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Purification and characterization of β-D-xylosidase from *Lactobacillus brevis* grown on xylo-oligosaccharides

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ABSTRACT

In the recent years there has been a growing interest in the use of oligosaccharides as prebiotics to modulate gut microbiota with an aim to improve the gut health. Though xylo-oligosaccharides (XOS) have been increasingly used as prebiotics, information pertaining to the enzymes used by lactobacilli to degrade these substrates is scanty. Present investigation reports the purification and characterization of β-D-xylosidase from *Lactobacillus brevis* NCDC01 grown on XOS. Three sequential steps consisting of ultra-filtration, DEAE cellulose ion-exchange and Sephacryl S-100 gel filtration chromatographies were employed to purify the enzyme to apparent homogeneity and it was found to be monomeric on SDS-PAGE with an apparent molecular mass of ~58.0 kDa. The pH and temperature optima were 6.0 and 40 °C respectively. The enzyme remained stable over a pH range of 5.5–7.5 and up to 50 °C for 30 min. Under optimum pH and temperature with p-nitrophenyl β-D-xylopyranoside as a substrate, the enzyme exhibited a K_m of 0.87 mM. The enzyme does not require any metal ion for activity or stability but is completely inhibited by Hg^{2+}, Pb^{2+}, p-chloromercuribenzoate (PCMB), oxalic acid and citric acid. This is perhaps the first report on the purification and characterization of β-D-xylosidase from *Lactobacillus brevis* NCDC01.

1. Introduction

Many of the oligosaccharides are not digestible in the small intestine but get fermented in the large intestine into short chain fatty acids (SCFA) and in turn they increase the beneficial bacterial population such as lactobacilli and bifidobacteria. The use of prebiotics (indigestible food components that promote the growth of beneficial bacteria) and probiotics (live bacteria that exert health beneficial effects upon consumption) as safe and effective agents that help in regulating the gut environment is rapidly gaining importance in scientific and industrial research. Xylo-oligosaccharides (XOS) are oligomers of 2–10 xylose residues linked together by β (1–4) linkage and are produced by the hydrolysis of xylan, the major component of plant hemicelluloses. XOS have attracted the attention of researchers in terms of resistance to both acids and temperature allowing their utilization in low pH juices and carbonated beverages (Modler, 1994; Wang, Sun, Cao, Tian, & Wang, 2009). Both *in vitro* and *in vivo* studies have shown them to be capable of imparting prebiotic effect by selectively stimulating the growth of bifidobacteria and lactobacilli in the colon (Campbell, Fahey, & Wolf, 1997; Jaskari et al., 1998; Wang, Sun, Cao, & Wang, 2010). Fermentation of XOS in colon results in increase in the production of SCFA such as acetic acid, propionic acid and butyric acid (Younes, Garleb, Behr, Remesy, & Demigne, 1995), decrease in the ceacal pH (Campbell et al., 1997) and in the number of aberrant crypt foci in the colon (Hsu, Liao, Chung, Hsieh, & Chan, 2004). In addition, XOS possess an array of biological activities such as antioxidant activity (Yuan, Wang, & Yao, 2004), antibacterial property (Christakopoulos et al., 2003), immunomodulatory action (Ebringerova, Kardosova, Hromadkova, Malovıkova, & Hrıbalova, 2002) and antidiabetic potential (Gobinath, Madhu, Prashant, Srinivasan, & Prapulla, 2010). Among the Lactobacillus species *Lactobacillus brevis* is reported to utilize XOS most effectively (Crittenden et al., 2002; Moura et al., 2007; Garde, Jonsson, Schmidt, & Ahring, 2002).

Utilization of XOS by the microorganism generally requires the action of three important enzymes, namely, β-xylosidases (EC 3.2.1.37) that cleave terminal xylose residue from the non-reducing end of short chain XOS, reducing end xylose-releasing exo-oligoxylanases (EC 3.2.1.156) and α-L-arabinofuranosidases (EC 3.2.1.55) that cleaves side chain arabinose substituents. There are few reports on the purification of β-xylosidases and α-L-arabinofuranosidases from bifidobacteria which include glycoside hydrolase family 51 (GH51) β-xylosidases from *Bifidobacterium breve* K-110 (Shin et al., 2003), GH8 reducing end xylose releasing exo-oligoxylanase, two β-xylosidases from

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Bifidobacterium adolescentis (Lagaert et al., 2007, 2011), α-L-arabinofuranosidase from *Bifidobacterium longum* B667 (Margolles & De Los Reyes-Gavilán, 2003) and arabinoxylan arabinofuranohydrolases from *Bifidobacterium adolescentis* DSM20083 (Van Laere et al., 1999). However there are no reports on the purification and characterization of β-xylosidase from any of the lactobacillus species.

Even though some information is available about the mechanism by which bifidobacteria utilize XOS the same is not true with respect to lactobacilli despite the commercial interest in this group of gram-positive bacteria. In order to understand the mechanism of XOS utilization by *L. brevis* we have purified and characterized the major xylanolytic enzyme produced by *L. brevis* NCDC01, grown on XOS as the sole carbon source. Our results provide an insight to the properties of β-D-xylosidase from *L. brevis* and its possible action on XOS thus helps to comprehend the metabolism of XOS by lactobacillus sps.

2. Materials and methods

2.1. Substrates and chemicals

p-Nitrophenyl (*p*NP) substrates, DEAE-cellulose, Sephacryl S-100, Ethylenediamine (EDTA), termamyl from *Bacillus licheniformis*, glucoamylase from *Aspergillus niger*, xylanase from *Thermomyces lanuginosus* and other chemicals were purchased from Sigma Chemical (St Louis, USA). Wheat bran (*Triticum aestivum*) was obtained from the local market. Protein molecular weight markers were obtained from Genie, Bangalore, India. Microbiological culture media and media ingredients were obtained from HiMedia, Mumbai, India. All other chemicals and solvents were of analytical grade.

Culture and maintenance: *L. brevis* NCDC01 was obtained from National Collection of Dairy Cultures, NDRI, Karnal, India and maintained on lactobacillus MRS broth (HiMedia, Mumbai, India).

2.2. Preparation of xylo-oligosacharides

Wheat bran (10 g) was suspended in sodium acetate buffer (100 ml, 50 mM, pH 4.8) and incubated with termamyl (~100 units/g of wheat bran) at 95 °C for 1 h. After cooling, glucoamylase (~1000 units/g) was added and incubated at 55 °C for 48 h to remove associated starch, if any. Destarched wheat bran (1 g) was taken in 50 ml acetate buffer (pH 4.8, 0.1 M) and incubated with xylanase (250 U/g of destarched wheat bran) at 50 °C for 3 h in a constant shaking water bath at 100 rpm. The reaction was stopped by adding three volumes of ethanol and the precipitated undegraded polysaccharides were removed by centrifugation at 3000 × *g* for 20 min. In order to remove monosaccharides from the crude XOS mixture it was concentrated and passed through charcoal-celite column (3.0 cm × 20 cm) (Whistler & Durso, 1950) and the monosaccharides were eluted with 700 ml of 1% (v/v) ethanol. The adsorbed oligosaccharides (XOS) was eluted with 600 ml of 50% (v/v) ethanol, concentrated and used as substrates for the growth of microorganisms.

2.3. Enzyme activity assay

The enzyme activity of β-D-xylosidase was determined by assaying the amount of p-Nitrophenol (*p*NP) released from p-Nitrophenyl-β-D-xylopyranoside (*p*NPX) at 37 ± 1 °C. The reaction mixture consisting of 5 mM pNPX in 50 mM phosphate buffer (900 μl, pH 5.7) was incubated with the 100 μl enzyme for 10 min in a total volume of 1.1 ml. The reaction was stopped by the addition of 100 μl saturated solution of sodium tetraborate and the amount of *p*NP released was determined by measuring the absorbance spectrophotometrically at 410 nm. One unit (U) of enzyme activity was defined as the amount of enzyme which liberated one micromole (μM) of *p*NP per minute at assay conditions. Specific activity was defined in terms of units per mg protein.

2.4. Enzyme purification

2.4.1. Culture conditions

L. brevis NCDC01 was precultured in MRS broth at 37 °C for 18 h without shaking and used for inoculum preparation. *L. brevis* NCDC01 inoculum (10^5 cfu/ml) was added to 500 ml MRS broth medium (dextrose was replaced with 0.5% (w/v) XOS) taken in a conical flask and incubated at 37 °C for 48 h.

2.4.2. Purification

Cells were harvested from the culture medium by centrifugation at 3000 × *g* for 20 min at 4 °C. The supernatant was concentrated to 10 ml, dialyzed; β-D-xylosidase activity was measured and designated as extracellular β-D-xylosidase activity. The pellet was washed twice with 50 mM sodium phosphate buffer pH 7.0 and the enzyme was extracted as per Wang, Geng, Egashira, and Sanada (2004). To the pellet lysis solution consisting of 50 mM sodium phosphate buffer (10 ml, pH 7.0) containing 0.9% NaCl and 2 mM (±)-dithiothreitol (DTT) and 1.3 mg of lysozyme (from chicken egg white) was added. After incubation at 37 ± 1 °C for 15 min with shaking in a water bath at 100 rpm, ultrasonication was carried out in ice for 60 s. The cell debris was removed by centrifugation (3000 × *g*, 20 min), the supernatant was used as crude extract, enzyme activity measured and designated as intracellular β-D-xylosidase activity. The crude extract was further concentrated and purified by ultrafiltration through a 10 kDa MWCO ultrafiltration membrane and the retained material was dialyzed against 20 mM phosphate buffer (pH 7.0). The dialysate was filtered through a 0.22 μm syringe filter and loaded to a DEAE cellulose column which was prequilibrated with 20 mM phosphate buffer (pH 7.0). The column was washed with the equilibrating buffer to remove any unbound proteins. The bound proteins were fractionated using a linear gradient of NaCl (0–0.5 M) in equilibrating buffer and monitored for protein concentration as well as β-D-xylosidase activity. The fractions containing β-D-xylosidase activity were pooled, dialyzed against 20 mM phosphate buffer (pH 7.0) and concentrated using a SpeedVac Concentrator (Sigma). The concentrated sample was further purified on Sephacryl S-100 column (0.9 cm × 90 cm) previously equilibrated with 20 mM phosphate buffer (pH 7.0) at a flow rate of 9.2 ml/h. Fractions showing β-D-xylosidase activity were pooled, dialyzed against 20 mM phosphate buffer (pH 7.0) and concentrated. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. The homogeneity of the purified enzyme was evaluated by native polyacrylamide gel electrophoresis (native-PAGE) as described by Laemmli (1970).

2.5. Zymogram analysis and molecular weight determination

Zymogram analysis was performed on native-PAGE polyacrylamide gel as per Bachmann and McCarthy (1991). After electrophoresis, the gel was washed with one volume of 50 mM Tris/HCl buffer and sandwiched to a 1% (w/v) agarose gel containing 10 mM pNPX, supported on a gel bound, sealed with cling and incubated at 37 °C for 1 h. Enzyme activity was detected as yellow band resulting from the release of pNP by β-xylosidase.

Molecular weight of the purified β-D-xylosidase was determined by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) using 12% running gel (25). The molecular weight markers (Genie) used as standard are phophorylase b (97.0 kDa),

Table 1
Summary of purification of β-D-xylosidase from *Lactobacillus brevis* NCDC01.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude enzyme	6.30	12.3	1.9	100	1
Ultrafiltration	4.60	11.2	2.4	91.0	1.26
DEAE cellulose	0.12	1.26	10.5	10.2	5.52
Sephacryl S-100	0.008	0.3	37.5	2.4	19.7

bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa) and aprotinin (6.5 kDa). The proteins were visualized by staining with silver stain.

2.6. Effects of pH, temperature, different metal ions and inhibitors on β-D-xylosidase activity

The optimum pH of purified β-D-xylosidase was determined with *p*NPX in different pH with an incremental level of 0.5 using 0.05 M sodium acetate buffer from 4 to 6, phosphate buffer from pH 6 to 7.5 and Tris–HCl buffer from pH 7.5 to 9. To determine pH stability, the purified β-D-xylosidase was incubated in the above mentioned buffers for 30 min at 37 ± 1 °C and the residual activity was determined. The original activity was taken as 100% and the relative activity was plotted against different pH.

The optimum temperature was determined by incubating the purified enzyme in 50 mM phosphate buffer (pH 6) at different temperatures (30–70 °C) at 5 °C interval. The thermostability was monitored by pre-incubating the enzyme without the substrate in 50 mM phosphate buffer (pH 6) at different temperatures for 30 min followed by addition of the substrate and measurement of the residual activity. The original activity was taken as 100% and the relative activity was plotted against different temperatures.

The influence of various metals ions ($CaCl_2$, $MgCl_2$, $MnCl_2$, KCl, $NiCl_2$, $PbCl_2$, $FeCl_3$ and $HgCl_2$) on β-D-xylosidase activity was investigated under standard assay conditions in 5 mM concentration and compared to the control (without metal ions). The enzyme activity control was taken as 100%. The effect of $HgCl_2$ on enzyme activity was studied in the concentrations range of 1.0–6.0 mM.

The effect of inhibitors such as DTT, EDTA, p-chloromercuribenzoate (PCMB), citric acid, boric acid and oxalic acid on β-D-xylosidase activity was determined in the concentration range of 2–10 mM. The purified enzyme was pre-incubated with the respective inhibitor for 1 h at 4 °C, followed by the standard enzyme assay as described above. The relative activity assayed in the absence of the protein inhibitors was taken as 100%.

2.7. Determination of kinetic parameters and specificity of β-D-xylosidase

To determine the kinetic parameters, the purified enzyme was incubated with various concentrations of pNPX (ranging from 0.2 to 1 mM) at the optimum assay conditions. The initial velocities were determined and used to estimate K_m and V_{max} from Lineweaver–Burk plot.

The substrate specificity of β-D-xylosidase was determined by using p-nitrophenyl derivatives and measured by the rate of p-Nitrophenol liberated during hydrolysis from 2 mM of the substrates in 50 mM phosphate buffer (pH 6.0) at 37 ± 1 °C for 10 min and detected spectrophotometrically at 410 nm.

2.8. Determination of nature of β-D-xylosidase

XOS (containing xylobiose and xylotriose) in 10 mg/ml concentration was incubated with 0.1 U/ml purified β-D-xylosidase in 50 mM phosphate buffer (pH 6.0) at 40 °C. Aliquots were withdrawn at every 30 min for 2 h, the reaction was stopped by boiling for 10 min and the hydrolysis products were analyzed by Thin layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany). The TLC plates were developed at room temperature with a solvent system of acetone, 1-butanol and water (8:1:1, v/v) and the spots were visualized as dark blue spots by spraying the plates with sulfuric acid–methanol–orcinol reagent and heating at 100 °C for 5 min.

2.9. Statistical analysis

All the experiments were performed in triplicates and the values were represented as mean values ± standard deviation (SD).

3. Results and discussion

In the current study β-D-xylosidase from *L. brevis* NCDC01 grown on XOS was purified and characterized. The cellular distribution of β-D-xylosidase was investigated in the cultures of *L. brevis* NCDC01. The activity of the enzyme was found to be higher (12.3 U) in the dialyzed cell free extract prepared by ultrasonication of cell pellets in lysis solution (intracellular β-D-xylosidase) as compared to the culture supernatant (1.1 U, β-D-xylosidase). Intracellular β-D-xylosidase was purified as summarized in Table 1. The crude enzyme was initially concentrated by ultrafiltration and followed by successive steps of purification using anion exchange and gel filtration chromatographies (Fig. 1a and b) resulting in the pure enzyme with an yield and fold purification of 2.4% and 19.7 respectively (Table 1). The identity of the purified enzyme as β-D-xylosidase was confirmed by Native PAGE as well as zymogram overlay analysis (Fig. 2b). The molecular weight of the enzyme was estimated to be ~58.0 kDa based on its mobility on SDS-PAGE and the enzyme was found to be monomeric. Most of the bacterial β-D-xylosidases are large dimeric or trimeric proteins with a molecular mass more than 100 kDa (Bachmann & McCarthy, 1991; Lee & Forsberg, 1987; Shao & Wiegel, 1992; Shao et al., 2011). However monomeric β-D-xylosidases with molecular mass below 100 kDa have also been reported (Ohta, Fujimoto, Fujii, & Wakiyama, 2010; Saxena, Fierobe, Gaudin, Guerlesquin, & Belaich, 1995; Shin et al., 2003). The apparent molecular weight of β-D-xylosidase of *L. brevis* NCDC01 in the present study is higher than the ones reported from *Bifidobacterium breve* K110 ((49 kDa), Shin et al., 2003) and *Clostridium cellulolyticum* ((43 kDa) Saxena et al., 1995).

The pH activity and stability curves of the purified enzyme are shown in Fig. 3a. The enzyme showed optimal activity at pH 6.0 and was stable in the pH range of 5.5–7.5. The enzyme remained stable at pH 6.0 for 48 h, thereafter lost its activity (data not shown). Above pH 7.0 the activity of the enzyme decreased and was completely lost at pH 9.0 and above. Most of the carbohydrate degrading enzymes have acidic amino acids such as aspartic acid and glutamic acid along with histidine in their active site pocket and known to exhibit similar type of pH profiles. The temperature optimum for the enzyme activity was found to be 40 °C (Fig. 3b) and it remained stable up to 50 °C for 30 min. Above 50 °C the enzyme rapidly lost its activity. This is in tune with the temperature optima and stability reported for several carbohydrate degrading enzymes. The enzyme exhibited apparent K_m and V_{max} values of 0.87 mM and 0.14 μmoles/min respectively (Fig. 4). The Arrhenius plot was

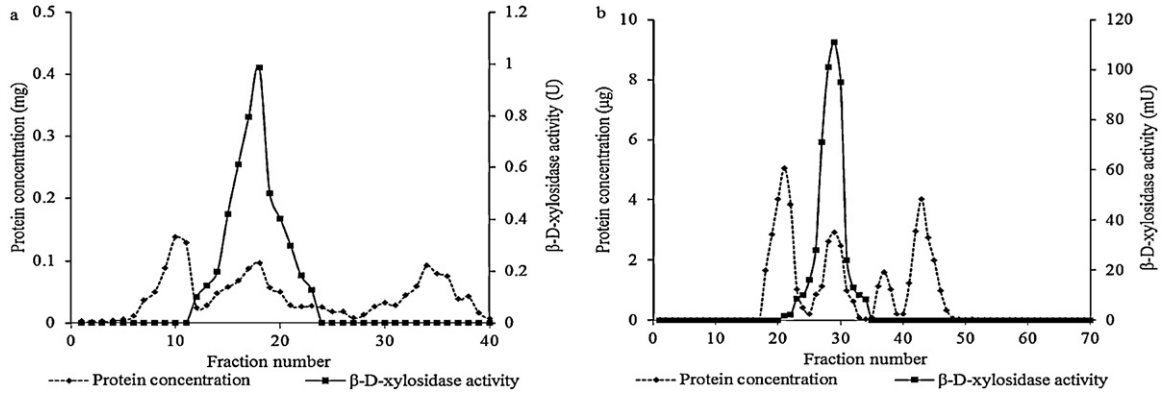

Fig. 1. Elution profiles of the β-D-xylosidase from *L. brevis* NCDC01 on DEAE-cellulose anion exchange (a) and Sephacryl S-100 gel filtration (b).

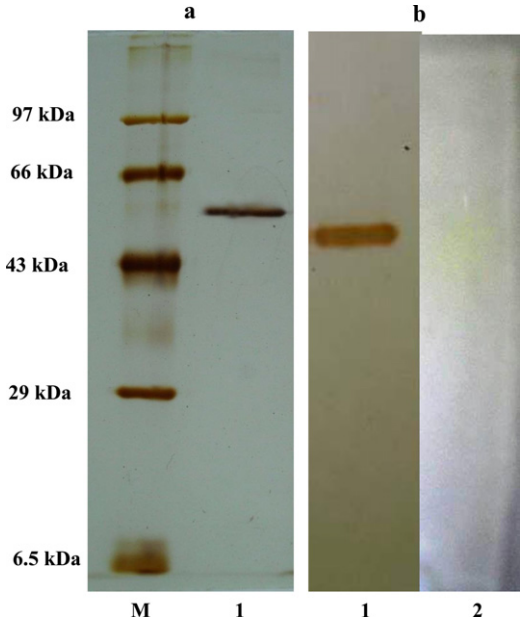

Fig. 2. (a) SDS-PAGE (12%) of the fractions obtained during the purification. Lane M, marker proteins with relative molecular masses indicated on the right; lane 1, purified β-D-xylosidase (b) 1, Native PAGE of purified β-D-xylosidase; 2, zymogram of purified β-D-xylosidase.

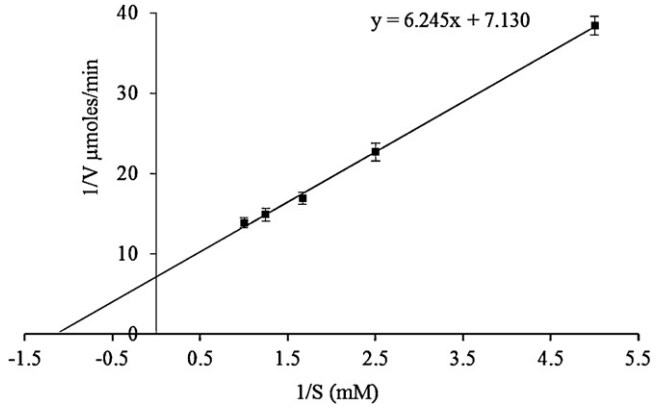

Fig. 4. Lineweaver–Burk plot for K_m and V_{max} values of the purified β-D-xylosidase in the presence of different concentrations of *p*NP-β-D-xylopyranoside. Data are given as means ± SD, $n = 3$.

linear over the range of 30–50 °C and activation energy (E_a) was found to be 28 kJ mol^{-1} (Fig. 5).

The effect of various metals ions on β-D-xylosidase activity was tested and the results are shown in Table 2. Ca^{2+} enhanced β-D-xylosidase activity by 26%. Positive effect of Ca^{2+} on enzyme activity may be attributed to the capacity of Ca^{2+} to form co-ordinate bonds with active site amino acids such as glutamic acid and aspartic acid. Hg^{2+} and Pb^{2+} inhibited 97 and 39% of xylosidase activity respectively. Other metal ions did not have any significant effect on xylosidase activity. EDTA, DTT and idoacetamide did not show any significant effect on enzyme activity. Similar result was reported for β-D-xylosidase, purified from *Bacillus thermantarcticus* (Lama, Calandrelli, Ganbacorta, & Nicolaus, 2004). PCMB and boric acid inhibited 78 and 58% of xylosidase activity respectively at 10 mM concentration. At similar concentration citric acid and oxalic acids completely inhibited the enzyme and proved to be more

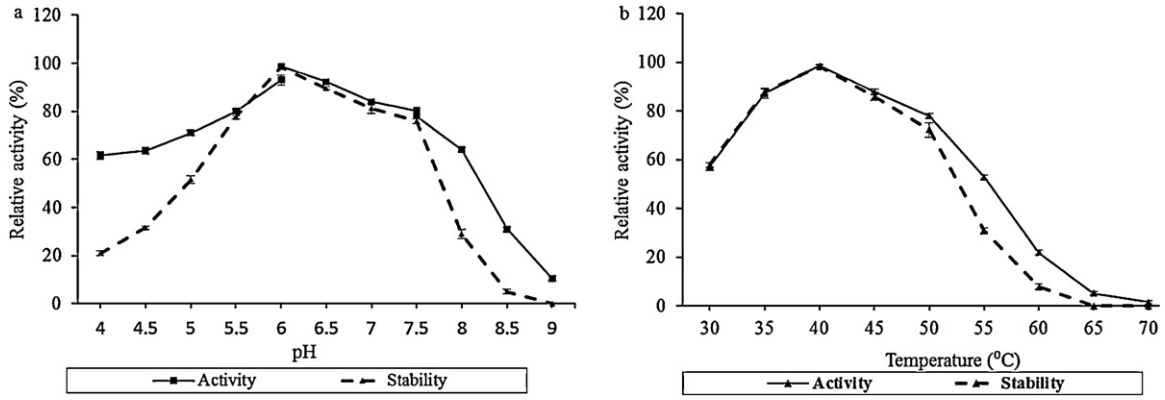

Fig. 3. Effects of different pH (a) and temperatures (b) on β-D-xylosidase activity and stability. Data are given as means ± SD, $n = 3$.

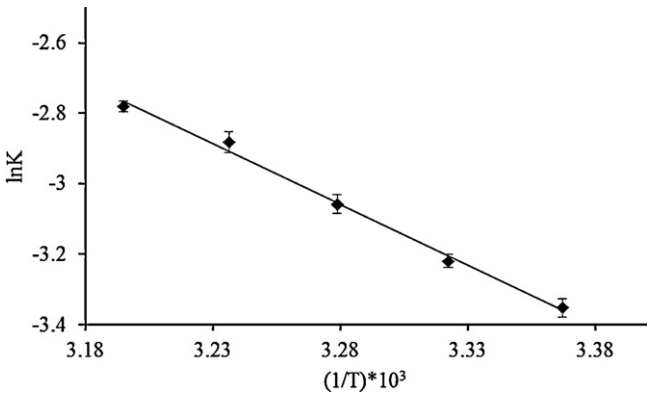

Fig. 5. Arrhenius plot. Reaction conditions: β-D-xylosidase activity at different temperatures (24–40 °C) with interval of 4 °C, *p*NP-β-D-xylopyranoside as substrate. Data are given as means ± SD, $n = 3$.

Table 2
Effect of different metal ions (5 mM) on the activity of purified β-D-xylosidase.

Metal ions	Relative activity (%)
None	100 ± 0[a]
Mg^{2+}	101 ± 2[a]
Mn^{2+}	101 ± 1[a]
K^{+}	100 ± 1[a]
Na^{+}	102 ± 1[a]
Ca^{2+}	126 ± 1[b]
Ni^{2+}	90 ± 2[c]
Pb^{2+}	65 ± 1[d]
Fe^{2+}	61 ± 1[e]
Hg^{2+} #	3 ± 1[f]

Data is given as means ± SD, $n = 3$; #: at 4 mM concentration; values not sharing common alphabets within the row are significantly different ($P < 0.05$).

effective inhibitors as compared to boric acid. The enzyme inhibition by Hg^{2+} and PCMB indicates the involvement of sulfhydryl groups in the enzyme activity as reported for β-D-xylosidase from the rumen anerobic fungus *Neocallimastix frontalis* (Hebraud & Fevre, 1990).

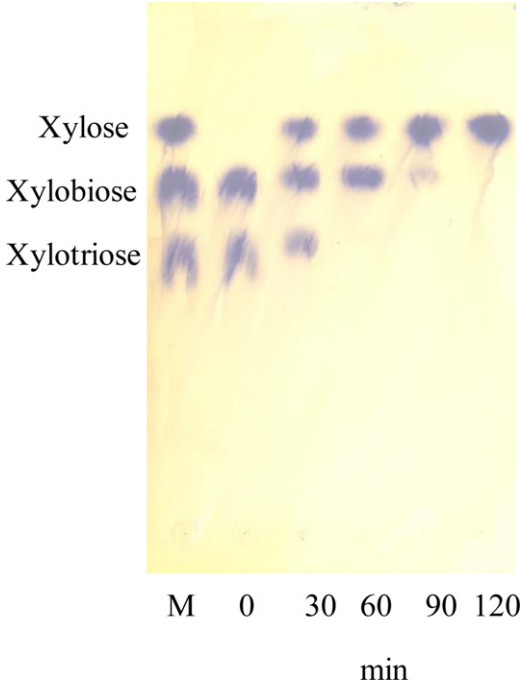

Fig. 6. TLC analysis of purified β-D-xylosidase treated xylo-oligosaccharides.

The enzyme showed high activity against *p*NP-β-D-xylopyranoside but did not have any activity on other *p*NP derivatives and birchwood xylan. Purified β-xylosidase treated XOS reaction products were determined by TLC with respect to time. The complete hydrolysis of XOS to xylose was achieved in 2 h (Fig. 6) indicating the enzyme to be an exo-xylosidase. Van Doorslaer, Kersters-Hilderson, and De Bruyne (1985) suggested that the affinity of xylosidases towards XOS decreases with the increase in degree of polymerization. β-D-xylosidases are usually unable to hydrolyze xylan however there are some reports of β-D-xylosidase which are able to attack xylan slowly to liberate xylose (Dekker & Richards, 1976). In general, arabinoxylans/XOS are preferred substrates for growth and fermentation than xylose or arabinose (Crittenden et al., 2002; Jaskari et al., 1998). Lambertus et al. (2005) suggest that these oligosaccharides might be first imported into the cell where they are hydrolyzed to their respective monosaccharides. Hence the presence of highly specific intracellular xylosidase might play a crucial role in facilitating the bacteria to utilize XOS.

4. Conclusion

This is perhaps the first report on the purification and characterization of β-D-xylosidase from *Lactobacillus brevis* NCDC01. We have purified a ~58.0 kDa β-D-xylosidase, which can completely hydrolyze XOS to xylose. This study provides an insight into the role of β-D-xylosidase in XOS metabolism. The presence of intracellular β-D-xylosidase in *L. brevis* suggests that XOS might be first imported into the cell by oligosaccharide transporters, followed by their degradation to xylose. Genomic studies are envisaged to understand in detail the metabolism of XOS by lactobacillus sp.

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