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ORIGINAL PAPER

Screening of a xylanase high-producing strain and its rapid separation and purification

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Abstract In this research, the xylanase high-producing strain of BE-91 (*Bacillus subtilis*) was selected. The enzyme activity in the fermentation liquor of BE-91 at 8 h reached 408 U/mL, which was 3.4 times higher than that of strain ACCC 10243. The xylanase was purified from BE-91 fermentation liquor with the ultrafiltration and gel chromatography, and its enzyme activity was up to 28,454 U/mg. Its recovery was above 69%, and the purification multiple of the enzyme activity was up to 18 times. The molecular weight of the purified xylanase was 22.54 kDa assayed with SDS-PAGE. The K_m and V_{max} were 0.5 mg/mL and 533 μmol/(min mL), respectively. The stabilizing pH and optimal pH of the xylanase were 4.6~6.4 and 5.8, respectively. And when the pH was 5.8, the stabilizing temperature and optimal temperature of the xylanase were 0°C~65°C and 60°C, respectively. Therefore it was considered that the strain BE-91 could be applied to the industrial production of xylanase.

Keywords Xylanase · High-producing strain · Rapid purification

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Introduction

Xylanases are hydrolytic enzymes which randomly cleave the β-1,4 backbone of the complex plant cell wall polysaccharide xylan (Collins et al. 2005), and which have a wide range of applications in papermaking, food processing, plant fiber extraction and feed additives (Liu 2007). The screening of xylanase-producing strains, and the separation and purification of xylanases, are currently of great interest (Nie et al. 2008).

The microorganisms thathave been reported to produce xylanase are mainly filamentous fungi such as *Trichoderma* (Breccia et al. 1998; Ratanakhanokchai et al. 1999), *Aspergillus* (Jiang et al. 1995; Kasuhiko and Nevins 1991; Kimura et al. 2000; Li et al. 2005; Biely 1993), *Penicillium* (Sunna and Antranikian 1997), and *Streptomyces* (Li et al. 2003; Shareck et al. 1991). As was reported by Bai et al. (2004), the activity of xylanase produced by fungi was higher than that produced by bacteria. Most studies of xylanase production by bacteria have concentrated on the *Bacillus* strains (Bernier et al. 1983; Honda et al. 1985; Khasin et al. 1993; Ratanakhanokchai et al. 1999; Tachaapaikoon et al. 2006; Yuan et al. 2005). However, these strains did not have a strong ability to produce xylanase. Mamo et al. (2006) reported that the activity of a thermostable alkaline active endo-1-4-xylanase from *Bacillus halodurans* S7 was 254 U/mL. So far, no natural bacteria have been reported to produce xylanase with more activity than this strain.

Research on methods of separating and purifying xylanase have mainly included ammonium sulfate precipitation (Julio et al. 2006), ion exchange chromatography, preparative paper chromatography (PPC) (Lequart et al. 1999), gel filtration chromatography, DEAE-cellulose chromatography (Gessesse 1998; Bim and Franco 2000), and hydrophobic interaction

chromatography (Breccia et al. 1998). Almost all these methods have problems such as a complicated extraction process, relatively strict purifying conditions and, moreover, relatively large loss of enzyme activity of about 50%.

This work reports a xylanase high-producing strain BE-91, and its rapid separation and purification by ultrafiltration and gel chromatography, which could contribute to the exploitation of xylanase.

Materials and methods

Strains

A total of 309 test bacteria were randomly selected from 3,150 natural bacterial samples collected by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences. The type strain *Bacillus subtilis* subsp. *subtilis* (Ehrenberg 1835) Cohn 1872, numbered ACCC 10243, was from the Agricultural Culture Collection of China (DSM 10).

Media

The medium, placed in Petri dishes, was composed of xylan 1.0%, peptone 0.5% and agar 1.8%. The seed medium was composed of glucose 1.0%, peptone 0.5%, yeast extract 0.2% and NaCl 0.5%. The fermentation medium was composed of xylan 0.5%, glucose 0.5%, peptone 0.5%, yeast extract 0.2% and NaCl 0.5%.

Prescreening

The sample strains were enriched at 30°C for 12 h in the medium: xylan 1.0%, peptone 0.5%, yeast extract 0.2% and NaCl 0.5%. The suspension, after serial dilutions, was spread onto the Petri dish with the following medium composition: xylan 1.0%, peptone 0.5% and agar 1.8%. The Petri dishes were incubated at 30°C for 24 h. The strains with large hydrolytic halos were chosen.

Table 1 The enzyme activity of the 11 bacteria of xylan degradation ability

Strain	Enzyme activity (U/mL)	Strain	Enzyme activity (U/mL)	Strain	Enzyme activity (U/mL)
T85-1	18±0.5797	K43	9±0.6212	BE-91	408±2.6928
T85-178	15±0.9401	T66	17±0.7133	CXJZ11-02	21±0.36
T85-191	12±0.6189	S13	3±0.5797	CXJZ11-03	26±0.6155
K42	8±0.6178	R26	5±0.6189	ACCC 10243	120±1.2732

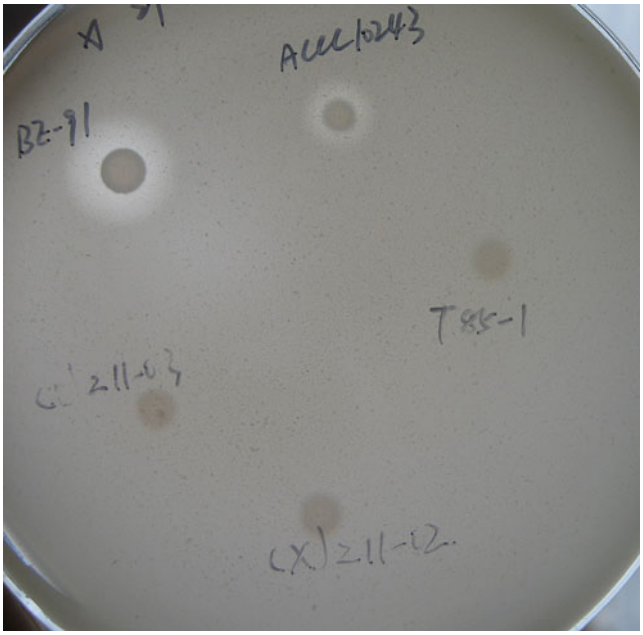

Fig. 1 Hydrolyzed circles of four different bacteria (BE-91, CXJZ11-03, CXJZ11-02 and T85-1) with relatively higher enzyme activities inoculated into selective Petri dishes of xylanase

Re-screening

The above selected strains were cultured in the seed medium and the fermentation medium, respectively, at 30°C for 6 h with a rotation rate of 150 rpm, and the suspensions, after serial dilution, were spread onto the Petri dishes which were incubated at 30°C for 24 h. The strains with large hydrolytic halos were the re-screened ones.

Separation, purification and detection of xylanase

The fermentation broth was successively fractionated by 50 kDa and 5 kDa membrane packages (Sartorius, Germany). The filtered solution was purified by a Sephadex G100 gel column chromatograph. Protein concentration was determined by the Coomassie brilliant blue method (Li et al. 1999). Xylanase

Table 2 Result of ultrafiltrate for protein and xylanase of BE-91 fermentation liquor

Fermentation liquor	Filtered volume (mL)	Total xylanase (U)	Ratio of xylanase (%)	Total protein (μg)	Ratio of protein (%)
Starting fermentation liquor	1,990	700,561	100	60,765	100
Ultrafiltrated liquor over 50 kDa	61	25,616	3.66	36,974	60.84
Ultrafiltrated liquor between 5 and 50 kDa	97	591,825	84.48	7,779	12.80
Ultrafiltrated liquor under 5 kDa	2,150	64,972	9.27	5,853	9.63

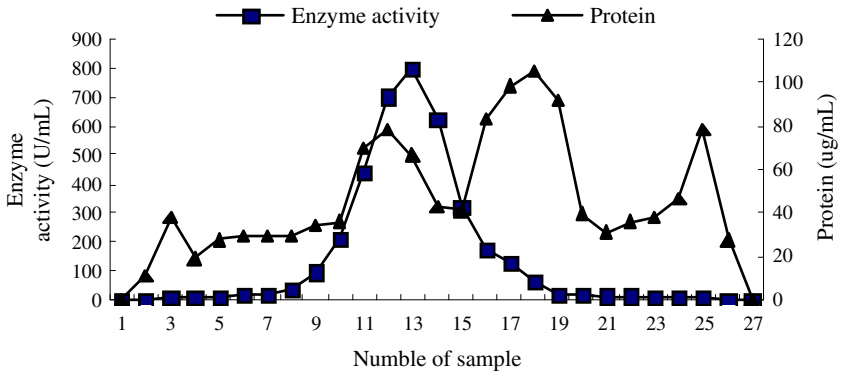

Fig. 2 Result of Gel-charomatography for protein and xylanase of the 5- to 50-kDa ultrafiltration sample

activity was determined by the DNS method buffered with citric acid–sodium citrate, pH 5.8, 60°C, and xylan concentration 0.8% as the substrate. The enzyme activity was defined as the amount of the enzyme required for 1 μmol xylose to be released from the degradation of a suitable concentration of xylan solution per minute. The buffer for purification was pH 5.8, 0.05 mol/L citric acid–sodium citrate solution (unless otherwise stated). Elution was carried out with the buffer for chromatography at the rate of 0.4 mL/min, and collected at the speed of 8 min/tube. SDS-PAGE was adopted for isolation of enzymatic protein components and molecular weight detection. Purification efficiency was calculated in accordance with conventional methods (Li et al. 1999).

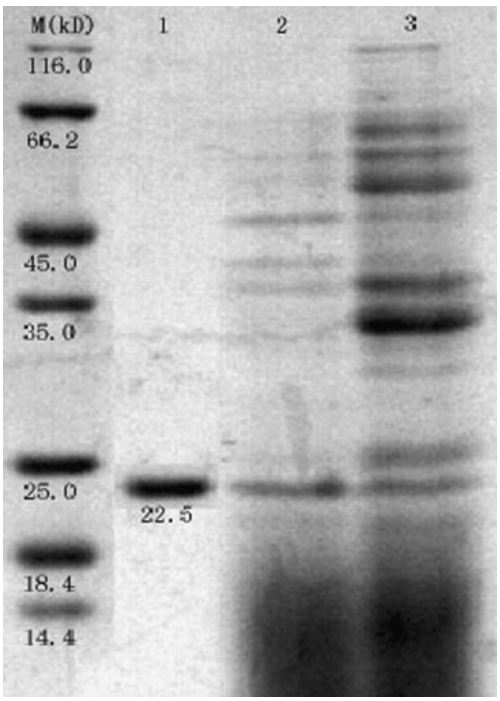

Fig. 3 The electrophoretic protein bands of the fermentation liquor, 5- to 50-kDa ultrafiltration diffluence liquor. *M* protein marker; *1* purified xylanase; *2* 5- to 50-kDa trapping solution; *3* fermentation liquid

Reaction temperature of xylanase

The enzyme activity was detected by DNS method (see above). The test conditions for the optimal enzymatic reaction temperature: pH 5.8, citric acid–sodium citrate buffer, temperature range 40°C~70°C. The temperature for thermal stability study was ranged from 0°C to 70°C, and the retaining time was 30 min.

Reaction pH value of xylanase

The reaction pH value of the enzyme was detected by the DNS method (see above). The conditions for the reaction: pH 3.5~7.5, citric acid–sodium citrate buffer, 60°C. The purefied enzyme was storaged at 4°C overnight.

The impact of metal ions on the enzymatic activity

The solutions of different metal ion (Co^{2+}, Mg^{2+}, Na^{+}, Fe^{3+}, Zn^{2+}, NH^{4+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, K^{+}) were mixed with equal quantities of enzyme, and the final concentration of metal ion was 1 mmol/L. The residual enzyme activity was measured to determine the impact of the metal ions on the enzymatic activity.

The determination of enzyme kinetic constant

The substrates of birch xylan and oat xylan with concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL were prepared with citric acid–sodium citrate buffer solution to determine the xylanase activity, and the K_m and V_{max} of the enzyme under different substrates were assayed with the method of Lineweaver–Burk plot.

The determination of molecular weight

Sephadex G-100 gel chromatography and SDS-PAGE method were adopted.

Table 3 The purification results by xylanase by ultrafiltration and chromatographic methods

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Fermentation liquor	939,580.5	594.63	1,580.1	100.0
Ultrafiltration	762,564.1	46.68	16,337.4	81.2
Gel chromatography	649,921	22.84	28,453.6	69.2

Results and discussion

Screening of the xylanase high-producing strain

After pre-screening, 11 xylanase-producing strains were obtained by comparing the size of the transparent circles. Results of the enzyme activity measurement of the 11 strains are shown in Table 1. From Table 1, it can be seen that *Bacillus subtilis* BE-91 strain (Xu et al. 2009) had the strongest ability to produce enzyme: its enzyme activity was 408 U/mL when fermented under appropriate conditions for 8 h, which was significantly different from other strains. The enzyme activity of the strain BE-91 was 3.4 times higher than that of the *Bacillus subtilis* type strain ACCC 10243. When the fermentation liquors of BE-91, CXJZ11-03, CXJZ11-02 and T85-1 strains with relatively higher enzyme activities were inoculated into selective Petri dishes of xylanase, it was shown by comparing the hydrolysis circles (Fig. 1) that the sizes of the hydrolysis circles of the four strains on the xylanase-screening Petri dishes completely coincided with the detection results of enzyme activity. Thus, BE-91 was determined to be the xylanase high-producing strain. The reported higher xylanase activity from fermentation liquor of *Bacillus subtilis* B10 and *Paenibacillus* sp. were 328.2 and 194.67 U/mL, respectively (Huang et al. 2006; Bao et al. 2008), and the reported xylanase activity from natural or genetically engineered bacteria was generally in the range 100~400 U/mL.

Separation, purification and detection of xylanase

The results of xylanase separation of the BE-91 high-producing strain are shown in Table 2. Much of the protein (60.8% of the total) in the fermentation liquor of BE-91 could be cut-off, but a small amount of the xylanase activity was cut-off by the 50-kDa membrane package, and about 10% of the protein and the xylanase were filtered by the 5-kDa membrane package. In contrast, 84.5% of the xylanase activity in the fermentation liquor could be concentrated in the ultrafiltration condensed liquor. This might indicate that the molecular weight of the xylanase was at 5~50 kDa and that the xylanase protein was nonlinear.

The test results of enzyme activity and protein content in the eluent of the 5- to 50-kDa ultrafiltration diffluence liquor through Sephadex G-100 gel column chromatography are shown in Fig. 2. It can be seen from Fig. 2 that four apparent protein peaks and one enzyme peak were separated from the 5- to 50-kDa ultrafiltration diffluence liquor. The protein peak of the eluent samples from the 9th to 18th tubes coincided with the enzyme peak.

From the SDS-PAGE (10% for resolution gel and 3% for stacking gel) test results (Fig. 3) of the fermentation liquor, four apparent electrophoretic protein bands could be seen in the 5- to 50-kDa ultrafiltration diffluence liquor (the same number as the protein components detected by chromatography of the 5- to 50-kDa ultrafiltration diffluence liquor), while only one protein band with a molecular weight of 22.5 kDa calculated on the electrophoretic mobility in the chromatographic samples of the 5- to 50-kDa ultrafiltration diffluence liquor could be found.

An amount of 2,400 mL of the fermentation liquor was taken for ultrafiltration and chromatographic purification in succession, volumes and concentrations of the samples of all levels were detected, and the purification results were calculated. From Table 3, it can be seen that the recovery of xylanase in BE-91 fermentation liquor through ultrafiltration separation and gel chromatography purification reached above 69%. The purification multiple of the enzyme activity was up to 18 times, and the enzyme activity of the purified samples by chromatographic reached 28,453.6 U/mg.

Thus, it can be seen that electrophoretically pure xylanasewas separated and purified from BE-91 fermentation liquor with ultrafiltration and chromatography with very high recovery of the enzyme activity. Compared with

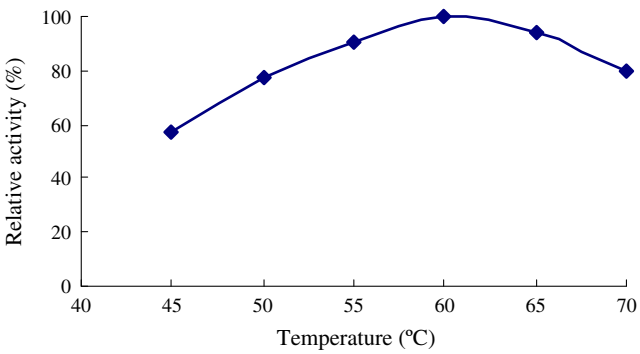

Fig. 4 The optimum temperature curve of xylanase detected by DNS method with the conditions of pH 5.8 in the citric acid–sodium citrate buffer

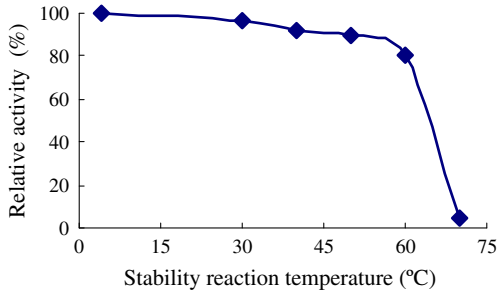

Fig. 5 The thermalstability curve of xylanase detected by DNS method with the conditions of pH 5.8 in the citric acid–sodium citrate buffer

other separation and purification methods reported, the method has advantages such as high efficiency, high speed, and easy operation.

The optimal temperature and thermal stability

The curve of enzyme activity varying with temperature, shown in Figs. 4 and 5, was made with temperature change as the abscissa and relative enzyme activity as the ordinate. It can be seen in Fig. 4 that the enzyme activity increased progressively as the temperature increased in the range of 45°C~55°C and decreased rapidly in the event that the temperature exceeded 60°C, and that the optimal temperature of the xylanase secreted by BE-91 strain was 60°C. It can be seen in Fig. 5 that xylanase was more stable at 0°C~65°C, 91% enzyme activity was retained when it was kept at 40°C for 30 min, and 80.5% enzyme activity was still retained when it was kept at 60°C for 30 min.

The optimal pH and its stability

The curve of the enzyme activity varying with pH increase, shown in Figs. 6 and 7, was made with pH as the abscissa and relative enzyme activity as the ordinate. It can be seen in Fig. 6 that the optimal pH of the xylanase secreted by BE-91 strain was 5.8, and the loss of enzyme activity was nearly 60% when the pH was 7.6. It can be seen in Fig. 7

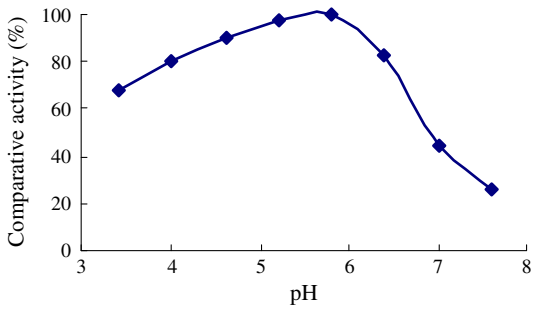

Fig. 6 The optimum pH of xylanase detected by DNS method with the conditions of pH 3.5~7.5 in the citric acid–sodium citrate buffer

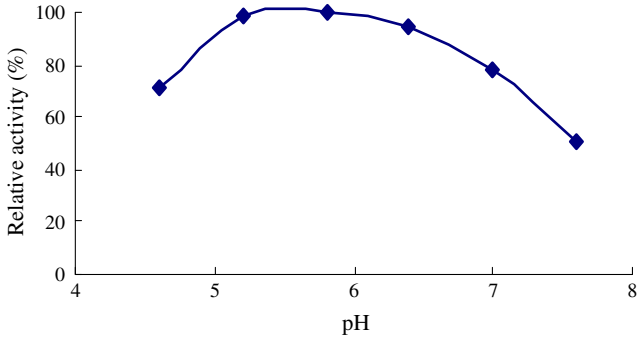

Fig. 7 The stability pH curve of xylanase detected by DNS method with the conditions of pH 4.5~7.5 in the citric acid–sodium citrate buffer

that the xylanase was stable when the pH was 4.6~6.4, and its activity decreased rapidly when the pH was more than 6.4, which showed that the xylanase secreted by the strain only acted as a catalyst in an acidic environment.

Effect of metallic ions on the enzyme activity

The results (Table 4) of the effect of metallic ions on the activity of xylanase secreted by BE-91 revealed that Fe^{2+} and Co^{2+} activated the enzyme activity by 59.21 and 38.69%, respectively, while Cu^{2+} intensively inhibited the enzyme activity by 92.5%.

Kinetic constant

K_m of the xylanase secreted by BE-91 was 0.5 mg/mL and V_{max} was 533 μmol/(min mL) with oat xylan as the substrate; K_m was 1.0 mg/mL and V_{max} was 500 μmol/(min mL) with birch xylan as the substrate. Thus, it canbe seen that the affinity of the enzyme with oat xylan was higher than that with birch xylan.

Conclusion

In this research, the xylanase high-producing strain of BE-91 (*Bacillus subtilis*) was selected from 3,150 bacteria resources collected by Institute of Bast Fiber Crops, Chinese Academy

Table 4 Effect of metallic ions (X±SE) on the xylanase activity

Metallic ions	Xylanase activity (U)	Metallic ions	Xylanase activity (U)	Metallic ions	Xylanase activity (U)
Blank	328.00	Fe^{3+}	279.56	Cu^{2+}	24.63
Co^{2+}	454.87	Zn^{2+}	338.76	Mn^{2+}	403.42
Mg^{2+}	334.58	NH_4^+	331.00	Ca^{2+}	389.00
Na^+	333.36	Fe^{2+}	522.2	K^+	332.41

of Agricultural Sciences. Compared with the *Bacillus subtilis* type strain ACCC 10243, the strain BE-91 had characteristics such as high enzyme activity, and short fermentation cycle. The enzyme activity in the fermentation liquor of BE-91 at 8 h could reach 408 U/mL, which was 3.4 times higher than that of strain ACCC 10243.

At the same time, xylanase was purified from BE-91 fermentation liquor with the ultrafiltration and gel chromatography, and its enzyme activity was up to 28,454 U/mg. Its recovery was above 69%, and purification multiple of the enzyme activity was up to 18 times. Furthermore, the molecular weight of the purified xylanase was 22.54 kDa assayed with SDS-PAGE. The K_m and V_{max} of the xylanase was 0.5 mg/mL and 533 μmol/(min mL), respectively, with oat xylan as the substrate. The stabilizing pH and optimal pH of the xylanase were 4.6~6.4 and 5.8, respectively. And when the pH was 5.8, the stabilizing temperature and optimal temperature of the xylanase were 0°C~65°C and 60°C, respectively. It could be activated by Fe^{2+} and Co^{2+}, and strongly inhibited by Cu^{2+}. Because of these, it can be said that the strain BE-91 is suitable for industrial production with crude conditions and also suitable to be applied in the feed industry for its narrow stabilizing pH range, playing a catalytic function in acidic conditions and being affected by a variety of metallic ions.

Based on the above data, it is considered that the strain BE-91 can be applied to the industrial production of xylanase.

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