Production and partial characterization of alkali-tolerant xylanase from an alkalophilic Streptomyces sp. CD3

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Of the 30 isolates screened, Streptomyces sp CD3, an alkalophilic isolate, showed maximum xylanase production. Organism efficiently used wheat bran and bagasse as substrates and produced 2.211 and 1.896 U/ml of xylanase, respectively. Soybean meal supplementation enhanced xylanase production substantially, while yeast extract and gelatin did so moderately. Although, optimum enzyme activity was at pH 8 and temperature (50°C), but enzyme retained considerable activity at higher pH (80% at 9-10) and temperature (60 % at 70-90°C). The enzyme was strongly inhibited by Hg^{2+}, while Fe^{3+}, Ca^{2+} and Zn^{2+} were slight inducers of xylanase. Zymogram analysis suggested the presence of three xylanases (mol wt, 69.18, 63.09 & 43.65 kDa). Purification (108-fold) was achieved by carboxymethyl sephadex chromatography. Enzyme obeyed Michaelis-Menten kinetics (K_m 3.9 mg/ml). Industrially desirable characteristics of the enzyme like thermostability and alkali-stability, and highly alkalophilic nature of the organism, and its ability to grow and produce enzyme on low value agricultural by-products reflects the potential commercial importance of this study.

Keywords: Streptomyces, Xylanase, Lignocellulosics, Thermostable, Alkalophilic, Alkali-tolerant, Zymogram

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Introduction

Lignocellulosics constitute one of the most abundant renewable resources in nature, and xylans, the most abundant of the hemicellulosic fraction of lignocellulosics, are the major components of the hardwoods and Graminaceous plants. Xylan (20-30 % of total dry weight of plant biomass) is heterogeneous polysaccharide consisting of a homopolymeric backbone of 1,4-linked β-D-xylopyranose residues and short chain branches including O-acetyl,α-L-arabinofuranosyl, α-D-glucuronyl residues. Due to structural complexity, biodegradation of xylans require synergistic action of endo-1, 4-β-xylanases (E.C. 3.2.1.8) and β-xylosidase (E.C. 3.2.1.37), and several accessory enzymes to hydrolyze the substituted xylans. Microbial xylanases have commercial applications in agriculture, industry and human food production. Xylanases are useful in bioconversion of lignocellulosics to fuel and chemicals, to improve silage for better digestion by ruminants, to improve quality of detergents, and also used for clarification of fruit juices, in flour improvements for bakery products and in controlling environmental hazards through biopulping. For biobleaching, candidate xylanase should be thermostable, alkali-tolerant, stable on kraft-pulps and its properties like effective molecular weight, net ionic properties and specific action pattern must suit process requirements. Moreover, to avoid damage to cellulose pulp, preparations should be free from cellulase activity.

Xylanases are produced by microorganisms including bacteria, fungi and actinomycetes. Actinomycetes are Gram-positive filamentous bacteria reported as producers of industrially important enzymes involved in lignocellulose degradation. Streptomyces, Chainia and Thermomonospora produce extracellular xylanases, which are mainly endotype. Further, many species of Streptomyces are reported to produce multiple xylanases. The enzymes are generally produced when the microorganism are cultivated in medium containing xylan or xylan hydrolysate as the carbon source. However, commercially available pure xylan is too expensive for use in industrial scale fermentation. Alternatively, agricultural by-products that contain cellulose, hemicellulose and lignin could serve as inexpensive sources for xylanase.

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production\textsuperscript{4,7}. Most of the xylanases are active below 50°C and act in acidic or neutral pH. However, in many industrial applications including enzyme assisted kraft pulp bleaching, the incoming pulp has high temperature and alkaline pH, necessitating the search for thermostable and alkali stable xylanases.

Approaches for getting economically viable, cellulase-free xylanases could be selective inhibition of cellulases by mercurials or by genetic engineering of the organism. However, keeping in view the broad biochemical diversity and cosmopolitan distribution of wood degrading microbes, the most practically feasible approach is to screen alkalophiles and thermophiles for production of cellulase-free xylanases active at high temperature and alkaline pH. The present study aimed at the isolation of xylanolytic bacteria from alkaline, hot and humid locations surrounding decaying organic matter and subsequently optimizing a fermentation process to cultivate the organism for maximum production of xylanase and finally, the characterization of enzyme.

Materials and Methods

Chemicals, Media and Media components

All chemicals, media and media components were of AR grade obtained from Sigma Chemicals Ltd and Merck & Co Inc., USA; and HiMedia Laboratories Ltd, Mumbai, and Ranbaxy Fine Chemicals Ltd and Qualigens Fine Chemicals Ltd, New Delhi, India.

Isolation and Screening of Bacteria for Xylanase Producing Ability

Samples of alkaline soil, sugar cane bagasse, cow dung, hay and paper industry waste were collected from different locations of Jammu city and suburban area. A total of 30 bacterial isolates were obtained from soil (AS1-AS4, RS1-RS4, BS1-BS4, CS1-CS4), cow dung (CD1-CD4), paper industry waste (PWA1-PWA3, PWB1-PWB2), hay (RH1-RH2) and sugar cane bagasse (MB1-MB3). Samples (\textasciitilde 5-10 %, w/v) were inoculated into nutrient broth (pH, 8-11) and incubated at 45°C with gyratory shaking at 200 rpm for 72 h. Periodically, the samples from enriched broth were withdrawn, appropriately diluted, and plated on basal salts agar medium containing oat spelt xylan (0.5 %, w/v).\textsuperscript{8} The plates were incubated at 45°C for 48 h and colonies developed were assayed for xylanase production by Congo red staining method\textsuperscript{9}. Plates were flooded with congo red (0.1 %) for 15 min and then washed with 1 M NaCl. The colonies showing halos around them were picked up and maintained on nutrient agar slants at 4°C with monthly transfer.

Fermentation Conditions for Xylanase Production

All the 30 bacterial isolates were tested for xylanase producing ability in production medium containing basal salts and xylan.\textsuperscript{8} The activated culture of the organism was inoculated into production medium (100 ml) contained in Erlenmeyer flasks (250 ml) and incubated under shaking at 45°C for 48-72 h. The enzyme activity was assayed periodically after different time intervals. Crude enzyme was obtained by centrifugation of a suitable volume of fermentation broth at 10,000 g for 5 min at 4°C and the supernatant was considered equivalent to crude enzyme. The isolate CD3, isolated from cow dung, was found to be the best producer of xylanase and used for further studies. Cultivation of the organism for xylanase production was carried out in basal salt medium containing xylan unless stated specifically in certain cases where medium was supplemented with certain additives.

Enzyme Assay and Protein Estimation

Xylanase activity was assayed by dinitrosalicylic acid (DNS) method\textsuperscript{10} using a calibration curve for D-xylose. Assay mixture [0.5 % xylan, 0.5 ml; phosphate buffer (50 mM, pH 7), 0.8 ml; and enzyme, 0.2 ml] was incubated at 50°C for 15 min and then 1.5 ml DNS reagent was added to terminate the reaction. Test tubes containing reaction mixture were stoppered using glass marbles and kept in a boiling water bath for 10 min and then cooled to room temperature and absorbance was recorded against reagent as blank at 575 nm keeping enzyme as control. One unit of xylanase activity was expressed as 1 \textmu mol of reducing sugar (xylose equivalent) released in 1 min. Similarly, cellulase activity was determined by measuring the release of reducing sugars from carboxymethyl cellulose (CMC) by DNS method using glucose as standard. All reaction conditions were same except that instead of phosphate buffer, acetate buffer (50 mM, pH 5) was used. One unit of cellulase activity (U) is defined as the enzyme necessary to release 1 \textmu mol of glucose equivalent per min at 50°C.

Protein content in the supernatant was estimated by Lowry’s method\textsuperscript{11} using bovine serum albumin (BSA) fraction V as standard. Protein content was also determined spectrophotometrically using Christian-Warburg method (Application 26: \textmu g/ml protein =...
1.55A_{280 \text{ nm}} - 0.77A_{260\text{nm.}}) while attempting protein purification.

**Effect of Environmental and Cultural Variables on Xylanase Production**

For studying xylanase production from crude lignocellulosics, xylan in the production medium was replaced by agricultural based crude carbon sources like sugar cane bagasse, wheat bran, paper industry waste, rice husk or wood husk. These substrates (1 %, w/v, each), used as sole carbon source, were tested for their ability to induce xylanase production, with and without steam treatment. The crude substrates were crushed to finer pieces and steamed for 30 min at 121°C in the autoclave before use. The effect of nitrogen on xylanase production was analyzed by supplementing production medium with different nitrogen sources (0.25 %, w/v, each) like yeast extract, soybean meal, gelatin, peptone, albumin, tryptone, urea, potassium nitrate and ammonium sulphate, and enzyme activity was assayed. The effect of pH on enzyme production was tested by adjusting the initial pH (8-11) of production medium using Na_{2}CO_{3} (2 %, w/v) and then carrying out the cultivation of organism for enzyme production at 40°C on shaker (200 rpm) for 96 h.

**Effect of Temperature, pH and Metal Ions on Enzyme Activity, and Thermostability of Enzyme**

The crude enzyme preparation obtained after cultivation of the organism in production medium was assayed for xylanase activity at different temperatures (40°C, 50°C, 60°C, 70°C & 90°C). For determining effect of pH on enzyme activity, different buffers (acetate, phosphate, glycine-NaOH) at a concentration of 50 mM and pH (4-10) were used in the enzyme assay reaction mixture to expose the enzyme to different pH values. Effect of various metal ions (Pb^{2+}, Hg^{2+}, Fe^{3+} & Ca^{2+}) on enzyme activity was studied by including them in the enzyme assay reaction mixture at the final concentration of 1 mM and 10 mM.

Thermostability of xylanase was determined by incubating the enzyme preparation between 50-90°C for different time periods before using it for assaying the residual activity.

**Zymogram Analysis**

Enzyme preparation obtained after ammonium sulphate saturation (30-60%) was used for zymogram analysis^{12}. Samples were subjected to discontinuous sodium dodesyl sulphate polyacrylamide electrophoresis (SDS-PAGE)^{13} containing (xylan 0.1%). After electrophoresis, the gel was washed three times for 30 min each at 4°C with 0.1 M phosphate buffer (pH 8) containing isopropanol (25%, v/v) to remove SDS. The gel was further washed twice with 0.1 M phosphate buffer (pH 8) and then submerged in the same buffer at 50°C and stained with Coomassie brilliant blue G-250. The gel was then destained and washed with 0.1 M Tris-HCl (pH 8) and zymogram was developed by soaking the gel in congo red for 15 min and washing with 1M NaCl. Molecular weight of protein markers ranged from 18.4 to 116 kDa (Fermentas).

**Partial Purification of Xylanase**

Partial purification of xylanase was carried out using ion exchange chromatography. The crude enzyme preparation was applied to carboxymethyl sephadex column, which was equilibrated with 50 mM phosphate buffer (pH 8). The unbound run through was collected, and after washing the column with 50 mM phosphate buffer (pH 8), elution was carried out using 0-0.3 M continuous sodium chloride gradient and fractions of 5.0 ml each were collected. Protein suspected to be still bound was eluted using 1 M NaCl (25 ml). Fractions collected were tested for xylanase activity and protein content.

**Determination of k_{m}**

Initial rates of xylan hydrolysis were determined at different substrate concentrations (4-20 mg/ml). Higher xylan concentration could not be used because of relative low solubility of xylan. Reaction rate vs. substrate concentration was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics.

**Results and Discussion**

**Xylanase Production by Different Isolates**

Isolate *Streptomyces* sp CD3, obtained from cow dung sample, produced maximum amount of enzyme (1.175 U/ml) after 72 h (Fig. 1) and was selected for further studies. Majority of isolates showed activity between 0.15-0.5 U/ml after different periods of incubation. Isolates AS1, AS2, and CS1 showed considerable enzyme activity after 24 h of incubation. Isolates CD2, CD4, CS1, RS4 and MB3 produced significant amount of enzyme activity after 48 h. Enzyme activity in case of CD3 increased appreciably after 72 h, while in case of other isolates the activity decreased. Vyas et al^{14} screened soil samples and obtained
Streptomyces sp V 5 as the good producer of xylanase. A large number of xylanolytic and thermophilic actinomycetes were isolated from composted grass and cattle manure and a few of the isolates like Actinomadura and Streptomyces sp were found to be high xylanase producers (4-12 U/ml). Kohli et al. isolated from alkaline soil of Thermoactinomyces thalophilus, which produced 23 U/ml of xylanase. Bacillus subtilis isolated from a hot spring produced about 12 U/ml of xylanase. A total of 162 actinomycete isolates from Brazilian soil were screened for xylanase activity and S. malaysiensis was found to be the best producer of xylanase (116 U/ml).

Xylanase Production using Agriculture based Crude Carbon Sources

Agricultural by products rich in hemicellulose constitute one of the most potential, economic and easily available substrate. Xylan of production medium was replaced by various agricultural based crude substrates like wheat bran, rice husk, sugarcane bagasse, wood husk and paper industry waste for enzyme production. Steam pretreated carbon sources gave considerable enzyme production, particularly in medium containing wheat bran (2.211 U/ml after 96 h) or sugarcane bagasse (1.896 U/ml in 24 h). The organism showed higher enzyme activity with these two substrates as compared to its enzyme activity in standard production medium containing xylan. In case of medium containing paper industry waste as carbon source, enzyme activity after 24 h was 0.434 U/ml, which is higher than that in production medium with xylan, but it decreased thereafter. Enzyme activity in case of media with rice husk and wood husk was lesser than that in xylan media (Fig. 2).

Agricultural based crude carbon sources serve as good substrates for xylanase production. Steam treatment of agricultural residues (corn cobs, bagasse) is reported to give enhanced production of xylanase. Sa-Pereira et al. obtained 3 U/ml of enzyme activity using wheat bran as carbon source while Qureshy et al. obtained 0.493 and 0.413 U/ml of xylanase activity on wheat bran and bagasse containing media, respectively.

The enzyme obtained from wheat bran containing production medium gave 0.33 U/ml of cellulase activity. Ideally, the enzyme preparation must not have cellulase activity particularly when the intended use is in biopulping industries. However, for other applications like biotransformations and enhancing silage quality, cellulase activity is useful. Xylanases from variety of different organisms get contaminated with cellulases. However, many studies reported cellulases-free xylanases from various microorganisms.

Effect of Nitrogen Sources on Enzyme Production

Soybean meal supplementation resulted in maximum enzyme production (4.253 U/ml after 72 h), followed by yeast extract supplemented medium (2.283 U/ml after 96 h). Enzyme activity was also quite high (1.872 U/ml after 96 h) in gelatin-supplemented medium. However, in case of urea, ammonium sulphate, potassium nitrate or tryptone-supplemented medium, no appreciable increase in enzyme activity was registered (Fig. 3). Sá-Pereira et al. reported that soyflour, a balanced source of
Fig. 2—Xylanase production from raw agricultural substrates

Fig. 3—Effect of different nitrogen sources on xylanase production
protein in terms of composition and accessibility, was the best nitrogen source for maximum xylanase production, while urea, being a recalcitrant source of nitrogen leads to repression in xylanase biosynthesis. Bakir et al.\textsuperscript{18} also reported soybean meal as best nitrogen source for maximum production of xylanase from \textit{R. oryzae}. However, Srinivasan et al.\textsuperscript{19} reported enhanced xylanase activity in yeast extract supplemented medium. Bakri et al.\textsuperscript{20} observed yeast extract as the best nitrogen source among various nitrogen sources tested. A combination of yeast extract and peptone as nitrogen sources led to the best xylanase production by \textit{Penicillium canescens} 10-10c.

Maximum specific activity of enzyme was found in medium containing soybean meal followed by yeast extract and gelatin containing medium (Fig. 4). Besides, specific activity in general corresponded to enzyme activity at a particular time in a particular broth, suggesting that the protein of interest constituted the major fraction of extracellular protein.

\textbf{Effect of Medium pH on Xylanase Production}

The organism was cultivated in production medium (supplemented with soybean meal @ 0.25%, w/v) adjusted at different pH 8-11, for 72 h at 40°C under shaking conditions, broth was harvested and enzyme was assayed in the supernatant (Fig. 5). Maximum enzyme activity was present in medium with pH 8 (4.2 U/ml) after 72 h, however, activity decreased thereafter. In medium with pH 9, maximum enzyme activity was achieved after 24 h (3.175 U/ml) while it decreased on further incubation. Sufficiently high activity was reported at pH 10 (2.423 U/ml) and 11(1.967 U/ml) after 48 h of growth. Whereas the activity in medium with pH 11 decreased sharply after 48 h, it remained more or less constant up to 72 h in medium with pH 10. These observations indicate that the organism is highly alkali-tolerant and produces significant levels of enzyme under alkaline conditions. The organism may have ability to produce multiple xylanases, which are induced at different pH values. Similar to present study, Bandivadekar & Deshpande\textsuperscript{21} reported optimum pH for xylanase production in \textit{Chainia} sp. as 7-8. Kohli et al.\textsuperscript{16} obtained maximum xylanase production by \textit{T. thalophilus} at media pH 8.5-9.0. Sá-Pereira et al.\textsuperscript{17} reported that the optimum pH for maximum enzyme production by \textit{B. subtilis} ranged from 6-7.

\textbf{Effect of Metal Ions on Enzyme Activity}

At 1 mM concentration of Hg\textsuperscript{2+}, enzyme activity was considerably reduced (75%), while at 10 mM concentration, activity reduction was more drastic (96%), suggesting involvement of disulphide bridges...
at the active or substrate binding site of enzyme (Fig. 6). In contrast, Pb\(^{2+}\) and Ca\(^{2+}\), at 1 mM and 10 mM, and Fe\(^{3+}\) at 10 mM enhanced enzyme activity (10-16%). Qureshy \textit{et al} reported that Hg\(^{2+}\) at 10 mM, inhibited xylanase activity (68 %), however, similar to present study, they reported Ca\(^{2+}\) and Fe\(^{3+}\) as stimulators of enzyme activity. In contrast, Ca\(^{2+}\) was reported as inhibitor by Sá-Pereira \textit{et al} \cite{17}, however, Fe\(^{3+}\) was found to have a stimulatory effect on enzyme activity, in agreement with present study.

**Effect of Temperature on Enzyme Activity**

Maximum enzyme activity (1.4 U/ml) was reported at 50°C (Fig. 7). Relative activity was less at 40°C (82.5%) and 60°C (75.47 %). At higher temperatures (70-90°C), enzyme activity was lesser (60 %). Thus, optimum temperature for enzyme activity was 50°C, but the enzyme retained considerable activity even at higher temperatures. Generally, microbial xylanases have temperature optima of about 50°C\textsuperscript{12}. Grabski & Jeffries\textsuperscript{23} reported xylanases from \textit{S. roisecleroticus} to have the temperature optima of 60°C, while certain strains of \textit{Actinomadura} have temperature optima of 70-80°C\textsuperscript{15}. Nascimento \textit{et al}\textsuperscript{4} documented optimum temperature of \textit{Streptomyces} sp. xylanase as 55-65°C, while Kohli \textit{et al}\textsuperscript{16} reported optimum temperature of xylanase from \textit{T. thalophilus} at 65°C. \textit{Bacillus coagulans} BL69 xylanase showed maximum activity between 45-75°C\textsuperscript{24}. For industrial applications, it is highly desirable that the enzymes must have higher temperature optimum.

**Effect of pH on Enzyme Activity**

Enzyme preparation obtained by culturing organism in soybean meal supplemented production medium was used for determining effect of pH on enzyme activity. Maximum enzyme activity (Fig. 8) was reported at pH 7 (3.315 U/ml) and 8 (3.354 U/ml). However, considerable amount of activity was observed (78-80 %) under high alkalinity (2.835 U/ml at pH 9; 2.670 U/ml at pH 10). Thus, enzyme has very high levels of alkali tolerance, which is one of the desirable features in biopulping industries. Kohli \textit{et al}\textsuperscript{16} reported optimum pH of 8.5-9.0 for maximum xylanase activity in \textit{T. thalophilus}, whereas pH of 6-7 is reported optimum for actinomycetes xylanases\textsuperscript{1,16,23}. In contrast, McCarthy & Bachmann\textsuperscript{25} reported pH...
between 5-9 as optimum for xylanase activity in case of *T. fusca*. The maximum xylanase activity by *B. coagulans* BL69 was obtained over a large range of pH (4.5–10)

**Thermostability of Xylanase**

Xylanase was fairly stable at 50°C up to 2 h (Fig. 9). Even after 20 h of incubation at 50°C, considerable activity (40 %) was retained. However, 30 min incubation of enzyme at 60°C caused drastic reduction in enzyme activity (80 %). Similarly, 15 min incubation of enzyme at 70°C and above caused severe loss of enzyme activity (72-78 %). Sá-Pereira *et al*\(^1\) reported that xylanase from *B. subtilis* was stable up to 60°C and stability decreased above 65°C. At 90°C, activity reduced progressively with complete thermal inactivation within 1 h. Kohli *et al*\(^2\) reported that xylanase from *T. thalophilus* was quite stable for 75 min at 65°C (retaining 100 % activity), while activity decreased steeply thereafter with complete inactivation after 175 min. *Streptomyces* sp. AMT-3 xylanase retained its original activity after 8 h (70 %) and 24 h (40 %) of pre-incubation at 55°C. However, enzyme activity was totally lost after 4 h of pre-incubation at 65°C.

**Zymogram Analysis**

Crude enzyme preparation was subjected to ammonium sulphate precipitation (30-60 % saturation) and concentrated by 1.7 times (xylanase specific activity in ammonium sulphate fraction was 24.27 U/mg protein vs 14.28 U/mg protein in crude preparation). This concentrated xylanase preparation was subjected to zymogram analysis, which suggested that enzyme preparation contained multiple xylanases as indicated by the presence of halos around three protein bands corresponding to molecular weights of 69.18, 63.09 and 43.65 kDa. Thus, the organism produces three different xylanases of varying molecular weight. Bakir *et al*\(^3\) reported 2.48-fold purification of *R. oryzae* xylanase by ammonium sulphate fractionation (45-75 % saturation). Nascimento *et al*\(^4\) reported a family of isoenzymes of xylanase by *Streptomyces* sp AMT-3 (mol wt, 170-700 kDA). Further, different substrates are reported to induce different xylanases production in the culture medium\(^5\). Jiang *et al*\(^6\) reported a novel, very large xylanolytic complex by *S. olivaceoviridis* E-86 having molecular weight of 1200 kDA.

**Partial Purification of Enzyme and Determination of \(K_m\)**

Partial purification of the enzyme using ion exchange chromatography resulted in a purification fold of 7.28 to 108.26 as determined by the specific activity of enzyme in different fractions (Fig. 10). Fractions with highest activity were pooled and used
to determine $K_m$. Determination of initial reaction rates at different substrate concentrations showed that enzyme obeys Michaelis-Menten kinetics and has a low $K_m$ (3.9 mg/ml). Tseng et al\textsuperscript{2} reported a $K_m$ of 18.5 mg/ml in case of xylanase produced by *R. oryzae* and the enzyme obeyed Michaelis-Menten kinetics. Similarly, Araki et al\textsuperscript{26} (1998) found that the $K_m$ of *Alcaligenes* sp. XY xylanase was 4 mg/ml.

Conclusions

*Streptomyces* sp CD3 has the potential to produce xylanase from crude substrates (wheat bran & bagasse) and soybean meal is the best nitrogen source for efficient xylanase production. The enzyme works optimally at pH 8 and temperature 50°C but retains substantial activity at higher pH and temperatures. Enzyme has low $K_m$ and obeys Michaelis-Menten kinetics. The enzyme is highly sensitive to inhibition by Hg\textsuperscript{2+}, while Pb\textsuperscript{2+}, Fe\textsuperscript{3+} and Ca\textsuperscript{2+} were slight stimulators of enzyme activity. Ion exchange chromatography with carboxymethyl sephadex resulted in 108-fold purification of the enzyme. Zymogram study showed the presence of 3 different xylanases of varying molecular weight in cultural broth. Highly alkalophilic nature of the organism and its ability to grow and produce enzyme on low value agricultural by-products, and enzyme being equipped with industrially desirable features including thermostability and alkali-stability, make this study not only interesting but prompts for further research on the enzyme and the organism for enhancing yield of the enzyme, understanding the molecular mechanisms of thermostability and alkali-stability of the enzyme; and finally the potential commercialization of the enzyme.

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References


