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ORIGINAL PAPER

Production and optimization of cellulase-free, alkali-stable xylanase by *Bacillus pumilus* SV-85S in submerged fermentation

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Abstract This paper reports the production of a cellulase-free and alkali-stable xylanase in high titre from a newly isolated *Bacillus pumilus* SV-85S using cheap and easily available agro-residue wheat bran. Optimization of fermentation conditions enhanced the enzyme production to 2995.20 ± 200.00 IU/ml, which was 9.91-fold higher than the activity under unoptimized basal medium (302.2 IU/ml). Statistical optimization using response-surface methodology was employed to obtain a cumulative effect of peptone, yeast extract, and potassium nitrate (KNO_3) on enzyme production. A 2^3 central composite design best optimized the nitrogen source at the 0 level for peptone and yeast extract and at the $-\alpha$ level for KNO_3, along with 5.38-fold increase in xylanase activity. Addition of 0.1% tween 80 to the medium increased production by 1.5-fold. Optimum pH for xylanase was 6.0. The enzyme was 100% stable over the pH range from 5 to 11 for 1 h at 37°C and it lost no activity, even after 3 h of incubation at pH 7, 8, and 9. Optimum temperature for the enzyme was 50°C, but the enzyme displayed 78% residual activity even at 65°C. The enzyme retained 50% activity after an incubation of 1 h at 60°C. Characteristics of *B. pumilus* SV-85S xylanase, including its cellulase-free nature, stability in alkali over a long duration, along with high-level production, are particularly suited to the paper and pulp industry.

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Introduction

Xylanase (E.C. 3.2.1.8) catalyzes the hydrolysis of xylopyranosyl linkages of β-1,4-xylan, the major hemicellulosic polysaccharide of hardwood [11, 34]. This enzyme is produced by a variety of microorganisms, including bacteria [19, 38], fungi [14], and actinomycetes [32]. From a commercial point of view, xylanases are an important group of hydrolases, with a worldwide market of US $200 million [23]. Xylan-degrading enzymes have attracted considerable attention because of their widespread application in industrial processes such as improving the digestibility of animal feed stocks [41], biobleaching of pulp [6, 25, 29, 38, 39, 44], textile industry [15], production of xylo-oligosaccharides [28], waste-water treatment [36], texture improvement in bakery products [40], clarification of juices and wine [7], debarking process [4], and bioconversion of lignocellulosic wastes into useful economical products such as ethanol, single-cell protein, sugar syrups, and liquid and gaseous fuels [16].

Members of the genus *Bacillus* produce a great variety of extracellular enzymes. Among them, xylanase has significant industrial importance [42]. In general, the fermentation profile of an organism is affected by nutritional and physiological factors such as carbon source, nitrogen source, additives, inoculum size, pH of the media, incubation temperature, agitation rate, and others. Hyperproduction of industrial enzymes by optimizing these growth parameters is of prime importance, because an improper optimization of these factors leads to a lower production of

the enzyme. In some cases, it is difficult to find the cumulative effect of more than two factors using the traditional one-variable-at-a-time approach. However, statistical or factorial design optimization and response-surface analysis fulfil this requirement. Response-surface methodology (RSM) is a collection of mathematical and statistical techniques widely used to determine the effect of several variables at a time to optimize the biotechnological processes [20, 21].

As the price of the substrate plays a crucial role in overall processing cost, cheap substrates such as agro-residues are used as carbon sources in enzyme production [37, 45]. Although xylanase production in submerged fermentation (SmF) using agro residues as substrates has been studied by several workers, very few reports are available on high-level production of cellulase-free and alkali-stable enzymes. This paper presents the production of an alkali-tolerant xylanase in high titre by *Bacillus pumilus* SV-85S under submerged fermentation using a cheap agro residue in order to establish an economical fermentation process.

Materials and methods

Bacterial strain

Bacillus pumilus SV-85S was isolated from soil samples collected from Ambala Cantt., Haryana, India. This strain was identified by the Institute of Microbial Technology, Chandigarh (IMTECH), India. The strain has been deposited to the Microbial Type Culture Collection and Gene Bank (MTCC) bearing the accession no. 9861. Stock cultures are being maintained on nutrient agar at 4°C by transfer onto a fresh medium after every 4–6 weeks. Morphological, physiological, and biochemical characteristics of the isolated bacterial strain are shown in Table 1.

Preparation of seed culture

First, the culture is grown in a tube having 5 ml of nutrient broth from plate culture. Then, a loop full ($\approx$0.01 ml, Himedia SS-4) of the culture from the tube is transferred to 50 ml nutrient broth (autoclaved at 1.05 kg/cm^2 for 20 min) and incubated at 37°C for 18 h under shaking at 200 rpm.

Enzyme production in SmF

Xylanase production in SmF was carried out using the basal medium containing (g/l) wheat bran 20.0; peptone 5.0; yeast extract 5.0; potassium nitrate (KNO_3) 5.0; potassium dihydrogen phosphate (KH_2PO_4) 1.0; magnesium sulfate ($MgSO_4$) 0.1, pH 7.0. The flasks were autoclaved at 1.05 kg/cm^2 for 20 min and cooled. They were then inoculated with 0.5% (w/v) overnight-grown culture and incubated at 37°C for 48 h under shaking at 200 rpm. Contents were centrifuged at 10,000*g* for 20 min at 4°C, and the clear cell-free supernatant was used for xylanase assay.

Enzyme assay

Xylanase activity was assayed according to the method of Bailey et al. [3] by measuring the amount of reducing sugars (xylose equivalent) liberated from xylan using 3,5-dinitrosalicylic acid [30]. The reaction mixture containing 490 µl of 2% birch-wood xylan (Sigma) 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 the reagents, but the reaction was terminated prior to the addition of enzyme. The contents were placed in a boiling-water bath for 10 min followed by cooling in ice-cold water. Absorbance of the resulting color was measured against the control at 540 nm in a spectrophotometer.

Cellulase activity [carboxymethyl cellulase(CMCase) and filter paperase (FPase)] was determined according to the method of Ghosh [18]. The reaction mixture for CMCase activity containing 500 µl of 2.0% carboxymethyl cellulose (Sigma) and 500 µl of crude enzyme was incubated at 50°C for 30 min. The reaction mixture for FPase activity containing Whatman no. 1 filter paper strip (1 × 6 cm), 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, cooled, and 20 ml of distilled water was added to it. A control was run simultaneously that contained all the reagents, but the reaction was terminated prior to the addition of enzyme. Absorbance of the resulting color was measured against the control at 540 nm in a spectrophotometer. One unit of xylanase or cellulase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of reducing sugar as xylose or glucose equivalent per minute under the specified assay conditions.

Effect of inoculum age and inoculum size

To study the effect of inoculum age on production, inoculum was prepared by inoculating 50 ml of nutrient broth with *B. pumilus* SV-85S and incubated at 37°C at 200 rpm. After every 6 h of incubation, 1.0 ml of inoculum was transferred to 50 ml of production media flask each time, which were incubated at 37°C for 48 h under shaking at

Table 1 Morphological, physiological, and biochemical characterization of *Bacillus pumilus* SV-85S, MTCC 9861

Tests	Results
Morphological tests	
Pigments	Cream
Configuration	Round
Elevations	Convex
Surface	Smooth
Gram stain	+
Cell shape	Rods
Size	2–4 μ
Arrangement	Short chains
Endospore	+
Position	Central
Shape	Ellipsoidal
Sporangia bulging	Nonbulged
Motility	+
Fluorescence (UV)	−
Biochemical tests	
Voges-Proskauer test, citrate utilization, gelatin hydrolysis, nitrate reduction, ornithine decarboxylase, lysine decarboxylase, catalase test and tween 40 hydrolysis	+
Growth on MacConkey, indole test, methyl red test, starch hydrolysis, H_2S production, starch hydrolysis, gas production from glucose, oxidase test and tween (20, 60, 80) hydrolysis	−
Physiological tests	
Temperature range	10–50°C
pH Range	5–10
Growth on NaCl	2–10 (%)
Acid production from carbohydrates	
Dextrose	+
Lactose	−
Mannitol	−
Growth under anaerobic conditions	+

200 rpm. Culture filtrate was centrifuged and used for the assay of xylanase activity. To study the effect of inoculum size, 50 ml of production media were inoculated at a level of 1.0, 2.0, 3.0, 4.0, and 5.0% (v/v) from an 18-h-old bacterial culture broth. After incubation at 37°C for 48 h, the culture filtrate was centrifuged at 10,000*g* for 20 min and xylanase activity was determined.

Effect of incubation period

Flasks containing 50 ml of production medium were inoculated with 1.0% seed culture and incubated at 37°C with constant shaking at 200 rpm. Following incubation for various time intervals (12, 24, 36, 48, 60, 72, 84, and 96 h), the culture filtrate was centrifuged and xylanase activity was determined.

Effect of pH and temperature on production

Xylanase production was studied at pH values ranging from 3.0 to 11.0. Each flask containing 50 ml production medium of different pH in the range of 3.0–11.0 was inoculated with 1.0 ml of 18-h-old inoculum and incubated at 37°C for 48 h in a rotary shaker incubator at 200 rpm. The enzyme was assayed in the culture filtrate after centrifugation. Xylanase production was studied at temperatures ranging from 30 to 55°C. Each flask containing 50 ml of production was inoculated with 1.0 ml of 18-h-old inoculum and incubated at different temperatures (30, 37, 40, 45, 50, and 55°C) for 48 h with constant shaking at 200 rpm. The enzyme was assayed in the culture filtrate.

Effect of carbon source

Enzyme production was carried out using the carbon sources viz. lactose, starch, glucose, maltose, mannitol, sucrose, fructose, galactose, cellulose, xylose, CM-cellulose, wheat bran, wheat straw, rice husk, paddy straw, birch-wood xylan, oat-spelt xylan, and saw dust, each at 1.0% (w/v). A control devoid of carbon source was also kept. Further, effect of the selected carbon source on enzyme production was investigated at its different concentrations.

Effect of nitrogen source

Enzyme production was monitored using various inorganic {[KNO_3, diammonium phosphate [$(NH_4)_2H_2PO_4$], sodium nitrate [$NaNO_3$], ammonium chloride [NH_4Cl], and ammonium sulfate [$(NH_4)_2SO_4$]} and organic (peptone, yeast extract, beef extract, tryptone, and casein hydrolysate) nitrogen sources at 0.5% both individually and in combinations. A control devoid of nitrogen source was also kept. Further, effect of the selected nitrogen source on enzyme production was investigated at its different concentrations.

RSM for optimization of nitrogen source

The best combination of nitrogen source, i.e. peptone, yeast extract, and KNO_3, was optimized via RSM. Using statistical software package Design Expert® 7.1.2, Stat-Ease, Inc., a 2^3 full factorial central composite design (CCD) with the above three factors and six replicates at the central points, leading to a set of 20 experiments, was used to optimize the nitrogen source for the production of xylanase from *B. pumilus* SV-85S. Each variable was used at five

Table 2 Experimental range and levels of each variable studied using Central composite design in terms of actual factors for the production of xylanase by *Bacillus pumilus* SV-85S

Independent variable	Symbols	Code level of variables				
		$-\alpha$	-1	0	1	$+\alpha$
Peptone (g/50 ml)	A	−0.14	0.1	0.45	0.8	1.04
Yeast extract (g/50 ml)	B	−0.14	0.1	0.45	0.8	1.04
KNO_3 (g/50 ml)	C	−0.14	0.1	0.45	0.8	1.04

KNO_3 potassium nitrate

coded levels ($-\alpha$, -1, 0, 1, $+\alpha$), as shown in Table 2. All variables were taken at a central coded value considered as zero. The 20 experiments of CCD included eight trails for factorial design, six trials for axial point, and six trails for replication of central point (Table 3). The response value from each experiment of CCD was the average of triplicates.

Effect of agitation

Each flask containing 50 ml of production medium was inoculated with 1 ml of 18-h-old inoculum and incubated at 37°C for 48 h under shaking at different agitation rates (50, 100, 150, 200, and 250 rpm) in a rotary-shaker incubator. One flask was also kept under stationary conditions. Xylanase activity was determined in the culture filtrates.

Effect of additives

The effect of various additives such as tween 20, tween 80, olive oil, glycerol, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetate (EDTA), Triton-X 100, and oleic acid was investigated on xylanase production. The additives were mixed into production medium at a concentration of 0.2% v/v (prior to autoclave), inoculated with 1.0 ml of 18-h-old inoculum, and incubated at 37°C for 48 h in a rotary-shaker incubator at 200 rpm.

Characterization

Effect of pH on activity and stability

The pH optima of xylanase was determined by measuring its activity at various pH values using different buffers, such as sodium citrate (pH 3–6), sodium phosphate (pH 6–8), Tris–hydrochloride (HCl) (pH 8–9) and glycine–sodium hydroxide (NaOH) (pH 9–11) each at 0.05 M. The pH stability was investigated by mixing equal aliquots of crude enzyme and different buffers in micro centrifuge tubes followed by incubation at 37°C. Buffer range was the same as for pH optima. Xylanase residual activity was calculated after different time intervals starting from 1 to 24 h. A crude enzyme without addition of any buffer was placed as control.

Effect of temperature on activity and stability

Xylanase activity was measured at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, and 75°C) under standard assay conditions using birch-wood xylan as substrate to determine the optimum temperature of the reaction. Thermo stability was determined by incubating the enzyme at different temperatures (25, 35, 40, 45, 50, 55, 60, 65, 70, and 75°C) in 0.05 M phosphate buffer, pH 6.0. Residual enzyme activity was measured after different time intervals (30, 60, and 120 min) by the standard assay.

Results and discussion

Under optimized submerged fermentation conditions, *B. pumilus* SV-85S produced 2995.20 ± 200.00 IU/ml of xylanase, which was 9.91-fold higher than the activity under unoptimized basal medium (302.2 IU/ml). Further, cellulase activity was undetectable in the cell-free supernatant, indicating that xylanase was cellulase free. The enzyme titre in our study was much higher than that reported earlier by Kiddinamoorthy et al. [25] in *Bacillus* sp. GRE7 under submerged fermentation using commercially available and expensive oat spelt xylan as a substrate. Poorna and Prema [35] reported production of 580 and 430 IU/ml xylanase by *B. pumilus* using oat spelt xylan and wheat bran, respectively. *Bacillus circulans* AB 16 [13] and *Bacillus subtilis* ASH [38] were able to produce 50 and 410 IU/ml, respectively, using wheat bran.

Inoculum size and age

Xylanase production was highest (456.3 IU/ml) when 1.0% (v/v) inoculum of 18-h-old culture was added to the production medium (Fig. 1a). The enzyme titre declined with increase in inoculum size beyond 1.0%. Several researchers have reported the use of 1.0–5.0% (v/v) inoculum for hyperproduction of xylanase [6, 22, 43]. Further, high concentration of inoculum is not preferred in industrial fermentation.

The effect of age on inoculum was studied by inoculating 50 ml of the production medium with 1% inoculum of 6- to 42-h-old culture of *B. pumilus* SV-85S (Fig. 1b). The production was highest during log phase, with a maximum of 629.6 IU/ml at 18 h [22 × 10^7 colony forming units (CFU)/ml] but showed a decline thereafter. Enzyme activity with 12-h-old inoculum (9 × 10^6 CFU/ml) was 223.3 IU/ml. These observations are in agreement with those of Sanghi et al. [38].

Table 3 Experimental design used in response-surface methodology by using three variables for xylanase production by *Bacillus pumilus* SV-85S under submerged fermentation

Standard order	Run	Peptone	Yeast extract	KNO_3	Xylanase activity (IU/ml)	
					Experimental value	Predicted value
1	19	0.10	0.10	0.10	538.84	509.05
2	17	0.80	0.10	0.10	1195.34	1202.25
3	1	0.10	0.80	0.10	1234.72	1223.57
4	13	0.80	0.80	0.10	311.67	317.53
5	11	0.10	0.10	0.80	210.20	163.65
6	14	0.80	0.10	0.80	1034.06	1024.52
7	20	0.10	0.80	0.80	1033.30	1005.7
8	2	0.80	0.80	0.80	238.22	247.32
9	12	0.00	0.45	0.45	625.92	670.06
10	16	1.04	0.45	0.45	635.00	615.14
11	8	0.45	0.00	0.45	430.55	152.20
12	3	0.45	1.04	0.45	398.75	399.36
13	5	0.45	0.45	0.00	1307.46	1314.48
14	9	0.45	0.45	1.04	959.09	981.04
15	10	0.45	0.45	0.45	1179.44	1178.60
16	7	0.45	0.45	0.45	1179.44	1178.60
17	6	0.45	0.45	0.45	1179.44	1178.60
18	4	0.45	0.45	0.45	1179.44	1178.60
19	15	0.45	0.45	0.45	1179.44	1178.60
20	18	0.45	0.45	0.45	1179.44	1178.60

KNO_3 potassium nitrate

Effect of incubation period

Xylanase activity was highest (594.07 IU/ml) after 36 h of incubation and declined on further increase in time of incubation (Fig. 2). Xylanases produced by *Bacillus* sp. were growth-associated, reaching a maximum after 24 h, and the enzyme production remained more or less the same up to 48 h [1]. On the other hand, *Bacillus amyloliquefaciens* secreted the highest xylanase activity in the culture supernatant after 48 h of growth [10]. *Bacillus* SSP-34 produced maximum xylanase activity (380 IU/ml) when grown for 96 h [43].

Effect of pH and temperature on production

The medium's pH is one of the regulatory parameters during fermentation [20]. The effect of varying pH of the production medium from 3 to 11 was studied on xylanase production. Substantial xylanase production was observed between pH 5 and 8, with highest at pH 6. Enzyme activity was 486.13, 604.99, 458.68, and 336.77 IU/ml at pH 5, 6, 7, and 8, respectively (Fig. 3a). Similar pH optimum for xylanase production was reported by Kohli et al. [26]. However, enzyme production by *B. subtilis* ASH [38], *B. circulans* AB 16 [13], *B. pumilus* ASH [6], and *Bacillus licheniformis* [2] was highest at pH 7.0.

Generally, microbes are known to produce high enzyme titre at their optimum growth temperature. The optimum temperature for xylanase production by *B. pumilus* SV-85S was found to be 37°C. Enzyme activity at 30, 37, and 40°C was 438.81, 665.03, and 380.12 IU/ml, respectively. At 45°C, the activity was negligible (Fig. 3b). A similar optimum temperature for enzyme production was reported for some *Bacillus* sp. [6, 17, 38].

Carbon source

Carbon source is one of the essential constituents of the microbial fermentation medium, which affects the overall cellular growth and metabolism. We tried different carbohydrates and agro residues to observe their effect on enzyme production. In controls, enzyme production was negligible. Among the different carbon sources, wheat bran supported highest xylanase (715.64 IU/ml) production followed by oat spelt xylan (690.45 IU/ml). Birch-wood xylan resulted in production of 650.32 IU/ml xylanase (Fig. 4). The enzyme titre in the presence of sugars was much lower compared with wheat bran or xylan. Although

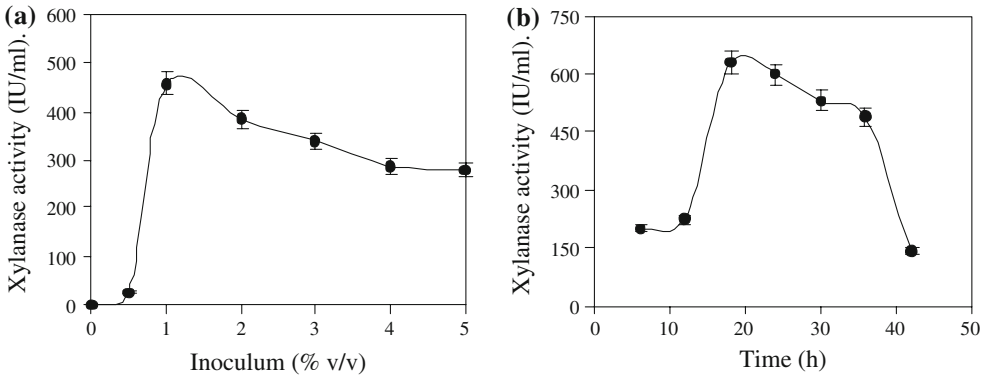

Fig. 1 Effect of inoculum size (**a**) and inoculum age (**b**) on enzyme production by *Bacillus pumilus* SV-85S. The fermentation medium contained 1.0% wheat bran as a carbon source, 0.5% each of peptone, yeast extract, and potassium nitrate (KNO_3) as nitrogen source and pH 7.0 incubated at 37°C with shaking at 200 rpm for 48 h

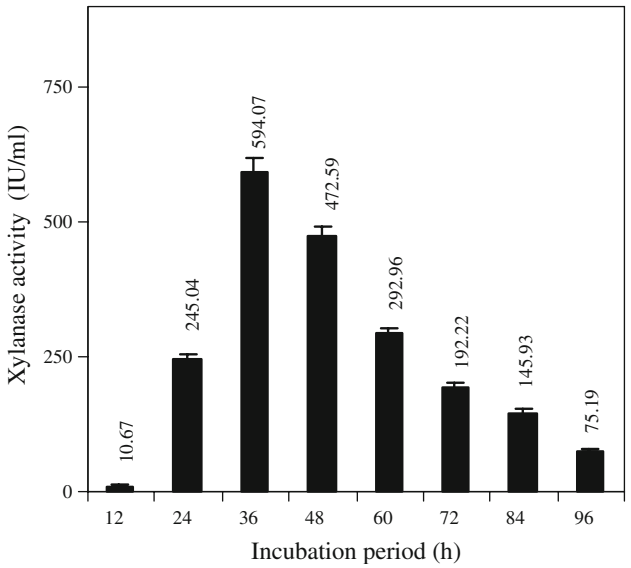

Fig. 2 Effect of incubation period on xylanase production by *Bacillus pumilus* SV-85S at 37°C, pH 7.0 under shaking at 200 rpm. The fermentation medium contained 1.0% wheat bran as a carbon source and 0.5% each of peptone, yeast extract, and potassium nitrate (KNO_3) as nitrogen source. The inoculum was 24 h old [11×10^7 colony forming units (CFU)/ml] used at 1.0%

hemicellulosic substrates such as wheat bran, rice straw, wheat straw, soybean flakes, rice bran, sugarcane bagasse, saw dust, ground nut shells, and others have been found to support xylanase production, the highest enzyme titre was obtained with wheat bran [20, 27, 35, 38, 42]. The higher level of production in the presence of wheat bran might be due to its high xylan content. As wheat bran is a cheap and easily available agro residue, it would affect the cost of the enzyme production directly.

Xylanase production was found to vary with change in the concentration of wheat bran. Enzyme activity was measured in the presence of 0.25–4.0% wheat bran. It was found to be highest with 2.0% (w/v) wheat bran, and there was a decline in xylanase production on increasing the concentration of wheat bran beyond 2.0% (Fig. 5). This could be due to formation of a thick suspension and improper mixing of the substrates in shake flasks.

Nitrogen source

Xylanase production was measured in the presence of several organic and inorganic nitrogen sources using 1.0% wheat bran as a substrate (Fig. 6). Organic nitrogen sources such as peptone (448.99 IU/ml), yeast extract (348.47 IU/ml), and beef extract (650.83 IU/ml) resulted in higher enzyme titre compared with inorganic compounds such as KNO_3 (88.72 IU/ml), $(NH_4)_2SO_4$ (271.46 IU/ml), and $NaNO_3$ (272.41 IU/ml). The effect of different combinations of nitrogen sources was also analyzed on enzyme production. It was observed that a combination of peptone, yeast extract, and KNO_3 resulted in highest enzyme production (1288.03 IU/ml). Similar results were reported by Battan et al. [6]. Highest xylanase production (251 IU/ml) by *Bacillus* SSP-34 occurred in a medium containing yeast extract and peptone each at 0.25% [43]. However, the best nitrogen source for xylanase production by *B. circulans* AB16 [13] and *Geobacillus thermoleovorans* [42] was tryptone. As it was difficult to perceive the cumulative effect of these three nitrogen source by one-variable-at-a-time approach, RSM was employed to optimize the concentration of the components of the above combinations.

RSM for optimization of nitrogen source

The concentrations of all the three components (peptone, yeast extract, and KNO_3) of the best nitrogen source combination were optimized via RSM. The experimental results of xylanase production by CCD with six replications of the central point and six axial points are shown in Table 3. Responses of the CCD design were fitted with a

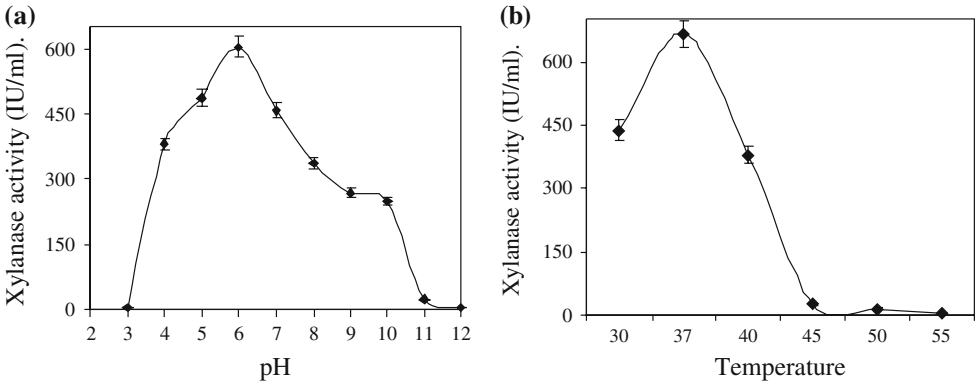

Fig. 3 Effect of pH (**a**) and temperature (**b**) on xylanase production by *Bacillus pumilus* SV-85S under shaking at 200 rpm. Fermentation medium contained 1.0% wheat bran as a carbon source and 0.5% each of peptone, yeast extract, and potassium nitrate (KNO_3) as nitrogen source

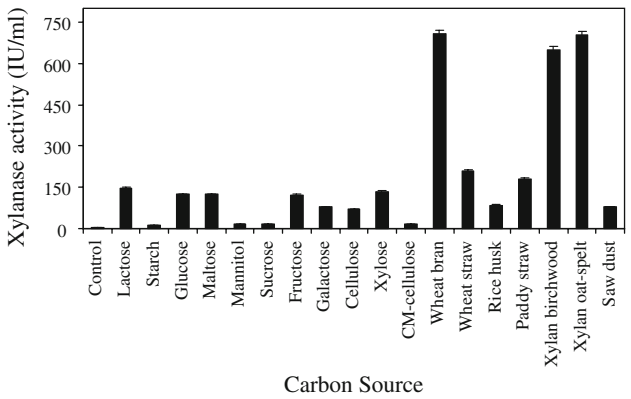

Fig. 4 Effect of various carbon sources on xylanase production in submerged fermentation by *Bacillus pumilus* SV-85S after 48 h of incubation, pH 7.0, at 37°C under shaking at 200 rpm with 0.5% each of peptone, yeast extract, and potassium nitrate (KNO_3) as nitrogen source. The inoculum was 18 h old and used at 1.0%

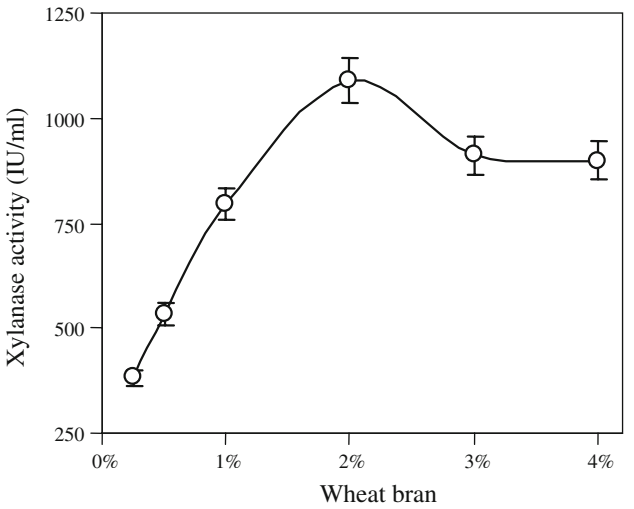

Fig. 5 Effect of wheat bran (%) on xylanase production in submerged fermentation by *Bacillus pumilus* SV-85S

polynomial quadratic equation (Eq. 1). The overall polynomial equation for xylanase production was:

$$\begin{aligned}\text{xylanase activity } (Y) &= +1178.60 - 16.29A \\ &- 15.67B - 98.90C - 399.81AB \\ &+ 36.92AC + 26.88BC - 188.63A^2 \\ &- 264.93B^2 - 10.85C^2 \end{aligned} \quad (1)$$

where, Y is xylanase activity; A is peptone; B is yeast extract and C is KNO_3.

The statistical significance of the model equation was evaluated by the F-test for analysis of variance (ANOVA), which showed that the regression was statistically significant, with a 99.9% confidence level. The model F-value of 493.39 as shown by Fisher's test indicated that the model was significant. The value of $P > F < 0.0500$ was desirable for a significant model, $P > F$ value of the model was 0.0001. Therefore, the model terms were also significant. It implied that the model was statistically significant for xylanase production. There was only a 0.01% chance that a 'Model F Value' was not significant, which could arise due to noise (Table 4). The determination coefficient (R^2) of the model was 0.997 (a value $R^2 > 0.76$ showed the aptness of the model). Moreover, R^2 value was in reasonable agreement with adjusted R^2 value of 0.995. The adjusted R^2 corrects the R^2 value for sample size and number of terms in the model. The Adeq Precision measures the signal to noise ratio. A ratio >4 was desirable. The ratio of 63.175 indicated an adequate signal (Table 5). Thus, the model was significant for the process.

Three-dimensional curves were helpful in observing interaction among these three factors and in determining their optimum values for maximum xylanase production. The interaction between peptone and yeast extract is shown in Fig. 7. The contour plot of peptone and yeast extract showed that the xylanase activity increased up to the mid range of concentration of both variables, followed by a decline on further increase in their concentrations (Fig. 7a). As the concentration of peptone increased, there was a corresponding decrease in yeast extract and vice versa (Fig. 7b). The activity was the lowest in response surface both at $-\alpha$ and $+\alpha$ levels, whereas concentrations of

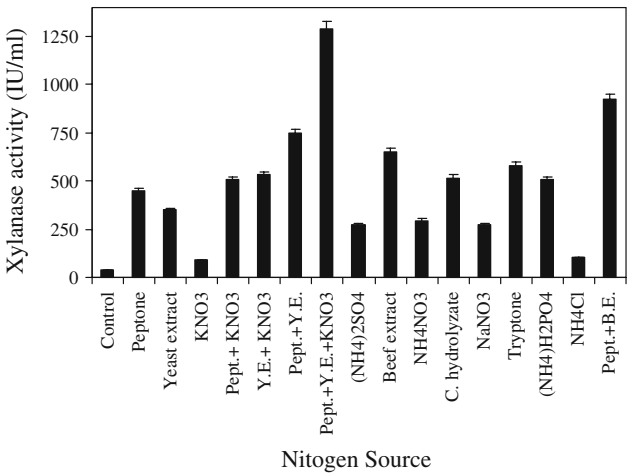

Fig. 6 Effect of nitrogen source on xylanase production in submerged fermentation by *Bacillus pumilus* SV-85S after 48 h of incubation, pH 7.0, at 37°C under shaking at 200 rpm with 1.0% wheat bran as a carbon source. The inoculum was 18 h old and used at 1.0%

peptone and yeast extract at the 0-level showed highest xylanase activity (Fig. 7c, d).

In Fig. 8 (a, b), contour plots depict that at the central point of peptone and yeast extract with minimum level of KNO_3 xylanase activity was highest. Response interactions of KNO_3 along with peptone and yeast extract suggested that a decrease in the concentration of KNO_3 leads to an increase in enzyme activity (Fig. 8c, d). In the RSM, xylanase activity was maximum at low concentration (-1 level) of KNO_3 along with other two factors at 0 level.

RSM has proven to be an advantageous tool for optimizing culture conditions and culture media composition. Response-surface plots suggested the following optimum values: peptone 0.45 g, yeast extract 0.45 g, and KNO_3 0.10 g for 50 ml of production medium. Under these conditions, xylanase activity was 1626.73 IU/ml. The result obtained in the experiment was satisfactory, as there was a 5.38-fold increase in xylanase activity using RSM compared with unoptimized basal medium. Concentrations of peptone and yeast extract were increased in the optimized medium, whereas the concentration of KNO_3 decreased compared with the initial medium.

There have been reports on the optimization of medium components using statistical approaches for a few fungal xylanase but not for high-level, alkali-stable bacterial xylanase under submerged fermentation. Statistical optimization of culture conditions for xylanase production by *B. circulans* D1 in submerged fermentation using a RSM with three-factor/three-level design resulted in a maximum activity of 22.45 U/ml, which was much higher compared with initial medium [9]. Different statistical experimental designs were combined by Pham et al. [33] to optimize the culture medium for xylanase production by *Bacillus* sp. I-1018. The highest xylanase production observed was 151 IU/ml.

Effect of agitation

Agitation and aeration are generally used to meet the oxygen demand and uniform mixing of nutrients during fermentation process. The highest enzyme titre (1779.12 IU/ml) was observed at an agitation of 150 rpm (Fig. 9). Further, enzyme production was higher under agitation than under stationary conditions. Lower enzyme production under stationary conditions might be due to reduced oxygen level in the medium, which in turn adversely affected the enzyme titre.

Effect of additives

Among the various additives used to enhance xylanase production, olive oil and tween 80 had a stimulatory effect. Compared with the control (1573.79 IU/ml), xylanase activity was 1710.10 and 1756.67 IU/ml after the addition of tween 80 and olive oil, respectively (Fig. 10). Further, enzyme production was monitored by varying the concentration of tween 80 and olive oil from 0.1% to 0.5%. The highest enzyme titre was obtained at 0.1% tween 80 and 0.2% olive oil, when added separately (Fig. 11a). The addition of 0.1% tween 80 alone to the production medium supported highest (2372.78 IU/ml) enzyme titre (Fig. 11b). A combination of both additives (at their optimal value) was tried in a production media, but the resulting activity (1430.38 IU/ml) was less compared with the control. Tween 80 (0.1%) was reported to enhance xylanase production by *G. thermoleovorans* also [42]. The stimulatory effect of tween 80 on xylanase production could be due to its favorable effect on cell membrane permeability.

Optimization studies revealed that xylanase production by *B. pumilus* SV-85S was highest (2995.20 ± 200.00 IU/ml) under optimized conditions, i.e., peptone 0.90%, yeast extract 0.90%, KNO_3 0.20%, wheat bran 2.0%, KH_2PO_4 0.10%, $MgSO_4$ 0.01%, tween 80 0.10%, pH 6.0, temperature 37°C, incubation time 36 h, agitation rate 150 rpm, and using 18-h-old (22×10^7 CFU/ml) 1.0% inoculum. Optimization of peptone, yeast extract, and KNO_3 through RSM markedly stimulated enzyme production. Optimization via RSM was beneficial, as it reduced the number of experiments and saved time.

Characterization of crude enzyme

In the paper and pulp industry, crude xylanase is used to bleach pulp. Enzymatic prebleaching requires a cellulase-free, thermostable, and alkali-stable xylanase. So, crude enzyme produced by *B. pumilus* SV-85S under submerged

Table 4 Result of regression analysis for response surface quadratic model

Source	Sum of squares	*df*	Mean square	*F* value	*P*-value prob. > *F*
Model	2844296.1	9	316032.9	493.39	<0.0001
A: peptone	3625.0	1	3625.0	5.66	0.0387
B: yeast extract	3353.7	1	3353.7	5.24	0.0452
C: KNO_3	133583.2	1	133583.2	208.55	<0.0001
Residual	6405.4	10	640.5		
Lack of fit	6405.4	5	1281.1		
Pure error	0.0	5	0.0		
Cor total	2850701.5	19			

KNO_3 potassium nitrate

Table 5 Analysis of variance (ANOVA) for response surface quadratic model for xylanase production

Term	Value	Term	Value
Standard deviation	25.31	R^2	0.9978
Mean	861.49	Adjusted R^2	0.9957
Coefficient of variation %	2.94	Predicted R^2	0.9829
PRESS	48858.40	Adeq precision	63.175

PRESS Predicted residual sum of squares

fermentation was characterized with respect to the effect of pH and temperature.

Effect of pH on activity and stability

Most xylanases known so far have their optimum pH around neutrality. Even xylanases produced by most alkaliphiles reported to date have their optimum pH around neutrality. In the activity assay using different pH buffers, highest activity of *B. pumilus* SV-85S was observed at pH 6 in 50 mM sodium phosphate buffer (Fig. 12). Enzyme activity at pH 6.5 and 7.0 was slightly less compared with that at pH 6.0. This is in agreement with the pH optima of xylanase from alkalophilic thermophilic *Bacillus* sp. [5]. The peak activity of *Bacillus* sp. GRE7 xylanase was observed at pH 7, and >60% activity was retained at pH 6 and 8 [25]. However, pH optima of 5.5 and 5.4 have also been documented [8, 46]. In contrast, pH optima of the alkaline xylanase from *Bacillus* sp. 41-M1 was 9.0; however, the activity was only 20 IU/ml [31]. Further, xylanase from *B. pumilus* exhibited two pH optima at 6.5 and 8.5 [35].

The pH stability studies revealed that the enzyme was alkali-stable. It was 100% stable at a pH range of 5–11 for

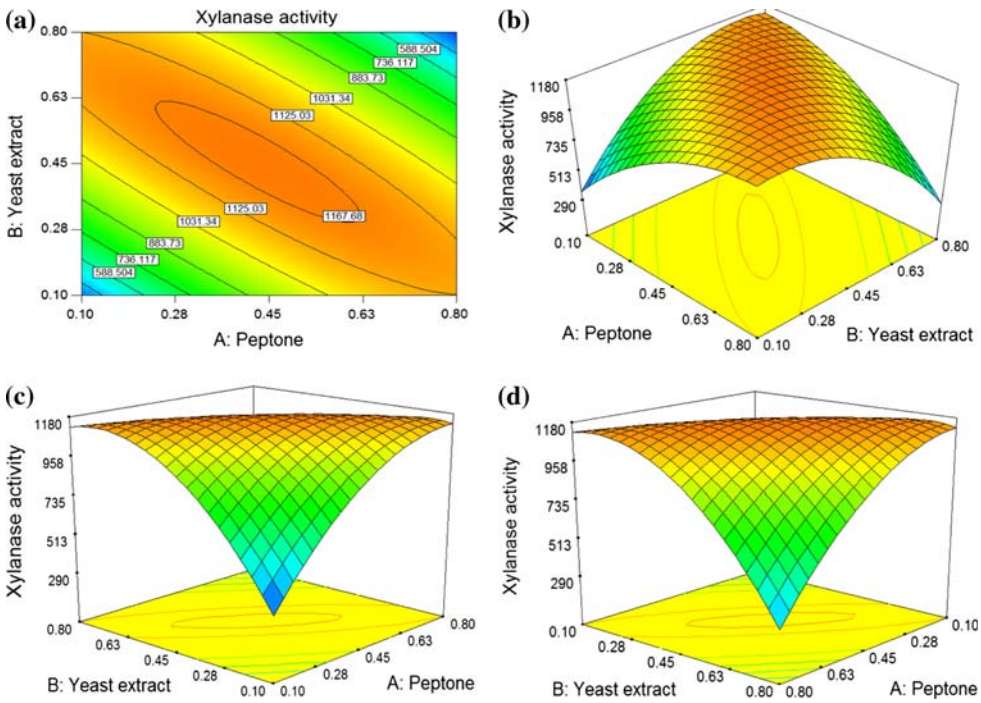

Fig. 7 Response-surface plots showing cumulative effect of peptone and yeast extract on xylanase production while keeping potassium nitrate (KNO_3) at 0 level; interaction between peptone and yeast extract. **a** Contour plots for the combined effects of peptone and yeast extract. **b** Highest activity at 0 level for both variables. **c** Decrease in activity at $-\alpha$ level for both variables. **d** Decrease in activity at $+\alpha$ level for both variables

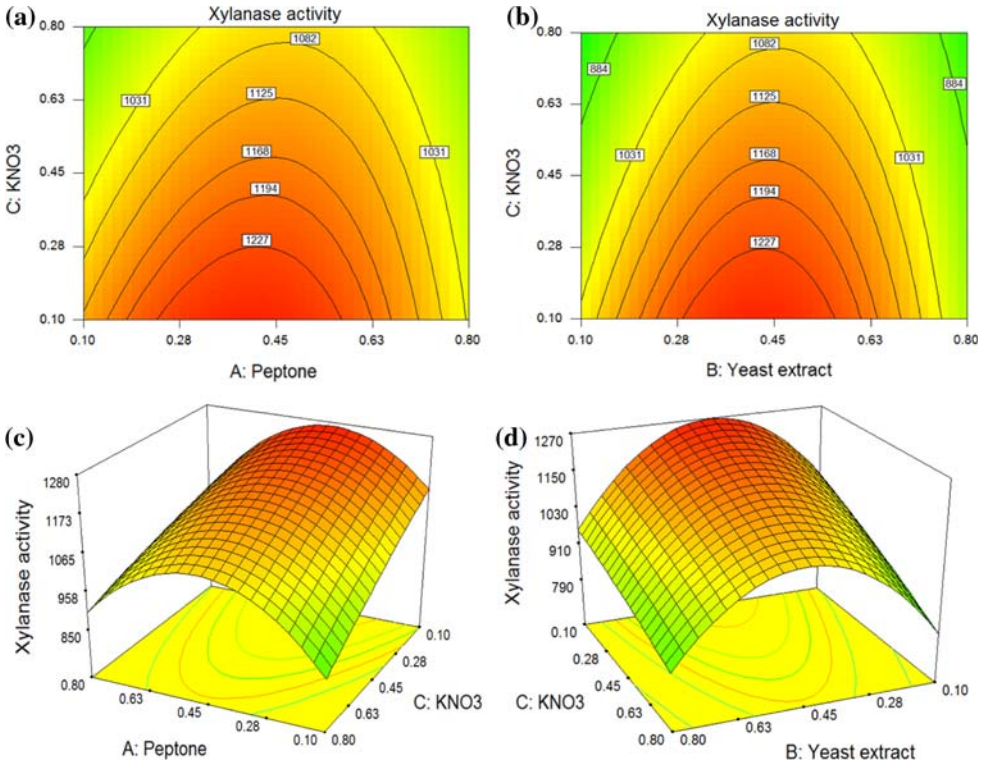

Fig. 8 Response-surface plots showing relative effect of two variables on xylanase production while keeping the third variable at 0 level; interaction between peptone and yeast extract along with potassium nitrate (KNO_3). **a** Contour plots for the combined effects of peptone and KNO_3. **b** Contour plots for the combined effects of yeast extract and KNO_3. **c** Response-surface plot for interaction of peptone and KNO_3. **d** Response-surface plot for interaction of yeast extract and KNO_3

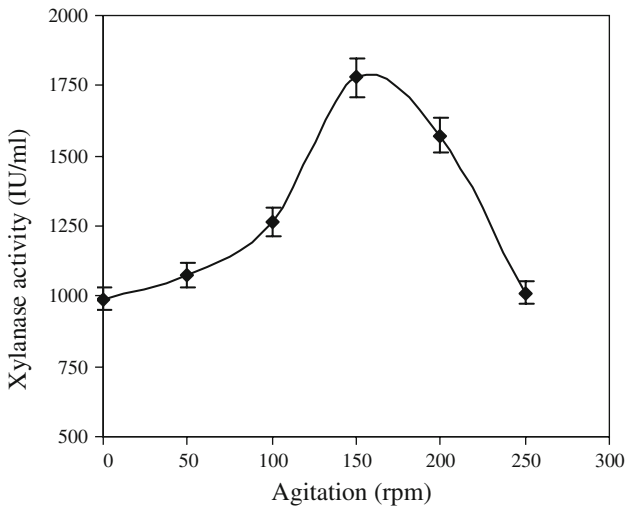

Fig. 9 Effect of agitation rate on xylanase production by *Bacillus pumilus* SV-85S at 37°C, pH 7.0. The fermentation medium contained 1.0% wheat bran as a carbon source and 0.5% each of peptone, yeast extract, and potassium nitrate (KNO_3) as nitrogen source. The inoculum was 18 h old and used at 1.0%

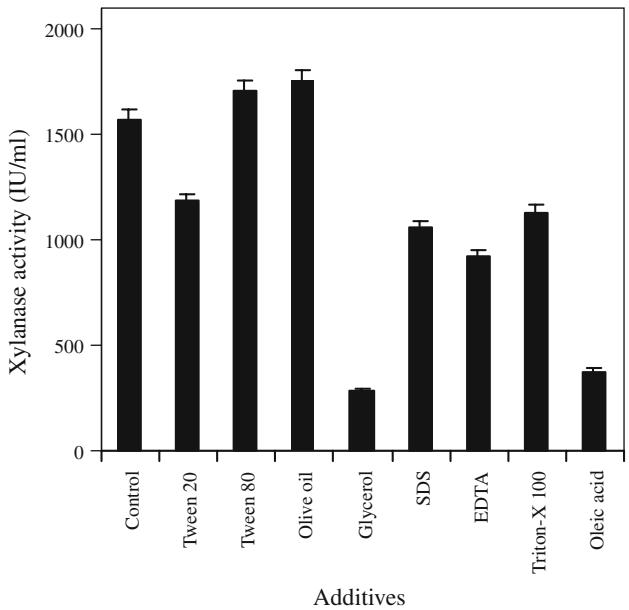

Fig. 10 Effect of various additives on xylanase production by *Bacillus pumilus* SV-85S after 48 h of incubation, pH 7.0, at 37°C under shaking at 200 rpm. Fermentation medium contained 1.0% wheat bran as a carbon source and 0.5% each of peptone, yeast extract, and potassium nitrate (KNO_3) as nitrogen source. The inoculum was 18 h old and used at 1.0%

1 h incubation at 37°C (Fig. 13). However, at pH 4, the enzyme showed 85% residual activity, and at pH 3 it was unstable. The crude enzyme was 100% stable, even after 3 h of incubation at pH 7, 8, and 9. Activity was 87.08, 84.79, 85.44, and 68.62% after an incubation of 3 h at pH 5, 6, 10, and 11, respectively. The enzyme was still stable at pH 7, 8, and 9 for 12 h, retaining 50% activity. Xylanase from *Bacillus* sp. GRE7 showed pH stability over pH range of 5–11 for 30 min. [25]. Xylanase from *Bacillus stearothermophilus* T-6 had stability at pH 6.5–10.0 for 5 min

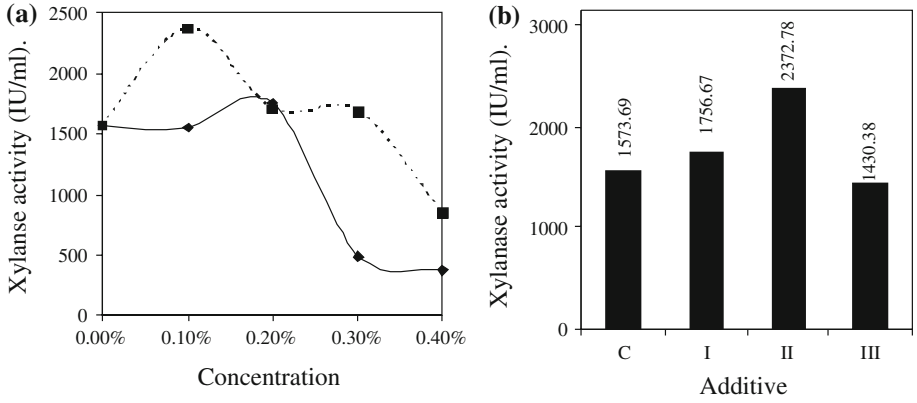

Fig. 11 **a** Effect of olive oil (*black diamond*) and tween 80 (*black square*) on xylanase production. **b** Xylanase production after addition of 0.1% olive oil and 0.2% tween 80 individually and in combination. **c** Control. *I* 0.2% olive oil, *II* 0.1% tween 80, *III* 0.2% olive oil + 0.1% tween 80

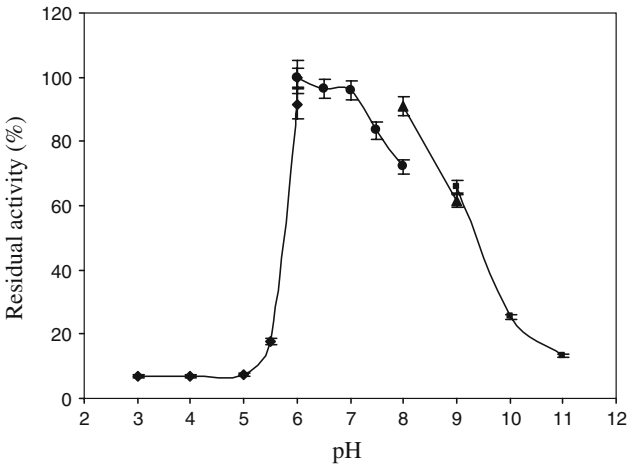

Fig. 12 Effect of pH on the activity of crude xylanase from *Bacillus pumilus* SV-85S using different buffers: pH 3–6, sodium citrate (*black diamond*); pH 6–8, sodium phosphate (*black circle*); pH 8–9, Tris–hydrochloride (HCl) (*black triangle*) and pH 9–11, glycine sodium hydroxide (NaOH) (*black square*) each at 0.05 M. Enzyme activity was measured using standard assay, except that pH was as indicated in the figure

[24]. Xylanase from alkalophilic *Bacillus* sp. strain 41 M-1 showed a broad pH activity profile, with pH range 4–11 [31]. In this study, xylanase exhibited substantial alkali tolerance, a desirable feature for its application in the paper and pulp industry. In addition, xylanase produced by *B. pumilus* SV-85S is likely to have potential in the textile industry where desizing and bioscouring treatments in bioprocessing of fabrics are performed at alkaline pH [15].

Effect of temperature on activity and stability

The effect of temperature on extracellular xylanase from *B. pumilus* SV-85S is shown in Fig. 14. The optimum temperature for xylanase activity was 50°C. At a temperature of 55, 60, and 65°C, the enzyme exhibited 94.0, 79.0, and 78.0% residual activity, respectively. The enzyme showed 50% activity even at 70°C. An identical temperature optimum was reported for the enzyme from *Bacillus* sp. [8, 12, 31, 35].

Thermal stability studies showed that the crude enzyme was stable at 37°C. However, an appreciable half-life of the enzyme was observed up to 60°C for a period of 60 min (Fig. 15). The enzyme exhibited 40% and 30% residual activity even at 65 and 70°C, respectively, after an incubation period of 30 min. As the xylanase produced by *B. pumilus* SV-85S exhibited significant activity at high temperatures and was alkali stable, it is likely to have immense potential in pulp bleaching [38] and the textile industry [15]. Poorna and Prema [35] demonstrated the stability of *B. pumilus* enzyme at 25–40°C for 1 h. Yang et al. [46] reported only 15–20% enzyme stability at 60°C after 30 min of incubation. The enzyme from *B. circulans* AB-16 was stable at 60°C after 2.5 h incubation [13]. Thermal stability of the enzyme from *Bacillus* sp. 41-M1 was low, losing 90% of the activity after 30 min heating at 60°C [31]. Xylanase from *B. pumilus* was stable at a wide range of temperatures (25–40°C) and retained 32% of activity at 60°C after 1 h of incubation [35]. Only a few xylanases are reported active and stable at both alkaline pH and elevated temperature [31, 43]. The enzyme in our study, in addition to having these properties, was produced in high titre and was stable for a longer period. The difference in pH and temperature stability for xylanase excreted may be due to the posttranscriptional modifications in xylanase excretion process, such as glycosylation, that improves stability in more extreme pH and temperature conditions [35].

Conclusions

B. pumilus SV-85S produced high levels of cellulase-free and alkali-stable xylanase, which was moderately active at elevated temperatures. The enzyme titre obtained in this study was much higher than the alkali-stable xylanases reported earlier. Optimization of fermentation conditions

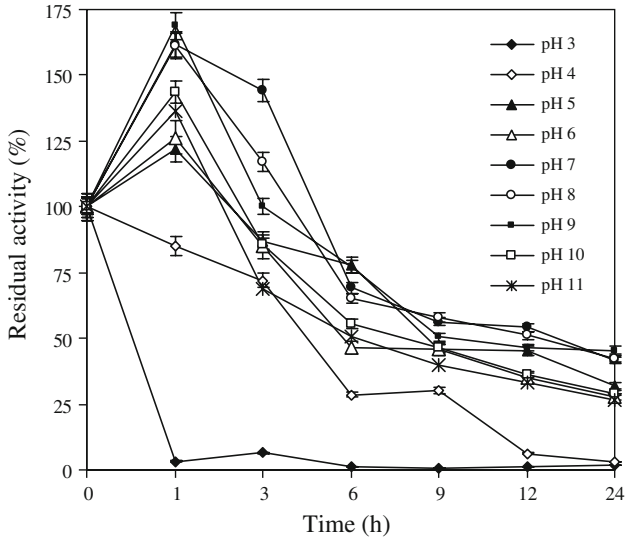

Fig. 13 Effect of pH on xylanase stability. The enzyme was mixed with an equal aliquot of different buffers viz. sodium citrate (pH 3–6), sodium phosphate (pH 6–8), Tris–hydrochloride (HCl) (pH 8–9) and glycine sodium hydroxide (NaOH) (pH 9–11), each at 0.05 M. Residual enzyme activity was measured using standard assay

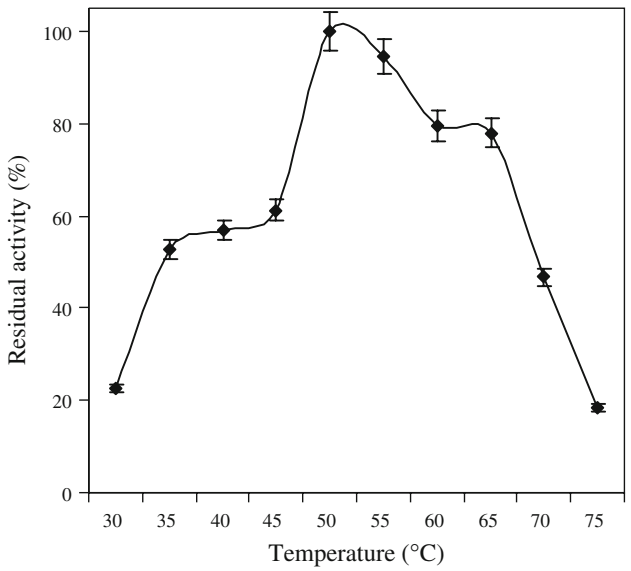

Fig. 14 Effect of temperature on the activity of xylanase produced by *Bacillus pumilus* SV-85S. Enzyme activity was measured using standard assay, except that the temperature of incubation was varied as indicated in the figure

enhanced the enzyme titre by 9.91-fold compared with the basal medium. The use of RSM for nitrogen source optimization stimulated enzyme yield. The enzyme was 100% stable for >1 h on a broad pH scale, and it lost no activity, even after 3 h of incubation at pH 7, 8, and 9. The optimum temperature for xylanase activity was 50°C, but the enzyme displayed 78% residual activity even at 65°C. The

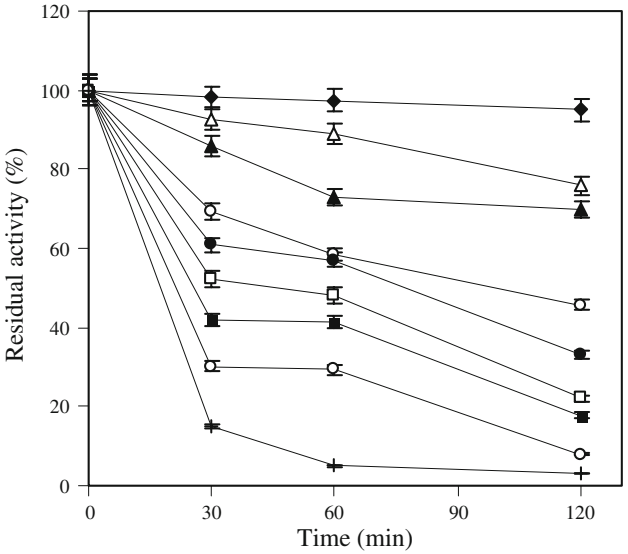

Fig. 15 Temperature stability of crude xylanase enzyme. The enzyme was diluted in sodium phosphate buffer (pH 6, 50 mM) and incubated at 37°C (*black diamond*), 40°C (*white triangle*), 45°C (*black trinagle*), 50°C (*white circle*), 55°C (*black circle*), 60°C (*white square*), 65°C (*black square*), 70°C (*white circle*), and 75°C (*plus sign*). Residual activity was assayed after time intervals of 30, 60, and 120 min

enzyme retained 50% activity after an incubation of 1 h at 60°C. Characteristics of the enzyme, such as its cellulase-free nature, alkali tolerance, and activity at elevated temperatures, together with its high titre, are particularly suitable for the paper and pulp industry.

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