Purification and Characterization of a recombinant β-Xylosidase from *Bacillus licheniformis* ATCC 14580 into *E. coli* Bl21

Asma Zafar[1*], Muhammad Nauman Aftab[2*], Asma Manzoor[3], Irfana Iqbal[4], Zia ud din[5], Afshan Kaleem[7], Ateeque Rahman Khooharo[6] and Mushtaq Ahmad Saleem[1]

[1]Faculty of life Sciences, University of Central Punjab, Lahore, Pakistan
[2]Institute of Industrial Biotechnology, GC University, Lahore, Pakistan
[3]Institute of Biochemistry and Biotechnology, University of Punjab, Lahore, Pakistan
[4]Department of Zoology, Lahore College for Women University, Lahore, Pakistan
[5]University of Baluchistan, Quetta, Pakistan
[6]Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan
[7]Center of Excellence in Marine Biology, University of Karachi, Karachi

Abstract: Present research work is aimed to purify and characterize a recombinant β-xylosidase enzyme which was previously cloned from *Bacillus licheniformis* ATCC 14580 in to *Escherichia coli* BL21. Purification of recombinant enzyme was carried out by using ammonium sulphate precipitation method followed by single step immobilized metal ion affinity chromatography. Specific activity of purified recombinant β-xylosidase enzyme was 20.78 Umg^{-1} with 2.58 purification fold and 33.75% recovery. SDS-PAGE was used to determine the molecular weight of recombinant purified β-xylosidase and it was recorded as 52 kDa. Purified enzyme showed stability upto 90°C within a pH range of 3-8 with and optimal temperature and pH, 55ºC and 7.0, respectively. The enzyme activity was not considerably affected in the presence of EDTA. An increase in the enzyme activity was found in the manifestation of Mg^{+2}. Enzyme activity was also increased by 6%, 18% and 22% in the presence of 1% Tween 80, β-mercaptoethanol and DTT, respectively. Higher concentrations (10 – 40%) of organic solvents did not show any effect upon activity of enzyme. All these characteristics of the recombinant enzyme endorsed it as a potential candidate for biofuel industry.

Keywords: β-xylosidase, *Bacillus licheniformis*, cloning, purification, characterization.

INTRODUCTION

The major component of plant cell wall comprises of cellulose and hemicellulose along with pectin, aromatic compounds such as lignin, proteins and various other interlinked polymer (Malherbe and Cloete, 2002). Cellulose is the most abundant polysaccharide occuring in nature, which comprises of solely 1,4-linked β- D-glucose units (Ahmed *et al*., 2009). In comparison to cellulose, hemicelluloses that are regarded as the second largest polysaccharide present on earth, are very complex polysaccharides and present in various structural types (Korner, 2003) such as mannans, xylans, β-glucans and xyloglucans (Ebringerova *et al*., 2005). Among all these types of hemicelluloses, xylan is considered as the most copious type of hemicellulose occuring in nature which comprises of β-1,4- linked xylose residues interlinked with sugar acid residues and sugars (da Silva *et al*., 2012).

For complete breakdown of hemicelluloses into xylose sugar, a series of strenuous action of hydrolytic enzymes is necessary (Coughlan and Hazlewood, 1993; Kulkarni *et al*., 1999; Cosgrove, 1997). The hydrolytic enzymes that are involved in hydrolysis of hemicelluloses into monosaccharide sugars, generally known as hemicellulases, which include endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), E.C.3.2.1.139, α- glucuronidase (α-glucosiduronase), acetylxylan esterase (E.C.3.1.1.72) and α-arabinofuranosidase (α-Larabinofuranosidase, E.C.3.2.1.55) (Juturu and Wu, 2012). Endoxylanases are involved in the generation of xylooligosacchrides while β-xylosidases cleave xylooligosacchrides to xylomonosacchrides (Biely, 1985). β-xylosidases split the terminal xylose units from non-reducing ends of dimers and trimmers of xylose that are generated by the action of xylanase (Peij *et al*., 1997; Perez-Gonzalez *et al*., 1998).

β-xylosidases are used in various industrial processes such as paper industry (Marques *et al*., 2003), wood pulp industry (Tsujibo *et al*., 2001), in bread making (Dornez *et al*., 2007), fruit juice production (Manzanares *et al*., 1999) and xylitol production (Polizeli *et al*., 2005). Xylan degrading enzymes are widely present in microorganisms (bacteria and fungi) (Khasin *et al*., 1993; Lee *et al*., 1986; Shao and Wiegel, 1992) but only few studies are present on thermotolerant xylan hydrolyzing enzymes (Dekker, 1983). To overcome the industrial demand for xylose and xylooligosacchrides, there is a need to explore more and more thermostable xylanolytic enzymes (Baba *et al*., 1994). Among xylan degrading enzymes, xylanases are vastly studied as compared to β-xylosidase and few

**Corresponding author:* e-mail: nauman535@yahoo.com, asma.zafar@ucp.edu.pk

reports are present on both xylanases and β-xylosidases (Nanmori *et al*., 1990; Sunna and Antranikian, 1996; Waino and Ingvorsen, 2003). The presence of both xylanolytic enzymes (xylanase and β-xylosidase) in the same microorganism was reported only in *Bacillus thermantarcticus* (Lama *et al*., 2004) and *Bacillus stearothermophilus* (Baba *et al*., 1994; Nanmori *et al*., 1990).

Due to ease of genetic manipulation, *Escherichia coli* is a very commonly used microorganism (Jarboe *et al*., 2007). By the application of gene manipulation techniques, much advancement has been made to generate a favorable environment for enzyme production at industrial scales (Zafar *et al*., 2014). *Bacillus licheniformis* is a spore producing, Gram-positive and slightly thermophilic bacterium that plays an important role in biotechnology industry to produce various kinds of enzymes including β-xylosidase and is capable of growing on cellulose as exclusive source of carbon (Rey *et al*., 2004).

We have published our related work earlier on the application of β-xylosidase enzyme in saccharification of plant biomass (Aftab *et al*., 2017). The present research work was carried out on characterization and purification of previously cloned β-xylosidase enzyme from *B. licheniformis*.

MATERIALS AND METHODS

Selection of microorganisms and plasmids

B. licheniformis ATCC 14580 strain was attained from the USDA ARS Culture and Collection, Peoria, USA. Lyophilized culture was inoculated in 10 ml of Tryptone Glucose Yeast extract (TGY) medium (0.1 % glucose, 0.5 % tryptone, 0.1 % K_2HPO_4 and 0.5 % yeast extract) and further maintained in LB (Luria-Bertani). *E. coli* BL21 (DE3) was acquired from culture bank of Institute of Industrial Biotechnology (IIB), Government College University, Lahore Pakistan, maintained in LB medium and used as expression system of cloned xylosidase gene. pET 21a (+) vector (Novegen) was used for the expression of cloned gene.

Cloning of β-xylosidase gene

β-xylosidase gene of *B. licheniformis* was cloned in pET 21a (+) and expressed into *E. coli* BL21 strain (Aftab *et al*., 2017). Genomic DNA of *B. licheniformis* ATCC 14580 was isolated by using standard protocol (Kronstad *et al*., 1983) and used for the amplification of *xynB* gene of *B. licheniformis* ATCC 14580. DNASTAR software was used to design the primers against the sequence (Accession No. Q65MB6) retrieved from NCBI database. Restriction sites of *Hind*III and *Nde*I shown in bold were added in sequences of reverse and forward primers, respectively. The sequences of the both primers are given below:

Xylo F' GCCATATGATGAGCGGTGAACACACA
Xylo R'GCAAGCTTATATGCAATATAGCGGAACC AGTC

Double restriction of amplified products of *xynB* gene and pET 21a(+) vector, was carried out by using *Hind*III and *Nde*I (Thermo Scientific) in 10X Tango buffer. Qiagen DNA purification kit was used to purify DNA. After purification, purified double restricted fragment of *xynB* was inserted within *Hind*III and *Nde*I restriction sites of vector pET 21a (+) by using T4DNA ligase in the presence of buffer of T4 DNA ligase and incubated for 1 hour at 22ºC and transformed into newly prepared competent cells of BL21 (DE3) strain of *E. coli* according the of Sambrook *et al*. (2001). The $CaCl_2$ method of Cohen *et al*. (1972) was used for the preparation of competent cells. Screening of positive clones was performed by double digestion of plasmid with restriction enzymes (*Hind*III and *Nde*I) as well as by colony PCR. Cell lysis method was used for plasmid isolation (Birnboim and Doly, 1979).

Expression of recombinant xynB gene

Expression of cloned *xynB* gene from *B. licheniformis* ATCC 14580 was analysed by culturing the recombinant *E. coli* BL21 (DE3) colony in LB medium supplemented with ampicillin (100µg/µl) at 37ºC until its optical density reached to 0.5 to 0.7 at 600 nm. Cells were then induced with IPTG (isopropyl-D-thiogalactopyranoside) (0.5mM) and incubated at 37ºC for further 4 hours. After 4 hours, cells were separated by centrifugation at 6000 rpm and 4ºC for 10 minutes. Supernatant was separated and cells were sonicated in a sonicator (Hielscher, Ultrasound Technology) after resuspension in 5ml of Tris-Cl buffer (50 mM) of pH 7.5-8. Again, it was centrifuged at 6000 rpm for 10 minutes at 4ºC and cell lysate was separated. Cloned *xynB* gene expression was analysed in both extracellular as well as intracellular fractions by running on SDS-PAGE (12%) and then visualizing after staining with Coomassie Brilliant Blue dye (Laemmli, 1970).

Enzyme assay

Activity of enzyme was determined by using *p*-nitrophenyl-β-D-xylopyranoside (1 mM) as substrate in sodium acetate buffer (50 mM, pH 5.0). Reaction mixture was prepared by adding 0.5 ml enzyme sample and 0.5 ml substrate solution (1 mM), incubated at 50ºC for 10 minutes and then 1 ml sodium carbonate (1M) was added to terminate the reaction. A control was run in parallel in which enzyme was added after incubation. The amount of *p*-nitro phenol liberated during reaction was calculated by observing absorbance at 400 nm in a spectrophotometer. Enzyme unit was defined as "the quantity of enzyme used to liberate 1 µmol *p*-nitro phenol per minute under standard assay parameters".

Purification of recombinant β-xylosidase enzyme

Ammonium Sulfate Precipitation

Partial purification of β-xylosidase enzyme was performed by using ammonium sulfate precipitation method (de Moraes *et al*., 1999). 50 ml of crude enzyme was saturated by ammonium sulfate (10-100%) along with continuous stirring with magnetic stirrer at 4ºC. After an hour of continuous stirring, it was centrifuged for 20 minutes at 12000 rpm and 4ºC. Pellet attained was resuspended in 1 ml sodium citrate buffer (50 mM) of pH 7.5. Dialysis was carried out for 24 hours against 50mM sodium citrate buffer having pH 7.5 with constant stirring by using membrane with molecular weight cut off 12 kDa.

Affinity chromatography

Final purification of enzyme was achieved by immobilized metal ion affinity chromatography (IMAC). A purification kit, Protino® Ni-TED, packed columns were used for purification. The columns were equilibrated with 1X LEW buffer (Lysis Equilibrium Wash Buffer) (4 bedvolumes) and endorsed to drain by gravity. 2 ml recombinant β-xylosidase enzyme was loaded at the column and endorsed to drainunder gravitational force. LEW buffer (1 X) was used to wash the column (eight bed volumes). Elution buffer (3 X) was used in order to elute the column bounded proteins.

Characterization of purified enzyme

Purified recombinant β-xylosidase enzyme was characterized by studying different parameters like; pH stability, thermostability, effect of EDTA and metal ions upon activity and stability of enzyme, organic solvents and inhibitors's effect upon the activity and stability of recombinant purified enzyme, substrate specificity of the enzyme and calculation of molecular weight of the purified recombinant β-xylosidase enzyme.

RESULTS

Cloning and expression of β-xylosidase gene

Transformed colonies were screened by restriction analysis of recombinant plasmid as well as by colony PCR. The restricted products were analysed on 1% agarose gel and observed the separation of cloned β-xylosidase gene from plasmid pET 21a (+) plasmid (fig. 1) which showed the successful cloning of β-xylosidase gene in vector pET 21a (+). Expression of cloned β-xylosidase gene in pET 21a (+) was determined by SDS-PAGE in both extra cellular and intracellular fractions after induction with IPTG. In intracellular fraction the enzyme activity was found to be 2.41 U/ml/min, while in extra cellular fractions no enzyme activity was found. Expression of recombinant β-xylosidase enzyme as determined by SDS-PAGE is represented in fig. 2.

Enzyme purification

β-xylosidase enzyme was partially purified by ammonium sulphate precipitation at 4ºC with gentle stirring. The fractions having high β-xylosidase activity were combined, dialyzed and subjected to immobilized metal affinity chromatography for further purification. The active fractions were pooled and analyzed by SDS-PAGE. Purified enzyme showed 2.58 purification fold having 33.75% yield with 20.78 U/mg specific activity. Appearance of single band of 52 kDa on SDS-PAGE indicated the successful purification of β-xylosidase enzyme (fig. 2). Result of all purification steps are summarized in table 1.

Characterization of purified recombinant β-xylosidase

Molecular weight determination

Mass of recombinant β-xylosidase was calculated by SDS-PAGE. A discrete band of 52 kDa was detected in purified fraction but such band was not observed in controls like simple uninduced vector, induced vector without any gene, cell extract of wild host strain and non-induced recombinant vector which contain β-xylosidase gene as shown in fig. 2.

Thermostability

Temperature stability of purified recombinant β-xylosidase was determined by examining the residual activity after pre-incubation of enzyme for 1-4 hours at varying temperature (40-90ºC). Recombinant β-xylosidase enzyme was stable at high temperatures and retained almost 100% activity after treatment of 4 hours at 40ºC and 50ºC. The enzyme showed very good stability and retained 75% of its residual activity at 60ºC after 4 hours of incubation (fig. 3A). The activity of β-xylosidase was declined as the incubation proceeded and was reduced to 58%, 49% and 45% at 70ºC, 80ºC and 90ºC after 4 hours of incubation. After 3 hours of incubation at 60ºC, 70ºC, 80ºC and 90ºC the residual activity of enzyme was 81%, 63%, 55% and 48%, respectively. However, after 2 hours of incubation enzyme was found to be very stable and retained upto 54% stability at 90ºC (fig. 3A).

Stability of purified β-xylosidase at various pHs

pH stability of cloned β-xylosidase enzyme was examined in buffers with varying pH range (3 - 8) for 1-4 hours at room temperature. The enzyme showed very good stability at pH 6 and 7 and retained almost 100% activity after 4 hours of incubation (fig. 3B). The enzyme stability was found to be decreased when incubation proceeded at pH 3, 4, 5 and 9 and after 1 hour of incubation the enzyme showed 55%, 69%, 82% and 70% stability respectively. However, reasonable enzyme activity (22%, 36%, 41% and 32%) was sustained after 4 hours of incubation at pH 3, 4, 5 and 9 (fig. 3B).

Substrate specificity

Specificity of purified β-xylosidase enzyme against different substrates was investigated by using 1% substrates (4-nitrophenyl-α-L-arabinofuranoside, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl β-

xylopyranoside and birch wood xylan). The enzyme showed highest activity against 4-nitrophenyl β-xylopyranoside (3.29 U/ml/min) (fig. 3C).

Inhibitor's effect on β-xylosidase activity

Effect of various inhibitors was determined on the activity of β-xylosidase enzyme against different concentrations of inhibitors (1-4%) at room temperature. Results showed

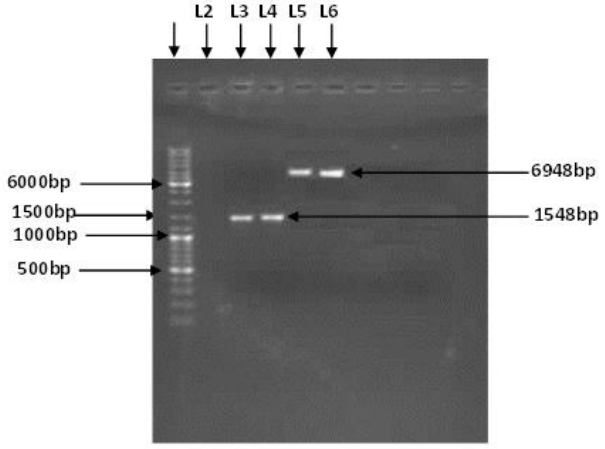

Fig. 1: Cloning of β-xylosidase enzyme: In lane 1 GeneRuler™ DNA marker is present, lane 2 is empty while in lane 3 and 4 amplified products of β-xylosidase gene (1420 bp) is present. Lanes 5 and 6 show the recombinant expression vector pET-21a (+) containing β-xylosidase gene (6820 bp).

that recombinant β-xylosidase enzyme was very stable in the presence of all inhibitors used (SDS, DMSO, NaN3, β-mercaptoethanol, DTT and Tween 80) and enzyme activity was increased to 118% and 129% in the presence of 1% β-mercaptoethanol and DTT respectively. Enzyme also showed significant stability 72%, 64% and 73% after treatment with 1% DMSO, SDS and NaN3, respectively. However, with higher concentrations of SDS, DMSO and NaN3 enzyme activity was seemed to be slightly decreased (fig. 3D).

EDTA and metal ion's effect

EDTA and metal ion's effect on β-xylosidase activity was determine by pre-incubation of enzyme with 1 to 10 mMof metal ions (Co^{+2}, Na^{+2}, $Mg^{+2,}$ Mn^{+2}, Ca^{+2}, Cu^{+2} and Hg^{+2}) as well as EDTA. Enzyme activity was found to be stable in the presence of most of the metal ions instead of Cu^{+2}, Ni^{+2}, Co^{+2} and NH_4^{+1} at higher concentrations as shown in fig. 3E. An increase in enzyme activity (119%) was observed with 10 mM Mg^{+2}, however, no considerable effect of EDTA was observed on recombinant β-xylosidase enzyme.

Organic solvent's influence on β-xylosidase stability

Influence of organic solvents upon β-xylosidase enzyme was checked by determining the residual activity of enzyme after incubation with different organic solvent for 1 hour at room temperature. The results revealed that recombinant enzyme was highly stable in the presence of all organic solvents even at higher concentration (40%). In the manifestation of 10% methanol, isopropanol, acetone, ethanol and n-butanol the enzyme activity was 93%, 91%, 97%, 82% and 88%, respectively.

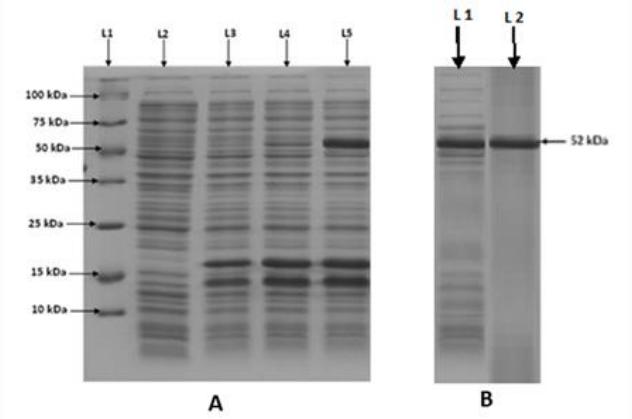

Fig. 2: Expresion and purification analysis of cloned β-xylosidase gee. (A) Lane 1 to 5 represents: protein marker, cell extract of wild *E. coli*, pET 21a (+) vector only induced, recombinant vector containing β-xylosidase gene (non-induced) and induced recombinant vector containing β-xylosidase gene. (B) Lane 1 to 2 represents β-xylosidase enzyme (partially purified) and purified β-xylosidase showing a distinct band of 52 kDa.

DISCUSSION

β-xylosidase is one of the hemicellulases which is considered as a necessary component for the saccharification process of hemicellulose components of plant cell wall. The present research work concerned with purification and characterization of a recombinant β-xylosidase enzyme from *Bacillus licheniformis* ATCC14580 into *E. coli* BL21 which can be used for the saccharification process of plant biomass along with other cellulases and hemicellulases of the mesophilic origin.

β-xylosidase gene of *Bacillus lichniformis* ATCC 14580 was cloned and successfully expressed into *E. coli* BL21 strain. pET 21a (+) was used as an expression vector to get the maximum expression of β-xylosidase gene under T7 promoter system. Pontonio *et al*. (2016); Sumarsih *et al*. (2015) and Umemoto *et al*. (2008) also cloned β-xylosidase genes from *Lactobacillus rossiae*, *Bacillus megaterium* and *Vibrio* sp. by using pHIS1525 and pET22b (+) as expression vectors, respectively. The expression of cloned gene was checked in both intracellular as well as extracellular fractions by using SDS-PAGE as well as by checking the enzyme activity in both fraction using standard enzyme assay method. In this study the expression of cloned β-xylosidase gene was found in intracellular fraction (fig. 2), which was obtained after cell lysis by heat sonication system.

Table 1: Summary of purification steps of β-xylosidase

Purification Step	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Percentage yield	Purification Fold
Crude extract	25.24	3.14	8.03	100	1
$(NH_4)_2SO_4$ Precipitation	18.24	1.2	15.2	72.26	1.89
Affinity Chromatography (IMAC)	8.52	0.41	20.78	33.75	2.58

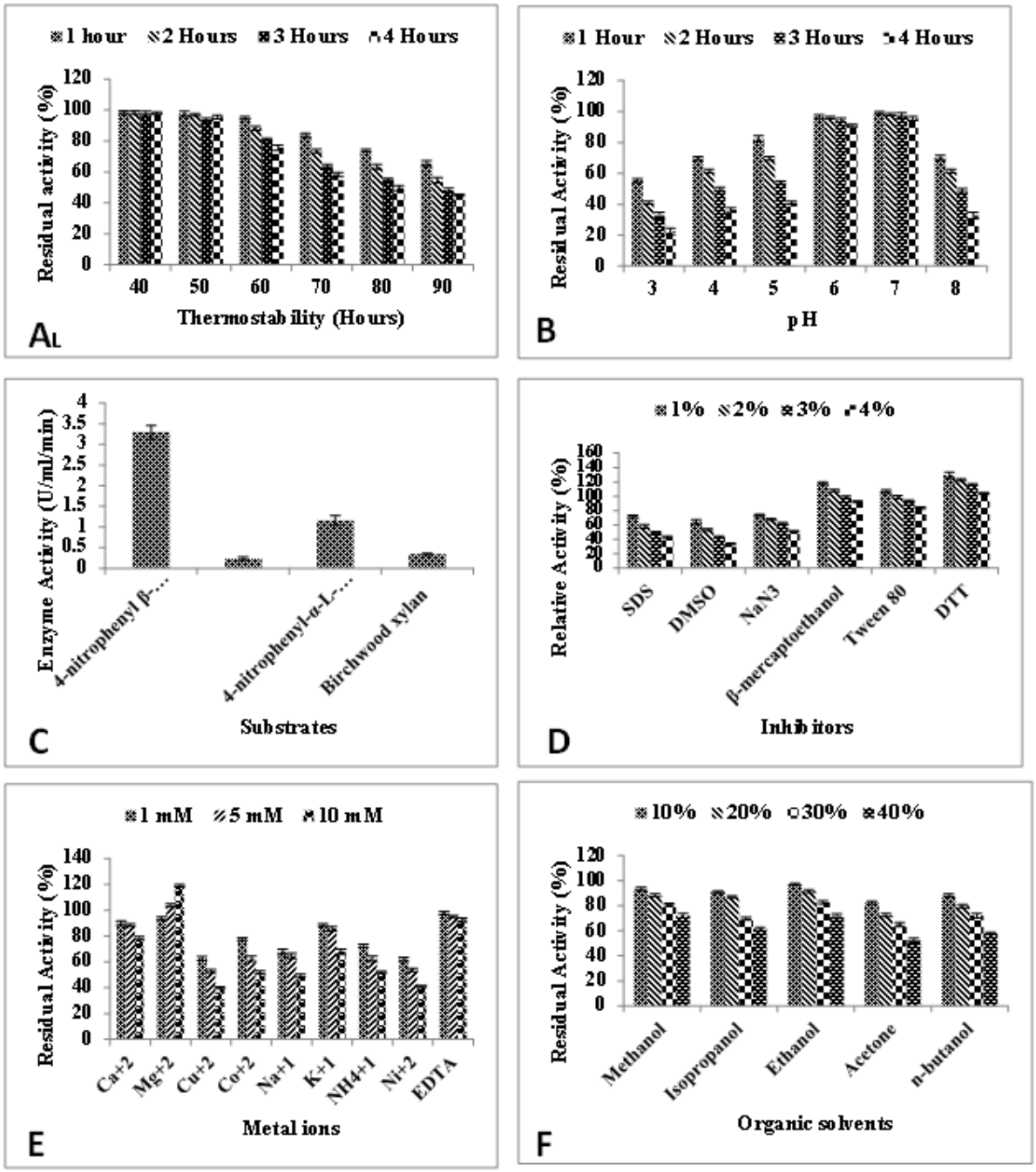

Fig. 3: Characterization of recombinant β-xylosidase; A: Thermostability B: pH stability C: Substrate specificity D: Effect of inhibitors on enzyme activity and stability E: Effect of EDTA and metal ions on enzyme activity and stability F: Effect of organic solvents on enzyme activity and stability.

To characterize the purified β-xylosidase enzyme for use in industrial processes, various parameters likes thermostability, pH stability, stability of enzyme in the presence of various inhibitors as well as organic solvents and metal ions were checked. Purified recombinant β-xylosidase enzyme was found to be stable upto 90°C and retained almost 75% of its activity after 4 hours of incubation at 60°C as shown in fig.3A. This is the most important characteristic of the enzyme which makes it a better candidate for the saccharification of plant biomass. Our results are in accordance to the Bhalla *et al*. (2014) who reported a recombinant thermostable β-xylosidase from *Geobacillus* sp. into *E. coli* which was stable at 50 – 70ºC. However, Lama *et al*. (2004) and Saha (2003) studied β-xylosidases from *Bacillus thermantarcticus* and *Fusarium proliferatum* which were stable at 60ºC for 1 hour and at 50ºC for 30 min, respectively. pH stability of the β-xylosidase enzyme was checked at a broad pH range (3-8) and it was found from the results that enzyme showed good stability even after 4 hours upto pH 8 which makes it a better choice for industrial use. Quintero *et al*. (2007) reported a β-xylosidase enzyme from *Geobacillus pallidus* which was stable at wide range of pH (3-9). However, a recombinant β-xylosidase from *Paenibacillus woosongensis* into *E. coli* was reported by Kim and Yoon (2010) which was stable at pH 6 – 8 and showed residual 76% activity while Shi *et al*. (2013) reported a recombinant β-xylosidase from *Thermotoga thermarum* which was stable at pH 5.0 – 7.5.

The purified recombinant β-xylosiadse enzyme gave maximum activity with 4-nitrophenyl β-xylopyranoside among various other substrates checked as shown in fig. 3C. In previous studies, β-xylosidases of *Paenibacillus woosongensis*, *Thermoanaerobacter ethanolicus* and *Aeromonas caviae* were also reported to exhibit maximal activity against 4-nitrophenyl β-xylopyranoside (Kim and Yoon, 2010; Shao and Wiegel, 1992 and Suzuki *et al*., 2001) but Kirikyali *et al*. (2014) expressed a recombinant β-xylosidase in *Pichia pastoris* from *Aspergillus oryzae* which showed maximum activity against 4-nitrophenyl β-xylopyranoside but also gave some activity against 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl-α-L-arabinofuranoside. Various inhibitors like SDS, DMSO, NaN3, β-mercaptoethanol, DTT and Tween 80 were used to checked the stability of cloned β-xylosidase enzyme against inhibitors. The enzyme was not only found to be stable in the presence of all these inhibitors but enzyme activity was increased to 118% and 129% in the presence of 1% β-mercaptoethanol and DTT, respectively, as shown in fig. 3D. It is reported in various studies that β-xylosidase from *Paecilomyces thermophila*, *Aspergillus oryzae* and *Bacillus thermantarcticus* was slightly inhibited by SDS and β-mercaptoethanol (Yan *et al*., 2008; Kirikyali *et al*., 2014; Lama *et al*., 2004). However, Lama *et al*. (2004) reported 100% stability of β-xylosidase from *Bacillus thermantarcticus* in the presence of DTT.

Organic solvents did not considerably affect the enzyme activity and results showed that recombinant β-xylosidase enzyme is very good stable in the presence of various organic solvents even at very high concentration (40%) as shown in fig. 3F. The enzyme showed upto 60% activity in the presence of 40% methanol, isopropanol, ethanol, acetone and n-butanol. Effect of different metal ions and EDTA was also determined on purified recombinant β-xylosidase enzyme by using various concentrations (1 to 10 mM) of different metal ions and EDTA. Enzyme showed very good stability against most of the metal ions even at higher concentration and enzyme activity was also found to be increased (119%) in the presence 10 mM Mg^{+2} as shown in fig. 3E. EDTA did not affect the activity of the enzyme. These results are in accordance to the Yan *et al*. (2008), Lama *et al*. (2004) and Kirikyali *et al*. (2014) who reported β-xylosidases from *Paecilomyces thermophila*, *Bacillus thermantarcticus* and *Aspergillus oryzae*, respectively. On the basis of the all these studies, it is suggested that this recombinant purified β-xylosidase enzyme from *Bacillus licheniformis* ATCC 14580 could be a potential candidate for use in many industrial applications, particularly in saccharification of plant biomass due to its adaptive capability to withstand unreceptive conditions used in industrial methods.

CONCLUSIONS

A recombinant thermostable β-xylosidase enzyme from *Bacillus licheniformis* was purified by using ammonium sulphate precipitation and immobilized metal ion affinity chromatography to homogeneity and further characterized for use in various industrial applications. The recombinant enzyme showed most of its activity toward p-nitrophenyl-xylopyranoside and showed tolerance to high temperature and pH. It was also stable in the presence of various organic solvents, showed resistance to the inhibitors and did not considerable affected by metal ions as well as EDTA. These characteristics of recombinant β-xylosidase enzyme nominated it as a potential candidate for industrial applications.

REFERENCES

Aftab MN, Zafar A and Awan AR (2017). Expression of thermostable β-xylosidase in *Escherichia coli* for use in saccharification of plant biomass. *Bioengineered*, **8**(5): 665-669.

Ahmed S, Riaz S and Jamil A (2009). Molecular cloning of fungal xylanases: An overview. *Appl. Microbiol. Biotechnol*., **84**(1): 19-35.

Baba T, Shinke R and Nanmori T (1994). Identification and characterization of clustered genes for thermostable xylan-degrading enzymes, beta-xylosidase and xylanase, of *Bacillus stearothermophilus* 21. *Appl. Environ. Microbiol.*, **60**(7): 2252-2258.

Bhalla A, Bischoff KM and Sani RK (2014). Highly thermostable GH39 β-xylosidase from a *Geobacillus* sp. strain WSUCF1. *BMC Biotechnol*., **14**(1): 963.

Biely P (1985). Microbial xylanolytic systems. *Trends Biotechnol*., **3**(11): 286-290.

Bimboim H and Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*., **7**(6): 1513-1523.

Cohen SN, Chang AC and Hsu L (1972). Nonchromosomal antibiotic resistance in bacteria:

genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Nat. Acad. Sci*., **69**(8): 2110-2114.

Cosgrove DJ (1997). Assembly and enlargement of the primary cell wall in plants. *Annu. Rev. Cell Dev. Biol*., **13**(1): 171-201.

Coughlan M and Hazlewood GP (1993). Beta 1, 4-D-xylan-degrading enzyme systems: Biochemistry, molecular biology and applications. *Biotechnol. Appl. Biochem*., **17**(3): 259-289.

da Silva AE, Marcelino HR, Gomes MCS, Oliveira EE, Nagashima Jr T and Egito EST (2012). Xylan, a promising hemicellulose for pharmaceutical use *Products and Applications of Biopolymers*: In Tech.

de Moraes LM, Astolfi Filho Sand Ulhoa CJ (1999). Purification and some properties of an α-amylase glucoamylase fusion protein from *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol*., **15**(5): 561-564.

Dekker RF (1983). Bioconversion of hemicellulose: Aspects of hemicellulase production by *Trichoderma reesei* QM 9414 and enzymic saccharification of hemicellulose. *Biotechnol. Bioeng*., **25**(4): 1127-1146.

Dornez E, Gebruers K, Cuyvers S, Delcour JA and Courtin CM (2007). Impact of wheat flour-associated endoxylanases on arabinoxylan in dough after mixing and resting. *J. Agri. Food Chem*., **55**(17): 7149-7155.

Ebringerova A (2005). Structural diversity and application potential of hemicelluloses. Paper presented at the Macromolecular symposia.

Gramany V, Khan FI, Govender A, Bisetty K, Singh S and Permaul K (2016). Cloning, expression and molecular dynamics simulations of a xylosidase obtained from *Thermomyces lanuginosus*. *J. Biomol. Struc. Dyna*., **34**(8): 1681-1692.

Jarboe LR, Hyduke DR, Tran LM, Chou KJ and Liao JC (2008). Determination of the *Escherichia coli* S-nitrosoglutathione response network using integrated biochemical and systems analysis. *J. Biologic. Chem*., **283**(8): 5148-5157.

Juturu V and Wu JC (2012). Microbial xylanases: engineering, production and industrial applications. *Biotechnol. Adv*., **30**(6): 1219-1227.

Khasin A, Alchanati I and Shoham Y (1993). Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. *Appl. Environ. Microbiol*., **59**(6): 1725-1730.

Kim YA and Yoon KH (2010). Characterization of a *Paenibacillus woosongensis* β-xylosidase/α-arabinofuranosidase produced by recombinant *Escherichia coli*. *J. Microbiol. Biotechnol*., **20**(12): 1711-1716.

Kirikyali N, Wood J and Connerton IF (2014). Characterization of a recombinant β-xylosidase (xylA) from *Aspergillus oryzae* expressed in *Pichia pastoris*. *AMB Express*, **4**(1): 68.

Korner C (2003). Carbon limitation in trees. *J. Ecol*., **91**(1): 4-17.

Kronstad J, Schnepf H and Whiteley H (1983). Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bacteriol*., **154**(1): 419-428.

Kulkarni N, Shendye A and Rao M (1999). Molecular and biotechnological aspects of xylanases. *FEMS Microbiol. Rev*., **23**(4): 411-456.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**(5259): 680.

Lama L, Calandrelli V, Gambacorta A and Nicolaus B (2004). Purification and characterization of thermostable xylanase and β-xylosidase by the thermophilic bacterium *Bacillus thermantarcticus*. *Res. Microbiol*., **155**(4): 283-289.

Lee H, Biely P, Latta RK, Barbosa MF and Schneider H (1986). Utilization of xylan by yeasts and its conversion to ethanol by *Pichia stipitis* strains. *Appl. Environ. Microbiol*., **52**(2): 320-324.

Malherbe S and Cloete TE (2002). Lignocellulose biodegradation: Fundamentals and applications. *Rev. Environ. Sci. Biotechnol*., **1**(2): 105-114.

Manzanares P, Ramon D and Querol A (1999). Screening of non-Saccharomyces wine yeasts for the production of β-D-xylosidase activity. *Int. J. Food Microbiol*., **46**(2): 105-112.

Marques AR, Coutinho PM, Videira P, Fialho AMand Sa-Correia I (2003). *Sphingomonas paucimobilis* beta-glucosidase Bgl1: A member of a new bacterial subfamily in glycoside hydrolase family 1. *Biochemic. J*., **370**(Pt 3): 793.

Nanmori T, Watanabe T, Shinke R, Kohno A and Kawamura Y (1990). Purification and properties of thermostable xylanase and beta-xylosidase produced by a newly isolated *Bacillus stearothermophilus* strain. *J. Bacteriol*., **172**(12): 6669-6672.

Peij NN, Brinkmann J, Vrsanska M, Visser J and Graaff LH (1997). B-Xylosidase Activity, Encoded by xlnD, is Essential for Complete Hydrolysis of Xylan by *Aspergillus Niger* but not for Induction of the Xylanolytic Enzyme Spectrum. *FEBS J*., **245**(1): 164-173.

Perez-Gonzalez JA, van Peij NN, Bezoen A, Maccabe AP, Ramon D and de Graaff LH (1998). Molecular cloning and transcriptional regulation of the *Aspergillus nidulans* xlnD gene encoding a β-xylosidase. *Appl. Environ. Microbiol*., **64**(4): 1412-1419.

Polizeli M, Rizzatti A, Monti R, Terenzi H, Jorge JA and Amorim D (2005). Xylanases from fungi: Properties and industrial applications. *Appl. Microbiol. Biotechnol*., **67**(5): 577-591.

Pontonio E, Mahony J, Di Cagno R, Motherway MOC, Lugli GA, O'Callaghan A, De Angelis M, Ventura M, Gobbetti M and van Sinderen D (2016). Cloning, expression and characterization of a β-D-xylosidase from *Lactobacillus rossiae* DSM 15814 T. *Microb. Cell Fact*., **15**(1): 72.

Quintero D, Velasco Z, Hurtado-Gomez E, Neira JL and Contreras LM (2007). Isolation and characterization of a thermostable β-xylosidase in the thermophilic bacterium *Geobacillus pallidus*. *BBA-Proteins and Proteomics*, **1774**(4): 510-518.

Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, Zaretsky EJ, Tang M, de Leon AL, Xiang H, Gusti V and Clausen IG (2004). Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related Bacillus species. *Genome Biol*., **5**(10): r77.

Saha BC (2003). Purification and properties of an extracellular β-xylosidase from a newly isolated *Fusarium proliferatum*. *Biores. Technol*., **90**(1): 33-38.

Sambrook J and Russell DW (2001). Molecular cloning: A laboratory manual. 2001: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Shao W and Wiegel J (1992). Purification and characterization of a thermostable beta-xylosidase from *Thermoanaerobacter ethanolicus*. *J. Bacteriol*., **174**(18): 5848-5853.

Sumarsih S (2015). Expression of b-xylosidase encoding gene in PHIS/*Bacillus megaterium* MS system.*Indonesian J. Tropic. Infec. Dise*., **2**(1): 25-29.

Sunna A and Antranikian G (1996). Growth and production of xylanolytic enzymes by the extreme thermophilic anaerobic bacterium *Thermotoga thermarum*. *Appl. Microbiol. Biotechnol*., **45**(5): 671-676.

Suryani, Kimura T, Sakka, K and Ohmiya K (2004). Sequencing and expression of the gene encoding the *Clostridium stercorarium* β-xylosidase Xyl43B in *Escherichia coli*. *Biosci. Biotechnol. Biochem*., **68**(3): 609-614.

Suzuki T, Kitagawa E, Sakakibara F, Ibata K, Usui K and Kawai K (2001). Cloning, expression, and characterization of a family 52 β-xylosidase gene (xysB) of a multiple-xylanase-producing bacterium, *Aeromonas caviae* ME-1. *Bio Sci. Biotechnol. Biochem*., **65**(3): 487-494.

Tsujibo H, Takada C, Tsuji A, Kosaka M, Miyamoto K and Inamori Y (2001). Cloning, sequencing and expression of the gene encoding an intracellular β-D-xylosidase from *Streptomyces thermoviolaceus* OPC-520. *Biosci. Biotechnol.Biochem*., **65**(8): 1824-1831.

Umemoto Y, Onishi R and Araki T (2008). Cloning of a novel gene encoding β-1, 3-xylosidase from a marine bacterium, *Vibrio* sp. strain XY-214, and characterization of the gene product. *Appl. Environ. Microbiol*., **74**(1): 305-308.

Wagschal K, Franqui-Espiet D, Lee CC, Kibblewhite-Accinelli RE, Robertson GH and Wong DW (2007). Genetic and biochemical characterization of an α-L-arabinofuranosidase isolated from a compost starter mixture. *Enzyme Microb. Technol*., **40**(4): 747-753.

Waino M and Ingvorsen K (2003). Production of β-xylanase and β-xylosidase by the extremely halophilic archaeon *Halorhabdus utahensis*. *Extremophiles*, **7**(2): 87-93.

Yan Q, Wang L, Jiang Z, Yang S, Zhu H and Li L (2008). A xylose-tolerant β-xylosidase from *Paecilomyces thermophila*: characterization and its co-action with the endogenous xylanase. *Biores. Technol*., **99**(13): 5402-5410.

Zafar M, Ahmed S, Khan MIM and Jamil A (2014). Recombinant expression and characterization of a novel endoglucanase from *Bacillus subtilis* in *Escherichia coli*. *Mol. Bio. Rep*., **41**(5): 3295-3302.