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Full Length Research Paper

Production and some properties of the thermostable feruloyl esterase and xylanase from *Bacillus pumilus*

Yemin Xue[1]*, Ruili Wang[2], Jingjing Zhang[2], Chunying Xu[2] and Huihui Sun[2]

[1]Department of Food Science and Nutrition, GinLing College, Nanjing Normal University, Nanjing, PR China 210097, China.
[2]Department of microbiology, College of Life Science, Nanjing Normal University, Nanjing, PR China 210046, China.

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This paper reports the enhanced production of extracellular thermostable feruloyl esterase and xylanase from *Bacillus pumilus* by optimization of inducing carbon sources. Batch studies were evaluated by varying the ratio of xylan and wheat bran on the medium for enzyme production by the bacterial culture. The feruloyl esterase and xylanase production were highest in culture filtrates from *B. pumilus* grown in media containing 1% xylan and 3% wheat bran, which were enhanced 2.4-fold and 3.6-fold compare to single xylan medium, respectively. The crude feruloyl esterase and xylanase were thermostable with the maximum activities at 50 and 55°C, and pH 5.8 and 7.4, respectively. Activity at temperatures of 40 to 45°C was high and stable, suggesting that these enzymes have a potential application in obtaining ferulic acid from agro-industrial waste materials in this moderate temperature range.

Key words: *Bacillus pumilus*, feruloyl esterase, xylanase, production, partial characterization.

INTRODUCTION

Xylans, the major components of hemicellulose fractions of agricultural residues, are the second most abundant renewable polysaccharide. Complete degradation of xylan requires the action of xylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, and feruloyl esterase. Among those enzymes, feruloyl esterases (EC 3.1.1.73., FAE), that are able to hydrolyze the ester bonds linking ferulic acid to L-arabinofuranose-containing polysaccharides, such as L-arabino-D-xylans and L-arabinans. Previous studies demonstrated that complete release of ferulic acid requires synergistic action of feruloyl esterases and β-(1,4)-endoxylanases from plant cell wall (Topakas et al., 2007; Nagar et al., 2010; Faulds et al., 1995; Yu et al., 2002; Huang et al., 2011). The main product of enzyme hydrolysis, ferulic acid (4-hydroxy-3-methoxy-cinnamic acid), is known to be an antioxidant (Graf et al., 1992; Levasseur et al., 2005), which has been found to possess some activity toward peroxynitrite (Pannala et al., 1998) and oxidized low-density lipoprotein *in vitro* (Schroeter et al., 2000). Although, ferulic acid producation has been previously carried out via chemical, physical, and biological methods, microbial feruloyl esterases have become important materials with considerable roles in biotechnological processes for many industrial and medicinal applications (Huang et al., 2011). Thus, discovery of new FAEs with novel properties continues to be an active research sector. *Bacillus pumilus* has been reported to produce a number of highly xylan degrading enzymes for example, xylanase, β-xylosidase, and α-L-arabinofuranosidase (Pei et al., 2008; Qu et al., 2010). However, there exists no report on *B. pumilus* feruloyl esterase.

Pioneering studies suggested that the feruloyl ester bond is necessary to induce FAE yield. Among agricultural by-products, wheat brans are cheap and easily available agricultural by-products according to its high amounts of ferulic acid in the cell wall of approximately 1% (wt/wt) (Topakas et al., 2007; Nagar et al., 2010; Faulds et al., 1995; Yu et al., 2002). Thus, xylan and wheat bran were used as inducing carbon source for esterase and xylanase production by *Bacillus pumilus* in this study. We report here the utilization of agricultural by-

*Corresponding author. E-mail: xueyemin@njnu.edu.cn. Tel: +86-25-85794123. Fax: +86-25-83598901.

products for increased production of feruloyl esterase and xylanase and some properties of the crude feruloyl esterase and xylanase.

MATERIALS AND METHODS

Microorganisms and culture conditions

A bacterial culture of *B. pumilus* (Pei et al., 2008) was used in this study. It was maintained on xylan agar slants containing (g/L) xylan 5.0 [Oat spelt xylan, Sigma Chemicals Co.], peptone 4.0, yeast extract 2.0, K_2HPO_4 3.0, NaCl 2.0 and agar 20.0 (pH 7.0) at 4°C. This medium without agar and inducing carbon resource was designated as basal medium.

Shake-flask cultivation was performed in 300 ml flask containing 50 ml medium without agar. The bacterium cell was grown at 45°C on a rotary shaker (200 rpm). At the end of cultivation the culture broth was centrifuged to clear supernatant which was used for extracellular enzyme characterization studies. The resulting pellets were resuspended in 20 ml of 20 mM Tris/HCl buffer (pH 7.9), and sonicated for 45 s three times. The cell extracts were cooled in an ice bath, and centrifuged (9600×g, 4°C, 30 min), the resulting supernatant was used as intracellular enzyme for all assays.

Effect of medium carbon source level on enzyme production

The influence of carbon source concentration on the enzyme production was evaluated by varying the ratio of xylan and the de-starch wheat bran (2 + 0%, 0 + 3%, 1 + 2%, 1 + 3%, 2 + 2%, 2 + 3%, w/v). The bacterial strain was cultivated as above mentioned basal medium with different inducing carbon source at 45°C for 48 h. The culture broth was centrifuged, and the resulting supernatant was determined for enzyme activity. The level of basal medium was maintained constant in all experiments. Each culture condition was performed in duplicate.

Enzyme assays and analytical methods

Unless otherwise mentioned, all enzyme activities were determined at 50°C. Wheat bran was provided by ARDC (Agro-Industries Development Corporation, Nanjing, China). They were destarched by using Amylase (Novoxymes, WuXi, China), and subjected to heat treatment at 121°C for 30 min, and washed three times with water and air-dried, the resulting destarched wheat bran (DSWB) was milled to a fine powder of a particle size less than 5 mm prior to use as substrate for the determination of FAE activities.

Esterase activity was assayed by measuring the amount of ferulic acid released from 3% (wt/vol) DSWB. The released ferulic acids were determined spectrophotometrically at 320 nm (Rybka et al., 1993). The enzyme was incubated with a DSWB solution (3% [wt/vol] DSWB, 50 mM citrate-phosphate buffer (CPB, pH 5.5) at 60°C for 20 min. The reaction was terminated by boiling for 3 min. Ferulic acids were assayed by adding 4 volumes of free water ethanol and removed insoluble pellets by centrifugation, and measuring the absorbance at 320 nm. A standard curve was prepared by using ferulic acid (Sigma). The amount of free acid released was quantified against standard curves. One unit of activity (1 U) is defined as the amount of liter of culture supernatant (or pellet) releasing 1 µmol of free acid per minute under the defined conditions.

Xylanase activity was determined by the 4-hydroxybenzoic acid hydrazide method (Lever et al., 1972). Oat spelt xylan (OSX, Sigma X0627) was used as substrate for enzyme activity. The reaction mixture containing 100 µl 0.5% (w/v) OSX in water, 90 µl 50 mM CPB (pH 6.0) and 10 µl crude enzyme was incubated at 50°C for 10 min. Reducing sugars were assayed by adding 600 µl of 4-hydroxybenzoic acid hydrazide, boiling for 10 min, cooling, and measuring the absorbance at 410 nm. One unit of xylanase activity was defined as the amount of enzyme releasing 1µmol reducing sugar per min. Protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA, sigma) as a standard. All assays were performed by using blanks to correct any backgrounds in enzyme and substrate samples, and were prepared and analyzed in duplicate, with <10% standard error for each set of results.

Effect of temperature and pH on esterase and xylanase activities

To determine optimal temperature under the conditions used, samples were incubated at various temperatures (30 to 70°C) for different time periods, and esterase and xylanase activities were assayed. Optimal pH was determined by using CPB (pH 4.5 to 8.0) and barbitone buffer (BB, 8.0-9.0) using standard conditions (See, Materials and methods section 2.3). The temperature thermostability was tested in the range of 30 to 50°C. Aliquots were preincubated at the designated temperature and after cooling at 0°C, residual esterase and xylanase activities were then assayed as previously indicated the materials and methods. The pH stability was determined by incubating the enzyme solution in CPB (pH 5.5 to 8.0) and BB (8.0-9.0). All incubations were performed at 50°C for 60 min, and then the remaining activities were assayed at optimum conditions. The activity determined prior to the preincubation was taken as 100%.

RESULTS AND DISCUSSION

Enzyme production using agro-industrial residues and effect of the ratio of xylan and wheat bran

The effect of the ratio of xylan and wheat bran on the medium for enzyme production is shown in Figure 1. Among all the tested ratio of xylan and wheat bran, the highest feruloyl esterase (34.8 U L^{-1}) and□β□ xylanase activity (3300 U L^{-1}) were produced on 1% xylan and 3% wheat bran media, which were enhanced 2.4-fold and 3.6-fold compare to single xylan media, respectively. At this point, decrease of 1% wheat bran or increase of 1% xylan resulted in the reduction of 36% of maximum for xylanase activity, and the corresponding amounts of feruloyl esterase appeared a slight change. The results showed that the change of the ratio of xylan and wheat bran on the medium obviously influenced enzyme production more for xylanase than that for feruloyl esterase, and monotonous carbon sources consisting of only xylan (without esterified FA) or wheat bran (lack of xylan) decreased the production of feruloyl esterase and xylanase. This study demonstrated that appropriate ratio of xylan and wheat bran on the medium was of great importance for the production of xylanase and feruloyl esterase because these substrates not only serves as a source of carbon and energy for the cell growth, but also provides the necessary inducing compounds for the enzyme production. Previous studies showed that complex carbon sources that contain high amounts of

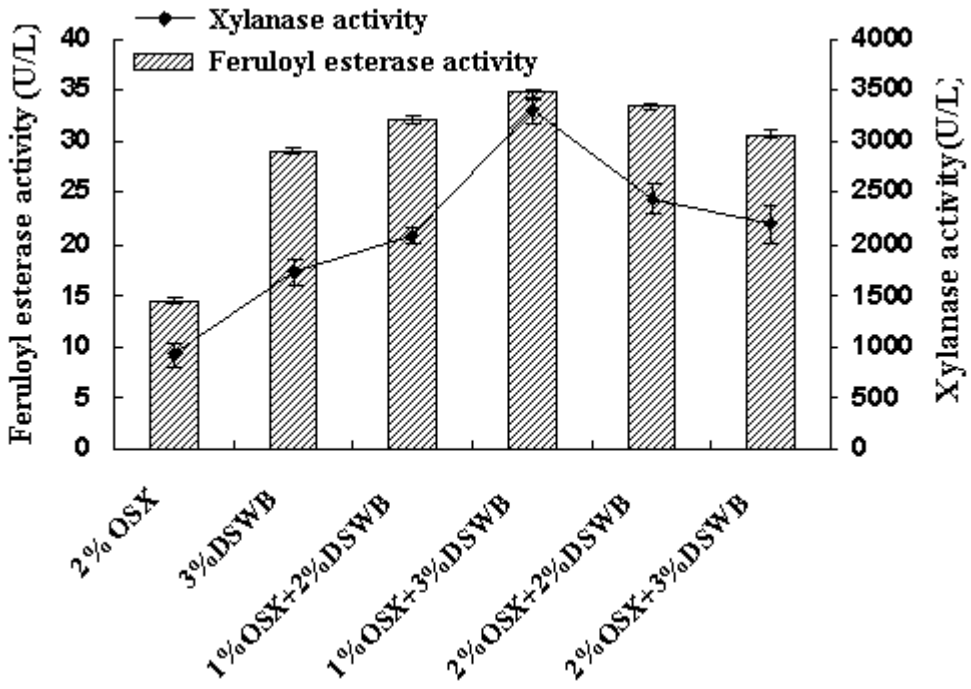

Figure 1. The effect of the ratio of xylan and wheat bran on the medium for enzyme production. Fold line represented xylanase activity, and histogram represented feruloyl esterase activity.

esterified FA, induces high levels of mainly feruloyl esterase, when purified xylans were used as substrates, low levels of feruloyl esterase production were observed, where the level of FAE production from *Schizophyllum commune*, *Aspergillus niger* and *Streptomyces avermitilis* CECT 3339 increased by 2.5, 1.7 and 0.3-fold compared to oat spelt xylan, respectively (Faulds et al., 1997; MacKenzie and Bilous, 1988; Garcia et al, 1998).

Time course of enzyme production on the medium containing optimized ratio of xylan and wheat bran

Bacillus pumilus was grown on the medium containing 1% xylan and 3% wheat bran. The time course of growth, feruloyl esterase and xylanase production from extracellular and intracellular are shown in Figure 2. The cell density increased rapidly during the initial 20 h of cultivation, reaching a peak at 27 h, after this point it declined slightly (Figure 2a). The extracellular enzyme activities increased very rapidly up to 20 h, after which feruloyl esterase continued to increase more slowly than xylanase up to 27 h. Although cell biomass declined slightly after 27 h, the extracellular enzyme activities continued to increase reaching maximum values at which feruloyl esterase and xylanase activity were 50.8 U L^{-1} and 2128 U L^{-1}, respectively (Figure 2b). The results show that the synthesis of enzyme activity was closely associated with the cell growth and continued into stabilization state of growth. In contrast to extracellular enzyme, the intracellular enzyme activity almost was not detected for feruloyl esterase and very low for xylanase, indicating that feruloyl esterase and xylanase from *B. pumilus* were the inducible extracellular enzymes, which are similar to Gram-positive bacteria reported previously, where some *B. subtilis* strains exhibited the crude feruloyl esterase activity (with methyl ferulate as the substrate) from cells from 1.29 to 34.14 mU mg $protein^{-1}$ in methyl-ferulate-containing liquid medium (Donaghy et al., 1998).

pH and temperature optima of feruloyl esterase and xylanase

The optimal pH and temperature of the crude feruloyl esterase and xylanase are shown in Figure 3. The optimal pH for the feruloyl esterase and xylanase were observed at pH 5.8 and 7.4, respectively; at pH 5.4 and 8.2, feruloyl esterase retained 78.7 and 69.6% of its maximum activity, respectively while xylanase retained 28.8 and 33.3% activity at the respective pH. Feruloyl esterase showed wider curves on the acidic and basic side, and xylanase showed the pH-activity curves narrowed on the basic side (Figure 3a). The optimal pH of the xylanase from *B. pumilus* which we reported herein (pH7.4) was higher than those of the recombinant xylanase of this organism (pH 6.6) (Qu et al., 2010) and *B. pumilus* SV-85S xylanase (pH 6.0) (Nagar et al., 2010).

The effect of temperature on the enzyme activity was

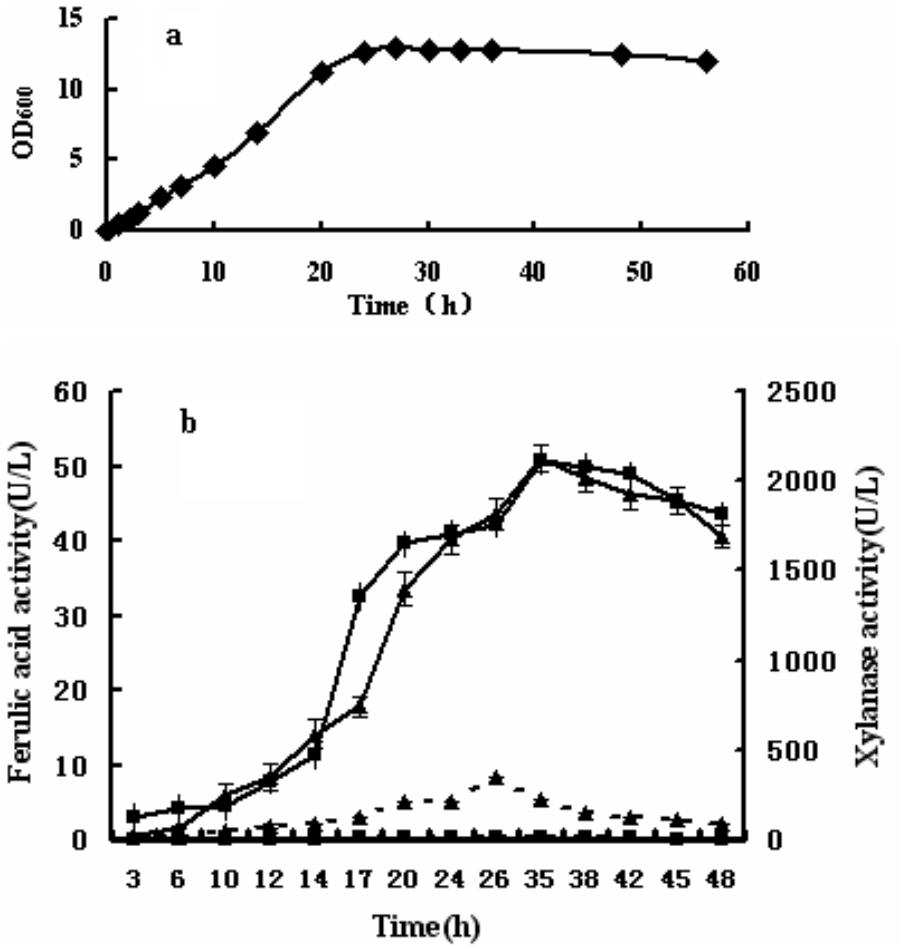

Figure 2. The time course of growth (A), and extra (—) and intracellular (…) enzymes of feruloyl esterase (■) and xylanase (▲) of the production (B) by *Bacillus pumilus,* grown on the optimized medium at 45°C.

assessed (Figure 3b). The feruloyl esterase activity was optimally active at 45 to 50°C (pH 5.8) with a retaining of 69.3% at 55°C and about half of maximum at 40°C. These results are the same to those obtained with most other FAEs, whose optimum temperatures were around 40 to 55°C. The activities of the feruloyl esterases from *Streptomyces avermitilis*, *Clostridium thermocellum*, *Fusarium oxysporum*, and *Lactobacillus acidophilus* (Garcia et al., 1998; Blum et al., 2000; Topakas et al., 2003; Wang et al., 2004) were maximal at 50°C (pH 6.0), 55°C (pH 5.0), at 45 (55)°C (pH 7.0), and at 37°C (pH 5.6), respectively. The crude xylanase optimum temperature was 55°C, which was 5°C higher than those of the recombinant xylanase of *B. pumilus* (Qu et al., 2010) and *B. pumilus* SV-85S xylanase that exhibited activity optimum temperature at 50°C (Nagar et al., 2010).

pH and thermal stability

The stability of the crude enzymes at different pH is shown in Figure 3c. The pH stability was found to lie in the range from pH 5.8 to 8.2 for the feruloyl esterase, and pH 6.2 to 8.2 for xylanase, respectively. The feruloyl esterase displayed a pH value at 5.8 resulting in a drastic increase in stability.

Correlations between heat stability and enzyme activity for the crude enzymes are shown in Figure 4. At 45°C and 50°C for 30 min, the feruloyl esterase retained 91.3% and 40.5%, respectively a slight increase in activity at 45°C for 15 min and 50°C for 5 min were observed (Figure 4a). The *B. pumilus* feruloyl esterase is highly stable at 45°C compared to the previous study, in which the feruloyl esterase from the intestinal bacterium *Lactobacillus acidophilus* lost as much as 68% of the activity at 45°C for 5 min (Wang et al., 2004). The crude xylanase retained 77.8% at 45°C and 34.8% at 50°C for 30 min, had half lives of over 20 min at 55°C (Figure 4b), its temperature stability was lower than those obtained for the recombinant xylanase of this organism previously (Qu et al., 2010). Possible reasons are that the thermal stability of this recombinant xylanase was affected by fusing his-tag

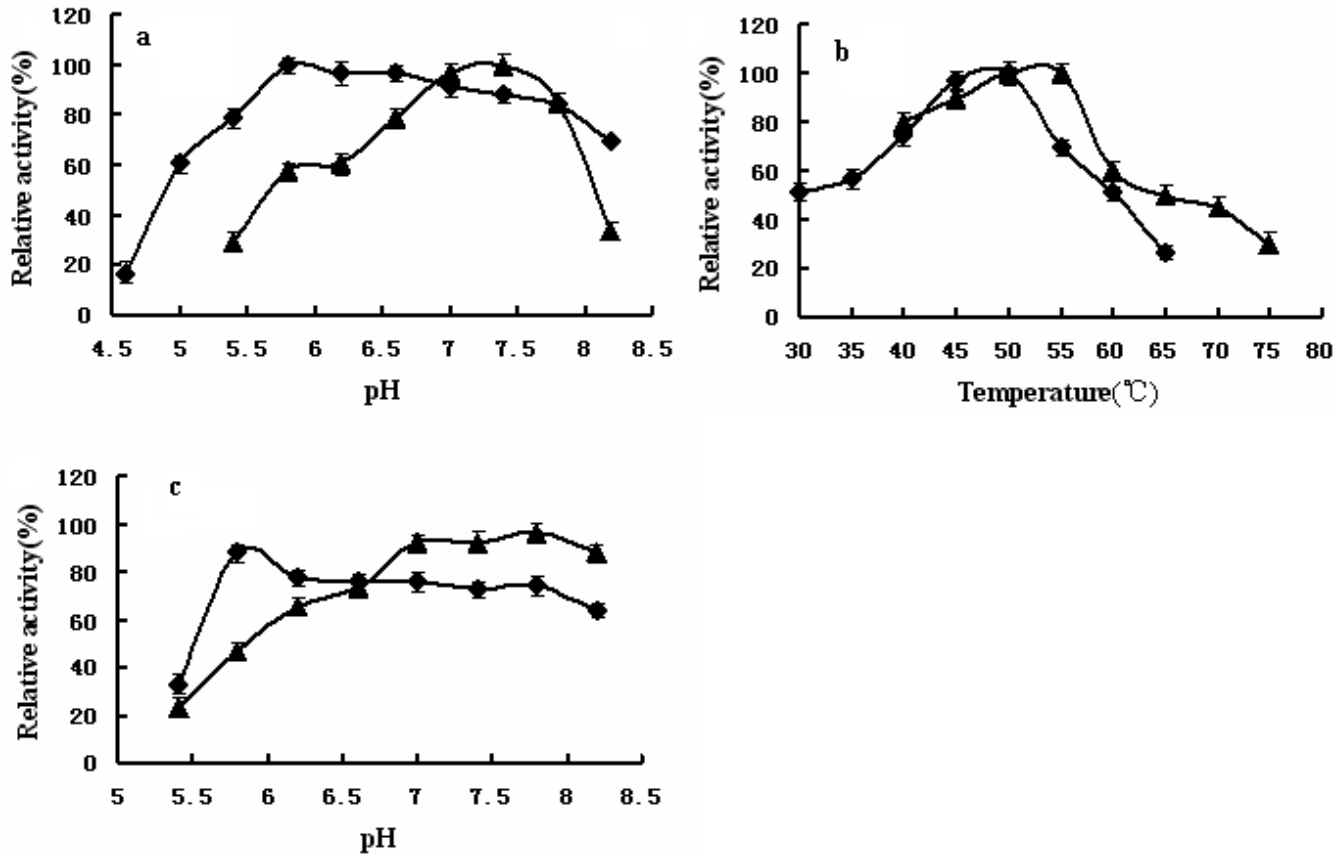

Figure 3. Effect of temperature (a) and pH (b) on the activity feruloyl esterase (◆) and xylanase (▲) from *Bacillus pumilus* and their pH stability profiles (c).

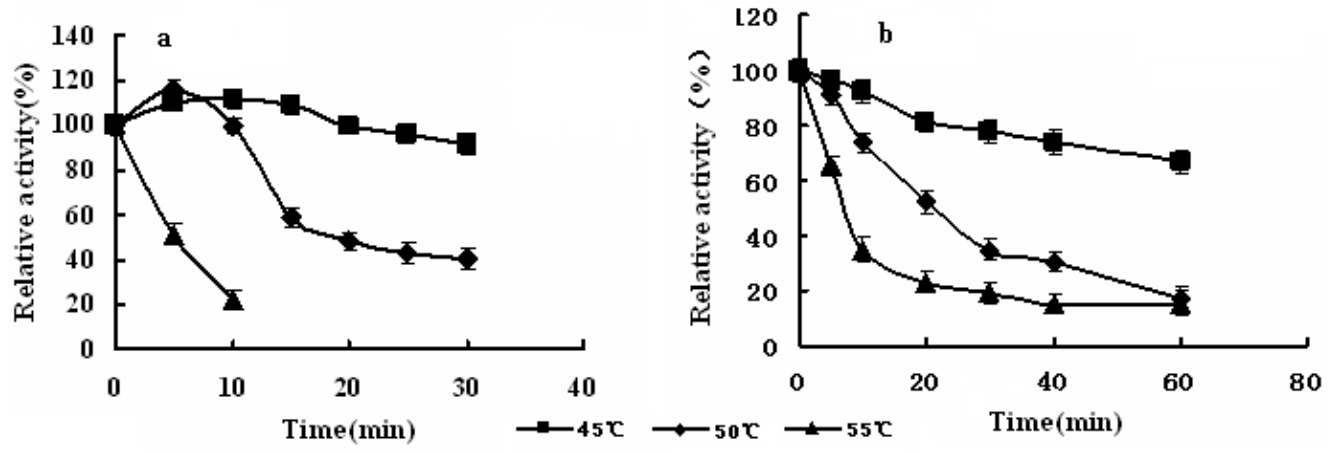

Figure 4. Effect of temperature on the stability feruloyl esterase (a) and xylanase (b) from *Bacillus pumilus*.

(added six extra histidines at the amino-terminal of the enzyme) (Xue et al., 2009).

Complete release of ferulic acid requires synergistic action of feruloyl esterases and β-(1, 4)-xylanases from plant cell wall. Optimization of the ratio of xylan and wheat bran on the medium for the cultivation of *B. pumilus* led to the utilization of agricultural by-products for increasing the production of feruloyl esterase and xylanase. The yields of the two enzyme and their properties corresponding to the thermostability and pH stability make the *B. pumilus* enzymes attractive for potential application in obtaining FA from agro-industrial waste materials.

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