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Significance of metal ions, solvents and surfactants to improve the xylan degrading behavior of β-1,4-D-xylanohydrolase from *Geobacillus stearothermophilus* KIBGE-IB29

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ABSTRACT

Biochemical characterization of the partially purified β-1,4-D-xylanohydrolase from *Geobacillus stearothermophilus* KIBGE-IB29 revealed its higher catalytic activity and stability in the presence of industrially used metal ions, surfactants and organic solvents. The β-1,4-D-xylanohydrolase displayed 1.85, 1.28 and 1.14 fold increase in its catalytic activity when incubated with K^+, Na^+ and Cs^+ ions, respectively for 60.0 min. The enzymatic activity enhanced up to 171% and 242% when 1.0 mM concentration of Ca^{2+} and Mg^{2+} ions was used respectively. An increase in concentration up to 5.0 mM stimulated the β-1,4-D-xylanohydrolase activity up to 3.6 fold in case of ethanol and 1.95 fold with methanol. Formaldehyde, chloroform, Tween 80 and Triton X-100 drastically enhanced the β-1,4-D-xylanohydrolase activity. Furthermore, 2.9 fold increase in activity was noticed with 1.0 mM SDS whereas, EDTA completely inhibited the enzymatic activity. Such an excellent characteristics of β-1,4-D-xylanohydrolase indicate its broad potential to be used in paper and textile industries.

1. Introduction

Xylan is the major hemicellulose found in plants cell wall as a polysaccharide component. β-1,4-D-Xylanohydrolase (Xylanase, EC 3.2.1.8) arbitrarily cleaves the β-1,4-glycosidic bonds present within the backbone of xylan and releases xylose with xylooligosaccharides of different lengths (Viikari et al., 2007). This hydrolase has attracted considerable research interest due to its broad applications in food, textile and paper industries. β-1,4-D-Xylanohydrolase is also used to accelerate the baking process of bread by degrading the polysaccharides in the dough and making cream crackers lighter with improved texture and palatability (Beg et al., 2001; Courtin and Delcour, 2002). It is employed to increase digestibility and nutritive quality of feed as well as to extract plant oils and coffee (Bajaj and Singh, 2010; Butt et al., 2008; Polizeli et al., 2005; Wong and Saddler, 1992). The growing areas of β-1,4-D-xylanohydrolase applications require more exploration for novel catalytic protein and new microbial producers with improved productivity, specific activity and other valuable properties (Ustinov et al., 2008). High pH, temperature, activators and different inhibitors are mostly used in industrial bioprocess that can alter the conformational structure of enzymes. The substrate catalyzing property and stability of enzymes against various physio-chemical conditions over long time period are important for their commercialization as well (Celestino et al., 2006; Gummadi and Panda, 2003). A variety of microbes are known to synthesize β-1,4-D-xylanohydrolase extracellularly with different catalytic properties and molecular weight (Amel et al., 2016; Sanjivkumar et al., 2017; Kumar et al., 2017). Different species of *Bacillus* have been used for the hyper production of β-1,4-D-xylanohydrolase but only few reports are available on the isolation of various metal ions tolerating β-1,4-D-xylanohydrolases that can survive extreme industrial condition (Beg et al., 2001). The exploration of an enzyme with unique catalytic properties from microorganisms and cost effective production approaches are required to fulfill the industrial requirement (Panwar et al., 2014). Considering the broad utility of β-1,4-D-xylanohydrolase the current study was designed to examine the biochemical properties and stability of enzyme from a thermophile *G. stearothermophilus* KIBGE-IB29 in the presence of different concentrations of industrially used metal ions, detergents and organic solvents.

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2. Material and methods

2.1. Synthesis and partial purification of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

A previously isolated *G. stearothermophilus* KIBGE-IB29 [GenBank Accession: KF241865.1] was used in the current study to produce β-1,4-D-xylanohydrolase extracellularly. The bacterial isolate was grown into culture broth containing ($g\,L^{-1}$): xylan, 5.0; meat extract, 1.0; yeast extract, 2.0; peptone, 5.0; potassium dihydrogen phosphate, 0.5; di-potassium hydrogen phosphate, 2.5; ammonium sulfate, 0.5 and calcium chloride, 0.1 with pH-6.0. The culture was kept at 60 °C for 24 h in order to achieve high production yield of β-1,4-D-xylanohydrolase (Bibi et al., 2014). The cells were removed from enzyme containing supernatant through centrifugation at 13,440 × *g* for 10.0 min at 4 °C. Afterwards, the supernatant was allowed to pass through filter membrane having 0.22 μm pore size (Nitrocellulose, Millipore, Germany). The cell free filtrate (CFF) was subjected to partial purification using 40% ammonium sulfate. The salt was gradually added into CFF at 4 °C with continuous stirring and kept at same temperature for 12.0 h to achieve complete equilibration. The precipitated β-1,4-D-xylanohydrolase fraction was separated by centrifugation at 13,440 × *g* for 10.0 min at 4 °C and dialysis was carried out using dialysis tubing with 12,000 kDa cut-off value (Servapor®). The partially purified β-1,4-D-xylanohydrolase was stored at 4 °C for further analysis.

2.2. β-1,4-D-Xylanohydrolase activity assay

The catalytic behavior of β-1,4-D-xylanohydrolase was monitored by calculating the amount of released xylose as an end product after xylan degradation through 3′5′ dinitrosalicylic acid (DNS) method (Miller, 1959). The freshly prepared xylan solution (20.0 $g\,L^{-1}$ xylan dissolved in potassium phosphate buffer having pH-7.0 and 25.0 mM ionic strength) was mixed with partially purified enzyme (0.1 ml) and incubated at 50 °C for 5.0 min. Subsequently, 1.0 ml DNS reagent was added into reaction mixture and incubated in water bath at 100 °C for 5.0 min. The deionized water (9.0 ml) was incorporated into reaction mixture and optical density was measured using spectrophotometer at 546 nm. One unite of β-1,4-D-xylanohydrolase represents *"the quantity of enzyme that is needed to liberate 1.0 μmol of xylose per minute under aforementioned test environment"*.

2.3. Total protein assay

The quantity of protein was estimated during purification steps by Lowry's method using Bovine serum albumin (BSA) as a reference protein (Lowry et al., 1951).

2.4. Impact of metal ions on the catalytic behavior of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

In order to examine the influence of various monovalent ions (K^+, Na^+, Cs^+), divalent ions (Ba^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Ni^{2+}, Hg^{2+}, V^{2+}, Mg^{2+}) and trivalent ions (Al^{3+}) on β-1,4-D-xylanohydrolase activity, the enzyme was incubated at 37 °C with 1.0 mM and 5.0 mM concentrations of metal ions without substrate. The samples were retrieved after 30.0 and 60.0 min and the catalytic activity assay was performed at 50 °C. The metal ions used in this experiment were the chloride salts. Percent relative activity of β-1,4-D-xylanohydrolase was calculated and was compared with control, which was not treated with any metal ion.

All the experiments were conducted in triplicates and the data presented are a mean value of three observations.

2.5. Impact of solvents on the catalytic behavior of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

The partially purified β-1,4-D-xylanohydrolase was incubated with 1.0 mM, 5.0 mM and 10.0 mM concentrations of different organic solvents including ethanol, methanol, isopropanol, chloroform, DMSO and formaldehyde to determine the impact of organic solvents on β-1,4-D-xylanohydrolase activity. The reaction was conducted at 37 °C for 30.0 min and the enzyme assay was performed at 50 °C. Percent relative activity was calculated and compared with the control. Control was taken as 100% and was not treated with any organic solvent.

2.6. Impact of surfactants and metal ion chelator on the catalytic behavior of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

The effect of different surfactants and a metal ion chelator including sodium dodecyl sulfate (SDS), triton X-100, tween 80 and ethylenediaminetetraacetic acid (EDTA) was investigated by incubating the partially purified β-1,4-D-xylanohydrolase with 1.0 mM and 5.0 mM concentrations at 37 °C for 30.0 min. The enzyme assay was conducted at 50 °C and percent relative activity was calculated. Enzyme without any treatment with surfactant was taken as control and considered as 100%.

3. Result and discussion

3.1. Partial purification of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

The β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 was partially purified using ammonium sulfate precipitation approach and fold purification was calculated after dialysis. It was observed that percent recovery of enzyme precipitates increased to 52% after dialysis. The partial purification of β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 yielded 10.0 fold purified enzyme.

3.2. Impact of metal ions on the catalytic behavior of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

Optimization of metal ions concentration is an important parameter that can directly affect the catalytic activity of enzyme. Metal ions influence the activity by accepting or donating electrons, act as electrophiles, eliminate unnecessary side chains, hold the reacting groups in the required three dimensional orientations, develop coordinate bonding to bring together enzyme and substrate, stabilize the catalytic active conformation of enzymes (Palmer, 2001). Various concentrations (1.0 mM and 5.0 mM) of monovalent, divalent and trivalent metal ions were evaluated under standard assay conditions for their effect on partially purified β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29. It was found that β-1,4-D-xylanohydrolase activity improved in the presence of monovalent ions such as K^+, Na^+ and Cs^+ (1.0 mM) and brought up to 1.85, 1.28 and 1.14 fold increase in the catalytic activity of enzyme, respectively after 60.0 min. Enhanced activity was also obtained at 5.0 mM concentration of aforementioned metal ions as the time increased from 30.0 to 60.0 min (Table 1). Stimulation of enzyme activity in the presence of K^+ and Na^+ ions has been reported for β-1,4-D-xylanohydrolase from *Bacillus* sp. PKD-9 (Panwar et al., 2014). Endoxylanase from *Geobacillus thermodenitrificans* TSAA1 also showed 100% residual activity in the presence of 10.0 mM Na^+ ions and 1.18 fold increase was observed with K^+ ions bound to catalytic site of enzyme to promote activity (Anand et al., 2013). Ions specific effect of Na^+ and K^+ was investigated on the catalytic properties of HIV protease. It was noticed that Na^+ ions are more strongly attracted to the surface of protein as compared to K^+ ions because of high interaction with carboxylate side chains found in glutamic and aspartic acid. However, higher concentration of Na^+ and K^+ ions at the

Table 1
Effect of metal ions on the catalytic activity of β-1,4-D-xylanohydrolase produced from *G. stearothermophilus* KIBGE-IB29.

	Relative activity (%)			
	1.0 mM		**5.0 mM**	
Metal ions[a]	**30.0 min.**	**60.0 min.**	**30.0 min.**	**60.0 min.**
Control	100.0	100.0	100.0	100.0
K^+	162.0 ± 8.1	185.0 ± 9.2	185.0 ± 9.2	205.0 ± 10.2
Na^+	112.0 ± 5.6	128.0 ± 6.4	130.0 ± 6.5	156.0 ± 7.8
Cs^+	105.0 ± 5.2	114.0 ± 5.7	135.0 ± 6.7	195.0 ± 9.7
Ba^{2+}	91.0 ± 4.5	71.0 ± 3.5	71.0 ± 3.5	62.0 ± 3.1
Co^{2+}	75.0 ± 3.7	42.0 ± 2.1	35.0 ± 1.7	20.0 ± 1.0
Mn^{2+}	100.0 ± 5.0	100.0 ± 5.0	105.0 ± 5.2	112.0 ± 5.6
Zn^{2+}	100.0 ± 5.0	100.0 ± 5.0	115.0 ± 5.7	137.0 ± 6.8
Cu^{2+}	100.0 ± 5.0	100.0 ± 5.0	108.0 ± 5.4	112.0 ± 5.6
Ca^{2+}	135.0 ± 6.7	171.0 ± 8.5	127.0 ± 6.3	137.0 ± 6.8
Ni^{2+}	105.0 ± 5.2	114.0 ± 5.7	124.0 ± 6.2	139.0 ± 6.9
Hg^{2+}	28.0 ± 1.4	14.0 ± 0.7	14.0 ± 0.7	ND
V^{2+}	129.0 ± 6.4	142.0 ± 7.1	152.0 ± 7.6	186.0 ± 9.3
Mg^{2+}	150.0 ± 7.5	242.0 ± 12.1	160.0 ± 8.0	350.0 ± 17.5
Al^{3+}	82.0 ± 4.1	71.0 ± 3.5	61.0 ± 3.0	32.0 ± 1.6

ND: Not detected.

[a] The metal ions were incubated with β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 at 37 °C without substrate. The samples were retrieved after 30.0 and 60.0 min and the catalytic activity assay was performed at 50 °C for 5 min. All the experiments were conducted in triplicates and the data presented are a mean value of three observations.

active sites leads to a less increase of catalytic performance that may appear due to the presence of alkali cations at the catalytic site to decrease the substrate binding capability (Heyda et al., 2009). In the presence of divalent cations such as Mn^{2+}, Zn^{2+} and Cu^{2+} having 1.0 mM concentration, β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 retained 100% activity after 30.0 and 60.0 min while by increasing the concentration up to 5.0 mM activity enhanced and 112%, 137% and 112% relative activity were obtained from Mn^{2+}, Zn^{2+} and Cu^{2+}, respectively after 60.0 min. The β-1,4-D-xylanohydrolase activity was enhanced up to 171%, 114%, 142% and 242% when 1.0 mM concentration of Ca^{2+}, Ni^{2+}, V^{2+}, and Mg^{2+} was used respectively after 60.0 min. It was observed that when 5.0 mM concentration of Ca^{2+} used, a slight decrease in activity occurred that might be due to the unnecessary metal ions binding at the active site of enzyme. Enzyme activation has been reported for xylanase from *Bacillus altitudinis* DHN8 in the presence of Mn^{2+} and Ca^{2+} salts where 126% and 168% relative activity were obtained, respectively (Adhyaru et al., 2014). It has been found that Cu^{2+} ions stimulated the catalytic activity of thermostable endoxylanase from *Geobacillus thermodenitrificans* TSAA1 and 118% residual activity was achieved at 10.0 mM concentration (Anand et al., 2013). A noticeable inhibition was observed at 1.0 mM concentration of Ba^{2+} and Co^{2+} salt that showed about 71% and 42% relative activity, respectively after 60.0 min and more decline was observed as concentration increased up to 5.0 mM. It was also observed that Hg^{2+} strongly inhibited the β-1,4-D-xylanohydrolase activity (14%) at 1.0 mM concentration and no activity was found at 5.0 mM concentration after 60.0 min, suggesting that sulfhydryl group containing amino acid such as cysteine present at the catalytic site of enzyme which significantly caused the conformational changes within the enzyme after oxidation with cations and resulted complete enzyme denaturation (Meissner et al., 2000). Trivalent cation Al^{3+} showed 71% and 32% activity at 1.0 and 5.0 mM concentrations after 60.0 min, respectively. This result indicated that Al^{3+} acts as an inhibitor of β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 up to some extent.

Table 2
Effect of various solvents on the catalytic activity of β-1,4-D-xylanohydrolase produced from *G. stearothermophilus* KIBGE-IB29.

	Relative activity (%)		
Solvents	**1.0 mM**	**5.0 mM**	**10.0 mM**
Control	100.0	100.0	100.0
Ethanol	180.0 ± 9.0	360.0 ± 18.0	205.0 ± 10.2
Methanol	100.0 ± 5.0	195.0 ± 9.7	97.0 ± 4.8
Isopropanol	190.0 ± 9.5	ND	ND
DMSO	ND	ND	ND
Formaldehyde	270.0 ± 13.5	10.0 ± 0.5	ND
Chloroform	250.0 ± 12.5	10.0 ± 0.5	ND

ND: Not detected.

*The solvents were incubated with β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 at 37 °C without substrate. The samples were retrieved after 30.0 min and the catalytic activity assay was performed at 50 °C for 5 min. All the experiments were conducted in triplicates and the data presented are a mean value of three observations.

3.3. Impact of solvents on the catalytic behavior of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

The impact of different solvents was examined on the catalytic activity of β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 using 1.0, 5.0 and 10.0 mM concentrations. Ethanol and isopropanol were found to improve the activity of enzyme which revealed 180% and 190% activity at 1.0 mM concentration, respectively after 30.0 min. However, the enzyme maintained its 100% activity at the same concentration of methanol. An increase in concentration up to 5.0 mM stimulated the activity up to 3.6 fold in case of ethanol and 1.95 fold in methanol (Table 2). Whereas isopropanol was proved to be a β-1,4-D-xylanohydrolase inhibitor at same concentration. Further increase in the concentration up to 10.0 mM of ethanol and methanol significantly declined the activity. These results indicated that the organic solvents produced specific effect on the activity and stability of β-1,4-D-xylanohydrolase. Similar results were reported earlier with xylanase from *Streptomyces* sp. CS428 that showed better activity with 1.49, 1.43, 1.45 fold increase using 0.25% ethanol, methanol and isopropanol, respectively (Pradeep et al., 2013). It was also observed that xylanase from *Bacillus altitudinis* DHN8 displayed 1.08, 1.17 and 1.26 fold increment in activity when ethanol, methanol and isopropanol used respectively (Adhyaru et al., 2014). The process of biocatalysis improves in the presence of organic solvents and it enhances the solubility of hydrophobic substrate, catalytic efficiency and reduces the microbial contamination (Sardessai and Bhosle, 2004). It was found that Dimethyl sulfoxide (DMSO) caused complete loss of β-1,4-D-xylanohydrolase activity. Same outcome was also reported where, the activity of thermostable β-1,4-D-xylanohydrolase from *Streptomyces* sp. CS428 inhibited in the presence of DMSO (Pradeep et al., 2013). These results suggested that polarity of different solvents extremely affects the catalytic property of enzymes. It was observed that highly polarized solvents inactivate the enzymatic proteins by absorbing the water molecules surrounding the catalytic site of enzyme. Solvents that have a log P value < 4 are recognized as toxic due to high degree of partitioning into aqueous layer. The catalytic proteins that have the property to remain stable in an organic phase can be employed to catalyze various biochemical reactions and to generate bioremediation processes (Klibanov, 2001). Moreover, formaldehyde and chloroform (1.0 mM) drastically enhanced the catalytic activity of β-1,4-D-xylanohydrolase that showed 270% and 250% relative activity, respectively after 30.0 min. However, 5.0 mM concentration caused inhibitory effect and 90.0% reduction in the catalytic activity of enzyme was noticed.

Table 3
Effect of surfactants and metal ion chelator on the catalytic activity of β-1,4-D-xylanohydrolase produced from *G. stearothermophilus* KIBGE-IB29.

	Relative activity (%)	
Substance[a]	**1.0 mM**	**5.0 mM**
Control	100.0	100.0
SDS	290.0 ± 14.5	8.0 ± 0.4
Triton X-100	175.0 ± 8.75	18.0 ± 0.9
Tween 80	245.0 ± 12.2	187.0 ± 9.3
EDTA	ND	ND

ND: Not detected.

[a] The substances were incubated with β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 at 37 °C without substrate. The samples were retrieved after 30.0 min and the catalytic activity assay was performed at 50 °C for 5 min. All the experiments were conducted in triplicates and the data presented are a mean value of three observations.

3.4. Impact of surfactants and metal ion chelator on the catalytic behavior of β-1,4-D-xylanohydrolase from G. stearothermophilus KIBGE-IB29

The impact of surfactants and metal ion chelator on the catalytic activity of β-1,4-D-xylanohydrolase from *G. stearothermophilus* KIBGE-IB29 was also examined for future commercial use. It was noticed that β-1,4-D-xylanohydrolase activity significantly improved in the presence of Triton X-100 and Tween 80 (1.0 mM) and up to 1.75 and 2.45 fold increase in catalytic activity was achieved, respectively (Table 3). It was evident that further increase in concentration of Tween 80 and Triton X-100 generated a negative effect on the activity of β-1,4-D-xylanohydrolase and only 1.87 fold increase was attained in case of Tween 80. It was reported earlier that Tween 80 and Triton X-100 mostly do not involve in the deactivation of enzyme and in the case of xylanase from *Aspergillus awamori* VTCC-F312, the catalytic activity was enhanced up to 28% and 16% in presence of Triton X-100 and Tween 80 (Pradeep et al., 2013; Do et al., 2012). The partially purified xylanase from *G. stearothermophilus* KIBGE-IB29 showed 2.9 fold increase in its activity after mixing with 1.0 mM SDS and as the concentration increased up to 5.0 mM, a remarkable decline in activity was attained. It was observed previously that SDS showed its inhibitory effect on xylanase from *Paenibacillus campinasensis* G1-1 and it retained only 20% activity (Zheng et al., 2012). The xylanase activity from *Jonesia denitrificans* BN-13 was enhanced up to 117% in the presence of 20.0 mM SDS (Boucherba et al., 2014). It was found that EDTA completely inhibited the catalytic activity of xylanase from *G. stearothermophilus* KIBGE-IB29 as reported earlier (Chapla et al., 2012).

4. Conclusion

It is concluded that partially purified xylanase from *G. stearothermophilus* possess excellent catalytic properties for commercial use. Most of the reported xylanases show limited stability and catalytic efficiency within a variety of metal ions, surfactants and organic solvents. However, xylanase from *G. stearothermophilus* exhibited significant stability profile when incubated with different industrially used metal ions, surfactants and solvents even after 60.0 min. Such characteristics reveal the potential utility of xylanase in different bioprocesses of paper and textile industries.

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Competing interests

This publication is approved by all authors and they do not have any conflict of interest regarding any financial, personal or other relationships with any other people or organizations.

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