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ORIGINAL ARTICLE

Production and purification of high levels of cellulase-free bacterial xylanase by *Bacillus* sp. SV-34S using agro-residue

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Abstract Xylanase produced from the isolated bacterial strain *Bacillus* sp. SV-34S showed a 8.74-fold increase in enzyme activity under optimized submerged fermentation conditions. Cultivation using wheat bran as the carbon source and beef extract and $(NH_4)H_2PO_4$ as the nitrogen source resulted in productivity of 3,454.01 IU/mL xylanase. Xylanase was purified by 12.94-fold, with a recovery of 13.4 % and a specific activity of 3417.2 IU/mg protein, employing ammonium sulphate fractionation followed by cation-exchange chromatography using CM-Sephadex C-50 column chromatography, with a product of 27 kDa. The purified xylanase showed an optimum temperature and pH of 50 °C and 6.5, respectively although it was active even at pH 11.0. The thermostability study revealed that *Bacillus* sp. SV-34S was thermotolerant, being stable up to 50 °C; the residual activity at 55 and 60 °C was 96 and 93 %, respectively. The enzyme was stable between pH 6.0 and 8.0, although it retained >100 % activity at pH 8.0 and 9.0, respectively, following pre-incubation for 24 h. Xylanase activity was inhibited by various metal ions added to the assay mixture, with maximum inhibition observed in the presence of $HgCl_2$. The K_m and V_{max} values of the purified xylanase using birch wood xylan as substrate were 3.7 mg/mL and 133.33 IU/mL, respectively. The isolated bacterial strain produced high levels of extremophilic cellulase-free xylanase. The fact that it can be used in crude form and that it can be produced cheaply with renewable carbon sources make the process economically feasible. The characteristics of the purified enzyme suggest its potential application in industries such as the paper and pulp industry.

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Introduction

Enzymes are central to every biochemical process and are the focus of intensive research worldwide. They play an important role in myriads of biochemical reactions in many manufacturing processes, functioning under both ambient and extreme conditions. As such, they are eco-friendly and often the best alternative to polluting chemical technologies. Enzymatic treatment provides the same level of yield as that achieved with standard methods requiring environmentally unfriendly chemicals. During the latter half of the twentieth century our understanding of microorganisms, their metabolic products, and their enzymes underwent an unparalleled expansion in terms of both basic research and their prospective industrial applications.

In the industrial sector, xylan and its hydrolytic enzymatic complex have recently been the focus of great interest. Xylan is the next most abundant hemicellulosic polysaccharide after cellulose, with both present in the plant cell wall. Xylanases (endo-1, 4-β-**D**-xylanohydrolase; EC 3.2.1.8) are xylan-degrading enzymes which play an important role in converting xylan into xylo-oligosaccharides and xylose. They are produced by diverse genera and species of microorganisms but have been studied mostly in bacteria (Anuradha et al. 2007), fungi (Okafor et al. 2007), and actinomycetes (Ninawe et al. 2006). Among those bacteria producing xylanases, members of the genus *Bacillus* have been extensively studied as they are the most common producers of these enzymes. The use of xylanase in the pulp and paper industry has significantly increased in recent years, but xylanases have many other viable industrial applications and are used as additives to agricultural silage and

grain feed (Kuhad and Singh 1993) and poultry feed to improve nutritional properties to wheat flour for improving dough handling and the quality of baked products, and to fruit juices in combination with pectinase and cellulase for clarification of the juices (Biely 1985) and degumming of plant fibers such as flax, hump, jute, and ramie (Kapoor et al. 2001).

Although several thermophilic bacteria have been isolated and their enzymes produced and characterized, there is still a need for novel strains capable of producing enhanced levels of enzyme in an economically feasible culture system. The suitability of any enzyme in an industrial application depends upon its thermal and pH stability and other kinetic properties. A thermophilic bacterium which produces a high level of cellulase-free alkali-stable xylanase is the more suitable microorganism for use in industry. The primary aim of our study was therefore to produce high levels of cellulase-free extremophilic xylanase which could be used in the paper and pulp industry under extreme conditions. A secondary aim was to elaborate the kinetic properties and various characteristics of purified xylanase for a better understanding of its physicochemical properties in order to make its industrial applications more profitable.

Materials and methods

Chemicals

All chemicals were purchased from Sigma Chemicals (St. Louis, MO), Hi-Media Laboratories India (Mumbai, India), and Pharmacia (Uppsala, Sweden).

Organism and strain cultivation

The organism was isolated from decaying wood, which was collected from the Chennai Sea, India. It was identified as *Bacillus* sp. SV-34S on the basis of morphological characteristics and biochemical tests performed in the laboratory of the Biochemistry Department, Kurukshetra University, Kurukshetra. The bacterium was cultivated on medium containing (w/v) 0.5 % peptone, 0.3 % beef extract, 2.5 % bacteriological agar, and 0.5 % birch wood xylan. The medium was sterilized before use at 121 °C, 15 lb/inch^2 for 20 min. The strain was routinely sub-cultured on the same medium and was able to grow well at 50 °C. It was stored at 4 °C as a stock.

Preparation of inoculum

Inoculum was prepared in 250 mL Erlenmeyer flasks by inoculating a loop (approx. 0.01 mL, Hi-Media SS-4) of culture into 50 mL of nutrient medium containing (w/v) 0.5 % peptone, 0.3 % beef extract. The medium was sterilized before use at 121 °C, 15 lb/inch^2 for 20 min. The inoculum was incubated for 18 h at 37 °C and 200 rpm in a shaker incubator before being used for xylanase production.

Production of xylanase under submerged fermentation

The Erlenmeyer flasks (250 mL) used for studying xylanase production contained 50 mL of modified Horikoshi medium containing (w/v) 0.5 % peptone, 0.6 % yeast extract, 0.5 % KNO_3, 0.1 % KH_2PO_4, 0.01 % $MgSO_4.7H_2O$, 1 % wheat bran; the pH was 7.0. The autoclaved flasks were inoculated with freshly prepared 2 % inoculum and incubated at 200 rpm at 37 °C in a shaker incubator for 48 h. The material was centrifuged at 10,000 *g* for 20 min to separate out the cells, and this cell-free culture supernatant was used as the crude enzyme for xylanase assay.

Xylanase and cellulase assay

The xylanase activity was assayed according to the method of Biely et al. (1985) by measuring the amount of reducing sugars (xylose equivalent) liberated from xylan using 3,5-dinitrosalicylic acid (Miller 1959). The reaction mixture containing 490 μL of 2 % birch wood xylan (Sigma Chemicals) as substrate and 10 μL of appropriately diluted enzyme extract was incubated at 55 °C for 5 min. The reaction was then terminated by adding 1.5 mL of 3,5-dinitrosalicylic acid reagent. A control was run simultaneously that contained all of the reagents, but the reaction was terminated prior to the addition of the enzyme. The contents were placed in a boiling water bath for 10 min followed by cooling in ice-cold water. The absorbance of the resulting color was measured against the control at 540 nm in a spectrophotometer.

Cellulase activity [carboxymethyl cellulase (CMCase) and filter paper activity (FPase)] was determined according to the method of Ghosh (1987). The reaction mixture for CMCase activity, which contained 500 μL of 2.0 % carboxymethyl cellulose (Sigma Chemicals) and 500 μL of crude enzyme, was incubated at 50 °C for 30 min. The reaction mixture for FPase, which contained a Whatman No. 1 filter paper strip (1 × 6 cm; Whatman, New York, NY), 1.0 mL citrate buffer, pH 4.8, and 0.5 mL enzyme, was incubated at 50 °C for 60 min. In both cases, the reaction was terminated by adding 3 mL of dinitrosalicylic acid reagent. The reaction mixture was boiled for 5 min in a boiling water bath and then cooled prior to the addition of 20 mL of distilled water. A control was run simultaneously that contained all of the reagents, but the reaction was terminated prior to the addition of enzyme. The absorbance of the resulting color was measured against the control at 540 nm in a spectrophotometer.

One unit (IU) of xylanase or cellulase activity was defined as the amount of enzyme that catalyzes the release of 1 mol of reducing sugar as xylose or glucose equivalent per minute under the specified assay conditions.

Protein estimation

The protein concentration of the chromatographic eluents was estimated by measuring the absorbance at 280 nm. At the end of the purification step total soluble protein content was estimated by the Lowry method (Lowry et al. 1951) using bovine serum albumin (BSA) as the standard.

Optimization of xylanase production

Effect of inoculum size

The effect of inoculum size was studied by inoculating 50 mL of production medium with a different amount of inoculum (1–4 %) and then incubating the culture at 37 °C for 48 h at 200 rpm. Xylanase was extracted from the culture filtrates and its activity determined.

Effect of carbon source

Different carbon sources (1 % w/v), namely, glucose, xylose, sucrose, lactose, maltose, cellulose, CM-cellulose, starch, birch wood xylan, oat spelt xylan, and lignocellulosic agro-residues, such as wheat bran, were tested. A control devoid of carbon source was also run. In addition, the effect of wheat bran as a carbon source for xylanase production was studied at different concentrations (range 1– 6 %).

Effect of nitrogen source

Xylanase production was monitored by varying the nitrogen source (0.5 % w/v). To this end, KNO_3, $(NH_4)_2H_2PO_4$, NH_4Cl, $(NH_4)_2SO_4$, peptone, yeast extract, beef extract, tryptone, and different combinations thereof were added to the medium. A control devoid of nitrogen source was also run.

Effect of incubation period

Flasks containing 50 mL of production medium were inoculated with 1.0 % 18-h-old inoculum and incubated for 0–72 h under shaking conditions at 200 rpm at 37 °C. The samples were harvested at regular intervals of 6 h and centrifuged. Xylanase activity was determined in the clear supernatant.

Effect of temperature

The effect of temperature was studied by inoculating 50 mL of production medium with 18-h-old inoculum and incubated at different temperatures (range 30–55 °C) under shaking conditions at 200 rpm. The samples were harvested after 48 h for the estimation of xylanase activity.

Effect of agitation rate

The medium was incubated under shaking conditions at different agitation rates (range 50–250 rpm) in a shaker incubator. Similarly, one flask was also kept under stationary conditions. Xylanase was extracted from the culture filtrates, and its activity was determined.

Effect of additives

Medium was supplemented with different additives (0.1 % w/v), such as Tween 20, Tween 80, olive oil, oleic acid, glycerol, triton X-100, ethylenediamine tetra acetic acid (EDTA) and detergent (sodium dodecyl sulfate, SDS). After 48 h of incubation, samples were harvested for the estimation of xylanase activity.

Effect of pH

Production medium at different pH (range of 3–11) was inoculated with 1 % (18 h old) inoculum of *Bacillus* sp. SV-34S and incubated at 37 °C for 48 h under shaking conditions at 200 rpm. The cell-free extract was obtained after centrifugation and xylanase activity was determined in the extract.

Purification of xylanase

The crude extract of xylanase was used for the purification of xylanase. The enzyme was purified by ammonium sulphate precipitation followed by cation exchange chromatography on a CM-Sephadex column. The purification was carried out at 4 °C, and the crude extract was subjected to ammonium sulphate (AMS) precipitation to 0–80 % saturation. The pellet obtained after centrifugation at 10,000 rpm was dissolved in 0.05 M phosphate buffer, pH 6.0, and dialyzed overnight. The dialyzed sample was applied onto the CM-Sephadex C-50 column (30 × 3 cm) that had been pre-equilibrated with 0.05 M phosphate buffer, pH 6.0. The column was run with 0.05 M phosphate buffer, and 5-mL fractions were collected at a rate of 40 mL/h. The proteins bound to the CM-Sephadex C-50 column were eluted using a continuous salt gradient of 0–1.0 M NaCl, and the fractions were monitored for their protein content by measuring the absorbance at 280 nm (A_{280}) of the eluent against 0.05 M phosphate buffer as the blank. The protein-containing fractions were analyzed for xylanase activity, and the fractions containing xylanase activity were pooled. The activity and

protein content were determined in the pooled extract and used for further experiments.

Electrophoresis

The predominant xylanase of *Bacillus* sp. SV-34S was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) in a 10 % acrylamide gel. Protein bands were stained by silver staining as described by Blum et al. (1987). The protein molecular weight standards used were phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa), and aprotinin (6.5 kDa).

Characterization of xylanase

Temperature optimum and stability

The optimum temperature for the xylanase activity was determined by incubating the reaction mixture at different temperatures (30–65 °C) using birch wood xylan as substrate. The thermostability was determined by pre-incubating an aliquot of the enzyme at different temperatures (30–65 °C) for 15 min followed by the measurement of xylanase activity at 55 °C under standard assay conditions.

pH optimum and stability

The effect of pH on xylanase activity was studied by using assay buffers of different pH ranging from pH 4 to 11. The pH stability of the enzyme was determined by pre-incubating an aliquot of the enzyme with buffers (0.05 M) of different pH, namely, citrate buffer (pH 3–5), phosphate buffer (pH 6– to 8), and glycine-NaOH (pH 9–11), for 24 h at 37 °C followed by the measurement of xylanase activity after varying time intervals. The residual activity (%) at each pH was calculated.

Effect of substrate concentration

Xylanase activity was measured at different concentrations of birch wood xylan (range 1–20 mg/mL), and a Lineweaver–Burk plot was drawn to determine the K_m (substrate concentration at which the reaction rate is half of V_{max}) and V_{max} [maximum rate achieved at maximum (saturating) substrate concentrations] of the enzyme.

Effect of additives

The effect of various additives, such as EDTA and SDS (30 mM), and metal ions (1 %), such as NaCl, KCl, $MgCI_2$, $CaCI_2$, $CoCl_2$, $MnCI_2$, $HgCl_2$, $FeCI_3$, $ZnSO_4$, $BaCl_2$, and $CuSO_4$ was tested by incubating each additive with the purified enzyme for 60 min at room temperature. The xylanase activity was then measured under standard assay conditions after 30 and 60 min. The residual activity (%) was then calculated.

Result and discussion

The *Bacillus* sp. SV-34S isolated from decaying wood is a Gram-positive rod having a single endospore, motile, and penicillin-resistant. Other characteristics are summarized in Table 1. *Bacillus* sp. SV-34S produced a cellulase-free xylanase that was found to be thermotolerant, had a maximum growth temperature of 55 °C, and was alkali stable, being capable of growing at pH up to 11.0. The optimal conditions determined for maximum xylanase production were: an incubation time of 48 h, pH 7.0, temperature of 37 °C, 1 % inoculum of an 18-h-old culture, shaking at 200 rpm, 2 % wheat bran, and 0.5 % each of beef extract and ammonium dihydrogen phosphate. A comparison of xylanase production measured under these optimized fermentation conditions (3,454.01 ± 80.89 IU/mL) with that under non-optimal conditions (394.74 ± 20.96 IU/mL) revealed an 8.74-fold increase in activity. As this strain is able to produce large-scale amounts of xylanase under convenient conditions, it can be an inexpensive industrial source of xylanase.

Effect of inoculum size

The highest production of xylanase production (1,178.7 IU/mL) was measured when 1 % (v/v) inoculum of 18-h-old (16×10^7 CFU/mL) culture was added to the production medium (Fig. 1). Enzyme production declined with increases in inoculum size beyond 1 %. Various research groups have reported the use of 1–5 % (v/v) inoculum for hyper-production of xylanase (Archana and Satyanarayana 1998; Battan et al. 2007; Subramaniyan et al. 2001). Moreover, the use of 10 % inoculum size for maximum xylanase production by *Bacillus* sp NCIM 59 has been reported by Kulkarni and Rao (1996).

Effect of carbon source

Xylanase production was lower when easily available agro-residues were used as the carbon source as compared to pure xylan. Among the different substrates tested, wheat bran favored maximum xylanase production, followed by oat spelt xylan. The enzyme activity obtained with wheat bran, oat spelt xylan, and birch wood xylan was 1,359, 1,239, and 1,658 IU/mL, respectively (Fig. 2a). Xylanase production was induced by xylan but repressed by the presence of the other polysaccharides (starch and cellulose), monosaccharides (glucose and xylose), and disaccharides (maltose, lactose and sucrose) tested in this study.

Table 1 Morphological and physiological characteristic of *Bacillus* sp. SV-34S

Characteristics	SV34S	Characteristics tested	SV34S
Cell shape	Rods	Colony shape	Round
Arrangement	Short chains	Growth temperature	10–50ºC
Spore	+	Growth pH	5–10
Position	Central	Growth at NaCl	1–10 %
Motility	+	Catalase test	+
Gram staining	+	Growth under anaerobic conditions	+
Colony color	White	Identified as	*Bacillus* sp.

Wheat bran has been reported to be a good substrate for xylanase production (Battan et al. 2007; Gupta et al. 2000). In our study, wheat bran was the best substrate (both qualitatively and quantitatively) for xylanase production as it contained sufficient nutrients and was able to remain loose in moist conditions, thereby providing a large surface area (Babu and Satyanarayana 1995). Many microorganisms produce higher quantities of xylanase when cultured with pure xylan compared to lignocelluloses (Archana and Satyanarayana 1998; Qureshy et al. 2002; Rizzati et al. 2001). However, the use of purified xylan as a substrate is uneconomical for the large-scale production of xylanase. Agricultural residues offer cost-effective alternative substrates for xylanase production.

Xylanase production was found to vary with changes in the concentration of wheat bran. The highest enzyme activity was found at 2 % (w/v) wheat bran, and there was a decline in xylanase production when the concentration of wheat bran was increased beyond 2 % (Fig. 2b). This could be due to the formation of a thick suspension and/or insufficient mixing of the substrates in shake flasks, as suggested by Kuhad et al. (1998).

Effect of nitrogen source

Among the various nitrogen sources used, beef extract and ammonium dihydrogen phosphate favored the highest xylanase production (1,830 ± 32.76 IU/mL) followed by peptone, beef extract, and ammonium dihydrogen phosphate (1,804 IU/mL), peptone, yeast extract, and KNO_3 (1,652 IU/mL), and beef extract (1,525 IU/mL). Enzyme production in the presence KNO_3, $(NH_4)_2H_2PO_4$, and $(NH_4)_2SO_4$ was 339, 1,309, and 1048 IU/mL, respectively (Table 2). The highest xylanase production (251 IU/mL) by *Bacillus* SSP-34 occurred in a medium containing yeast extract and peptone, each at 0.25 % (Subramaniyam and

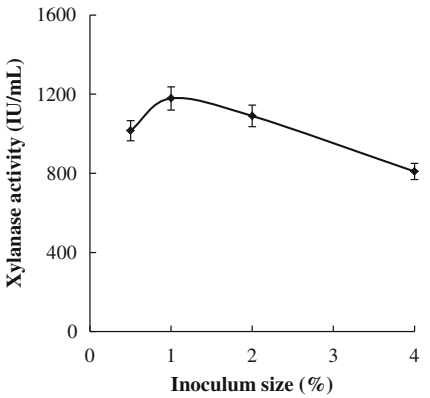

Fig. 1 Effect of inoculum size (%) on xylanase production in a submerged fermentation (SmF) system by *Bacillus* sp. SV-34S

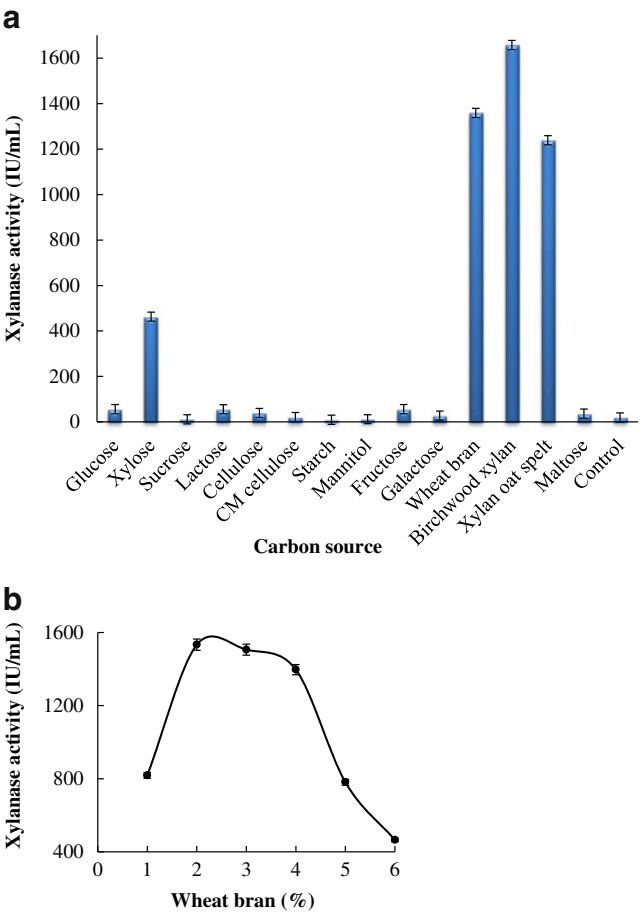

Fig. 2 **a** Effect of carbon source (1 % w/v) on xylanase production in a SmF system by *Bacillus* sp. SV-34S. **b** Effect of wheat bran concentration on xylanase production in a SmF system. *CM* Carboxymethyl

Table 2 Effect of nitrogen source (w/v) on xylanase production in a submerged fermentation system

Nitrogen source	Xylanase activity (IU/mL)	Nitrogen source	Xylanase activity (IU/mL)
Peptone	839.7 ± 15.56	$(NH_4)_2SO_4$	1,048.4 ± 24.89
Yeast extract (Y.E.)	998.7 ± 17.31	Tryptone	754.0 ± 14.23
KNO_3	339.5 ± 5.67	$(NH_4)H_2PO_4$	1,309.6 ± 26.03
Peptone + KNO_3	1,004.4 ± 23.43	NH_4Cl	923.8 ± 16.09
Yeast extract + KNO_3	1,039.1 ± 24.65	Peptone + Y.E. + KNO_3 + $(NH_4)H_2PO_4$	1,245.5 ± 25.89
Peptone + yeast extract	980.0 ± 16.98	Beef extract + $(NH_4)H_2PO_4$	1,830.5 ± 32.76
Peptone + Y.E. + KNO_3	1,652.2 ± 30.21	Peptone + B.E. + $(NH_4)H_2PO_5$	1,804.4 ± 31.78
Beef extract (B.E.)	1,525.4 ± 29.32	Control	180.5 ± 4.45
Peptone + B.E.	1,457.3 ± 26.49		

Prema 2002). However, the best nitrogen source for xylanase production by *B. circulans* AB16 (Dhillon et al. 2000) was tryptone. To date, there has been no published report of beef extract and ammonium dihydrogen phosphate combination as a nitrogen source.

Effect of incubation, temperature, agitation, and additives

The production of xylanase could be detected after 12 h of incubation, reaching a maximum (1,970.2 IU/mL) at 48 h and declining thereafter (Fig. 3). An identical incubation time for highest xylanase production has been reported for *Bacillus* sp. (Anuradha et al. 2007). However, *B. circulans* Teri-42 and *B. pumilus* ASH 7411 exhibited maximum xylanase activity at 24 h (Battan et al. 2007; Qureshy et al. 2002).

Maximum xylanase production occurred at 37 °C, although *Bacillus* sp. SV-34S was able to produce enzyme even up to 50 °C. The enzyme activity at 37 °C and 50 °C was 1,970.2 and 1,104.2 IU/mL, respectively (Fig. 4a). *B. circulans* produced 400 IU/mL xylanase at 30 °C under optimized conditions (Ratto et al. 1992) and the activity of xylanase in the our study was much higher than the 1.1 IU/mL produced by *B. circulans* Teri-42 at 37 °C (Qureshy et al. 2002).

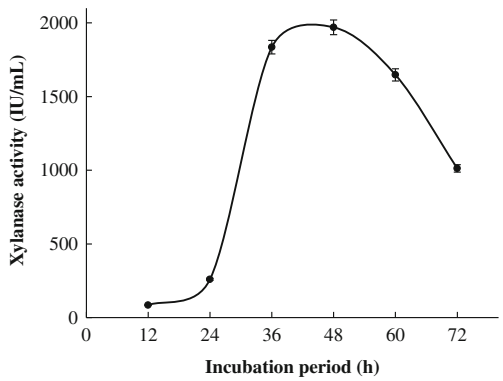

Fig. 3 Time course of xylanase production by *Bacillus* sp. SV-34S in a SmF system using 2 % wheat bran as a substrate

Agitation of the inoculated medium has a profound effect on enzyme production since it promotes the growth of the microorganisms by facilitating a uniform distribution of nutrients and a sufficient supply of oxygen. The xylanase titer under shaking conditions was found to be higher than than under stationary conditions. Shaking at 200 rpm resulted in maximum xylanase production (1,970.2 IU/mL), while enzyme production under stationary conditions was about 35 % of that with shaking at 200 rpm (Fig. 4b). Mechanical agitation may be effective in mixing the contents of the medium, in uniformly distributing air, and preventing cell clumping. Similar to our observation, maximum xylanase production has been reported in shake flask culture with agitation at 200 rpm by several researcher groups (Archana and Satyanarayana 1998; Battan et al. 2007; Shah et al. 1999). In other studies, however, the highest xylanase titer was reported to occur with agitation at 100 (Rizzati et al. 2001), 150 (Kohli et al. 2001), 250 (Okazaki et al. 1985), and 400 rpm (Yoshida et al. 1994), respectively.

Xylanase production was reduced by the addition of various compounds to the production medium (Fig. 4c) and had a maximum activity (1,970.0 IU/mL) without any additive. A similar result was observed by Nagar et al. 2012. The maximum reduction in enzyme activity was recorded following the addition of olive oil and oleic acid. However, the addition of 0.1 % (v/v) Tween 80 to the production medium supported the highest enzyme yield in *Bacillus pumilus* SV-85S (Nagar et al. 2010).

Effect of pH

The initial pH of the production medium plays a vital role in the metabolic activity of the microorganism and hence on its growth. It was observed that *Bacillus* sp. SV-34S produced detectable amounts of xylanase beginning at pH 6.0 (1,353.6 IU/mL), with the highest

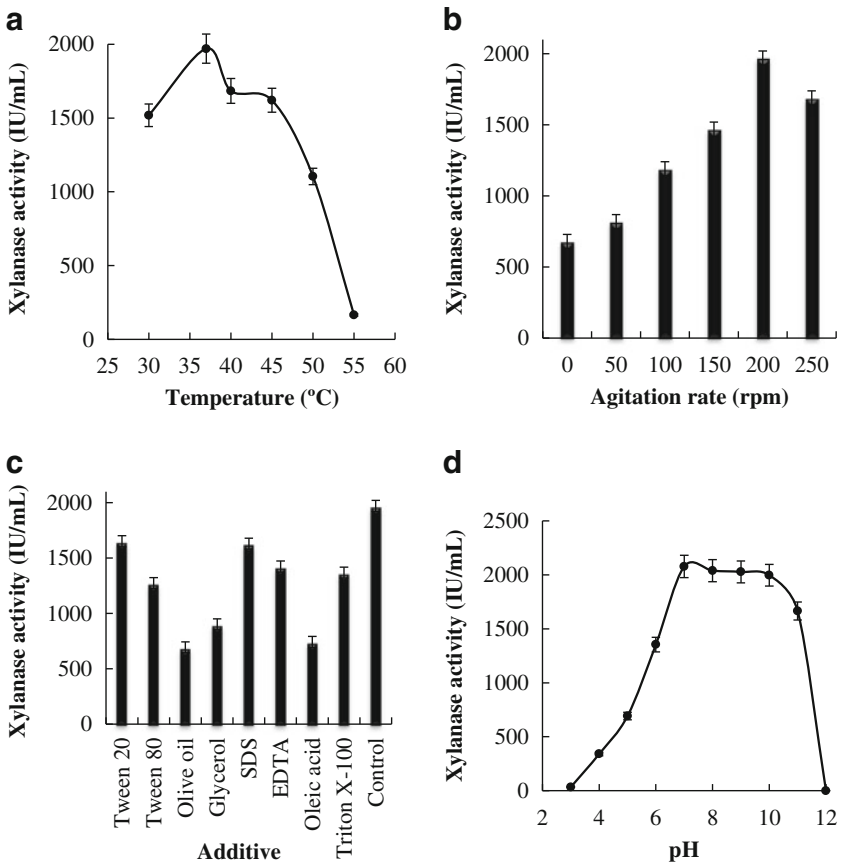

Fig. 4 Effect of temperature (°C) (**a**), agitation rate (rpm) (**b**), additives (%) (**c**), and pH (**d**) on xylanase production by *Bacillus* sp. SV-34S in a SmF system

production at pH 7.0 (2,077.0 IU/mL), and that it was capable of producing xylanase even up to pH 11.0 (1,664 IU/mL), as shown in Fig. 4d. These results indicate that the isolated bacterium is alkalophilic.

Maximum enzyme production at pH 7.0 has also been reported by *B. circulans* AB-16 (Dhillon et al. 2000), *B. pumilus* (Poorna and Prema 2006), and *B. licheniformis* A-99 (Archana and Satyanarayana 1998). In some microorganisms, maximum xylanase production was found at pH ranging from 8.0 and 8.5 (Anuradha et al. 2007; Battan et al. 2007). Among alkalophilic xylanase-producing microorganisms, *Bacillus* NCIM 59 was found to produce xylanase at pH 10.0 (Dey et al. 1992) and *Bacillus* sp TAR-1 produced xylanase at pH 10.5 (Nakamura et al. 1994).

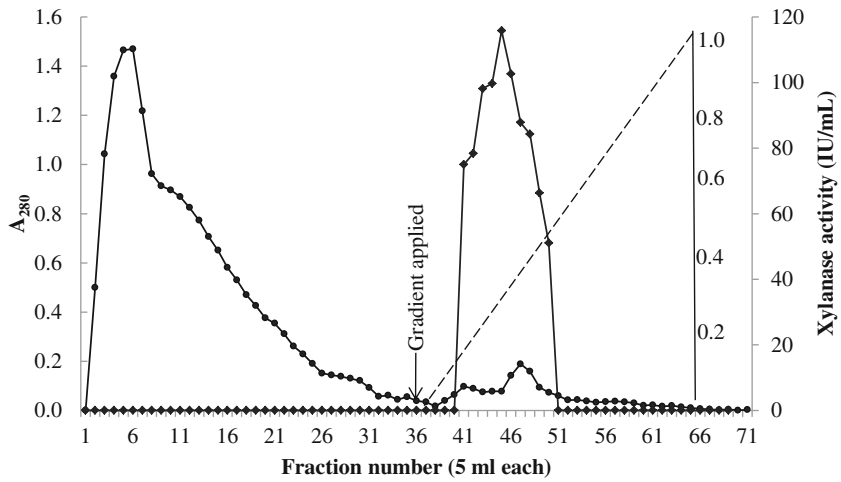

Fig. 5 Elution profile by cation exchange chromatography through a CM-Sephadex C-50. *Filled circle* Absorbance at 280 nm, *filled diamond* xylanase activity, *broken line* NaCl gradient applied

Table 3 Purification of xylanase from *Bacillus* sp.SV-34S

Purification step	Total activity (IU)	Volume (mL)	Total protein (mg)	Specific activity (IU/mg)	Recovery (%)	Purification fold
Crude extract	12,8902.00	40	488.13	264.07	100.00	1.00
Ammonium sulfate precipitation	11,3334.00	2	40.33	2,803.10	88.00	10.62
Carboxymethy-Sephadex C-50	17,325.00	20	5.07	3,417.20	13.44	12.94

Purification of xylanase

Xylanase was purified from the culture filtrate by ammonium sulphate precipitation followed by ion-exchange chromatography through a CM-Sephadex C-50 column. The enzyme protein was precipitated from the crude extract by ammonium sulfate (80 % saturation). The elution profile of xylanase through CM-Sephadex C-50 cation exchange column is shown in Fig. 5. The enzyme was purified to 12.94-fold, with a recovery of 13.44 % (Table 3).

Qureshy et al. (2002) reported purification of xylanase from *B. circulans* Teri-42 using Q-Sepharose and Sephadex G-50 column chromatography with a twofold purification. Xylanase from *Arthrobacter* sp. was purified to 21-fold with a 14 % yield using $(NH_4)_2SO_4$ fractionation, Sephadex G-200, DEAE-Sepharose FF, and CM-Sepharose FF chromatography (Khandeparker and Bhosle 2006). Xylanase from *Streptomyces cyaneus* SN32 was purified to 2.25-fold using $(NH_4)_2SO_4$ fractionation and DEAE-Sepharose chromatography (Ninawe et al. 2007). The specific activity of xylanase from *Laetiporus sulphureus* using DEAE, Mono Q, and Sephadex 75 columns was found to be 72.4 IU/mg, which is ninefold higher than that of the crude culture solution (Lee et al. 2009).

SDS-PAGE revealed a single band, indicating that the xylanase was purified to apparent homogeneity. The electrophoresis product showed a sharp band corresponding to a 27-kDa protein estimated from the migration of molecular weight standards after electrophoresis (Fig. 6).

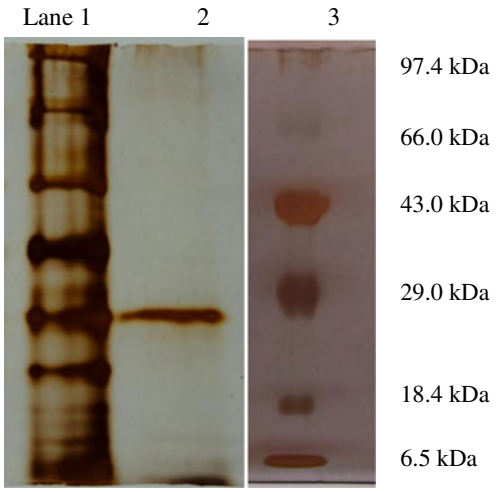

Fig. 6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified *Bacillus* sp. SV-34S xylanase. *Lanes*: *1* ammonium sulphate, *2* purified xylanase, *3* molecular weight markers

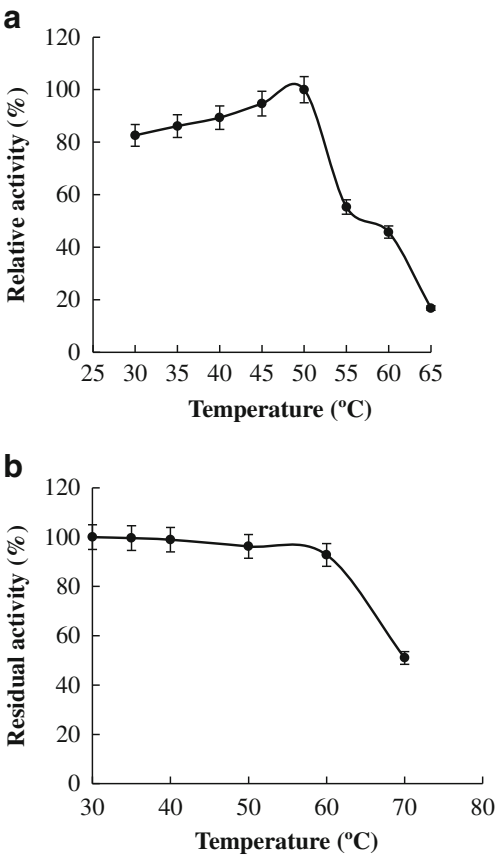

Fig. 7 **a** Temperature optimum of purified xylanase obtained by varying temperature during the assays followed by calculating relative activity. **b** Effect of temperature on the stability of xylanase determined by residual activity calculated after 15 min of pre-incubation at different temperatures

Characterization of the purified xylanase

Temperature optimum and stability

The measurement of xylanase activity at different temperatures showed that the activity increased up to 50 °C and then declined progressively (Fig. 7a). The optimum temperature of the purified xylanase was considered to be 50 °C as the enzyme exhibited maximum activity at this temperature. At 65 °C, the residual enzyme activity was about 17 %. Our thermostability study of the purified enzyme revealed that it was stable up to 50 °C. At 55 and 60 °C, the residual activity was 96 and 93 %, respectively (Fig. 7b). Thus, we concluded that this enzyme is thermotolerant.

Purified xylanases with almost a similar temperature optimum and stability have been reported from *B. circulans* Teri-42 (Qureshy et al. 2002) and *Bacillus* sp. (Sapre et al. 2005). However, the optimum temperature for xylanase activity in the range of 60–80 °C has been reported from *Bacillus* sp. SPS-0 (Bataillon et al. 2000), *Thermomyces lanuginosus* CBS (Li et al. 2005), *S. cyaneus* SN32 (Ninawe et al. 2007), and *Laetiporus sulphureus* (Lee et al. 2009). In contrast, enzymes with low temperature optimum of 25–40 °C have also been reported (Qinnghe et al. 1993)

pH optimum and stability

The profile of xylanase activity as a function of pH is shown in Fig. 8a, which shows that the enzyme displayed maximum activity at pH 6.5. The enzyme was active even at alkaline pH but to a lesser extent as compared to pH 7.0. The enzyme activity was 62 and 44 % at pH 9.0 and 10.0, respectively, indicating that enzyme was alkalophilic. Maximum pH stability of purified xylanase was between pH 6.0 and 8.0. The purified enzyme retained >100 % activity after pre-incubation at pH 8.0 and 9.0, respectively, for 24 h followed by assay at pH 7.0 (Fig. 8b). The results of our study are in agreement with those reported earlier in *B. circulans* Teri-42 (Qureshy et al. 2002) and *Hermomyces lanuginosus* CBS (Li et al. 2005). Bataillon et al. (1998) found that xylanase from *Bacillus* sp. had a pH optimum at 6.0 was stable up to pH 9.0. Xylanase from alkalophilic *Bacillus* sp. strain 41 —1 showed a broad pH activity profile with pH range of 4–11 (Nakamura et al. 1993). The pH stability of the purified xylanase of *Bacillus* sp. was 6–10.5 (Sapre et al. 2005).

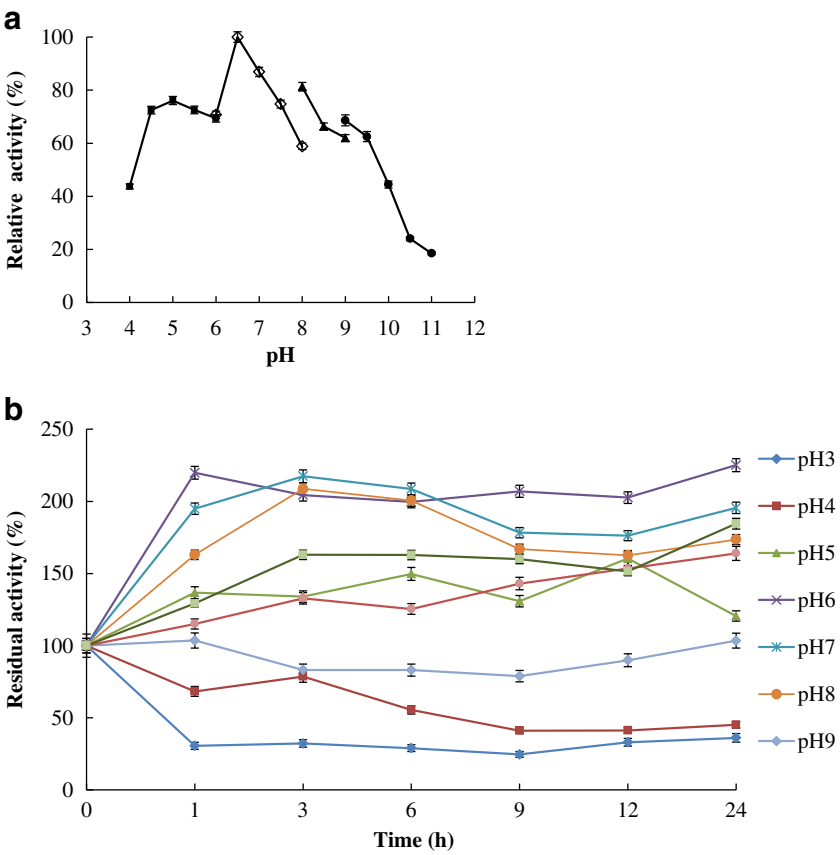

Fig. 8 **a** Optimum pH of xylanase obtained by assaying relative activity with varying buffers (0.05 mM each) at different pH. *Open diamonds* Citrate buffer (pH 3–6), *filled squares* sodium phosphate buffer (pH 6–8), *filled triangle* Tris-HCl buffer (pH 8–9), *filled circle* glycine NaOH buffer (pH 9–11).**b** Stability of xylanase assayed at different pH for 24 h at regular interval of 3 h at pH 6.5, 50 °C

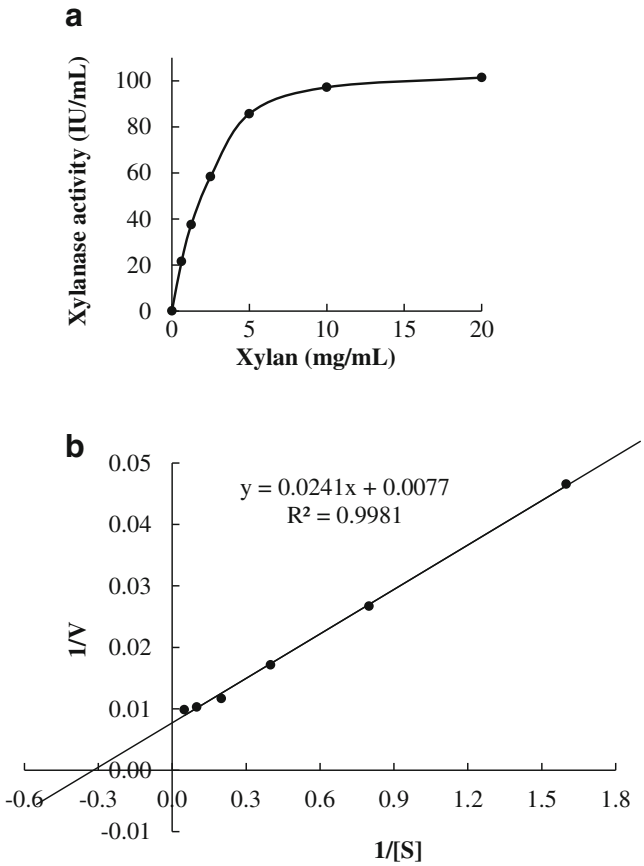

Fig. 9 **a** Kinetics of xylanase by substrate saturation curve showing that enzyme activity followed Michaelis–Menton equation. **b** Lineweaver–Burk plot of initial velocity data for xylanase for calculating K_m and V_{max}

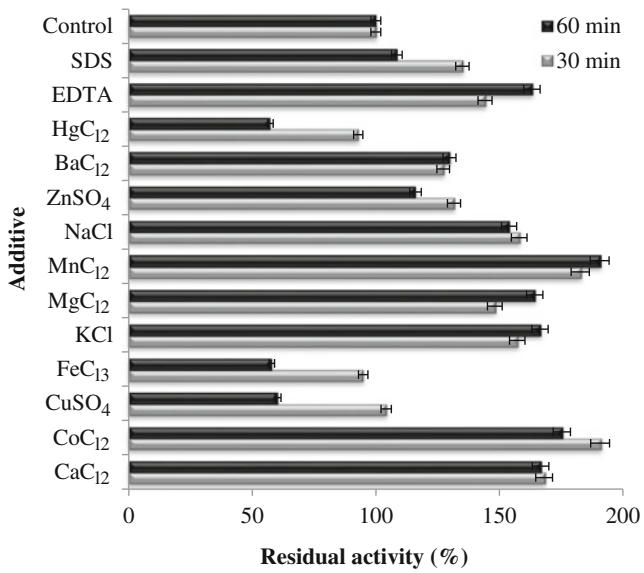

Fig. 10 Effect of various additives (30 mM) assayed for 1 h after an interval of 30 min, at pH 6.5, 50 °C

Determination of K_m and V_{max}

The K_m of xylanase depends on the type of hemicellulose used for enzyme assay. The activity of purified xylanase, measured using various concentrations of birch wood xylan, exhibited a rectangular hyperbolic response, indicating that it obeys Michaelis–Menten kinetics (Fig. 9a). K_m and V_{max} values obtained from the Lineweaver–Burk plot were 24.67 mM (3.7 mg/mL) and 133.33 IU/mL, respectively (Fig. 9b). Some xylanases have been reported to have a high affinity for substrates with a lower K_m (range 0.025–1.7 mg/mL; Khandeparker and Bhosle 2006; Sapre et al. 2005). In contrast, xylanase from *S. cyaneus* SN32 exhibited a K_m of 11.1 mg/mL (Ninawe et al. 2007).

Effect of various additives

Some of the metal ions added to the assay mixture inhibited xylanase activity; maximum inhibition was observed in the presence of $HgCl_2$ (Fig. 10). Inhibition of enzyme activity by $HgCl_2$ (Bataillon et al. 2000; Qureshy et al. 2002) and EDTA has been reported earlier (Gupta et al. 2000).

Conclusion

We isolated *Bacillus* sp. SV-34S from decaying wood and tested its ability to produce xylanase under different culture conditions. Under optimal conditions, xylanase production was increased by 8.74-fold with the optimized activity of 3,454.01 IU/mL. Beef extract in combination with ammonium dihydrogen phosphate added to the production media resulted in higher enzyme activity than the addition of peptone and yeast extract; the latter have often been used in previous studies. The xylanase was purified up to 12.94-fold with a specific activity 3417.2 IU/mL in just a two-step process as compared to the earlier reported data which used multi-step processes. Such a simple procedure saves time and is efficient in terms of purification. Also, the thermotolerance, alkali stability, and affinity towards the substrate, as shown by the low K_m, suggest that *Bacillus* sp. SV-34S is a relatively cheap enzymatic option in industries such as the pulp and paper and fruit industries.

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