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Production and partial purification of xylanase from a new thermophilic isolate

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ABSTRACT : Ten thermophilic xylanase producing microbes were isolated from degraded wood, cow feed sample, sugarcane bagasse and soil sample on potato dextrose agar plates, pH 5.5 at 40°C. These isolates were screened for xylanase production and the hyper-xylanase producer isolate (120U/ml) from cow feed sample (*Bacillus pumilus* MTCC 8964) was selected for further study. Maximum production of xylanase with this isolate was observed in late log phase at pH 6.0 with 1% inoculum at 40^{0}C. Maltose (1%) and yeast extract (1%) were selected as best carbon and nitrogen source respectively for xylanase production by this organism. High activity of enzyme was recorded with acetate buffer at pH 6.0 and 60°C (241.4U/ml). Molecular mass of this enzyme was estimated to 14.3KDa with SDS-PAGE after gel exclusion chromatography.

Keywords: Thermophilic xylanase, *Bacillus pumilus* MTCC 8964, production optimization, partial purification

INTRODUCTION

Hemicellulose is the second most abundant plant fraction available in nature and waste residues contain up to 40% hemicellulose formed by pentose sugars (Magge and Kosaric, 1985). There is a great interest in the enzymatic hydrolysis of xylan, the major constituent of hemicelluloses, due to possible applications in feedstock, fuel, chemical production and paper manufacturing (Coughlan and Hazelwood, 1993). â-1,4 xylanases (1,4 â -xylan-xylanohydrolase, E.C. 3.2.1.8) catalyzes the hydrolysis of xylan to xylo-oligosaccharides and xylose. A variety of microorganisms, including bacteria, yeasts and filamentous fungi have been reported to produce xylanases (Coughlan and Hazelwood, 1993; Nascimento *et al*., 2002; Poorma and Prema 2007; Bakri *et al*., 2008). The use of cheaper lignocellulosic residues viz. wheat bran, wheat straw, corn cob and sugarcane bagasse can be used as growth substrates in culture to produce xylanase and also to replace the xylan as a inducer for cost reduction in production. Xylanase obtained from thermophilic microorganisms have unique physico-chemical and catalytical properties with its major application in paper and pulp industry for pre-bleaching of Kraft pulp (Bajpai, 1999; Beg *et al*., 2001; Christov *et al*., 1999). Other applications include; extraction of coffee, plant oils and starch, improvement of nutritional properties of agricultural silage and grain feed, and clarification of fruit juices in combination with pectinase and cellulase, and recovery of fermentable sugars from hemicelluloses (Kuhad and Singh, 1993; Lama *et al*., 2004; Wang and Saddler, 1992).

Bacterial xylanase is generally induced by the microorganisms cultivated in medium containing xylan or xylan-hydrolysate as a carbon source (Fogarty and Ward, 1973; Simpson, 1956). Considering the industrial importance of xylanase, the objective of present study was to evaluate the isolation, production and partial purification of a thermostable xylanase obtained from a new thermophilic strain *Bacillus pumilus* MTCC 8964.

MATERIAL AND METHODS

Isolation and screening of microorganism

Samples of degraded wood, sugarcane bagasse, cow feed and soil sample were collected from various sites of Solan and Shimla (H.P.), India, in sample containers and stored at 4°C for further processing. Samples were enriched in nutrient medium supplemented with xylan (0.5%) at 40°C for 24h at pH 5.5 in an incubator shaker at 135rpm and plated on PDA containing oat-spelt xylan (0.5% w/v) at 40°C for 24h. All the isolates were screened for xylanase release ability in production medium containing potato 5.0%, dextrose 2.0%, yeast extract 0.5%, oat spelt xylan 0.5% (pH 5.5) and incubated at 40°C for 48h at 150rpm. Only the hyper-xylanase producer isolate (X1) was selected for further optimization and characterized according to the method given by Harrigan (1998).

Xylanase assay

The supernatant obtained after centrifugation of fermentation broth at 5000rpm for 15 min at 4°C was used as a crude enzyme. Xylanase activity was determined by

measuring the reducing sugar by the dinitrosalicylic acid (DNS) method (Miller, 1959) using D-xylose as the standard. The enzyme assay was carried out at 40°C using 0.5% (w/v) oat spelt xylan (50mM Acetate buffer, pH 6.0) as substrate. One unit of xylanase activity was expressed as 1ìg of reducing sugar (xylose equivalent) released/ml/min under assay conditions.

Optimization of xylanase production and activity. The *Bacillus pumilus* MTCC 8964 was cultivated in 50ml of production medium with 1% (v/v) inoculum at different pH (4.0-10.0) and temperatures (30°C-70°C) for 48h under standard shaking conditions to assay the enzyme produced. Effect of carbon and nitrogen sources on production was studied by substituting potato extract (5%) and dextrose (2%) in the production medium with various synthetic and natural carbon sources (1% w/v) and yeast extract with organic and inorganic nitrogen sources (1% w/v) to assess their suitability to promote the xylanase by this microorganism. Xylanase production was studied upto 7 days in production medium supplemented with 0.5% oat spelt xylan using 1% (v/v) inoculum of 24h old culture (OD at 540 nm = 1.0) at 40°C in a incubator shaker (Scigenics Pvt. Ltd., India) at 155rpm. Xylanase activity was assayed at different pH of buffer (50mM) ranging from 5.0-11.0 and at various temperatures (30-70°C) to determine the optimal activity under reaction conditions.

Partial purification and molecular mass estimation To 100ml of crude xylanase solution previously filtered through 0.45µm filter (Sartorious, Germany), pre chilled ethanol was added slowly up to 70% saturation with gentle stirring and left overnight at 4°C. The precipitate formed were collected by centrifugation at 10,000g for 30min and dissolved in 1 ml of buffer (50mM Acetate, pH 6.0). The enzyme sample was loaded on to gel exclusion column (KT 30, Bangalore Genei) and eluted with the same buffer. Protein fractions (OD at 260nm) were collected and pooled together and used further for molecular mass estimation by Shi and Jackowski (1998) method. Molecular mass of the partially purified enzyme was calculated by using low molecular weight calibration kit of Bangalore Genei, India.

RESULTS AND DISCUSSION

Ten thermophilic bacteria were isolated from various samples collected from different sites around Solan and Shimla districts of Himachal Pradesh, India (Table 1). Growth was observed in the plates and colonies which degraded xylan were detected by halo zones around them. The isolates (X1) obtained from cow feed samples produced maximum amount of enzyme during screening and was further characterized (Table 2) on the basis of morphological, biochemical and physiological characteristics as per the methods of Harrigan (1998). This isolate was found to be rod shaped, spore former and non-motile It was facultative thermophile growing at temperatures of 30-45°C and identified as *Bacillus pumilus* MTCC 8964 by Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India.. There are also reports on isolation of xylanase producing microorganism from baggase (Rezende *et al*., 2002) and soil (Nascimento *et al*., 2002; Kuhad *et al*., 2006).

Table 1 : Screening of microorganisms for xylanase production at 40°C.

Sample habitat	Isolate No.	Xylanase activity (U/ml)
Cow feed	X1X2	40.00± 0.1006.00± 0.100
Degraded wood	X3X4X5X6	3.30± 0.1001.60± 0.10026.9± 0.20017.6± 0.100
Soil	X7X8	4.47± 0.08530.1± 0.025
Sugarcane baggase	X9X10	1.27± 0.08520.2± 0.025

*One unit of xylanase activity was defined as the amount of enzyme required to liberate 1µg of xylose/ml/min under assay conditions.

Table 2 :Morphological and biochemical characteristics of Bacillus pumilus MTCC 8964.

Morphological characteristics*	
Configuration	Circular
Surface	Mucoid
Pigment	Cream
Opacity	Opaque
Gram's reaction	Positive
Cell shape	Long rod
Spore	Positive
Shape	Ellipsoidal
Arrangement	Filamentous
Motility	Non-motile

Biochemical tests	Results
Indole test	Negative
Methyl test	Negative
Voges Proskauer test	Negative
Casein hydrolysis	Positive
Starch hydrolysis	Negative
Catalase test	Positive
Oxidase test	Positive
Acid from glucose	Positive
Acid from lactose	Positive
Acid from sucrose	Positive

*Morphological tests were performed in the laboratory and other tests were carried out at MTCC, IMTECH, Chandigarh, India.

The organism secreted xylanase at broad range of temperature and pH. Temperature has profound effect on xylanase production. Maximum production was observed at 40°C (43.85U/ml) (Fig. 1). Optimum xylanase production was reported earlier at 37°C with wheat bran (Battan *et al*., 2006). The isolate had shown maximum growth and production at pH 6.0(113.20U/ml) (Fig. 2). Kuhad *et al*. (2006) observed maximum production of xylanase by *Bacillus* sp. RPP-1 at pH 7.0.

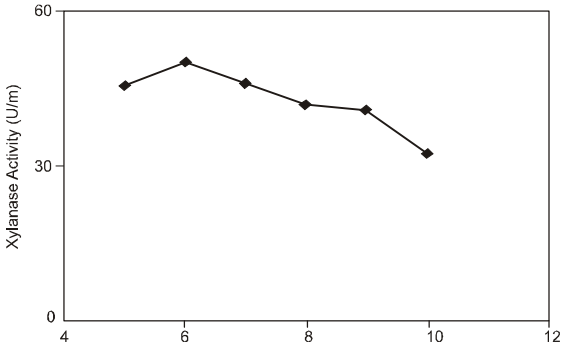

Fig 1. Production of xylanase by *Bacillus pumilus* MTCC 8964 at different pH of medium [After 48h of incubation at 40°C]

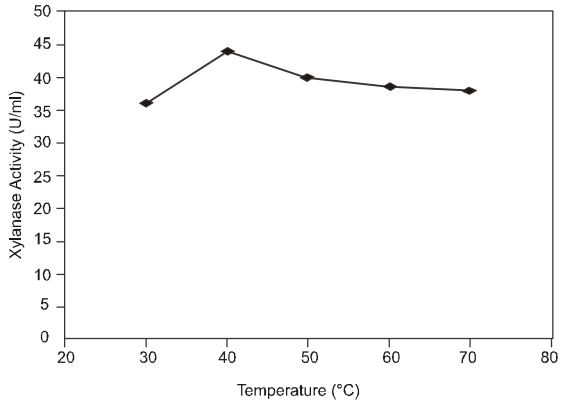

Fig. 2. Production of xylanse by *Bacillus pumilus* MTCC 8964 at different temperatures [After 48h incubation in production medium at pH 6.0]

The Bacillus pumilus MTCC 8964 utilized all carbon sources tested (Table 3) and induced highest level of xylanase production with maltose (60.20U/ml) followed by saw dust and cellulose filter paper. The use of purified xylan as an inducer increases the cost of enzyme production. For this, reason, different lignocellulosic residues, can be used as growth substrates in cultures to produce xylanases (Reis *et al.*, 2003).

Of the various organic nitrogen sources tested, yeast extract (128.07U/ml) was found to be the best nitrogen source for xylanase production from the organism. Very low level of enzyme was produced in the presence of inorganic nitrogen (Table 3). Beef extract and peptone were similarly reported earlier the best nitrogen source for xylanase production (Kheng and Omar, 2005). Similar results were observed for xylanase production by *Bacillus* sp. RPP-1(Kuhad *et al*., 2006). As, some amount enzyme is required to hydrolyse natural substrates before their entry in to the cells, and this could be the reason for low levels of xylanase production by this organism in natural carbon and nitrogen sources. However, organic nitrogen sources are generally reported better than inorganic nitrogen sources for xylanase production by microorganisms (Kuhad *et al*., 2006). There are also reports on use of xylan (Lopez *et al*., 1998), soyameal waste (Heck *et al*., 2002) and rice bran (Virupakshi *et al*., 2005) as a substrate for the xylanase production by *Bacillus* spp.

Table 3 : Production of xylanase by Bacillus pumilus MTCC 8964 in the presence of various carbon and nitrogen sources.

Carbon source (1% w/v)	**Xylanase activity(U/ml)**
Dextrose	42.1 ± 0.050
Maltose	60.2 ± 0.095
Xylose	39.65 ± 0.125
Wheat bran	36.23 ± 0.0650
Saw dust	48.37 ± 0.085
Filter paper	45.35 ± 0.025
Nitrogen source (1% w/v)	
Urea	95.0 ± 0.100
Peptone	107.4 ± 0.100
Yeast extract	128.0 ± 0.050
Ammonium nitrate	69.6 ± 0.100
Ammonium sulphate	62.0 ± 0.100

The time course of xylanase production was studied in shake flasks using under optimized condition. Enzyme was assayed after every 4h. Xylanase production increased exponentially up to 48 h (158U/ml) with optimum production at late log phase and had shown slight decrease in activity up to 72 h of incubation (Fig. 3). Maximum xylanase production was achieved in 72 h with *Bacillus pumilus* ASH (Battan *et al*., 2006).

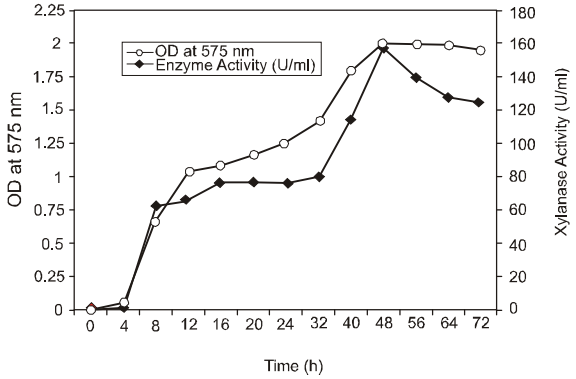

Fig. 3. Time course of xylanase production by *Bacillus pumilus* MTCC 8964 at 40°C [The optical density and xylanase activity in the supernatant were determined at regular intervals of 4h up to 24h and afterwards 8h intervals up to 72h]

Enzyme activity was assayed in the presence of different range of buffers (50mM). The enzyme exhibited a broad pH profile (5.0-11.0) for the hydrolysis of oat spelt xylan (Fig. 4). The optimum pH for the activity of xylanase was 6.0 (220.6U/ml) and xylanolytic activity decreased remarkably after pH 11.0. However, the optimum activity was recorded earlier at pH 8.0 (Sharma and Bajaj, 2005). The maximum activity of xylanase of *Bacillus pumilus* MTCC 8964 was observed at 60ÚC (241.4U/ml) and decreased with increase in temperature to 70°C (Fig. 5). There was a gradual increase in activity from 50-60°C and considerable decrease below and above these temperatures.

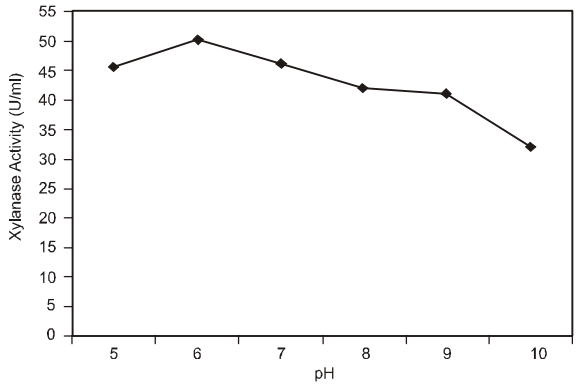

Fig. 4. Xylanase activity at different pH of assay buffer [The pH of the reaction buffer was altered using Acetate buffer 50mM (pH5.0-6.0); Tris HCl 50mM (pH 7.0-9.0) and Glycine-NaOH (pH 10.0) at 40°C]

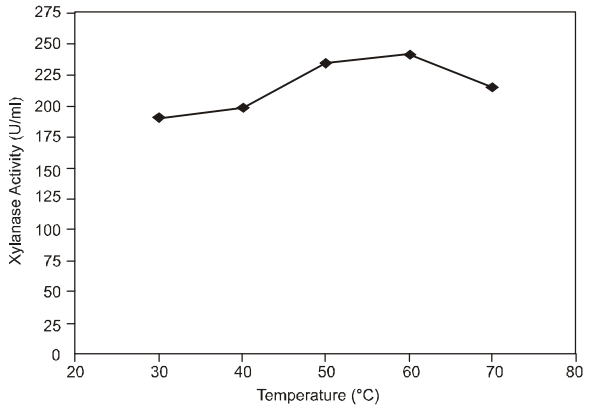

Fig. 5. Effect of temperature on xylanase activity [Enzyme reaction was carried out at different temperatures under assay conditions at pH 6.0]

The solvent precipitation steps were carried out at 4°C and the enzyme migrated as a single band on SDS-PAGE (Fig. 6). The apparent molecular mass of the partially purified enzyme was very low (14.3KDa). It has been reported that solvent precipitation yields a product of higher purity and activity than does salt precipitation.

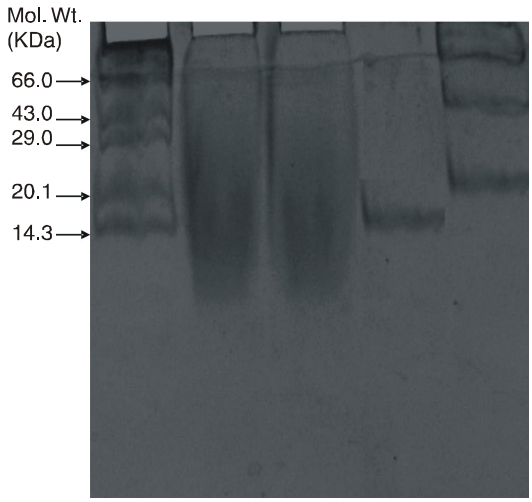

Fig. 6. SDS-PAGE of partially purified xylanase after gel exclusion chromatography [Lane 1 and 5, protein molecular marker; lane 2 and 3, crude enzyme; lane 4, ethanol precipitated enzyme]

CONCLUSIONS

In conclusion, the results obtained from the present work indicates significant amount of xylanase production from a new isolate of *B. pumilus* MTCC 8964 using selective growth and nutrient conditions. The xylanase produced by the test organism owing to its broad activity, temperature range and cellulase free nature seem to be of considerable use in paper and pulp industry. Detailed characterization of the enzyme is in process.

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