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Full Length Research Paper

Sawdust and digestive bran as cheap alternate substrates for xylanase production

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Xylanases are a major group of enzymes, mostly produced from microbial fermentation processes, and have wide industrial and biotechnological applications. The production cost of xylanase is the major factor limiting its use, thus indicating the need for low cost production systems for market of this enzyme. In this study, therefore, sawdust and digestive bran were investigated as substrates for xylanase production by *Bacillus* strains. The xylanase titre ranging between 30.849 to 45.206 nkat/ml and 6.633 to 22.717 nkat/ml was produced by these *Bacillus* strains, using sawdust and wheat bran as the substrate, respectively. The optimum temperature for the production of xylanase was found to be between 45 and 55°C, while the optimum pH was 8.0 for all the strains tested. The xylanases produced by these *Bacillus* strains were found to be stable over a wide range of temperature tested (40 to 90°C). Up to 98 and 95% of the initial activity was retained by the crude extract of two of the *Bacillus* strains tested, while 10.3 to 56% loss in activity was observed for the other isolates after one hour incubation at 70°C. Addition of metal salts or additives to the crude extract was found to inhibit the enzyme activity to a varying degree, with the following order Hg^{2+}> EDTA > Na > Urea > Mg^{2+} > Ca^{2+} observed. Findings from this study indicate the potential use of sawdust and digestive bran as cheaper alternatives for the production of xylanases.

Key words: *Bacillus* sp, digestive bran, sawdust, thermostability, xylanase.

INTRODUCTION

Organic wastes from agricultural residues comprise cellulose, hemicellulose and lignin in an average ratio of 4:3:3, with hemicellulose being the second most abundant fraction available in nature (Taiz and Honigman, 1976; Abdel-Sater and El-Said, 2001). Xylans, the major portion of the hemicellulose of plant cell walls, are hetero-polymers consisting principally of xylose and arabinose (Biely, 1985; Coughlan and Hazlewood, 1993) and is amenable to degradation by different microorganisms, including bacteria, yeasts, and fungi. Several types of enzymes appear to be involved in the degradation of native xylan. Endoxylanases, which attack the linear polyxylose chain in a manner analogous to that of endoglucanase on cellulose, are the most important and have accordingly received the most attention (Sunna and Antranikian, 1997). β-xylosidase hydrolyzes xylobiose and oligosaccharides to complete the conversion of xylan to xylose and probably relieves the end product inhibition of endoxylanase activity (Srinivasan and Rele, 1999; Subramaniyan and Prema, 2000; Chávez et al., 2006). This activity appears to be extracellular in fungi but cell-associated in bacteria, although relatively few of these enzymes have been studied in detail (Esteghlalian et al., 2008).

Other enzymes play an important role in the removal of side groups from polymeric xylan to create more sites for subsequent enzymatic hydrolysis and possibly contribute to lignin solubilization by attacking the covalent bonds responsible for the integrity of the lignocarbohydrate matrix (Bachmann and McCarthy, 1991; Contreras et al., 2008). The choice of an appropriate substrate is of great

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importance for the successful production of xylanases. The substrate not only serves as carbon and energy source, but also provides the necessary inducing compounds for the organism, preferentially for an extended period of time, for an increased overall productivity of the fermentation process (Duff and Murrayh, 1996; Haltrich et al., 1996). Purified xylans are frequently used for small-scale experiments and are considered as excellent substrates not only because of high yields of xylanase obtained, but also because they cause a selective induction of xylanase with little or no concomitant cellulase activities (Biswas et al., 1990; Gilbert et al., 1992). However the high cost of xylan and other good substrates has limited their applications for larger-scale production processes, hence, the need to search for cheaper alternatives.

Several inexpensive substrates, mainly insoluble lignocellulosic material, such as barley husk, corn cobs, sugarcane bagasse have been used (Camassola and Dillon, 2009). Although poorly investigated, the use of soluble sugars for the production of xylanases has been shown to circumvent problems associated with the use of high concentrations of insoluble substrates, such as media viscosity and difficulties with agitation. In addition, xylose, which can be easily obtained from the xylan portion of lignocellulosic material, has been described as an effective inducer of xylanase activity in several organisms (Haltrich et al., 1996). Biotechnological applications of xylanases have broadened markedly since they are now widely employed as supplements in paper manufacturing, animal feeds, biobleaching of pulp and paper, and in the production of bioethanol (Beg et al., 2000; Subramaniyan and Prema, 2000; Techapun et al., 2003). The most important industrial application of xylanase is in the pre-bleaching of Kraft pulp. This allows for a lower consumption of chemicals during the bleaching process, and also results in a brighter product than can be achieved without the enzymatic treatment (Viikari et al., 1994; Ninawe and Kuhad, 2006). Most of the commercial xylanase is produced by microbial fermentation process.

The production cost of xylanase is the major factor preventing its use, thus indicating the need for low cost production systems for market of this enzyme. Therefore, the main objective of this study is to investigate the potential of cheap raw substrates such as sawdust and digestive bran for the production of xylanases by *Bacillus* sp. The thermostability and pH stability of the xylanases produced by these organisms on the raw substrates was also investigated to determine their potential industrial applications.

MATERIALS AND METHODS

Organisms and sawdust used in this study

Bacillus strains used in this study, namely; *B. subtilis* (S1), *B. chitinosporus* (S2), *B. licheniformis* (S3), *B. stearothermophilus* (S4), *B. coagulans* (S5), *B. uniflagellatus* (S6), *B. laterosporus* (S7), and *B. pumilus* (S8) were obtained from the culture collections of the University of KwaZulu-Natal and Durban University of Technology, South Africa. Pure cultures of the *Bacillus* strains were preserved on nutrient agar slants and stored at 4°C. Sawdust used in this study was donated by Country Wood (Pinetown), South Africa and it is composed of Balau deck boards (20%); Beech wood (20%); Kiaat (12%), Mahogany (12%); SA pine (5%); Blackwood (4%), Cherry (4%), Meranti (4%), Saligna (4%); Cotton wood (2%), Cypress (2%), Imbuia (2%), Oak (2%), Rosewood (2%); Iroko (1%), Mable hard prime (1%), Panga Panga (1%), Spruce (0.5%), Tatajuba (0.5%), Teak Rhodesian prime (0.5%), and Yellow wood (0.5%).

Preliminary screening for xylanase production

The bacterial strains were inoculated onto modified xylan agar plates, consisting (in g/l): Digestive bran or sawdust, 10.0; yeast extract, 5.0; peptone, 5.0; K_2HPO_4, 1.0; $CaCO_3$, 2.0; $(NH_4)_2SO_4$, 2.0 (pH 8.0) (Kapoor et al., 2008) and 1.8% (w/v) bacteriological agar. Pure colonies obtained were tested for xylanase production by flooding plates with 0.1% Congo red for 15 min and destained with 0.1 M NaCl for 30 min (Mamo et al., 2006). The zones of hydrolysis were revealed by finally flooding the plates with 0.5% (v/v) acetic acid. Isolates with clearing zone were considered xylanase positive.

Xylanase production and assay

Erlenmeyer flasks (250 ml) containing 100 ml of modified xylan medium (pH 8.0) were inoculated with 5 ml of standardized cultures (OD of 1 at $\lambda_{600\ nm}$) and incubated at 35°C in a rotary shaker (200 rpm) for 4 days. The cultures were harvested by centrifugation at 6000 × g for 5 min and the cell free supernatant was then used as an enzyme source. Xylanase activity was measured by the DNS (3,5-dinitrosalicylic acid) assay (Miller, 1959), that determines the amount of reducing sugars liberated from oats-spelt xylan (OSX) (5 g/l, Sigma) solubilized in phosphate buffer (0.05 M, pH 6.0), according to Bailey et al. (1992). This reaction mixture of an enzyme (100 µl) and a substrate (900 µl) was incubated for 15 min at 50°C, before stopping the reaction by the addition of 1.5 ml DNS solution. The samples were boiled in test tubes for 10 min, cooled under running water for colour stabilization and the optical density was measured at 540 nm. Xylanase activity was determined from a calibration curve constructed with varying concentrations of D-xylose (sigma) and expressed in nkat. One nkat (0.0599 IU) was defined as the amount of enzyme that releases 1 nmol xylose/s, at 50°C.

Effect of temperature and pH on xylanase production

To determine the optimal conditions for the production of xylanolytic enzymes, pH of cultivation media (4 to 12) and incubation temperature (30 to 70°C) were varied and their effect on xylanase production investigated by determining xylanase activities under different conditions as previously described.

Temperature and pH profile of xylanases

The optimum temperature for the enzyme activity was determined by incubating the crude enzyme at different temperatures ranging from 40 to 90°C for 15 min. The pH profile of crude xylanase was evaluated by incubating the enzyme for 15 min in the presence of appropriate buffers: 0.05 M citrate buffer (pH 4); phosphate

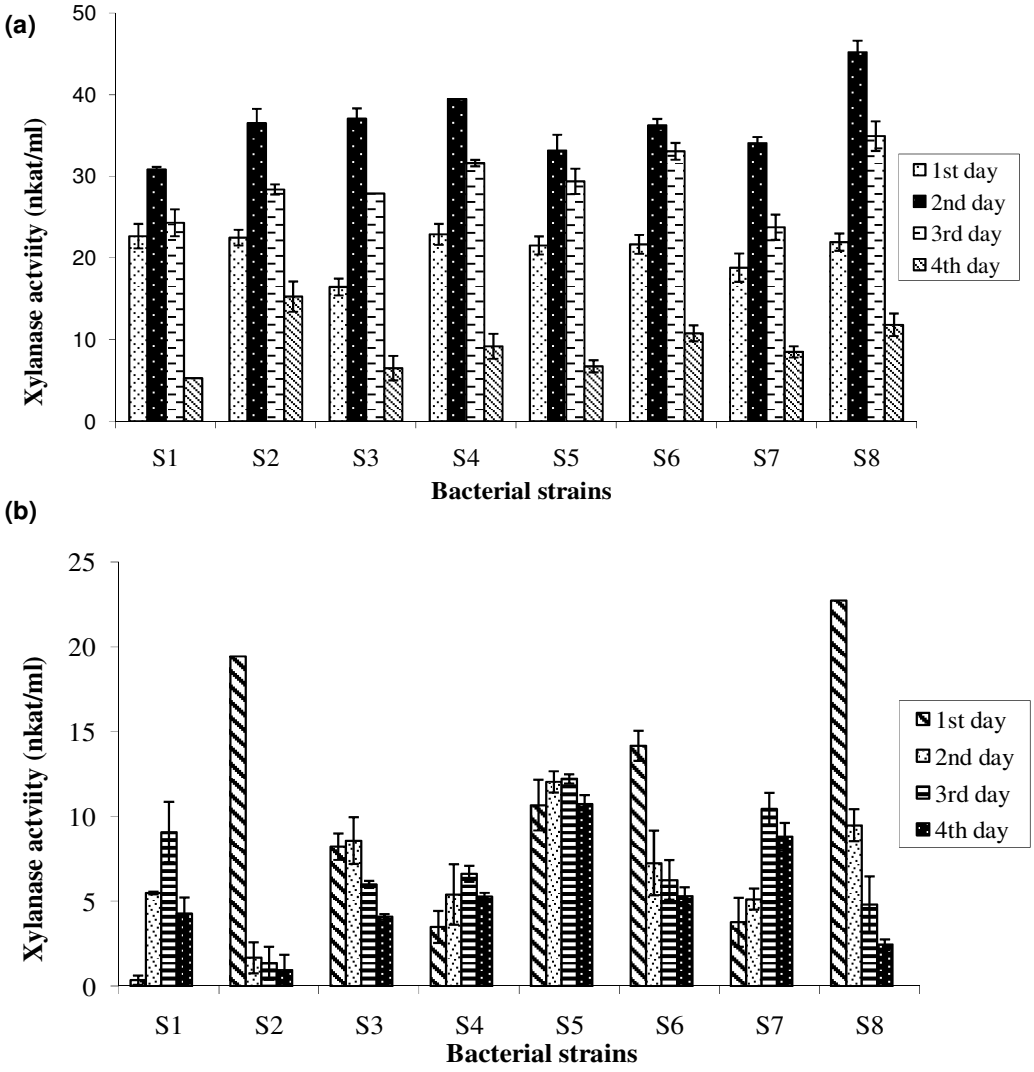

Figure 1. Time-course production of xylanase by the *Bacillus* strains using (a) sawdust and (b) bran as the substrate.

buffer (pH 6); Tris-HCl buffer (pH 8) and glycine-NaOH (pH 10) (Sa´-Pereira et al., 2002; Anuradha et al., 2007) at the pre-determined temperature.

Xylanase activity was determined by using the DNS assay as previously described.

The effect of metal salts or additives on xylanase activity

The effect of metal salts or additives was determined by incubating the crude enzyme (100 µl) with 10 µl of 0.1 M salt solution ($MgCl_2$, $HgCl_2$, EDTA, NaCl, $CaCl_2$, and Urea) (Bataillon et al., 2000) before measuring xylanase activity.

Thermostability and pH stability of xylanases

Temperature stability studies were performed by incubating the crude enzyme at an optimum pH of 6 and at temperatures ranging from 40 to 90°C for 1 to 4 h (Sa´-Pereira et al., 2002; Anuradha et al., 2007). The pH stability was determined by incubating the crude enzyme in appropriate buffers:

Citrate buffer (pH 4); phosphate buffer (pH 6.0 and 7.0); Tris-HCl buffer, [pH 8.0], and glycine-NaOH, (pH 10 and 12) (Anuradha et al., 2007).

Xylanase activity was determined every hour using DNS assay as previously described and the relative activity of the enzyme determined by comparing activity at time (t) to the initial activity obtained before incubation.

RESULTS

Xylanase production on digestive bran and sawdust

The xylanase production profile of the *Bacillus* strains using digestive bran and sawdust as substrate over a 4

day period is shown in Figure 1. The xylanase activity of the crude enzymes obtained from the *Bacillus* strains grown on sawdust was optima after 2 day and ranged between 30.849 to 45.206 nkat/ml (Figure 1a), while the activity peaked between days 1 and 3 for the different strains when digestive bran was used as a substrate and ranged between 6.633 to 22.717 nkat/ml (Figure 1b). Sawdust was observed to be a better substrate, resulting in higher amount of xylanase production by all the bacterial strains, compared to the digestive bran. *Bacillus* strain S8 was found to produce the highest amount of xylanase, irrespective of the substrate used.

Effect of temperature and pH on the production of xylanases

The effects of temperature and pH on the hydrolysis of oat-spelt xylan by the crude xylanase produced by these *Bacillus* strains were evaluated, in order to determine the optimum conditions. The optimum temperature for the production of xylanase was found to be observed by 55°C for the strains, except for isolates S2 and S5 where the optimum temperature was 45°C (Figure 2a). A decrease in xylanase production was observed above and below these optima. In all the strains, the optimum pH for the production of xylanases were pH 8 (Figure 2b). However, a slight increase in xylanase production was noted at pH 12 for isolates S1-S6, compared to pH 10.

Determination of the temperature and pH profile of xylanases on sawdust

The highest activity was obtained when the enzyme was incubated at 50°C, while increase in temperature from 60 to 90°C tend to strongly inhibit the enzyme activity, with up to 6-fold decrease in activity observed from 50 to 70°C (Figure 3a). The optimum pH for xylanase activity was achieved at pH 6, with 1.37 to 14 fold increase in activity observed at pH 10, compared to those at pH 8 for all the tested organisms (Figure 3b).

The thermostability and pH stability of xylanases

The xylanases produced by these *Bacillus* strains were found to be stable over a wide range of temperature tested (40 to 90°C). However, results presented in this paper are for the thermostability of the crude enzyme at 70 and 90°C. It is worth noting that xylanases produced by strain S1 and S6 retained up to 98 and 95% of the initial activity, respectively, with between 10.3 and 56% loss in activity observed for the other isolates after one hour incubation at 70°C (Figure 4a). Up to 25.80 to 60.61% activity was still retained by the crude enzyme extract of the strains, even after 3 h. At 90°C, the enzyme retained between 45 to 80% of its activity after one hour, after which a progressive decrease in activity was observed as the incubation time increases, with 86.05 to 98.68% loss in activity observed after 4 h (Figure 4b). The pH stability of xylanases produced by *Bacillus* strains was determined at pH 4; 6; 8; and 12 by using appropriate buffers. However only results for pH 6 to 10 are shown in this article.

Xylanases were less stable at pH 4 and lost most of its activity after 1 h. At pH 6, the enzyme was more stable for 2 h, with isolates S5 and S9 losing only 19% and 16% of its activity respectively after 1 h (Figure 5a). At pHs 8, 10 and 12, a drastic decrease in enzyme activity was observed after 2 h (Figures 5 b, c, and d), with most of the enzyme activity lost after 4 h (Figure 5d). However, xylanases produced by isolate S3 retained 73; 80 and 77% of its activity at pH8, 10 and 12, respectively.

Effects of metal salts or additives on xylanase activity

Addition of metal salts or additives to the crude extract was found to inhibit the enzyme activity to a varying degree (Table 1). Generally, HgCl and EDTA were found to have the most negative effect on the enzyme activity, with up to 90.34 and 94.32% reduction in activity, respectively, observed for the crude extract of strain S4. $CaCl_2$ and $MgCl_2$ only slightly inhibited the enzyme activity, with up to 96 and 87%, respectively, of the activity retained. The general pattern of enzyme inhibition follows the order of Hg^{2+}> EDTA > Na > Urea > Mg^{2+} > Ca^{2+}.

DISCUSSION

The production cost of xylanase is the major factor preventing their use, thus indicating the need for low cost production systems for this enzyme (Bae et al., 2008). Various substrates including, larchwood, beechwood, birchwood, wheat bran, oat-spelt, corn cob, sugar beet pulp, sugarcane bagasse, and various industrial wastes have been used for their production (Bailey et al., 1992; Maheshwari et al., 2000). In this study, digestive bran and sawdust were used as cheap alternate substrates for the production of xylanase by *Bacillus* strains. Sawdust is a material that exhibits a high polarity mainly due to the presence of different oxygen-containing functional groups such as alcohol and ether (Bledzki and Gassan, 1999; Álvarez et al., 2005). The cell walls of sawdust mainly consist of cellulose and lignin, and many hydroxyl groups, such as tannins or other phenolic compounds, which are active ion exchange compounds. The lignin content of hardwoods is usually in the range of 18 to 25%, whereas that of softwoods varies between 25 and 35% (Batzias and Sidiras, 2007).

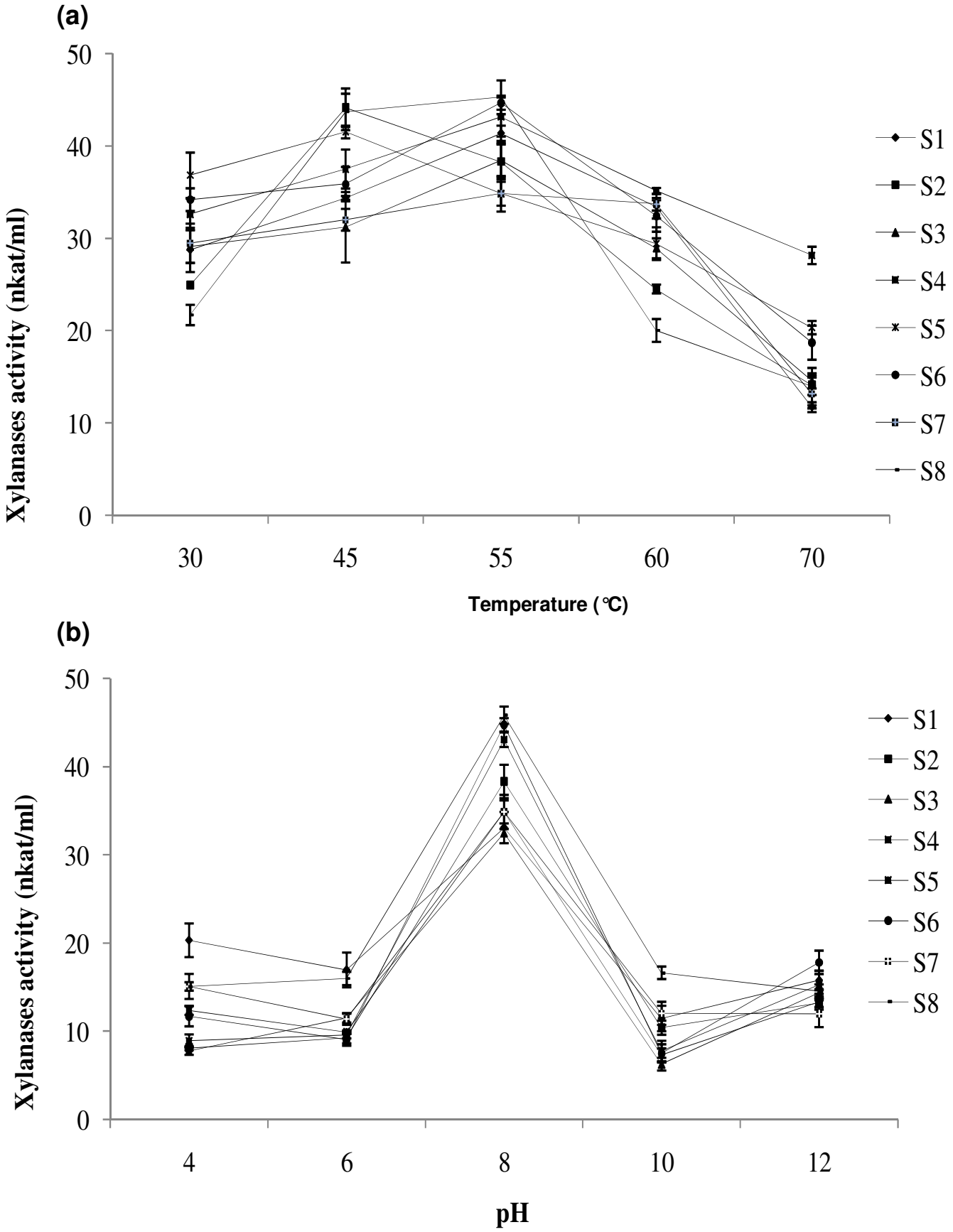

Figure 2. The effect of (a) temperature and (b) pH on the production of xylanase using sawdust as the substrate.

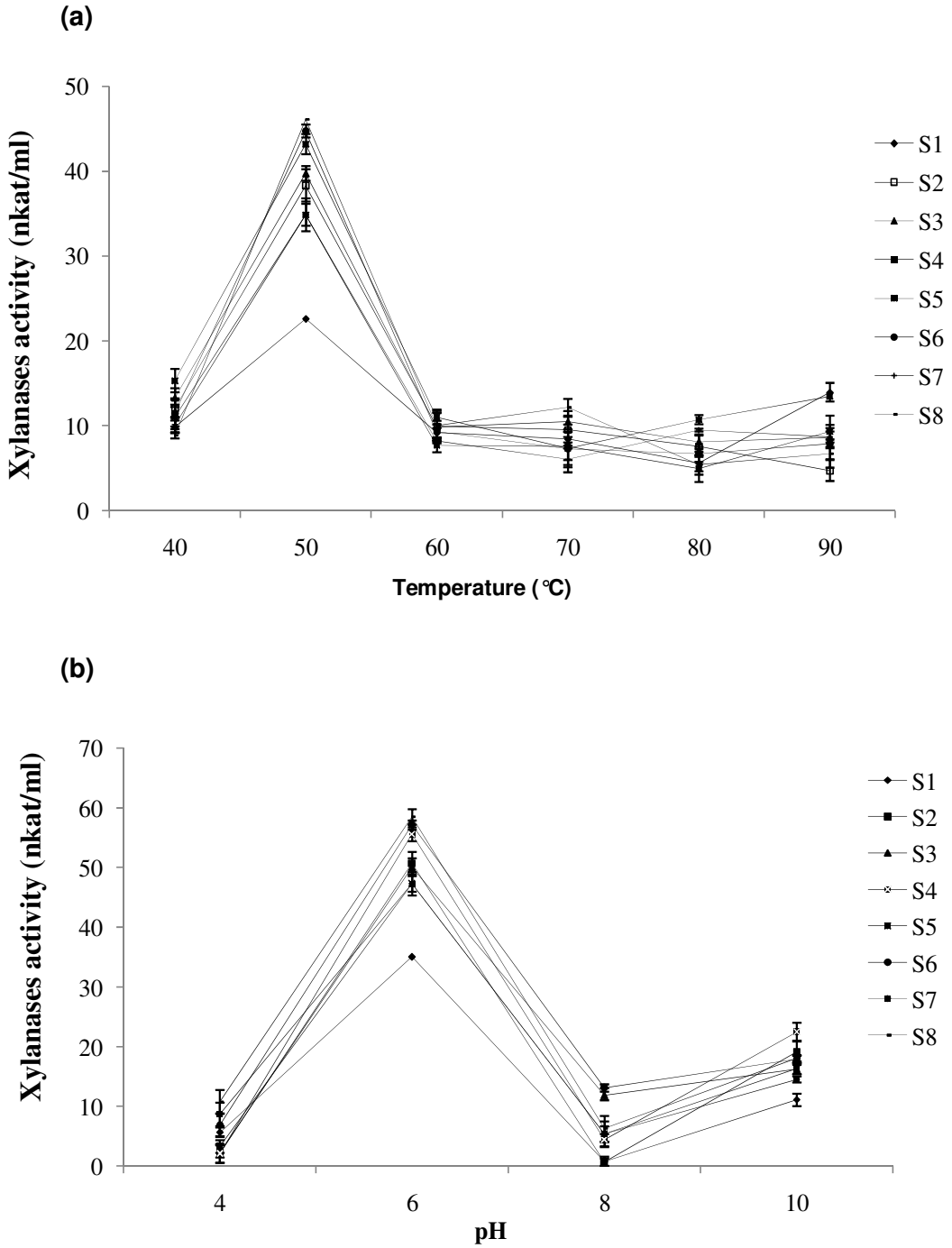

Figure 3. The effect of (a) temperature and (b) pH on xylanase activity of the crude enzyme produced by the Bacillus strains on sawdust.

Sawdust used in the present study had high beech wood (20%) content making it an ideal substrate for xylanase production, since beechwood has been reported as a good substrate for xylanase production (Bailey et al., 1992; Maheshwari et al., 2000). The composition of the sawdust is indicated under materials and methods. This could explain the high xylanase production on sawdust by all the isolates used in this study. The decrease in enzyme activity observed after the second day of incubation could possibly be due to the accumulation of metabolic waste and production of proteases which might inhibit xylanase activity (Gessesse and Mamo, 1999). Nawel et al. (2010) reported that *Jonesia denitrificans* BN-13 isolated from Algerian soil produced extracellular

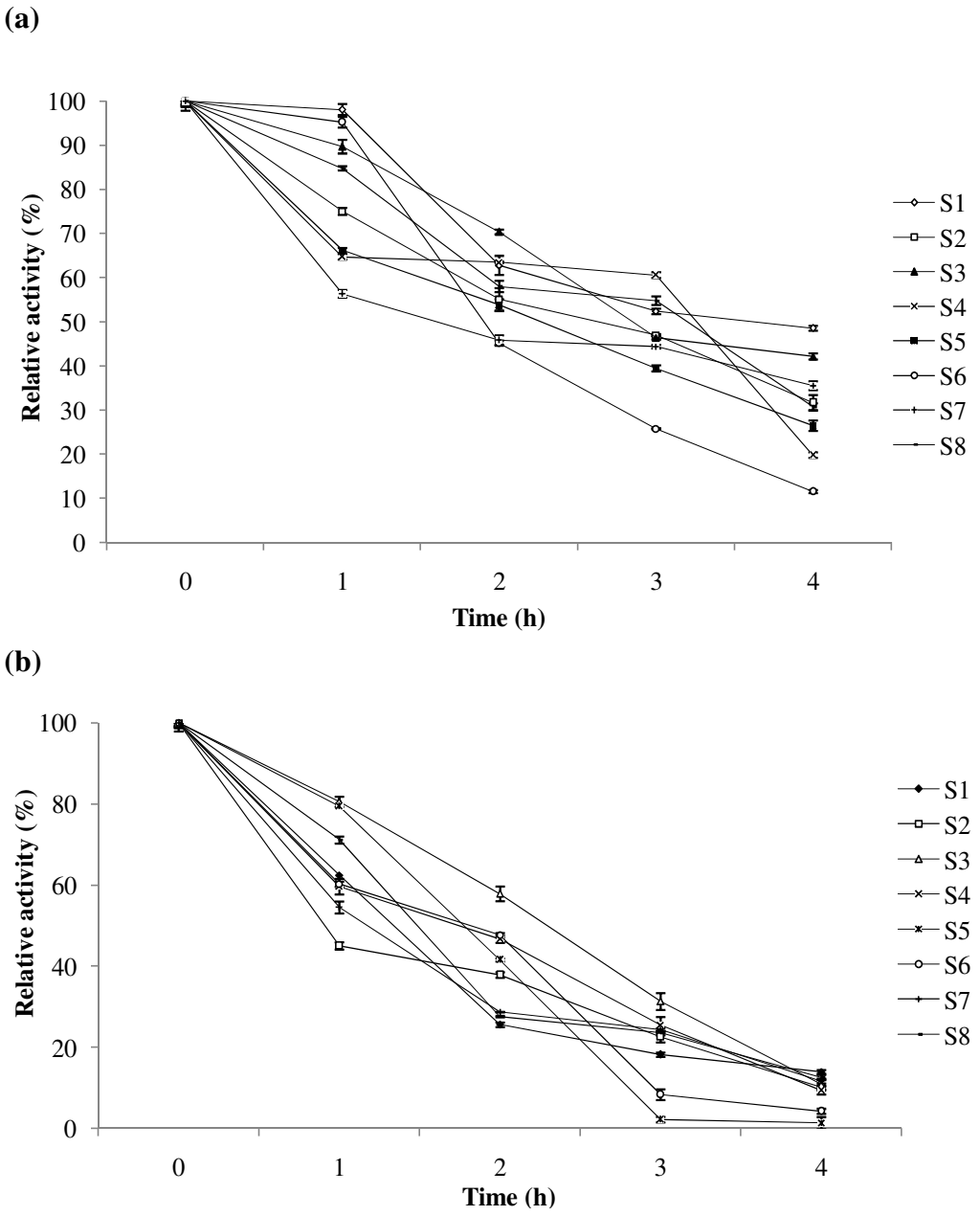

Figure 4. The thermostability of crude xylanase at (a) 70°C and (b) 90°C using sawdust as the substrate.

xylanases, and that the best xylanolytic activity was obtained using birchwood xylan (7mg/ml), yeast extract (2.5 mg/ml), NaCl (3 mg/ml), NH_4Cl (6 mg/ml), and $MgSO_4$ (0.3 mg/ml). Bacteria belonging to the genus *Bacillus* have been widely used for the production of hemicellulase and xylanases (Coughlan and Hazlewood, 1993; Pinaga et al., 1993), with variation in the production efficiency and thermostability of the enzymes produced (Ramesh and Lonsame, 1989; Asther and Meunier, 1990).

Pham et al. (1999) observed that up to 24 nkat/ml of xylanase was produced on lignocellulosic wastes within 36 and 42 h at 30°C using *Bacillus polymyxa*. This is much lower than the highest titre produced by isolates S2 (36.528 nkat/ml), S3 (37.074 nkat/ml), S4 (39.482 nkat/ml), S6 (33.075 nkat/ml), and S8 (45.206 nkat/ml) on sawdust in this study. Similarly, a high level of xylanase production by *Bacillus* sp. AR-009 has been observed when wheat bran was used as a solid support; with maximum enzyme production observed after 72 h (Gessesse and Mamo, 1998). Further incubation after this time did not show any improvement in the level of enzyme production, which is comparable to the findings of the present study. However, higher xylanase production of up to 1455 U xylanase/g deoiled seed cake

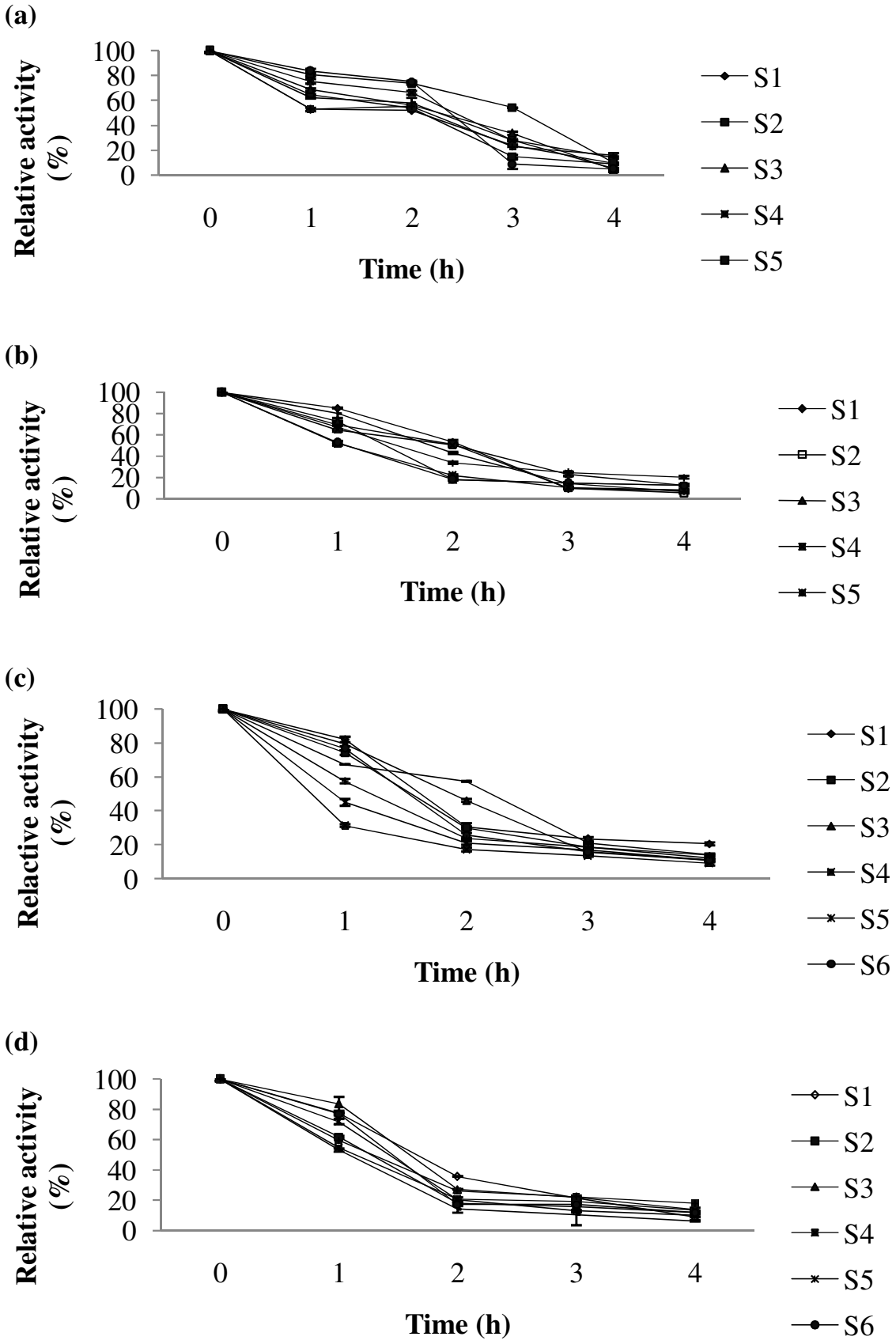

Figure 5. The stability of crude xylanases at (a) pH 6 (b) pH 8 (c) pH 10 and (d) pH 12 using sawdust as the substrate.

Table 1. Effects of metal salts or additives on xylanase activity.

Isolates	Xylanase activity (nkat/ml)						
	None	$MgCl_2$	$HgCl_2$	EDTA	NaCl	$CaCl_2$	Urea
S1	30.197±1.307 (100)	6.256±0.849 (22.186)	3.491±0.797 (11.560)	7.888±1.178 (26.122)	6.301±1.846 (20.866)	18.995±2.993 (62.904)	6.437±0.685 (21.317)
S2	33.256±2.159 (100)	14.779±0.490 (44.440)	4.035±4.215 (12.133)	2.312±0.283 (6.952)	6.528±0.471 (19.629)	10.381±2.468 (31.215)	7.117±2.117 (21.401)
S3	35.091±1.498 (100)	4.715±1.919 (13.436)	10.291±2.277 (29.327)	4.035±0.566 (11.499)	4.352±2.404 (12.402)	12.784±2.247 (36.431)	5.077±1.108 (14.468)
S4	39.536±0.906 (100)	7.344±1.173 (18.575)	3.627±1.836 (9.174)	2.131±1.157 (5.421)	6.891±0.965 (17.430)	10.109±1.516 (25.569)	7.117±2.452 (20.028)
S5	32.749±1.157 (100)	22.349±0.157 (68.243)	10.200±2.211 (31.146)	9.701±2.589 (29.622)	14.235±0.192 (43.467)	21.397±2.867 (65.336)	15.867±3.012 (48.450)
S6	36.099±1.886 (100)	20.717±2.631 (57.389)	4.669±1.439 (12.934)	9.157±1.529 (25.366)	5.712±2.879 (15.823)	29.875±2.211 (82.759)	17.816±0.593 (49.353)
S7	32.976±1.539 (100)	5.485±1.662 (16.633)	4.941±2.186 (14.984)	6.075±0.749 (18.422)	2.765±0.136 (8.385)	15.051±1.935 (45.642)	5.848±1.021 (17.734)
S8	43.744±1.778 (100)	23.936±2.345 (54.718)	9.429±2.406 (21.554)	5.259±0.757 (12.022)	20.173±4.491 (46.116)	25.296±1.533 (57.827)	11.243±1.623 (25.701)

Results are average of triplicate data ± standard deviation while values in parenthesis represent the percentage relative activity.

has been reported for the thermophilic fungus *Scytalidium thermophilum* by solid-state fermentation when *Jatropha curcas* seed cake was used as a substrate under optimized conditions (Joshi and Khare, 2010).

Temperature plays a crucial role in enzyme production and activity. The temperature and the pH optima of the bacterial or fungal enzymes often reflect the pH and temperature of its habitat. The highest enzyme production was observed between 45 and 55°C (Figure 2a), while the highest enzyme activity was obtained at 50°C

(Figure 3a), which was slightly lower than 60°C reported by Sá-Pereira et al. (2002) and Liu and Liu (2008). Hidayah et al. (2008) showed that endoglucanase from *B. pumilus* EB3 was maximally secreted at 37°C at the initial pH of 7 which is in contrast to the observation of this study. A decrease in activity was observed at high temperatures of 60 and 70°C. Enzymes have temperature optima for maximum activity, and a rise in temperature above this optimum could results in disruption of enzyme structure and subsequent loss in activity (Prescott et al., 1996). Bataillon et al. (2000) showed that xylanase from *Bacillus* sp were most active at 75°C and also stable at pH of 7 to 9, which is comparable to those of the current study as the optimum pH for xylanase production was at pH 8.

A further increase in enzyme production at pH

12 (Figure 2b) and activity at pH 10 (Figure 3b) could be attributed to the production of multiple xylanases, which has also been previously reported (Wong et al., 1988; Gessesse, 1998; Cacais et al., 2001). Battan et al. (2007) reported a peak xylanase production by *Bacillus pumulis* when grown in medium at pH 8.0. This is similar to the findings of this study since a pH of 8 was found to be optimum for xylanase production. The inhibition in xylanase activities upon the addition of the metal salts or additives observed in this study could be attributed to the fact that proteins unfold in certain solvents, which disrupt its non-covalent interactions thus reducing its activity (Archana and Satyanarayana 1997; Podar and Reysenbach, 2006; Turner et al., 2007). A pH of 8 was found to be optimum for the activity of xylanase obtained from an alkalophilic *Bacillus sp*. Sam-3 (Shah et al., 1999). Pham et al. (1998) showed that the two xylanases produced by *Bacillus polymyxa* were not stable at 60°C even after 15 min. Also, only 15 to 20% of residual activity of the crude xylanase from a *Bacillus* sp. was left at 60°C (Yang et al., 1995). This is in contrast to the present study because the crude enzyme extract from the organism retain up to 70% of its activity after 2 h.

Shah et al. (1999) reported retention of approximately 75% xylanase at 60°C at the end of 2 h of incubation, while the enzyme was found unstable at 70°C, with only 4% of the original activity retained after 45 min. This is in contrast to the current study where the enzyme retained 56 to 98% of its activity after one hour at 70°C (Figure 4a). Similarly, Roy and Belaluddin (2004) showed that alkaline xylanases from *Bacillus* sp. isolated from an alkaline soda lake were stable at the temperature of 40 to 80°C, with 60% loss in activity observed at 80% which is similar to the findings of this study.

In conclusion, for commercial production of xylanase enzymes, the focus should be on the utilization of agro-residual wastes along with development of efficient bioprocess strategies to obtain high enzyme titer (Biswas et al., 2010). The alternate substrates investigated in this study demonstrate a good potential for production of high titre of xylanase by the *Bacillus* strains. The enzyme also demonstrated high stability at a wide range of both alkaline conditions and high temperatures, thus making it a good candidate for use in Kraft pulp treatment. This could allow for direct enzymatic treatment of the alkaline pulp and avoids the cost incurring and time consuming steps of pH re-adjustment. Although sawdust differs in composition with respect to fibre structure and chemical composition, for example, contents of lignin, hemicelluloses, pectin and extractives, the present work has shown that sawdust could be considered as a promising material for enzyme production. Overall, the use of sawdust and digestive bran could provide a cheaper alternative for the large-scale production of xylanases, for various industrial applications, compared to oat-spelt and birchwood xylan currently being used in small scale experiments.

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