase showed maximum activity in 96-120 h but showed maximum activity after 72- 96 h of cultivation in case of H. lanuginosus. In this study, kinetic parameters for H. lanuginosus, were also determined for production of enzyme, cell mass formation and substrate utilization (Table 3-4). H. lanuginosus supported maximum volumetric xylanase productivity of 32.4 IU/f/h on wheat bran and 35 IU/f/h on xylan, 16.9 IU/f/h, on rice bran 26.5 IU/f/h, on wheat straw

Table 3: Production of Xylanase by *Humicola lanuigosus* from different substrates and sources at 45°C

Carbon Sources	Qp (IU/flask h)	Yp/s (IU/gs)	Yp/x (IU/g cell)	μ	qp (u/gh)
Wheat Bran	32.90	1159.83	3555.13	0.23	0.077
Rice Bran	16.90	631.99	2644.87	0.22	0.052
Xylan	35.30	1304.21	4130.00	0.25	0.081
Wheat Straw	26.50	1038.33	4346.50	0.25	0.071
Corn cobs	17.50	675.41	2397.06	0.25	0.075
Sugarcane Bagasse	9.90	393.43	1418.42	0.24	0.064
CMC	10.45	362.71	1601.20	0.24	0.062
α-Cellulose	9.50	402.11	2171.42	0.25	0.046
Arabinose	5.45	305.55	1312.50	0.25	0.055
Fructose	7.20	221.33	1077.63	0.23	0.055
Glucose	4.40	151.26	776.31	0.24	0.049
Galactose	3.90	828.90	976.66	0.24	0.056
Sucrose	2.31	231.69	533.33	0.23	0.046
Lactose	7.10	293.85	1340.24	0.33	0.057
Maltose	2.60	200.83	898.75	0.24	0.053

Table 4: Production of β-Xylosidase by Humicola lanuigosus from different substrates at 45°C

Carbon Sources	Qp (IU/flask h)	Yps IU/gs	Ypx (IU/g cell)	Yxs
Wheat Bran	3.97	169.06	518.66	0.33
Rice Bran	3.35	149.70	644.87	0.23
Xylan	5.49	231.31	732.50	0.32
Wheat Straw	4.78	180.27	754.65	0.28
Corn cobs	1.21	135.08	779.41	0.30
Sugarcane Bagasse	1.52	131.16	476.32	0.26
CMC	1.28	87.56	386.58	0.25
α-cellulose	0.39	63.22	341.42	0.18
Arabinose	0.10	95.50	410.22	0.23
Fructose	0.48	48.91	238.15	0.23
Glucose	0.86	44.05	214.47	0.20
Galactose	0.23	35.93	153.33	0.23
Sucrose	0.15	51.03	222.62	0.24
Lactose	1.04	29.14	132.92	0.17
Maltose	0.69	38.55	172.50	0.22

corncobs 17.6 IU/f/h, sugarcane bagass 9.9 IU/f/h, CMC 10.45 IU/f/h, α- cellulose 9.5 IU/f/h, but sugars showed less activity (Table 1) arabinose (5.45 IU/f/h). These productivity levels are higher than those reported in bacterial cultures [16].

Effect of Nitrogen Sources on Enzyme Production: It has been observed by Kolankaya [17] that by limiting the nitrogen sources, enzyme production can be improved. Regulation of xylanase production by nitrogen sources were observed by adding these into xylan, wheat bran and rice bran, wheat straw, corn cobs, CMC, a cellulose, sugarcanebagass because these substrate were the best inducer for *H. lanuginosus* but sugars showed low activity of xylanase. These showed enhanced protein and enzyme activity. This can be concluded that both these substrates have high crude protein level. In these studies corn steep liquor 2% was used, based on the previous findings [18]. The study of Pandey *et al.* [19] on *A. niger* following growth on rice bran showed that addition of ammonium ions can increase xylanase production but maximum production occurs on corn steep liquor.

Effect of Temperature on Xylanase Production: The temperature optimum for production of different xylanase from *H. lanuginosus* was mainly between 45°C and 50°C. The value on this temperature was 32.9 U/mL/min produced by solid state fermentation (Figure 1, 2). Temperature higher than 50°C suppressed production of xylanase due to excessive water losses. Similar findings have been reported for *A. awamori* [20] and *A. niger* [21]. When different parameters of xylanase were compared in this study, it was observed that the values of these parameters were higher on 45°C and 50°C. Xylanase in the presence of all other enzyme activities can help in biobleaching of pulps.

Effect of pH on Xylanase Production: Maximum activity was observed at pH 5.5 and pH 6 by *H. lanuginosus* (Figure 3). This is very important from practical point of view as pH control would not be critical as observed with many other enzyme preparations. For other xylanase producing organisms namely Clostridium sp. G0005 and *A. Terreus, A. nidulas* and *A. niger* [22], by following the changes in pH during growth of organism, it was found that maximum

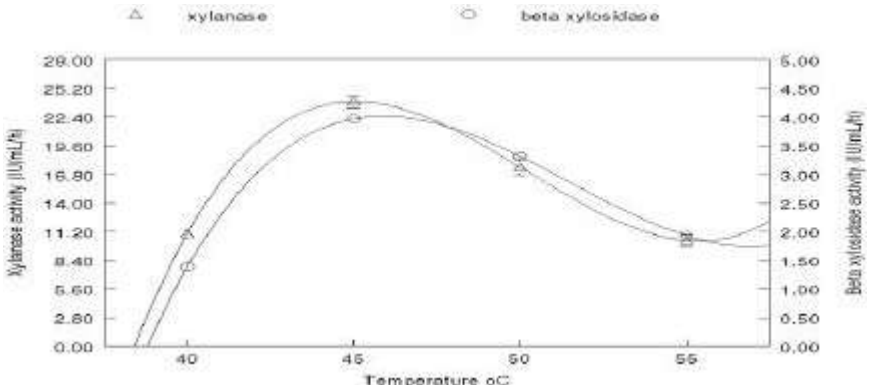

Fig. 1: Effect of increase in incubation temperature on xylanase production by *Humicola lanuginosus*

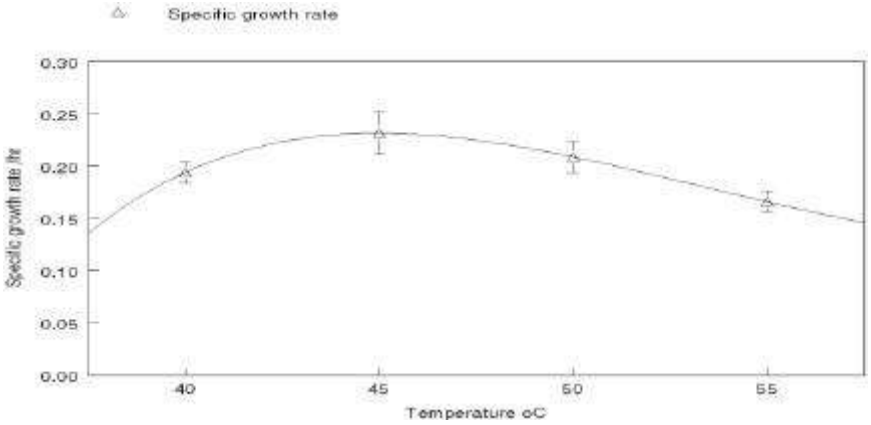

Fig. 2: Effect of increase in incubation temperature on β-Xylosidase production by *Humicola lanuginosus*

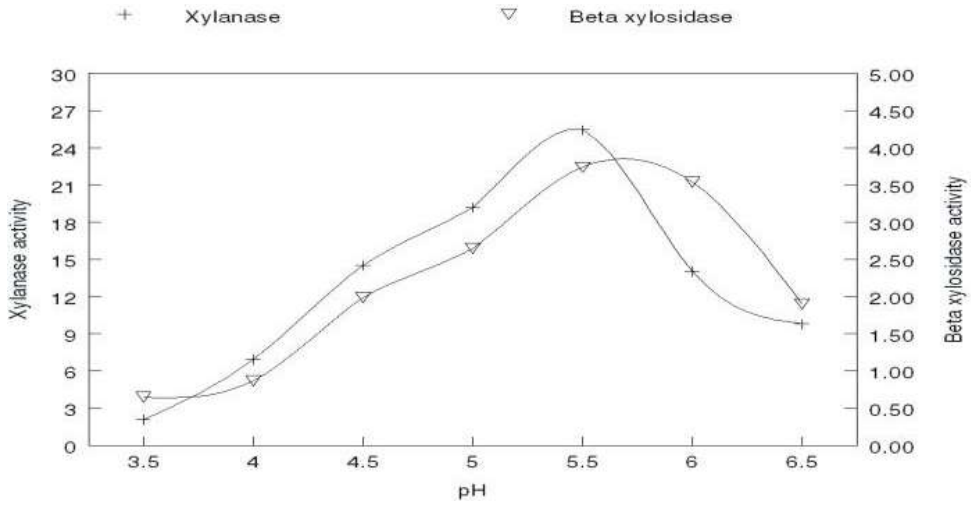

Fig. 3: Effect of different pH values of the medium on β-Xylosidase production by *Humicola lanuginsus*

xylanase and cell mass production occurred at pH 5.5. Different optimal pH values have been reported in *A. oryzae* (pH 4, 5) for growth and maximum enzyme production. Similar results have been reported by Nigam and Singh [23] and Bigelis [22].

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