Middle-East Journal of Scientific Research 8 (2): 357-363, 2011
ISSN 1990-9233
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Removal of Hemicelluloses (Reducing Sugars) from Lignocellulosic Substrates by the Treatment of Xylanase

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Abstract: *Humicola lanuginosus* produced xylanase 1100 U/ mL in liquid state culture at optimum condition like pH 5, temperature 35°C, inoculum size 2mL, time period 4 days, Vogel's media 160 mL, 1 g corn steep liquir as best nitrogen source using sugar cane bagasse (5g) as a substrate. Xylanase passing from gel filteration obtained with an average specific activity of 379 U/mg protein, purification fold 1.9 and recovery yield of xylanase 10%. Patially purified xylanase has optimum pH 5.0, stability range of pH 6 to 7 and optimum temperature 45°C, stability range of temperature 35 to 40°C for 24 hours incubation. Km and Vmax of partially purified xylanase oxidize xylan were obtained 2 mM, 450 mM/min. $MgCl_2$ enhanced the activity of partially purified xylanase but silver nitrate strongly inhibited. Crude xylanase (2-10 mL) removed the reducing sugar 20 mg/mL from rice polish as compared with lignocellulosic substrates such as rice husks, corn stover and corn cobs.

Key words: *Humicola lanuginosus*, Puriflcation, Characterization, Stability, Xylanase, Sugar cane bagasse

INTRODUCTION

The fungi constitute a most fascinating group of organisms exhibiting great diversity in form, structure, habit, life history and mode of nutritional and mycelial tropic stage adequately distinguishing the fungi from other groups and there is now universal acceptance of fungi as a separate kingdom [1-2]. Fungi are the most common industrial sources of hemicellulases such as glucanases, xylanases, galactanasese, mannases, glalactomannases and pentosanases. Lignocellulose consists primarily of three major polymers cellulose, hemicellulose and lignin. Among these, hemicellulose is the second largest component (20-40%) of lignocellulosic material after cellulose [3-4]. Hemicellulose does not have a homogenous chemical composition. It is primarily composed of three main groups of polysaccharides such as xylan, the mannans and glucomannans and the galactans a araboinrgalactans. Xylan is the major component (80%) of hemicellulose and is usually associated with the cellulose and lignin compounds of plant cell wall [5]. It comprises upto 30% of the cell wall material of annual plants, 15-30% hardwood and 7-10% of soft wood [6-7]. Xylans are heteropolymers consisting principally of D-xylose, D-mannose, D-galactose and L-arabinose [8]. In plants, xylans or hemicelluloses are situated between the lignin and the collection of cellulose fibers underneath.They were composed of a backbone of D-xylose unit variously substituted by L-arabinose and 4.0 methyl D-glucuranic acids.

Xylanase is an enzyme which hydrolyzes the β 1; 4-D-xylosidic linkage of higly polymerized and substituted β-1, 4-linked D-xylopyranosyl residues into xylobiose, xylotriose and substituents containing two or four xylose residues. The common substituents found in the β-1, 4-D xylopyranosyl residues are acetyl, arabinosyl and glucuronosyl residues [9, 2]. 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.

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Xylanases have been extensively studied and could potentially be employed for the production of hydrolysates for agro-industrial wastes [10], nutritional improvement of lignocellulosic foods (e.g. clarification of juices), increasing animal feed digestibility [11], agro fibre [12-13] and bioleaching of Kraft pulp [14]. Xylanases improve the cleaning ability of detergents. Treatment of forage with xylanase (along with cellulose as) results in better quality silage and improves the subsequent rate of plant cell wall digestion by ruminants.

Xylanases of thermophiles are generally inducible and have almost similar regulatory mechanisms as in mesophiles [15]. *Humicola lanuginosus* is a thermophilic fungus that produces xylanase without any accompanying cellulase activity [16]. The production and purification of cellulase free endoxyalnase by thermophilic fungus was investigated in liquid fermentation.

MATERIALS AND METHODS

Humicola lanuginosus were grown on vogel's medium. The slants were incubated at 40°C for 96 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 Liquid-state Fermentation: Experiments were performed using duplicate 500 mL Erlenmeyer flasks. Flasks contained 5g sugar cane bagasse in Vogal`s medium upto 100mL mark. The flasks were sterilized (121°C) in autoclave for 15 minutes and inoculated with 2mL conidial suspension. The inoculated flasks were subjected to liquid state fermentation at 35°C, pH 5 and corn steep liquor as a best nitrogen source for different time periods under continuous shaking conditions (120 rpm). Samples were harvested after every 48 hours.

Sample Harvesting in Liquid State Fermentation: After predetermined incubation period 48, 96, 144 and 192 h, experimental flasks in triplicate were harvested. 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 15000 rpm for 30 minute at 4°C.

Enzyme Assay: Activity of enzyme was determined by the method described by Tuncer [17] against oat spelt xylan.

Protien Determination: Protein of the sample was estimated by the method of Bradford [18].

Purification of Xylanase: Xylanase was obtained from *H. laouginousa* centrifuged at 15,000 rpm for 15 minutes at 4°C to increase clarity to maximum and then concentrated by freeze drying.

Fractional Precipitation and Dialysis: Xylanase was placed in ice bath and crystals of ammonium sulfate were added to attain initially 80% saturation at 0°C. The pellet was dissolved in minimum quantity of buffer and dialyzed against distilled water for 20 hours with four changes of equal intervals to remove ammonium sulfate. Total proteins and xylanase activity were determined before and after dialysis of ammonium sulfate precipitated and finally freeze dried.

Gel Filtration: A pooled fraction from Hiload - Q column was loaded on Sephadex G - 75 column to get further purification to homogeneity level. The 400 µL/run of sample in 100 mM phosphate, 0.15 M, pH, 5 was used as elution buffer. The flow rate was 0.5 ml min^{-1}. 1mL size fractions were collected.

Characterization of Xylanase: The purified Xylanase was subjected to characterization through kinetic studies by studying the following:

Effect of pH on Xylanase: Xylanase was assayed at different pH ranging from 2-9, pH 3-3.5 (100 mM succinate buffer, 100 mM sodium tartrate buffer), pH 4-5 (100 mM malonate buffer, 100 mM succinate buffer), pH 6-7 (100 mM citrate buffer, 100 mM phosphate buffer), pH 8-9 (100 mM phosphate buffer, 100 mM carbonate buffer).

Effect of Temperature on Xylanase Activity: Xylanase was assayed at different temperature ranging from 25-70°C at pH 5.

Effect of Substrate Concentration, Determination of Km and V Max: The Michalis-Menten kinetic constants (Km, Vmax) were determined by using different concentration of xylan were prepared ranging from 0.1-0.5 mM.

Effect of Metals Ions on Partially Purified Xylanase: Metal ions such as $Pb(NO_3)_2$, $MgSO_4$, $CuSO_4$ were studied ranging 2-10 mM. All the organic compounds studied

above and heavy metals having inhibitory effects reduce the activity of xylanase.

Application for Removal of Hemicelluloses: Lignocellulosic substrates such as rice polish, rice husks, corn cobs, corn stover and banana stalk treated with xylanase. Every substrate 100g/L was taken, 0.1 M phosphate buffer at pH 7. Lignocellulosic substrates were treated with different concentration of crude xylanase such as 2, 4, 6, 8 and 10 mL for 24 h at 45°C for 100 rev/min in shake flasks. The supernatants were assayed for sugars determination.

RESULT AND DISCUSSION

Optimization and Purification: Humicola lanuginosus was multiplied in sugar cane bagasse (5g) liquid state medium using varying, incubation time, temperature, pH and nitrogen sources. Xylanase activity of 550000U/500 mL after 4 days. After passing the xylanase from gel filteration obtained with an average specific activity of 379U/mg protein, purification fold 1.9 and recovery yield of xylanase 10% as shown in Table 1. Liquid state culture was optimized after 4 days of growth, optimum temperature 35°C, pH 5, inoculums size 2mL, Vogel's media 160 mL and best N sources corn steep liquir 1 g with 5 g sugar cane bagasse. Therefore routinely harvested to the fermented biomass and filter through filter paper (125 mm) filtration was centrifuged (14,000 rpm, 10 min, 4°C) and removed the fungal pellets to obtain the maximal xylanase activity. The proteins were precipitated from the cell-free fermentation broth with ammonium sulphate (80% saturation) obtained specific activity 300 U/ mg and purification fold 1.4. The precipitate was dissolved in 50 mM citrate buffer (pH 5) and the extra salt was removed by dialysis. Xylanase was partial purified by ammonium sulfate precipitation, molecular exclusion chromatography [19].

Gel Filtration Chromatography: The partially purified xylanase sample was applied to the column, equilibrated with 50 mM citrate buffer, pH 5. The partially purified xylanase was eluted monitored A 280. The concentrate sample in 50 mM citrate buffer pH 5 was applied to Sephadex 75. Fifteen major positive fractions were collected and lyophilized to reduce the volume as shown in Figure 1.

Effect of Ph on Activity and Stability of Partially Purified Xylanase: The optimum pH of xylanase was 5 and stability of xylanase 6-7 obtained from H. Launginousa. Xylanase retained 15% of its activity at pH 9 after 24 h incubation at 40°C. The optimal pH of xylanase was 8.0 and stability 5.0-7.0 [19-21]. The activity of xylanase was pH 4.5 and stability of pH from pH 3.5 to 7.5 [22].

Effect of Temperature on on Activity and Stability Partially Purified Xylanase: The optimum temperature of partially purified xylanase was observed at 45°C. The thermal stability of this enzyme was 35 to 40°C after 24 h incubation. Partially purified xylanase activity retained 24% at 60°C after incubation 24 h as shown in Figure 3. Xylanase activity was 60°C and stability 40 and 60°C [4-21]. The purified xylanase optimum activity was 55°C and pH 9 [23]. The optimum xylanase activity was 50°C and stability 40°C [24].

Effect of K_m and V_{max}: The kinetic parameters of V_{max} and K_m value of partially purified xylanase were 450 mM/min, 1.4 mM for oxidation of oat spel xylan as substrate (Figure 4). The Km and Vmax values of purified xylanase were 35.47 mg of xylan/ml and 1.05 ìmol/min/mg of protein [20-21]. Km values using soluble and insoluble arabinoxylans were 10.87 and 11.20 mg/mL [23-24].

Effect of Metals Ions on Partially Purified Xylanase: Various ions metals ion were studied, only 2 mM to 4 mM $MgCl_2$ enhanced the activity of partially purified xylanase. But the effect of $CuSO_4$ on xylanase activity 2 mM to 10 mM concentration remains constant as shown in Figure 5. While studying the effect of 2 to 10 mM concentration of $AgNO_3$ and $PbNO_3$ on activity of xylanase it was noted 75% inhibited activity (Figure5). Xylanase activity was induced by Ca^{+2} and decreased by Na^{+} and Zn^{+2} [21]. Fe^{+}, Ca^{+2} and Mg^{+2} enhanced the activity of purified xylanase [2-4]. Xylanase activity was increased 31 % by Mg^{2+} and 28 % by Al^{3+} [24].

Table 1: Purification of xylanase

Sr. No	Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
1	Crude Xylanase	500	550000	2700	203	100	1
2	$(NH_4)_2SO_4$ Precipitation	50	391000	1300	300	71	1.4
3	Dialysis	50	338589	1030	328	61	1.7
4	Sephadex-75	15	58899	155	379	10	1.9

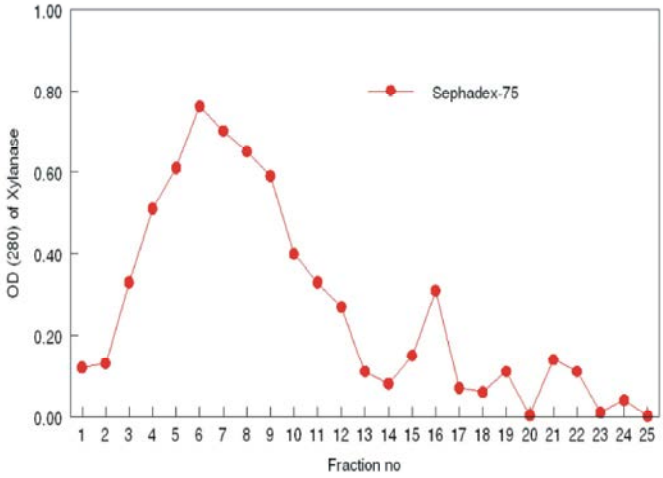

Fig. 1: Gel filteration chromatography of xylanase produced by *H. lanuginosus*

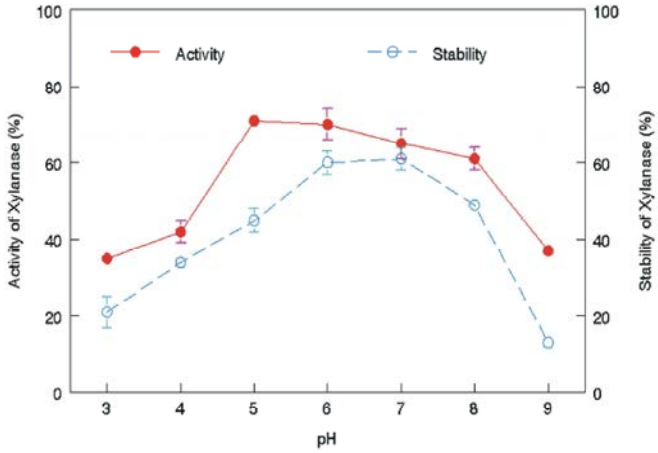

Fig. 2: Effect of pH on activity and stability of partially purified xylae produced by *H. lanuginosus*

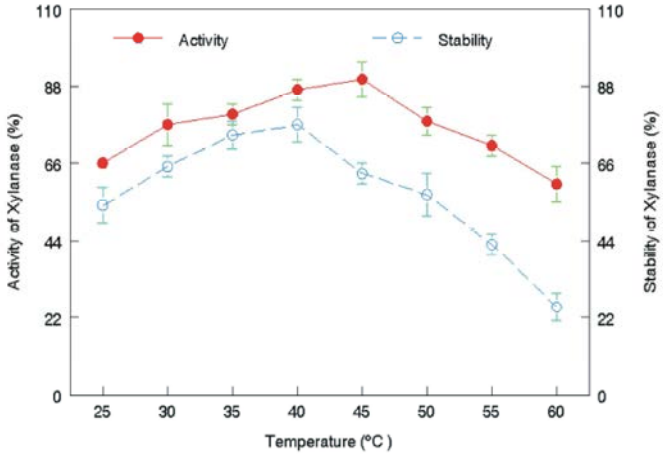

Fig. 3: Effect of temperature on activity and stability of partially purified xylanase produced by *H. lanuginosus*

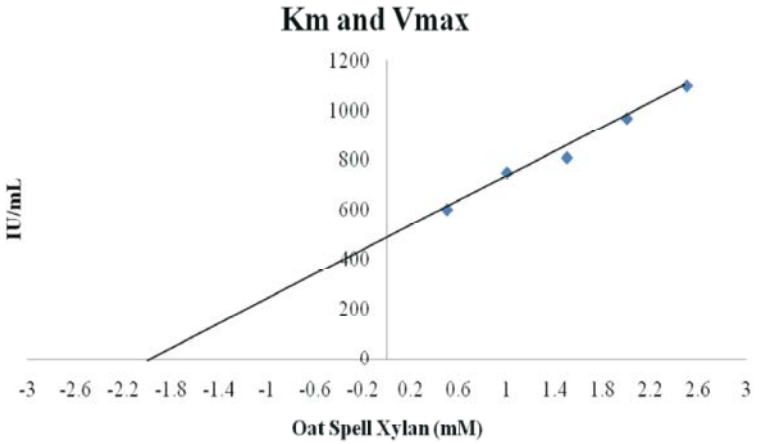

Fig. 4: Km and Vmax of partially purified xylanase

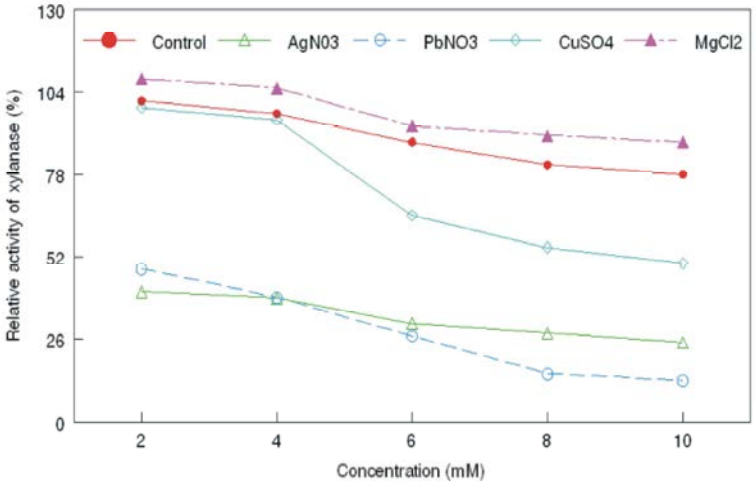

Fig. 5: Effect of metals ions of partially purified xylanase produced by *H. lanuginosus*

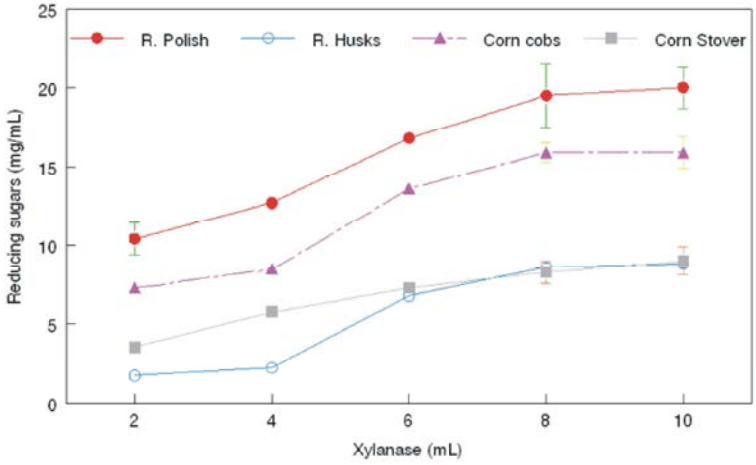

Fig. 6: Crude xylanase used to remove the hemicellulose from lignocellulosic substrates after 24 hours incubation

Application of Xylanase: Reducing sugars were released from lignocellulosic substrates by the treatment of xylanase after incubation of 24 h. Twenty mg/mL of sugars released from rice polish followed by the corn cobs 15 mg/mL after traetment of crude xylanase 10 mL as shown in Figure 6. Reducing sugars was released in the kraft pulp by addition of 25-100 U of xylanase after 6h incubation [25]. Xylanases improved the bread volume and in increasing shelf life of bread [26]. Xylanase used to remove hemicellulose from pulp bleaching [2, 27].

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