Effect of Selected Polyols And Salts on Stability of Xylanase Produced By *Bacillus pumilus*

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ABSTRACT

The objective of this study is to improve the stability of an alkaline xylanase with selected polyols and salts, which was produced by a locally isolated alkalo-thermophilic *Bacillus pumilus*. Xylanases with higher stability are used for the application in paper and pulp industry for bio bleaching. In the absences of additives at 55˚C, the xylanase retained 38(±1.0)% of its initial activity at 30min and retained 45.6 (±0.76) and 20.6(±0.84)% of its initial activity at pH 8.0 and 9.0 respectively. Xylanase which contained polyols such as 10mM Poly Ethylene Glycol (PEG)-8000, 1M glycerol and 2M sorbitol the enzyme retained 24.0(±0.34), 19.0(±0.84) and 53.8(±0.57)% of its initial activity respectively at 60min. Addition of 10mM NaCl lost all of its activity while the enzyme retained 88.4(±0.18)% of its initial activity in the presence of 10mM$CaCl_2$. Sorbitol and $CaCl_2$ were the best additives among the polyols and salts respectively. $CaCl_2$ and sorbitol of different concentrations were studied and the half-lives of the xylanase in presences of 10mM $CaCl_2$ and 2M sorbitol were 302 and 63min respectively. When both 10mM $CaCl_2$ and 2M sorbitol were used together, the enzyme retained more (95%) of its initial activity at 60min than that in presence of 10mM $CaCl_2$ (88%) and 2M sorbitol (53%) individually. At 55˚C half-life of the xylanase in presence of $CaCl_2$, sorbitol and $CaCl_2$ & sorbitol were 18, 47 and 552min respectively. The enzyme contained both $CaCl_2$ & sorbitol helped to retain 95, 88 and 18% of the initial activity at 55, 60 and 65^oC respectively; while at 70^oC it lost all of its activity at 120min. Xylanase from *B.pumilus* was stable at 60^oC for 2h with both $CaCl_2$ & sorbitol.

Key words: Half-life, Polyols, Stability, Salts, Xylanase

1. INTRODUCTION

An increasing concern over environmental pollution has new challenges for the development of Biotechnological processes. Due to the environmental friendly nature, xylanases are used for their application in paper and pulp industry [1, 2, 3] for bio bleaching. Application of xylanase in this industry was effective in decreasing the amount of chlorinating agents [4]. Stability of xylanase at high temperatures that is in the range of 60-70^oC is expected to increase its suitability for application in paper and pulp industry [5]. The stability of enzyme can also be increased by chemical modification, cross-linking, immobilization, treatment with additives and protein engineering [6]. Inclusion of additives to enzyme solution changes its microenvironment and provides a simple but practical means of increasing the stability of the enzyme [5, 7, 8]. In the present investigation, the effects of different additives such as polyols and some salts on the thermal stability of xylanase from *B.pumilus* were studied.

2. MATERIALS AND METHOD

Materials

Birchwood xylan was from Roth, Germany and peptone, yeast extract, PEG-8000, NaCl, $CaCl_2$, glycerol & sorbitol were from Sigma Chemical Company, USA.

Strain

In this study xylanase produced by locally isolated and identified *Bacillus pumilus* was used [9].

Culture Media and Production of xylanase

The Xylan Nutrient Agar plates and slants containing (gL^{-1}) nutrient agar, 28.0 and Birchwood xylan, 20.0 at pH 8.5 was used for the storage of the isolates and incubated at 40^oC for 24 h.

The activation medium contained (gL^{-1}) xylan, 20.0 and nutrient broth, 25.0 at pH 8.5. The bacterial colonies grown on the slant were transferred to 100mL conical flask containing 10mL of activation medium (1 loop/10 mL) and incubated in a reciprocal shaker water bath (120rpm) at 45°C and at pH 8.5, for 18h. Fermentation medium contained (gL^{-1}) xylan, 20.0; peptone, 2.0; yeast extract, 2.5; $CaCl_2.2H_2O$, 0.005; $MgCl_2.6H_2O$, 0.005; $FeCl_3$, 0.005; K_2HPO_4, 2.5; KH_2PO_4, 1.0; NaCl, 0.1 and $(NH_4)_2SO_4$, 2.0 at pH 8.5. Fermentation medium was inoculated with the inoculum (20%, v/v) and incubated at 45°C for 32h. Xylanase in the crude spent medium was separated from the cells by centrifugation (3500rpm) and the supernatant filtered through membrane filter (0.45µm) and used for the stability studies.

Preparation of inoculum

One loop of bacterial colonies grown on the slant were transferred 10mL of activation medium and incubated in a reciprocal shaker water bath (120rpm) at 45°C and at pH 8.5, for 18h and used as inoculum.

Xylanase activity assay

Assay mixture consisted of 0.25mL of diluted enzyme solution and 0.25mL of 20gL^{-1} xylan in 0.05M Tris-HCl buffer (pH 8.4). After incubating at 60°C for 4 min, the reducing sugar produced was determined by Dinitrosalicylic acid (DNS) method [10] with xylose as the standard.

One unit of xylanase activity is defined as the amount of enzyme that releases one µmol of reducing sugar equivalent to xylose per minute at 60°C and pH 8.4 with 20gL^{-1} xylan.

Effect of temperature on the stability of crude xylanase without additives

Effect of temperature on the stability was studied at 55 and 60˚C, and pH 8.4.

Effect of pH on stability of crude xylanase without additives

Effect of pH on the stability was studied by incubating the enzyme with Tris buffer at pH 8.0, 8.4 and 9.0 at 55˚C and the residual activity was monitored.

Effects of different additives on the thermal stability of xylanase

Effect of different polyols

Effect 10mM PEG-8000, 1M glycerol and 1M sorbitol on the stability of the xylanase was studied at 55°C and at pH 8.4. Crude enzyme without polyols was used as control.

Effect of different salts

Effect of 10mM NaCl and 10mM $CaCl_2$ on the stability of the xylanase was studied at pH 8.4 and 55°C. Crude enzyme without salts was used as control.

Effect of different concentrations of $CaCl_2$ and sorbitol

Effects of different concentrations of $CaCl_2$ (0-20mM) and sorbitol (0-3M) on the stability of the xylanase were studied at 55°C.

Combined effect of $CaCl_2$ and sorbitol

The stability of crude xylanase in presence of optimized amounts of $CaCl_2$ and sorbitol and their mixture was studied at pH 8.4 and 55°C.

Combined effects of $CaCl_2$ –sorbitol at different temperatures

Stability of crude xylanase was studied in presence of optimized amounts of $CaCl_2$ and sorbitol at 55, 60, 65 and 70˚C.

3. RESULTS AND DISCUSSION

The xylanases stable and active at high temperature are suitable for Biotechnological applications [8]. Therefore efforts have been made to improve the thermo-stability of xylanase. In this study, thermo-stability of the xylanase from *B.pumilus* was studied with different additives.

Thermal stability of crude xylanase without additives

The thermal stability of the enzyme was studied at 55 and 60°C because this xylanase showed better activity at 55°C. When the thermal stability of the xylanase was studied without additives at 30 min, it retained 5 (±0.92) and 38 (±1.0) % of its initial activity at 60 and 55°C respectively (Figure 1, pH 8.4) and the half-lives of the enzyme were 7 and 22 min respectively. Xylanase from *B.pumilus* which retained 30% of its activity at 1h and 60°C was reported by Asha, *et al*. [11]. Increase in temperature disturbs the secondary, tertiary and quaternary structures of enzyme proteins [12]. Since specificity and catalytic activity are dependent on the 3-D structure of the enzyme, high temperature would decrease and even abolish enzyme activity and stability [12]. Incorporation of suitable additives to enzyme solution may change its microenvironment and can provide improved thermal stability. The results indicated that 55°C is the best temperatures for the storage of the

enzyme. Therefore to improve the thermal stability of the enzyme, effects of different additives were studied.

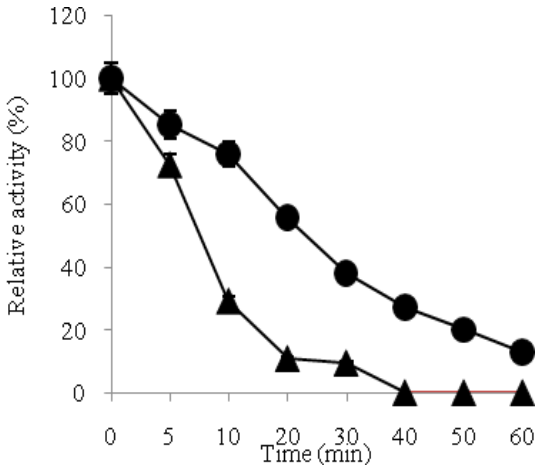

Fig 1: Thermal stability of xylanase (●) at 55 and (▲) at 60 ºC and pH 8.4 without additives.

pH stability

Besides thermo-stability, stability at alkaline pH is a desirable trait for biotechnological applications of xylanase. At 60min, the xylanase from *B. pumilus* preincubated at pH 9.0 lost all of its activity (55ºC) while when preincubated at pH 8.0 and 8.4 (55ºC) retained 8.4 and 24.9% of its initial activity respectively (Figure 2). Half life of the xylanase at pH 8.0, 8.4 and 9.0 were 17, 30 and 0 min Enzymes have different ionizable groups and thus the changes in pH of the medium would change the conformation of the enzyme and its active site, leading to the variation in binding to the substrate. Such effects might have altered the pH stability of the enzyme. The results indicated that pH 8.4 is most suitable to store the enzyme. Based on these results pH 8.4 and 55ºC were considered for the storage stability studies.

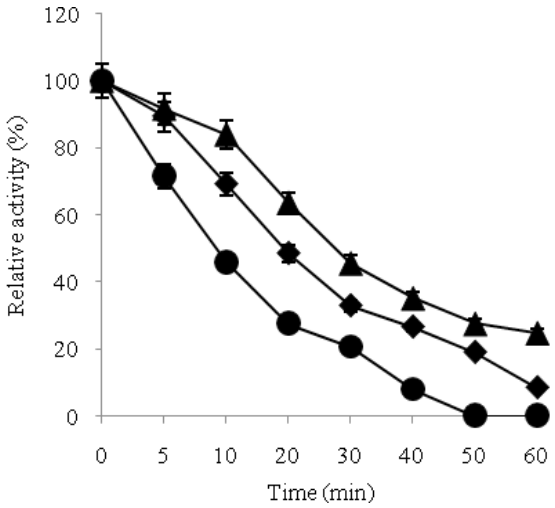

Fig. 2: Stability of xylanase at pH (◆) 8.0, (▲) 8.4 and (●) 9.0 at 55 ºC without additives.

Stability of crude enzyme in the presences of different additives

Effect of different polyols

All the polyols considered in this studies (Glycerol, sorbitol and PEG-8000) showed positive effects on xylanase stability and the enzyme retained 24.0(±0.34), 19.0(±0.84) and 53.8(±0.57)% of its initial activity respectively at 60 min (Figure 3). Xylanase in presence of PEG has lost all of its activity at 90 min while in presence 2M sorbitol and 1M glycerol it retained 33(±0.36) and 28 (±0.86)% of its initial activity at 120 min respectively. Half-life of the xylanase in presence of glycerol, sorbitol and PEG 8000 were 29, 67 and 25 min respectively. It has been reported that polyol (1M) additives such as ethylene glycol (2C) glycerol (3C) and sorbitol (6C) improved the stability of xylanase [13]. These compounds showed similar effects on xylanases isolated from *Thermomonospora* sp. [8] and *Arthrobactwer* sp. MTCC 5214 [14]. The protective effect increases with polyol chain length and related to the number of hydroxyl group per molecules [8, 15]. Some studies have shown that the thermo-stabilizing effect was proportional to the molecular size of the polyols, which can be correlated with the number of hydroxyl groups per polyol molecules [13]. Xylanase protection by ethylene glycol and glycerol was more effective with higher thermo-inactivation temperature [16]. An increase in the thermal stability of the enzymes can be achieved by increasing the viscosity of the solution by adding polyols [12]. It was expected that PEG might provide a better environment to the xylanase due to its high molecular weight and viscosity. Polyols have the capability to form hydrogen bonds with the amino acid residues of proteins and stabilize by supporting the native conformation [8].

The stabilizing effect of additives also depends on the hydrophilic and hydrophobic nature of the enzyme proteins [8]. This is also evident from our studies. When compared with glycerol and sorbitol, the polyethylene glycol was less effective even though the molecular weight of the polyethylene glycol was higher than that of glycerol and sorbitol. As the enzyme showed highest half-life with sorbitol it was selected for further studies.

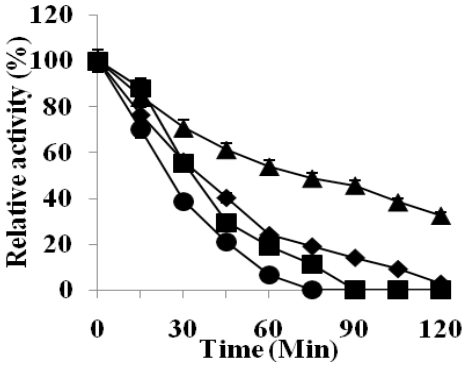

Fig. 3: Stability of xylanase from B.pumilus with (■) 10 mM PEG -8000, (▲) 2 M Sorbitol and (◆) 1M Glycerol, and (●) Control at 55 ˚C and pH 8.4.

Effect of different salts

Xylanase which contained 10mM $CaCl_2$ provided better stability than the control which did not contain the salts. In presence of 10mM NaCl, the xylanase retained 35.0(±0.74) % of its initial activity at 30 min and lost all of its activity at 60min. Addition of 10mM $CaCl_2$ helped to retain 85.3(±0.18) and 88.4(±0.18) % of the initial activity at 120 and 60 min respectively (Figure 4). $CaCl_2$ (10mM) was also effective in increasing the thermo-stability of the xylanase from *Chainia* sp. by doubling the half-life of the enzyme at 60°C [5]. It has been observed that binding of calcium increases the unfolding temperature of the protein [11] and calcium protects xylanase from proteinase and thermal unfolding [4]. The added divalent metal ions increase the thermal stability than monovalent metal ions. Furthermore the enzyme does not require monovalent metal ions for its activity. As $CaCl_2$ improved the enzyme stability, the effect of different concentrations of $CaCl_2$ on the thermal stability of the xylanase was studied.

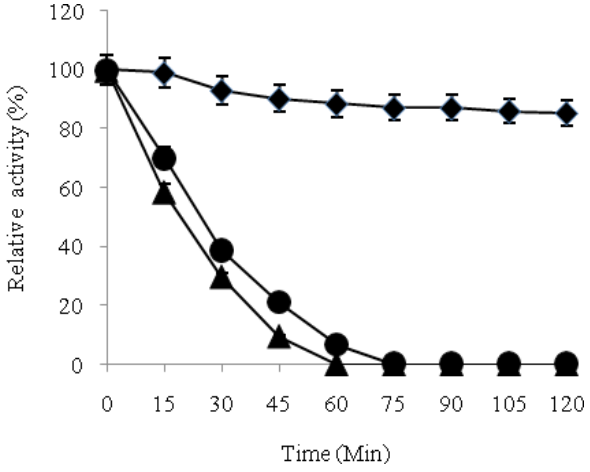

Fig. 4: Stability of xylanase from *B.pumilus* with (◆) 10 mM $CaCl_2$ and (▲) 10 mM NaCl, and (●) Control at 55 ˚C and pH 8.4.

Effect of different concentrations of $CaCl_2$ and sorbitol

At 60 min the enzyme retained 88.4 and 53.4% of the initial activity with 10mM $CaCl_2$ and 2M sorbitol respectively. Half lives of the xylanase with 10mM $CaCl_2$ was 307 min and with of 2M sorbitol was 65 min (Table 1). Breccias *et al*. [17] have shown that addition of sorbitol increased the half-life by 63 fold at 65°C and an increase in sorbitol concentration in the range from 250 to 400 $mgmL^{-1}$ led to exponential increase in thermal stability.

From the results, 10mM $CaCl_2$ and 2M sorbitol were selected to stabilize the xylanase. Polyols protect enzyme protein from heat denaturation, protective effect increases with concentration of the polyhydric alcohol [16]. 2M xylitol as well as sorbitol exhibited some important protective effect on xylanase from *Aspergilus awamori* [7]. Another study has shown that addition of 400 $mgmL^{-1}$ sorbitol increased the half-life of β-xylanase from *Bacillus amyloliquefacians* [17].

Table 1: Half-life and residual activity at 60 min of xyl anase produced by *B.pumilus* in presence of different concentrations of $CaCl_2$ and sorbitol at pH 8.4.

Sorbitol (M)	Relative activity (%)	$T_{1/2}$ (Min)	$CaCl_2$ (mM)	Relative activity (%)	$T_{1/2}$ (Min)
0	5.4	19	0	4.9	19
1.0	54.6	49	5	87.1	296
1.5	72.8	56	10	100	307
2.0	100	65	15	99.5	269
2.5	84.6	59	20	79.7	220
3.0	69.2	47	-	-	-

Combined effect of 10mM $CaCl_2$ and 2M sorbitol

In presences of 10mM $CaCl_2$, the enzyme retained 88 % of its initial activity at 60 min and with 2M sorbitol, the enzyme retained 53 % of its initial activity, but the enzyme with both 10mM $CaCl_2$ and 2M sorbitol retained 95% of its initial activity (Fig: 5).

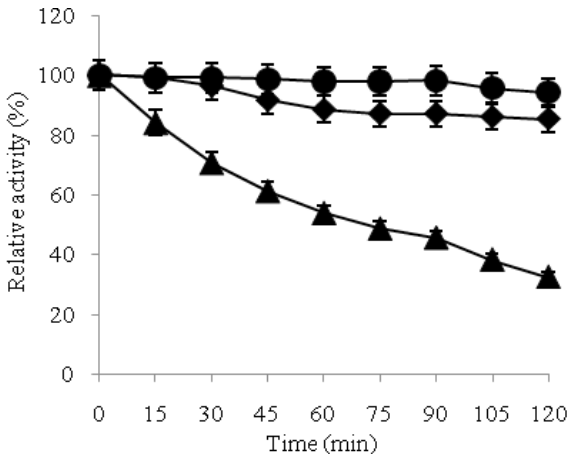

Fig: 5: Stability of xylanase from *B.pumilus* with (◆) 10 mM $CaCl_2$, (▲) 2 M Sorbitol and (●) 10 mM $CaCl_2$ & 2 M sorbitol at 55°C, pH 8.4.

Therefore this combined effect was selected for further stability studies. Half-life of the xylanase in the presences of 10mM $CaCl_2$, 2M sorbitol and 10mM $CaCl_2$ & 2M sorbitol was 302, 65 and 552 min respectively. The stability of xylanase increased with 10mM $CaCl_2$ and 2M sorbitol. It may be due to both the unfolding effect of $CaCl_2$ and reducing the water activity of the enzyme.

Combined effect of $CaCl_2$ & sorbitol at different temperatures

The enzyme with 10mM $CaCl_2$ & 2 M sorbitol retained 95, 88, 18% of its activity at 55, 60 and 65°C respectively at 60min (Table 2), while at 70°C the enzyme lost all of its activity at 30 min (Figure 6). When the temperature was increased from 60 to 65°C, in presence of 10mM $CaCl_2$ & 2M sorbitol, the xylanase stability was dramatically reduced (Figure 6). But further increase in temperature from 65-70°C made the enzyme to lose all of its activity. Half-life of the enzyme in presence of 10mM $CaCl_2$ & 2M sorbitol at 55, 60, 65 and 70°C were 2057, 552, 46 and 0 min Half-life of the enzyme in presence of 10mM $CaCl_2$ & 2M sorbitol at 60°C was 552 min while without additives it was 7 min.

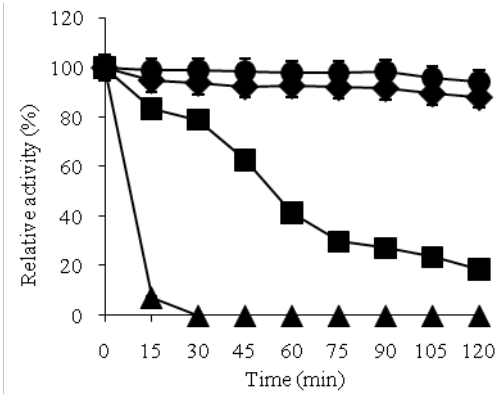

Fig. 6: Stability of xylanase from *B.pumilus* with 10 mM $CaCl_2$–2M sorbitol at different temperatures such as () 55, (◆) 60, (■) 65 and (▲) 70 °C and at pH 8.4.

4. CONCLUSION

Xylanase from *B.pumilus* showed better stability at 55°C than at 60°C without additives. To get better thermal stability of the xylanase, 10mM $CaCl_2$ and 2M sorbitol were more suitable and their mixture improved the half life at 55°C from 38 to 2057 min. Further the mixture of 10mM $CaCl_2$ and 2M sorbitol improved the thermal stability of the xylanase at 60°C from 35 to 572min.

Acknowledgement

The authors thank Sida/SAREC and International Science Programme in the Chemical Sciences (IPICS), Sweden for the financial support.

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