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PURIFICATION AND PROPERTIES OF A XYLANASE FROM *BACILLUS SUBTILIS* G1

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SUMMARY

The xylanase from *B. subtilis* G1 was purified 5.8- fold to homogeneity through purification scheme involving dialysis, gel filtration chromatography (Sephadex G-100) and ion exchange chromatography (DEAE-Sephadex A-50). The purified xylanase (specific activity at 91.7 UI/mg protein) was a single band protein with a molecular mass of 27.0 kDa as estimated by SDS-PAGE. The purified xylanase from *B. subtilis* G1 is active at 55°C and pH 6.0 - 8.0, stable at 37 - 40°C and highly active at pH 4 - 8. Organic solvents as methanol, acetone and isopropanol decreased the activity of xylanase from *B. subtilis* G1. Especially, acetone strongly decreased the activity by 92% after 2 hours incubation. In general, metal ions increased xylanase activity. Especially, ions Cu^{2+}, Ag^{+} and Ni^{2+} at the concentration of 10 mM increased the activity of xylanases from *B. subtilis* G1 up to 10 - 26%. The ions Mg^{2+}, Ca^{2+} additives did not increase but slightly decreased xylanase activity. EDTA treatment increased this enzyme activity after 2 hours incubation. When enzyme was incubated with SDS, its activity was strongly decreased at the concentrations of 1 - 2% and decreased to nearly zero after 2 hours incubation comparing with the control. Tween 80 and Trixton X100 at the concentration of 2% was slightly decreased xylanase activity.

Keywords: *Bacillus subtilis* G1, *characterization, chromatography, purification, xylanase*

INTRODUCTION

Xylanases are hemicellulases that hydrolyze xylan, which accounts for 20 to 30% of the dry weight in different hardwood species. Biodegradation of xylan, a major component of the plant cell wall, requires the combined action of several enzymes, among which xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) play a key role. Xylanases have increasing interest for saccharification of xylan, abundant in agricultural wastes, for fuel production. They have important applications in the pulp and paper industry, where alkaline xylanases are of special interest. In many fungi and bacteria, xylan is an important inducer of xylanase production while frequently xylose does not support good enzyme production.

A variety of microbes, including bacteria, yeast and filamentous fungi, has been reported to produce xylanases, in which the most potent producers are fungi (Haltrich *et al.*, 1996). A number of xylanases have been purified from a wide variety of microbes such as *Bacillus* strains (Bernier *et al.*, 1983; Blanco *et al.*, 1995; Sá-Pereira *et al.*, 2002; Ayyachamy, Vatsala, 2007; Yin *et al.*, 2010).

We report here the purification and characterization of the purified xylanase enzyme isolated from *Bacillus subtilis* G1.

MATERIALS AND METHODS

Chemicals

Birch wood xylan was purchased from Biochemika; 3,5-dinitrosalicylic acid (DNS) from Fluka (Germany). Sephadex G-100 and DEAE-Sephadex A-50 were supplied by Phamarcia Co. (Sweden); SDS from Sigma and Tween 80 from BioBasic Inc. (USA); Triton X-100 from Merck (Germany). All other chemicals were of analytical grade unless otherwise stated.

Bacterial strain and culture conditions

B. subtilis G1 strain was obtained from Molecular Microbiology Laboratory (Institute of Biotechnology) and Microbiology Laboratory (Vietnam Academy of Agricultural Sciences). It was cultivated in the MTK liquid medium containing: 2 g KH_2PO_4; 1 g $MgSO_4.7H_2O$; 0.5 g $FeSO_4.7H_2O$; 0,05 g $MnSO_4$; 0.1 g $CaCl_2.2H_2O$; 1 g CMC; 2 g xylan; 2.5 g casein and pH 7.0. The inoculated flasks were incubated for 24 hours at 30°C on a rotary shaker at 200 rpm. After growth, cells were removed by centrifugation and the supernatant was used as the source of crude xylanase.

Xylanase purification

Thirty ml of the crude enzyme extract (0.88

units) was dialyzed and then 3 ml of the dialyzed enzyme was applied to a Sephadex G-100 column (2.6 x 6 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 25 ml/h then washed with the same buffer. The eluate was collected with 1.5 ml per fraction. A highly active xylanase pool of 2 ml through Sephadex G-100 column was further applied to DEAE-Sephadex A-50 ion exchange chromatography and pre-equilibrated with 50 mM Tris HCl buffer pH 8 containing 50 mM NaCl (buffer A), then washed with the same buffer. The protein was eluted with 50 mM Tris HCl buffer pH 8 containing 1000 mM NaCl (buffer B) at a flow rate of 20 ml/h until OD_{280nm} < 0.01. The eluate was collected with 1.5 ml per fraction. The fractions containing high xylanase activity were pooled and used for characterization. All purification steps were carried out at 4°C, unless otherwise specified.

Xylanase activity estimation

The xylanase activity was calculated by measuring the amount of xylose released from 0.5% birchwood xylan according to the method described by Bailey et al. A volume of 0.1 ml of the crude or purified xylanase was incubated with 0.4 ml of the xylan solution [0.5% (w/v) birch wood xylan, 20 mM potassium phosphate buffer, pH 6.5] at 55°C for 5 min. To arrest the reducing sugar released in the reaction mixture, 3,5,-dinitrosalicylic acid (DNS) was added. The reduced sugars were determined by measuring the absorbance at 540 nm (Miller, 1959). One unit (IU) of xylanase activity was defined as the amount of enzyme that released 1 µM of xylose per minute under the standard assay conditions.

All measurements were repeated three times and the average value was taken.

SDS-PAGE and protein concentration

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by (Laemmli, 1970) with Bio-Rad equipment. SDS-PAGE was performed on gels containing 12.5% (w/v) acrylamide according to the manufacturer's recommendation. The gels were stained with Coomassie Brilliant Blue R-250 for protein. Protein concentration was estimated by Bradford method with bovine serum albumin as standard (Bradford, 1976).

Temperature and pH optima

The pH and temperature optima of xylanase were determined by measuring the activity as described above using 20 mM acetate buffer (pH 3 5) and 20 mM phosphate buffer (pH 6 - 8), and in the temperature range of 37 - 70°C, respectively.

Temperature and pH stability

For the determination of temperature and pH stability, purified enzyme (3.85 µg protein for each reaction) was preincubated at different temperatures 40 - 70°C, pH 6.5 for 1 - 8 hours and in pH range 3-8 (20 mM potassium acetate pH 3 5 and 20 mM potassium phosphate pH 6 - 8) for 4 hours at 30°C, respectively. The residual activity was then determined

Effect of metal ions, organic solvents, and detergents

Purified enzyme (3.85 µg protein for each reaction) was preincubated in presence of 10 mM of various metal ions (Fe^{3+}; Mg^{2+}; Fe^{2+}; Cu^{2+}; Ca^{2+}; Ag^{+}; Ni^{+}; K^{+}; EDTA and Zn^{2+} at 30°C for 2 hours, in presence of 10% (v/v) of various solvents (methanol, ethanol, isopropanol, acetone, n-butanol and ethyl acetate) at 30°C for 1 hour, and in presence of 1- 2% (w/v) of various detergents (SDS; Tween 80 and Triton X-100) at 30°C for 2 hours. The residual activity was then determined.

RESULTS AND DISCUSSION

Purification of *B. subtilis* G1 xylanase

The xylanase activity increased with increase of culture time and reached a peak of activity (about 0.88 IU/ml) on 24 hours then decreased. So, this collection time of the enzyme was determined for the purification. Xylanase of *B. subtilis* G1 grown in MTK medium for 24 hours was purified by three-step purification scheme involving dialysis, gel filtration chromatography (Sephadex G-100) and DEAE-Sephadex A-50 ion exchange.

The purified enzyme gained a specific activity of 91.7 U/mg (Table 1) and showed a single protein band on SDS-PAGE (Fig. 1B). The molecular weight of this protein was estimated to be 27 kDa. The result of the purification was shown in Fig. 1a and Fig. 1b and summarized in table 1.

Other xylanases from *Bacillus* strains were purified to homogeneity through a similar purification scheme involving ammonium sulfate precipitation, dyalyse, gel filtration

chromatography (Sephadex G-200, G-100, G-75), ion exchange chromatography (DEAE-Sephadex A-50, DEAE Sepharose), affinity chromatography Phenyl Sepharose 6 Fast Flows, hydrophobic interaction chromatography and Sephacryl S-100 HR chromatograph (Bernier *et al.*, 1983; Yang *et al.*, 1988; Morales *et al.*, 1993; Blanco *et al.*, 1995; Javier *et al.*, 1998; Sá-Pereira *et al.*, 2002; Yin *et al.*, 2010). Purified xylanases from different *Bacillus* strains had various molecular weights from 19 to 22 kDa on SDS-PAGE: 18.4 and 19.6 kDa from *Bacillus amyloliquefaciens* (Javier *et al.*, 1998), 19 kDa from *Bacillus* sp. YJ6 (Yin *et al.*, 2010), 20 kDa from *Bacillus* sp. CCMI 966 (Sá-Pereira *et al.*, 2002), and 22 kDa from *Bacillus* sp. (Paice *et al.*, 1986 ; Yang *et al.*, 1989); *Aeromonas caviae* W-61 (Nguyen *et al.*, 1991); and *Bacillus* sp. strain BP-23 (Blanco *et al.*, 1995). The xylanase from *Bacillus subtilis* G1 had also the same molecular weight of 27 kDa as it from *A. awamori K-1, A. caespitosus*.

Purification factor was 34 kDa for xylanase from *B. polymyxa* (Morales *et al.*, 1993), 44 kDa for xylanases from *B. stearothermophilus* T-6 (Gat *et al.*, 1994), 32 kDa for xylanase from *B. subtilis*, (Bernier *et al.*, 1983), *Bacillus* sp. strain BP-23 (Blanco *et al.*, 1995), 36 kDa for xylanase from *B. subtilis* (Annamalai *et al.*, 2009), higher than that (11) for xylanase from *Bacillus subtilis* G1.

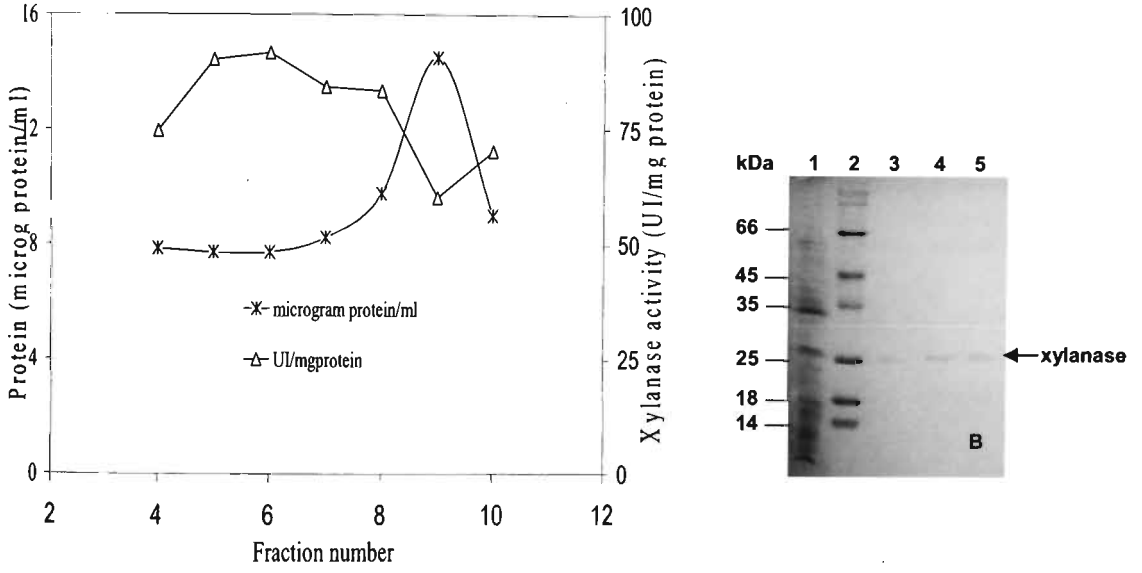


Figure 1. (A): DEAE-Sephadex A-50 ion exchange chromatography of the xylanase from *B. subtilis* G1 (*triangles*: xylanase activity U/mg protein, *stars*: protein µg/ml); (B): SDS-PAGE of purified xylanase from *B. subtilis* G1 after DEAE-Sephadex A-50 ion exchange chromatography (lane 1: the crude enzyme; lane 2: molecular weight marker; lane 3→5: fractions, respectively).

Table 1. The purification of xylanase from *B. subtilis* G1.

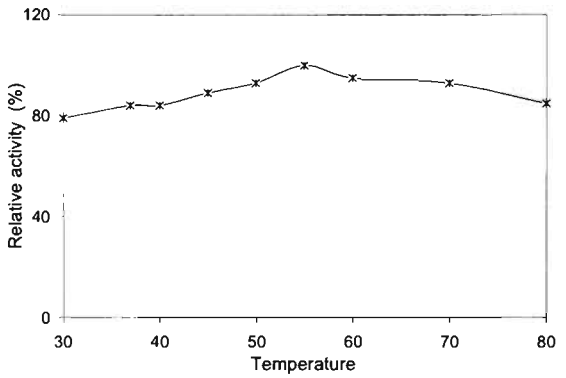
Steps	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (IU/mgprotein)	Yield (%)	Fold
Crude enzyme	0.056	0.88	15.6		
Dialyzed	0.064	1.39	21.8	157	1.4
Sephadex G-100	0.030	1.15	38.5	130	2.4
DEAE-Sephadex	0.0077	0.71	91.7	80	5.8

Temperature optimum and stability

The xylanase from *B. subtilis* G1 had an optimum temperature of 55°C (Fig. 3A) and showed high activity (≥80%) at a large temperature range of 40-70°C in comparison to the maximum activity.

The enzyme was stable at 37 - 40°C for 2 h with a residual activity of ≥ 80% (Fig. 3B) but the xylanase lost 27% of activity when it was treated at 60°C just for 1 - 2 h.

Most xylanases from other *Bacillus* strains had a similar optimum temperature ranged from 45°C to 60°C. The optimum temperature for *Bacillus* sp. CCMI 966 was 60°C (Sá-Pereira *et al.*, 2002). The optimum temperature for *Bacillus* xylanases was 50°C (*Bacillus* sp. YJ6 (Yin *et al.*, 2010); *Bacillus* sp.(Bernier *et al.*, 1983); *Bacillus amyloliquefaciens* (Javier *et al.*, 1998) ; *Bacillus* sp. strain BP-23 (Blanco *et al.*, 1995); 55°C *B.subtilis* (Annamalai *et al.*, 2009); 45°C *B.polymyxa* (Morales *et al.*, 1993)



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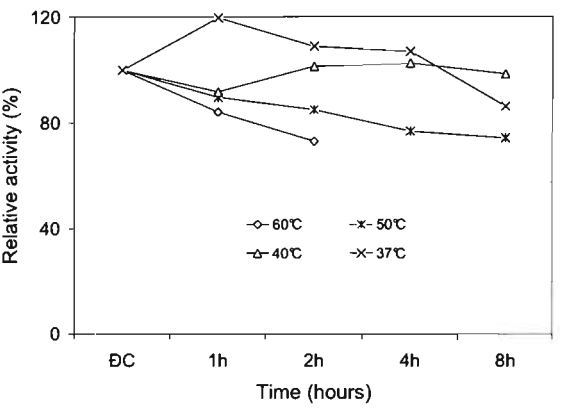

B

Figure 3. The effect of temperature on the xylanase activity (A) and thermal stabitity of xylanase (B). (◊: 60°C ; ∗: 50°C; Δ: 40°C; ×: 37°C).

pH optimum and stability

The xylanase from *B. subtilis* G1 had an optimum pH 4 - 8 (Fig. 4) and worked well in a pH range 5 7 with activity of ≥ 85% in comparison to the maximum activity.

We investigate the stability of xylanase activity in different with pH 3,0-8,0. After a certain time incubation in the medium at 4°C, we obtained the result as shown in Fig. 4. In general, the graphs of xylanase activity at pH range from 5,0 to 8,0 are similar to each other. Activity of xylanase from *B. subtilis* G1 is relatively stable. After 4 hours incubation, the remaining activity is 93% (at pH 4.0), 95% (at pH 5.0), 95% (at pH 6.0), 103% (at pH 7.0), 103% and (at pH 8.0).

The enzyme was stable in a pH range 5.0 8.0 for 4 hours with a residual activity of ≥ 80% (Fig. 4). Thus, xylanase from *B. subtilis* G1 is stable at pH range from 5.0 - 8.0.

This finding was coincident with most xylanases from *Bacillus* strains which showed an optimum pH around 5.0 in acid range: pH 5.0 and was stable at pH 5.0-9.0 (*Bacillus* sp. YJ6, (Yin *et al.*, 2010), *Bacillus subtilis* (Bernier *et al.*, 1983). In contrast, pH optimum for xylanases from *Bacillus amyloliquefaciens* strain was in alkaline range (pH 9.0) and optimum pH of activity was 6.8 - 7.0 (Javier *et al.*, 1998).

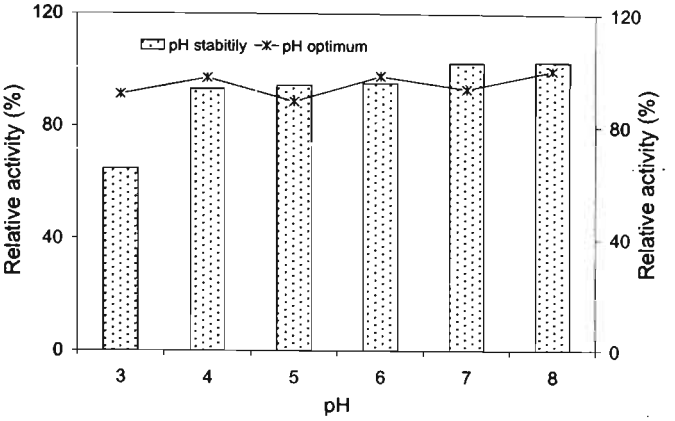

Figure 4. The effect of pH and stabitity on xylanase activity.

Effect of metal ions on xylanase activity

In general, metal ions increased xylanase activity. Especially, Cu^{2+} , Ag^{+} and Ni^{+} ions at the concentration of 10 mM increased the activity of xylanases from *B. subtilis* G1 up to 10-26%.

The Mg^{2+}, Ca^{2+} additives slightly decreased xylanase activity. EDTA treatment increased this enzyme activity after 2 hours incubation. Enzyme activity was not affected by Fe^{2+}, K^{+} and Zn^{2+} ions (Table 2).

Table 2. Effect of metal ions on activity of xylanase.

Metal ions (10 mM)	Enzyme activity (%)
Mg^{2+}	97
Fe^{2+}	106
Cu^{2+}	110
Ca^{2+}	92
Ag^{+}	123
Ni^{+}	126
K^{+}	102
EDTA	112
Zn^{2+}	104

The xylanase activity from *Bacillus amyloliquefaciens* was completely inhibited by Hg^{2+} ions and was reduced drastically in the presence of Cu^{2+} and Fe^{3+} ions. Mn^{2+} ions, EDTA, stimulated the enzyme activity (Javier *et al.,* 1998). In contrast, the purified xylanases from *Bacillus* sp. YJ6 was inhibited by Cu^{2+}, Fe^{3+} Hg^{2+} ions and activated by K^{+}, Na^{+}, Co^{2+}, Mg^{2+} ions (Yin *et al.,* 2010).

Effect of organic solvents on xylanase activity

In order to evaluate the effect of organic solvent on xylanase activity, enzyme extract was supplemented with organic solvents and was incubated for 2 hours at 30°C.

Obtained results in Fig. 5A show that organic solvents partly decreased the enzyme activity when enzyme was incubated at 30°C. Especially, Act strongly decreased and reached 92% after 2 hours incubation. Such solvents as EtOH, n-BtOH did not affect on the enzyme activity during 2 hours incubation at 30°C.

Effect of detergents on xylanase activity

Supplementation of Tween 80 and Trixton X-100 at the concentration of 1% (w/v) slightly decreased enzyme activity comparing with control after 2 hours incubation (Figure 5B). When sample was incubated together with Triton X-100 1% (w/v), enzyme activity was significantly increased. Trixton X-100 at the concentration of 1% decreased enzyme activity to 12% after 2 hours incubation comparing with the control (Fig. 5B). Especially, SDS at the same concentrations strongly decreased enzyme activity to nearly zero after 2 hours incubation comparing with the control. Fialho and Carmona (2004) showed that SDS strongly inhibited xylanase activity from *A. giganteus* (Fialho, Carmona, 2004).

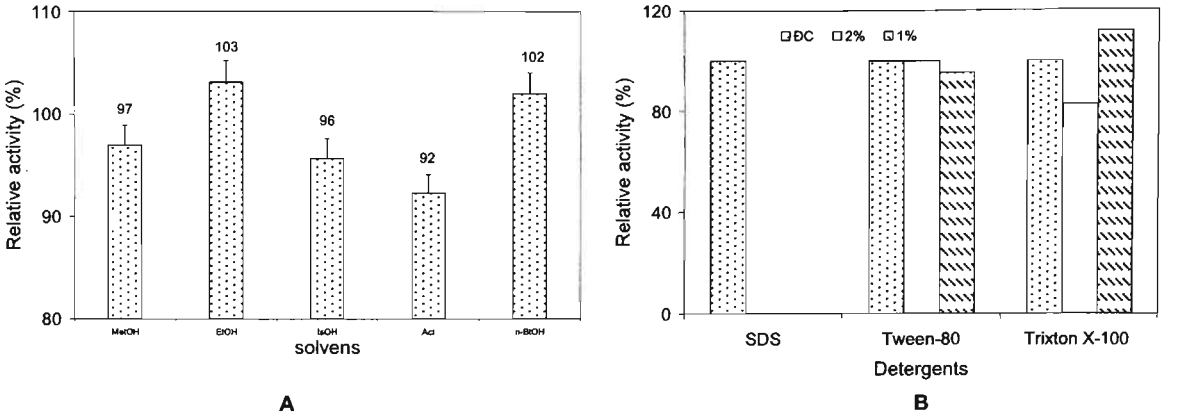

Figure 5. The effect of organic solvent on xylanase activity (A); The effect of detergent on xylanase activity (B).

CONCLUSION

Xylanase from *B. subtilis* G1 crude enzyme extract was purified to homogeneity by using some chromatography techniques with specific activity of 91.7 UI/mg protein, purification folds of 5.8. Purified xylanase was about 27,0 kDa as determined by SDS-PAGE.

The purified xylanase was more active and stable at acidic pH and alkaline and thermostable at 37-40°C. The data indicates that xylanase is suitable for use as enzyme feed in animal husbandry.

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TINH SẠCH VÀ ĐÁNH GIÁ TÍNH CHẤT HÓA LÝ CỦA XYLANASE TỪ CHỦNG *BACILLUS SUBTILIS G1*

Đỗ Thị Tuyên, Quyền Đình Thi*

Viện Công nghệ sinh học

TÓM TẮT

Xylanase đã được tách chiết và tinh sạch từ dịch nuôi tế bào của chủng *Bacillus subtilis G1* sau 24 h nuôi cấy trong môi trường khoáng MTK. Sau ba bước tinh sạch bằng thẩm tích và sắc ký lọc gel sephadex G-100, sắc ký trao đổi ion DEAE-sephadex A- 50, xylanase tinh sạch nhận được có độ sạch so với dịch enzyme ban đầu là gấp 5,8 lần. Điện di trên gel SDS-PAGE cho thấy băng protein có khối lượng phân tử ~27 kDa. Để có thể áp dụng xylanase trong công nghiệp, một số tính chất hóa lý của xylanase từ chủng *B. subtilis* G1 được đánh giá. Hoạt tính xylanase tối thích ở nhiệt độ phản ứng 55°C và pH 6,0 - 8,0. Enzyme bền ở nhiệt độ 37 - 40°C, ở 60°C hoạt tính giảm 27% sau 1 - 2 h ủ và ở pH 6. Các dung môi hữu cơ như methanol, acetone và isopropanol đều làm giảm hoạt tính của xylanase từ chủng *B. subtilis* G1. Đặc biệt dung môi acetone làm giảm hoạt tính của xylanase xuống còn 92% sau 2 h ủ. Nhìn chung, các ion kim loại đều làm tăng hoạt tính của xylanase. Đặc biệt ion Cu^{2+} , Ag^{+} và Ni^{2+} ở nồng độ 10 mM đã làm tăng hoạt tính của xylanase lên 10 - 26%. Việc thêm ion Mg^{2+}, Ca^{2+} đã không những làm tăng mà còn làm giảm nhẹ hoạt tính của xylanase. EDTA làm tăng hoạt tính của enzyme sau 2 h ủ. Khi enzyme được ủ với chất tẩy rửa SDS, hoạt tính xylanase giảm mạnh so với mẫu đối chứng và mất hoạt tính hoàn toàn sau 2 h ủ. Trixton X-100 ở nồng độ 2%, hoạt tính enzyme giảm mạnh.

Từ khóa: *B. subtilis G1, chất tẩy rửa, dung môi hữu cơ, ion kim loại, nhiệt độ, pH, xylanase*

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