Extremophiles (2012) 16:135–146
DOI 10.1007/s00792-011-0413-0

ORIGINAL PAPER

# Biochemical characterization of a novel thermostable xyloglucanase from an alkalothermophilic *Thermomonospora* sp.

**Dipali Pol · Vishnu Menon · Mala Rao**

Received: 29 August 2011 / Accepted: 10 November 2011 / Published online: 26 November 2011


**Abstract** Xyloglucanase from an extracellular culture filtrate of alkalothermophilic *Thermomonospora* sp. was purified to homogeneity with a molecular weight of 144 kDa as determined by SDS-PAGE and exhibited specificity towards xyloglucan with apparent $K_m$ of 1.67 mg/ml. The enzyme was active at a broad range of pH (5–8) and temperatures (40–80°C). The optimum pH and temperature were 7 and 70°C, respectively. The enzyme retained 100% activity at 50°C for 60 h with half-lives of 14 h, 6 h and 7 min at 60, 70 and 80°C, respectively. The kinetics of thermal denaturation revealed that the inactivation at 80°C is due to unfolding of the enzyme as evidenced by the distinct red shift in the wavelength maximum of the fluorescence profile. Xyloglucanase activity was positively modulated in the presence of $Zn^{2+}$, $K^+$, cysteine, $\beta$-mercaptoethanol and polyols. Thermostability was enhanced in the presence of additives (polyols and glycine) at 80°C. A hydrolysis of 55% for galactoxyloglucan (GXG) from tamarind kernel powder (TKP) was obtained in 12 h at 60°C and 6 h at 70°C using thermostable xyloglucanases, favouring a reduction in process time and enzyme dosage. The enzyme was stable in the presence of commercial detergents (Ariel), indicating its potential as an additive to laundry detergents.

**Keywords** Alkalothermophilic *Thermomonospora* sp. · Xyloglucanase · Purification · Thermostable · Biotechnological applications

Communicated by S. Albers.

D. Pol · V. Menon · M. Rao (✉)
Division of Biochemical Sciences,
National Chemical Laboratory, Pune 411 008, India
e-mail: mb.rao@ncl.res.in

## Introduction

The desire for improved methods of biomass conversion to fuels and feedstocks has re-awakened interest in the enzymology of plant cell-wall degradation. Xyloglucan is one of the major structural hemicellulosic heteropolysaccharides present in the primary cell walls of higher plants, consisting of about 20–25% of the dry weight in dicotyledonous angiosperms, 2–5% in grasses and 10% in soft woods. The xyloglucans form a complex network of hydrogen-bonded interactions with cellulose microfibrils that confers rigidity and extensibility to cell walls (Martinez-Fleites et al. 2006). Structurally xyloglucan consists of a $\beta$-(1-4)-linked $\beta$-D-Glcp ($\beta$-D-glucopyranose) backbone having up to 75% of the $\beta$-D-Glcp residues covalently linked to α-D-Xylp (α-D-xylopyranose) at the O-6 position. Depending upon the plant species and tissue localization, the xylose branches may in turn be substituted with combinations of galactopyranose, fucopyranose, arabinofuranose, and *o*-acetyl residues (Gloster et al. 2007). While the extent of xylose substitution varies considerably, the glucose backbone structure is typically substituted by 50 or 75% xylose (Powlowski et al. 2009). The most widely used xyloglucan in research and industry is derived from the seeds of *Tamarindus indica* Linn., an abundantly grown tree of Southeast Asia, India, Mexico and Costa Rica (Zhou et al. 2007; Goyal et al. 2007).

Many endoglucanases have been reported to hydrolyze xyloglucan as a substrate analogue, however, few glucanases have high activity towards xyloglucan, and they are virtually inert towards the non-substituted $\beta$-glucans and carboxymethyl cellulose (Menon et al. 2010b). Xyloglucanases (3.2.1.151) represent a class of polysaccharide degrading enzymes which can attack the backbone also at

substituted glucose residues (Benko et al. 2008). The distinction between these xyloglucanases and endocellulases may lie in the requirement for specific side groups. Xyloglucanases belong to the inverting and retaining glycoside hydrolyases (GHs) which constitute 113 protein families responsible for the hydrolysis and/or transglycosylation of glycosidic bonds. GHs present in the carbohydrate-active enzymes (CAZy) database have widespread importance for biotechnological and biomedical applications (Cantarel et al. 2009).

The xyloglucan and the enzymes responsible for its modification and degradation are of increasing prominence, reflecting the drive for diverse biotechnological applications in fruit juice clarification, textile processing, cellulose surface modification, pharmaceutical delivery, production of food thickening agents, paper and pulp industry, plant growth modulation, production of novel surfactant, synthesis of biocomposites and enzyme kinetic studies (Gloster et al. 2007; Wong et al. 2010). Xyloglucan-specific glycosidases with high substrate specificity are useful tools for analysing the xyloglucan structure. Xyloglucanases have been used to characterize molecular alterations in plant cell-wall mutants, engineer the physical properties and biochemical composition of plant fibres and link chemically modified xylo-oligosaccharides to xyloglucan polymers that can be used to impart novel functionality to cellulose microfibrils (Master et al. 2008). Recent advancements in the production of biofuel from plant biomass have also spurred enormous interest in accessory enzymes such as xyloglucanases (Menon et al. 2010c). The action of xyloglucanases enhances the hydrolysis of lignocellulose by making the cellulose more accessible to cellulase enzymes and also improves the economics of biomass conversion (Benko et al. 2008).

There are few reports on the xyloglucanases from various microorganisms; however, there is paucity of literature on specific xyloglucanases from thermophilic microorganisms. Xyloglucanases stable and active at high temperature and alkaline conditions are suitable for biotechnological applications. The present investigation reports the purification and biochemical characterization of a thermostable xyloglucanase from an alkalothermophilic *Thermomonospora* sp. The properties of the purified enzyme were studied to understand and evaluate the potential of xyloglucanase in biotechnological applications. Kinetic and thermodynamic studies on purified xyloglucanase were followed in order to understand the demand of enthalpy and entropy for thermal inactivation. The study also explores the effect of thermostable xyloglucanase in the hydrolysis of galactoxyloglucan (GXG) from tamarind kernel powder (TKP).

## Materials and methods

### Microorganism and culture conditions

Alkalothermophilic *Thermomonospora* sp. was maintained on Luria–Bertani wheat bran slants at pH 10 and 50°C according to George et al. (2001).

### Isolation of genomic DNA

DNA was extracted from actinomycetes grown in Luria broth (pH 9.0) for 48 h at 50°C. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The genomic DNA of the isolate was extracted by the method of Hopwood et al. (1985).

### Amplification of 16S rDNA gene and sequence analysis

The primers used for the identification of the isolate were universal eubacterial 16S rDNA primers: 16F27N (5′CCA GAG TTT GAT CMT GGC TCA G), 16F536 (5′GTG CCA GCA GCC GCG GTR ATA), 16R1525XP (5′TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC) commercially obtained from Life technologies, India. PCR was performed in the final volume of 25 µl containing genomic DNA 0.50 µl, 10× buffer 2.50 µl, 0.2 mM deoxyribonucleoside triphosphate (dNTP) 2.5 µl, forward and reverse Primers 10–20 pmol (1.25 µl each), distilled water 16.87 µl, and 1 unit of Taq DNA polymerase 0.25 µl (Banglore Genei). After an initial 30 s denaturation at 98°C the reaction was run for 35 cycles: denaturation was done at 98°C for 10 s, annealing at 58°C for 20 s and extension at 72°C for 90 s followed by a final extension step at 72°C for 7 min. Products were analysed on 1% agarose gel containing 0.7 µg/ml ethidium bromide and visualized under UV light.

PCR products were purified using a PCR purification kit (QiaGEN, Hilden). The sequencing reactions were carried out using Taq DNA polymerase dye terminator cycle sequencing using the 'ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit' (Perkin Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's protocol. The BLAST searches of the sequences were performed at the National Centre for Biotechnology Information (NCBI) GenBank data library (http://www.ncbi.nim.nih.gov/BLAST/) using BLASTN with default settings. The sequences were downloaded from the NCBI database and aligned using the CLUSTALW program (http://align.genome.jpg/sit-bin/clustalw). Sequences were trimmed using DAMBE software. A phylogenic tree was generated by the neighbour-joining method (Saitou and Nei 1987) using MEGA software (Kumar et al. 2001).

### Production of xyloglucanase in different media

*Thermomonospora* was grown on two different media (a) modified Reese medium (Mandels and Weber 1969) containing 1% yeast extract, 4% cellulose powder and 0.1% Tween 80 and (b) modified Reese medium with 0.5% yeast extract, 1% TKP, 1% cellulose powder and 0.1% Tween 80. The pH of the media was adjusted to 9 with 10% $Na_2CO_3$ after autoclaving. The culture was grown at 50°C for 120 h on a rotary shaker maintained at 200 rpm. Samples were withdrawn after an interval of 24 h, centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was checked for xyloglucanase activity.

### Enzyme assay

Xyloglucanase activity was measured by incubating 1 ml of assay mixture containing 0.5 ml of 5 mg/ml of tamarind xyloglucan and 0.5 ml of suitably diluted enzyme in 50 mM phosphate buffer (pH 7) for 30 min at 50°C (Menon et al. 2010b). Enzyme and reagent blanks were also simultaneously incubated with test samples. The reducing sugar formed was estimated by dinitrosalicylic acid (Mandels and Weber 1969). One unit of enzyme activity is defined as the amount of enzyme required to liberate 0.5 mg of reducing sugar per minute under assay conditions. The protein concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin as a standard.

### Purification of xyloglucanase

The culture filtrate obtained after 120 h of incubation in medium (b) was concentrated using ammonium sulphate (0–90% saturation). All purification steps were carried out at 4°C unless otherwise stated. The precipitate was dissolved in 0.05 M sodium phosphate buffer, pH 7, dialyzed against the same buffer with several changes for 8 h, and applied to a QAE-Sephadex column (3 × 20 cm) previously equilibrated with 0.05 M sodium phosphate buffer, pH 7. The elution was carried out by a linear gradient of sodium chloride (0–0.6 M) in same buffer. The fractions having maximum specific activity were pooled and concentrated by ultrafiltration through a Amicon UM-10 membrane. The concentrated sample was applied to a Bio-Gel P-100 column (2 × 100 cm) previously equilibrated with 0.05 M sodium phosphate buffer pH 7. Elution was carried out using the same eluent at a flow rate of 12 ml/h. The fractions (1.5 ml each) having maximum specific activity were pooled and concentrated. The purity of the enzyme was checked by SDS-PAGE followed by silver staining (Laemmli 1970).

### Determination of molecular weight of the enzyme

The molecular weight of xyloglucanase was determined by gel filtration chromatography using Sephacryl S-200 previously equilibrated with 50 mM phosphate buffer, pH 7. The column was calibrated using the following marker proteins; $\beta$-amylase (Mr 205,000), alcohol dehydrogenase (Mr 97,400), bovine serum albumin (Mr 66,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 29,000) obtained from Amersham. The void volume of the column was determined by the elution volume of blue dextran.

### Determination of optimum pH, temperature and stability of xyloglucanase

Estimation of xyloglucanase activity at different pH (4–10) and temperatures (40–100°C) were carried out under standard assay conditions to determine optimum pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 50 U/ml of enzyme at 50°C in buffer of desired pH for 1 h. The following buffer systems were used: 0.05 M acetate buffer (pH 4–5), 0.05 M phosphate buffer (pH 6–7), 0.05 M Tris–HCl buffer (pH 8) and 0.05 M carbonate–bicarbonate buffer (pH 9–10). The temperature stability was determined by incubating 50 U/ml of enzyme at different temperatures. The samples were removed at regular time intervals and the residual activity was estimated under standard assay conditions.

### Thermodynamics and fluorescence studies on thermal inactivation

Thermal inactivation of xyloglucanase was determined by incubating enzyme solutions (50 U/ml) in 0.05 M phosphate buffer (pH 7), at different temperatures (70–85°C). Aliquots were withdrawn at different time intervals, cooled by keeping in ice for 3 h and assayed for enzyme activity. Thermoinactivation rate coefficients ($k_d$) were determined by linear regression from the plot of natural logarithm of residual activity (ln ra) versus the time of incubation at the respective temperatures. $k_d$ was the absolute value of the slope in the regression line.

The thermodynamic parameters of xyloglucanase denaturation, free energy, enthalpy and entropy were calculated according to the following equations (Pal and Khanum 2010):

The half-life of xyloglucanase ($t_{1/2}$, $min^{-1}$) was determined from the relationship:

$$t_{1/2} = 0.693/k_d. \quad (1)$$

The $D_T$ values (decimal reduction time or time required to inactivate 90% of the original enzyme activity at a

constant temperature) was calculated from the following equation:

$$D_T = \ln 10/k_d. \quad (2)$$

Other parameters were calculated as given below:

$$E_a = -R(\text{Slope}) \quad (3)$$

$$\Delta H = E_a - RT \quad (4)$$

$$\Delta G = -RT \ln(k_d h/k_B T) \quad (5)$$

$$\Delta S = (\Delta H - \Delta G)/T. \quad (6)$$

where $E_a$ is the activation energy for denaturation (kJ mol$^{-1}$) and was determined by linear regression of the plot of the natural logarithm of $k_d$ (ln $k_d$) versus the reciprocal of the absolute temperatures (1/$T$, K) at which xyloglucanase was irreversibly inactivated by the first order rate constant, according to Eq. 3. The change in enthalpy ($\Delta H$, kJ mol$^{-1}$), free energy ($\Delta G$, kJ mol$^{-1}$), and entropy ($\Delta S$, J mol$^{-1}$ K$^{-1}$) were calculated using the Eqs. 4, 5 and 6, respectively, where $R$ the gas constant (8.314 J mol$^{-1}$ K$^{-1}$), $T$ the corresponding absolute temperature (K), $k_d$ the thermoinactivation coefficient (min$^{-1}$), $h$ the Plank constant ($11.08 \times 10^{-36}$ J mol$^{-1}$), $k_B$ the Boltzmann constant ($1.38 \times 10^{-23}$ J K$^{-1}$) (Leite et al. 2007). All the experiments were performed in triplicates and the mean of the experiments were considered for further calculations.

The fluorescence study was performed on a Cary Varian Eclipse fluorescence spectrophotometer connected to a Cary Varian temperature controller. Xyloglucanase was incubated at 50, 60, 70 and 80°C for 30 min and the fluorescence was measured with an excitation at 295 nm and emission from 300 to 400 nm at 25°C. The slit width on both excitation and emission were set at 5 nm, and the spectra were obtained at 1 nm/min. Corrections for the inner filter effect were performed as described by the formula (Lakowicz 1983) $F_c = F$ antilog $[(A_{ex} + A_{em})/2]$, where $F_c$ and $F$ are the corrected and measured fluorescence intensities, respectively, $A_{ex}$ and $A_{em}$ are the solution absorbance at the excitation and emission wavelengths, respectively. Background buffer spectra were subtracted to remove the contribution from Raman scattering.

### Effect of additives on thermostability

The thermostability of xyloglucanase at 80°C in presence of various additives was determined by incubating the reaction mixture (enzyme (50 U/ml) in 0.05 M phosphate buffer, pH 7) with additives and estimating the xyloglucanase activity at different time intervals. At the end of incubation period the enzyme was cooled for 5 min and the residual activity was determined. Suitable controls were also taken. The time at which 50% initial activity was reached corresponded to the half-life of the enzyme.

### Effect of additives and metal ions on xyloglucanase activity

The effect of additives on xyloglucanase activity was determined in the presence of various additives; alanine, cystein, glycine (final concentrations of 10 mM each) and polyols (sorbitol, mannitol and glycerol) at final concentration of 100 mM. The effect of metal ions on the xyloglucanase activity was determined by incubating the reaction mixture with various metal ions at final concentration of 0.1, 1 and 10 mM and xyloglucanases activity was determined. Xyloglucanase activity in absence of metal ions was taken as 100%.

### Determination of kinetic parameters and substrate specificity

The apparent $K_m$ and $V_{max}$ values of the pure enzyme were determined by measuring the activity with tamarind xyloglucan at concentrations ranging from 1 to 10 mg/ml. Kinetic constants were calculated for the data obtained by non-linear regression analysis and analysed by Lineweaver–Burk's reciprocal equation. The purified enzyme was used to hydrolyze different substrates (1%) with varying glycosidic bonds of glucose and xylan. The substrates used were xyloglucan, oat spelt xylan, laminarin, lichenan, avicel, $\beta$-glucan, carboxymethyl cellulose, $p$-nitrophenyl $\beta$-D-glucopyranoside (PNPG) and $p$-nitrophenyl $\beta$-D-xylopyranoside (PNPX).

### Thermostable xyloglucanase in hydrolysis of TKP

Galactoxyloglucan (GXG) was isolated from TKP according to Menon et al. (2010b). The hydrolysis of GXG was carried out at two different substrate concentrations (10 and 25 g/l) with varying concentrations of *Thermomonospora* xyloglucanase (20 and 40 U/ml) at various temperatures of 40, 50, 60, 70°C. The hydrolysis was carried out in a stoppered flask in 25 ml reaction volume containing appropriate concentrations of substrates in 0.05 M phosphate buffer, pH 7. Hydrolysis was terminated by boiling at 100°C for 5 min at the end of stipulated time intervals, filtered and the filtrate was assayed for reducing sugar by the DNS method (Mandels and Weber 1969). Appropriate substrate and enzyme controls were performed.

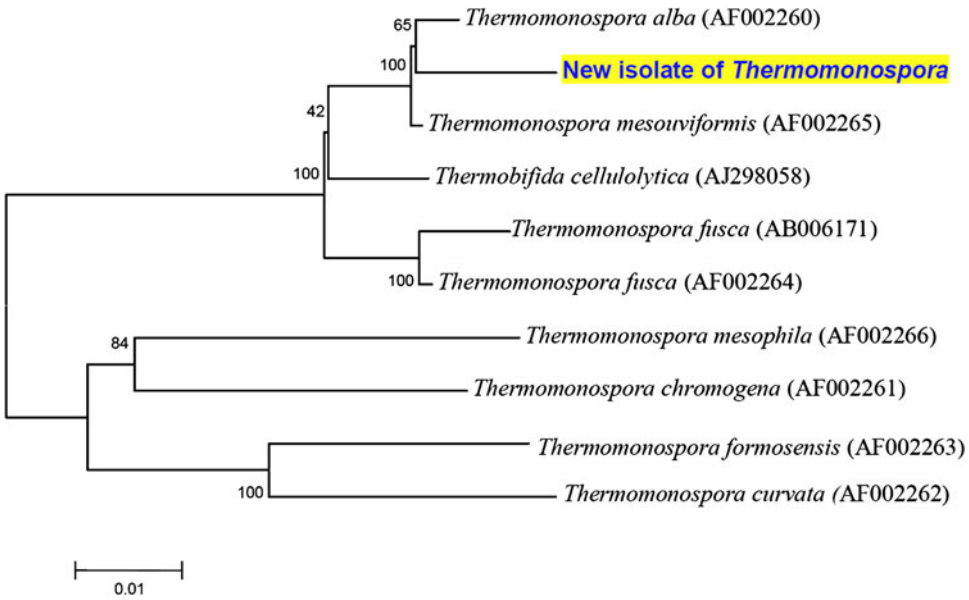


**Fig. 1** Phylogenetic tree showing the positions of the new isolate (new isolate of *Thermomonospora*) and related species based on 16S rDNA sequences

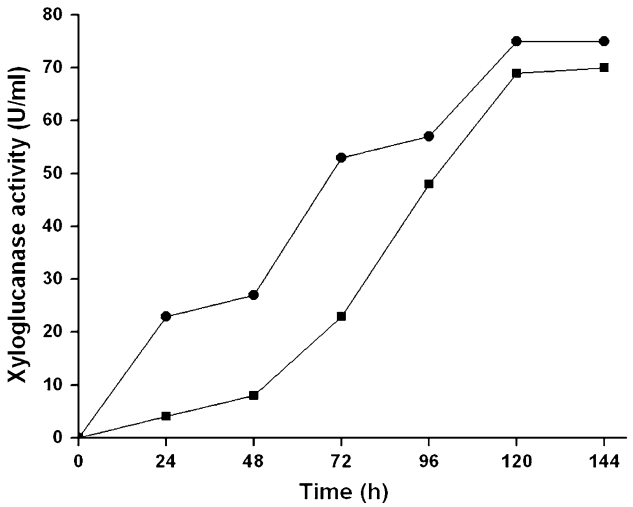


**Fig. 2** Time course of xyloglucanase production by alkalothermophilic *Thermomonospora* sp. in two different media; Medium a: modified Reese medium with 4% cellulose powder (*filled square*), Medium b: modified Reese medium with 4% cellulose powder and 1% TKP (*filled circle*). pH of the media was adjusted to 9 with 10% $Na_2CO_3$

### Stability of xyloglucanase in commercial detergents and organic solvents

Stability of xyloglucanase in the presence of commercial detergents (0.7 mg/ml) and organic solvents (25% v/v) was investigated at 50°C. The residual activity of the enzyme was quantified under the standard assay conditions.

## Results and discussion

### Identification of actinomycetes based on sequence analysis based on 16S rDNA

BLAST searches indicated that the 16S rDNA sequence of the new isolate is closely related to the homologous sequences of various *Thermomonospora* sp. as shown in phylogenetic tree (Fig. 1). It revealed that the isolate of *Thermomonospora* showed 98% homology with *Thermomonospora mesouviformis*. Since *T. mesouviformis* grows optimally at neutral pH and the present isolate is an

**Table 1** Purification of xyloglucanase from *Thermomonospora* sp.

| Steps | Total volume (ml) | Total protein (mg) | Total xyloglucanases activity (U) | Specific activity (U/mg) | Recovery (%) | Fold purification |
|---|---|---|---|---|---|---|
| Culture filtrate | 100 | 635 | 7,500 | 11.81 | 100 | 1 |
| Ammonium sulphate precipitation | 10 | 458 | 5,960 | 13.01 | 79.47 | 1.10 |
| QAE-Sephadex | 12 | 46.36 | 2,959 | 63.84 | 39.46 | 5.41 |
| Bio-Gel P 100 | 5 | 4.81 | 584 | 121.35 | 7.46 | 10.28 |

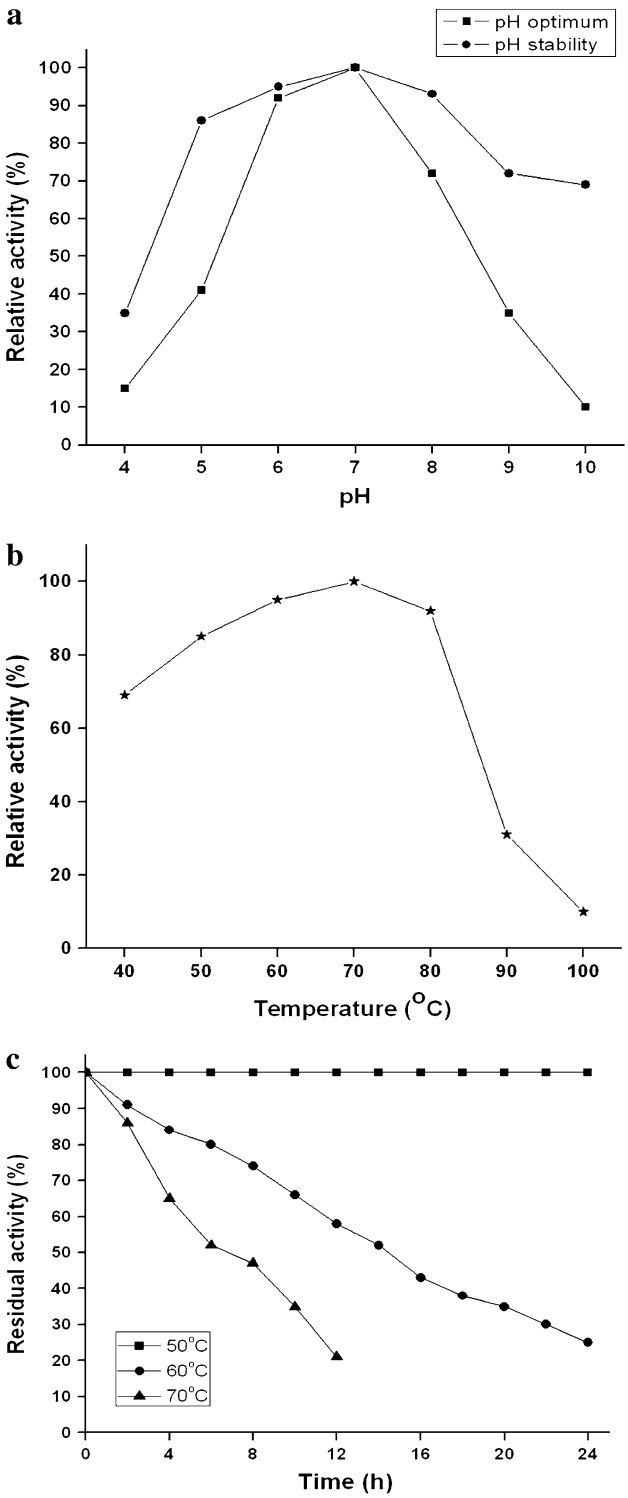


**Fig. 3** **a** pH optima and pH stability of xyloglucanase at 50°C. The following buffer systems were used: 0.05 M acetate buffer (pH 4–5), 0.05 M phosphate buffer (pH 6–7), 0.05 M Tris–HCl buffer (pH 8) and 0.05 M carbonate–bicarbonate buffer (pH 9–10). **b** Temperature optima of xyloglucanase: 50 U/ml of xyloglucanase was incubated in 0.05 phosphate buffer (pH 7) at different temperatures in the range (40–100°C) and the residual activity was determined. **c** Thermal stability of xyloglucanase: 50 U/ml of xyloglucanase was incubated in 0.05 phosphate buffer (pH 7) at different temperatures for different intervals and residual activity was estimated under the standard assay conditions

obligate alkalophile (optimum growth at pH 9), the isolate was classified to be a new species of *Thermomonospora* (Anish 2007).

## Production of xyloglucanase

The time course of xyloglucanase production by the alkalothermophilic *Thermomonospora* sp. in the extracellular culture filtrate is shown in Fig. 2. A maximum xyloglucanases activity of 75 U/ml was obtained at 120 h using TKP as the carbon source. Cellulose powder was also an alternative carbon source for xyloglucanase production (68 U/ml). *Geotrichum* sp. M128 (Yaoi and mitsuishi 2004) and *Paenibacillus* sp. strain KM21 (Yaoi et al. 2005) are reported to produce xyloglucanase in a medium supplemented with xyloglucan (carbon source) from tamarind wherein TKP was found to be a suitable carbon source for xyloglucanase production from *Thermomonospora* sp. TKP being a low cost industrial by-product, can be an economical carbon source for the commercial production of xyloglucanase.

## Purification of enzyme

The purification of xyloglucanase from the extracellular culture filtrate of medium (b) was carried out by ammonium sulphate precipitation (0–90% saturation) followed by chromatography on QAE-Sephadex ion exchange column and Bio-Gel P-100 gel filtration column. Xyloglucanase from *Thermomonospora* sp. was purified to homogeneity with tenfold purification, specific activity of 121 U/mg and percent recovery of 7.46 (Table 1). SDS-PAGE revealed a single homogeneous band of pure protein with a molecular mass of 144 kDa. A sharp peak of an average molecular mass of 144 kDa was obtained by gel permeation chromatography. The reported molecular weights for xyloglucanase range from 75 to 105 kDa (Grishutin et al. 2004; Yaoi et al. 2005; Sinitsyana et al. 2010). The yield of the purified xyloglucanases from alkalthermophilic *Thermomonospora* sp. is 4.8 mg/100 ml of culture broth. The work is ongoing on the identification of xyloglucanase gene and overexpression in a suitable host. This is the first report of a high molecular weight xyloglucanase from an alkalothermophilic *Thermomonospora* sp.

## Kinetic parameters and substrate specificity

The apparent $K_m$ and $V_{max}$ of the purified xyloglucanase were 1.67 mg/ml and 36.4 U/ml, respectively. The enzyme was active towards xyloglucan (121.35 U/mg). The enzyme had no activity towards oat spelt xylan, laminarin,

lichenan, Avicel, β-glucan, carboxymethyl cellulose, PNPG and PNPX. Xyloglucan-specific endoglucanases have also been reported from various microorganisms having higher catalytic activity towards xyloglucan and no or negligible activity towards other linear polymers (Grishutin et al. 2004; Master et al. 2008).

## Optimum pH and stability of xyloglucanase

The enzyme was active in a broad pH range (5–9) with maximum activity at pH 7. It retained 72 and 35% of its activity at pH 8 and 9, respectively. Xyloglucanase was stable in the pH range 5–8 with more than 80% residual activity. At pH 9 and 10, it exhibited 72 and 65% residual activities, respectively (Fig. 3a). Xyloglucanases are active in broad pH ranges; however, the reported optimum pH range is 4–6 (Grishutin et al. 2004; Sinitsyana et al. 2010). Considering substantial stability of *Thermomonospora* xyloglucanase at neutral and alkaline pH, it may find potential application in laundry detergents compositions and for cellulose fibre processing industries.

## Thermal stability

The xyloglucanase showed a maximum activity at 70°C; with 90 and 92% activities at 60 and 80°C, respectively (Fig. 3b). The xyloglucanase was exceptionally stable at 50°C, with 100% activity up to 60 h and had a half-lives of 14 and 6 h at 60 and 70°C, respectively (Fig. 3c). At 80°C the enzyme had half-life of 7 min. The reported optimum temperature for xyloglucanase activity is from 50 to 65°C (Yaoi and Mitsuishi 2004). The xyloglucanases of

**Table 2** Biochemical properties of xyloglucanase from different microbial sources

| Microorganisms | Optimum conditions | | | | MW (kDa) | Specific activity (U/mg) | $K_m$ (mg/ml) | Substrate specificity | Temperature stability | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | Growth | | Activity | | | | | | | |
| | pH | Temp (°C) | pH | Temp (°C) | | | | | | |
| *Aspergillus japonicus* | – | – | 5 | – | 32 | 98 | 0.67 | Xyloglucan β-glucan[c] | – | Grishutin et al. (2004) |
| *Trichoderma reesei* | – | – | 5.3 | – | 75–105 | – | 0.30 | Xyloglucan, β-glucan, CMC | – | |
| *Chryosporium lucknowense* | 6.5 | 28 | 6 | – | 78 | 75 | 0.31 | Xyloglucan, β-glucan, CMC | – | |
| *Geotrichum sp. M128* | 6 | 30 | 5.5 | 55 | 80.5 | 68 | – | Xyloglucan | 90% activity for 10 min at 50°C | Yaoi and Mitsuishi (2004) |
| *Aspergillus terreus* | 7 | 45 | 4 | 60 | 78 | 45 | 2.5[a] | Barley β-glucan, xyloglucan, lichenan, CMC | 79% activity for 1 h at 60°C | Nazir et al. (2009) |
| *Penicillium canescenes* XG A | – | – | 4 | 65 | 25 | 21 | 1.33 | Tamarind seed xyloglucan | $t_{1/2}$ of 1 h at 60°C | Sinitsyana et al. (2010) |
| *Penicillium verruculosum* XG 25 | – | – | 4.6 | 60 | 25 | 52 | 0.28 | Tamarind seed xyloglucan, barley β-glucan[c] | $t_{1/2}$ of 1 h at 60°C | |
| *Penicillium verruculosum* XG 70 | – | – | – | 55 | 70 | 38 | 0.29 | Tamarind seed xyloglucan, barley β-glucan, CMC[c] | $t_{1/2}$ of 45 min at 60°C | |
| *Aspergillus oryzae RIB 40* | 5 | 30 | 4.5–5.5 | 60 | 29 | 883 | 1,250[b] | Tamarind xyloglucan, AZCL xyloglucan, xylan[c], CMC[c] | 20 min at 50°C | Hakamada et al. (2011) |
| *Thermomonospora* sp. | 9 | 50 | 7 | 70 | 144 | 121 | 1.67 | Tamarind xyloglucan | $t_{1/2}$ of 14 h at 60°C and 6 h at 70°C | Present work |

*CMC* carboxymethyl cellulose, *AZCL* xyloglucan–azurine-crosslinked xyloglucan

[a] mM/min

[b] Units/mg

[c] Trace activity

*Penicillium* sp, *Aspergillus japonicus* and *Trichoderma reesei* were stable at 50°C, retaining 100% at 24 h (Yaoi et al. 2005); however, *Thermomonospora* xyloglucanase retained 100% activity for 60 h. Table 2 lists the biochemical properties of xyloglucanases from various microbial sources.

## Thermodynamic and fluorescence studies of xyloglucanase inactivation

The purified xyloglucanase was incubated at temperatures ranging from 70 to 85°C. The semi-logarithmic plots of residual activity versus incubation time were linear, indicating that the inactivation could be expressed as first-order kinetics. The slope of these plots was the $k_d$ values applied in the plot of ln $k_d$ versus the reciprocal of the absolute temperature. It was observed that the rate of enzyme deactivation ($k_d$) increased with increase in temperature. The half-life of xyloglucanase was 360 min at 70°C and 1 min at 85°C. With increase in temperature a decrease in $t_{1/2}$ was observed. The time needed to reduce the enzyme activity by 90%, i.e., $D_T$ value, also decreased with increase in temperature.

Activation energy for irreversible inactivation ($E_a$) of the xyloglucanase was 396 kJmol$^{-1}$ as determined from the Arrhenius plot (Fig. 4a). The enthalpy of activation of thermal denaturation ($\Delta H$) of xyloglucanase was 393.26 kJ mol$^{-1}$ at 70°C. The values marginally decreased with rise in temperature, indicating that lesser energy is required to denature enzyme at elevated temperatures. The observed change in $\Delta H$ also indicates that the enzyme undergoes considerable change in conformation at higher temperatures. The Gibbs free energy ($\Delta G$) of thermal inactivation was found to decrease with increase in temperatures from 113.91 to 101.50 kJ mol$^{-1}$. The $\Delta G$ value is intimately related with the protein stability, as higher the $\Delta G$ the higher will be the enzyme stability (Longo and Combes 1999). When the incubation temperature was increased from 70–85°C there was a significant reduction of $\Delta G$, indicating the destabilization of the protein with rise in temperature (Table 3). The values of $\Delta S$ did not show significant alterations with the increase in temperature, since the increase in entropy by denaturation is thought to be due to the exposure of the hydrophobic chains during the unfolding of proteins. The positive values of $\Delta S$ indicate that there is no significant process of protein aggregation (Marin et al. 2003). However, the thermal denaturation at higher temperature (80°C) is due to the unfolding of xyloglucanase as observed from fluorescence spectrum.

The fluorescence of xyloglucanase exhibited emission maxima ($\lambda_{max}$) at 342 nm, which is characteristic of partial shielding of the tryptophan residues from aqueous solvent

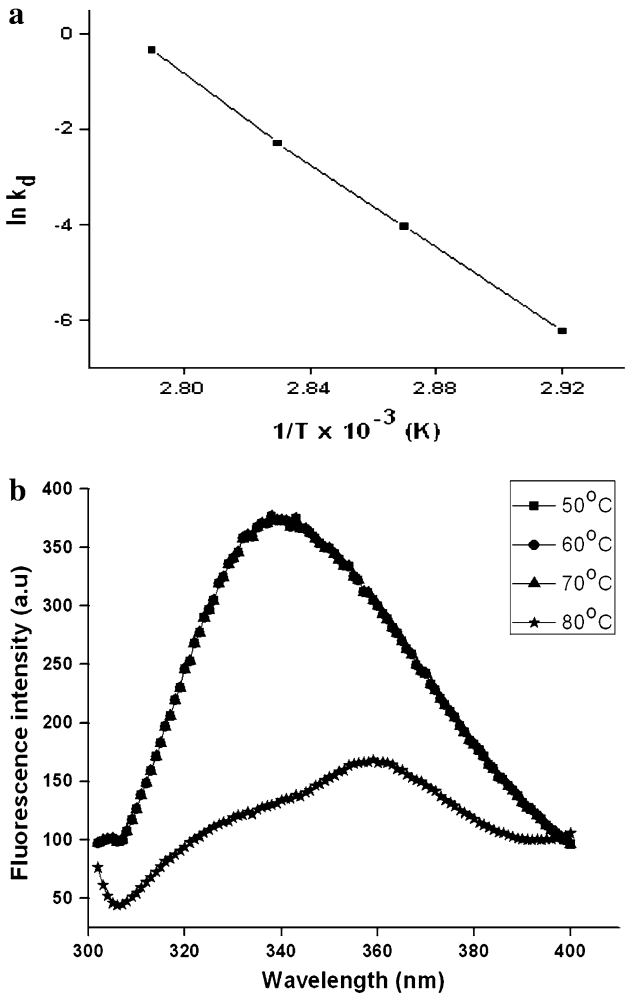


**Fig. 4** **a** Effect of temperature on first order thermoinactivation rate coefficients of xyloglucanase from *Thermomonospora* sp. Thermal inactivation was determined by incubating enzyme solution (50 U/ml) in 0.05 M phosphate buffer, (pH 7) at different temperatures (70–85°C). **b** Fluorescence emission spectra of xyloglucanase at different temperatures. Xyloglucanase was incubated at 50, 60, 70 and 80°C for 30 min and the fluorescence was measured on a Cary Varian Eclipse fluorescence spectrophotometer connected to a Cary Varian temperature controller with an excitation at 295 nm and emission from 300 to 400 nm at 25°C

(Cho et al. 1994). The enzyme retains its conformational stability and at the same time remains catalytically intact and active at 50, 60 and 70°C. However, at 80°C the unfolding of the enzyme was evidenced by a distinct red shift in the wavelength maximum and a corresponding decrease in the fluorescence intensity (Fig. 4b).

## Effect of additives on thermostability

In order to improve the thermostability of xyloglucanase at higher temperature, different compounds were added to the enzyme solution. The addition of alanine (0.5 M), tween 80

**Table 3** Thermodynamic parameters for irreversible thermal inactivation of xyloglucanase at different temperatures

| Temperature (K) | $k_d$ (min$^{-1}$) | $t_{1/2}$ (min) | $D_T$ (min) | $\Delta H°$ (kJ mol$^{-1}$) | $\Delta G°$ (kJ mol$^{-1}$) | $\Delta S°$ (J mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|---|---|
| 343 | 0.001925 | 360 | 1195 | 393.26 | 113.91 | 814.43 |
| 348 | 0.0173 | 40 | 133 | 393.21 | 109.26 | 815.95 |
| 353 | 0.099 | 7 | 23 | 393.17 | 105.75 | 814.22 |
| 358 | 0.693 | 1 | 3.3 | 393.13 | 101.50 | 814.60 |

$k_d$, First-order rate constant for thermal inactivation; $t_{1/2}$, half-life; $D_T$ decimal reduction time; $\Delta H$, variation in enthalpy; $\Delta G$, variation in free energy; $\Delta S$, variation in entropy

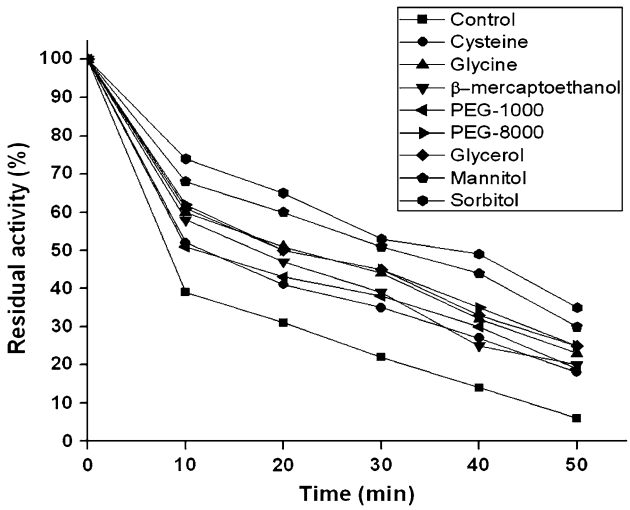


**Fig. 5** Thermal stability of xyloglucanase at 80°C in presence of various additives: 50 U/ml of xyloglucanase was incubated in 0.05 M phosphate buffer (pH 7) in the presence of additives. At the end of incubation period enzyme was cooled for 5 min and the residual activity was determined

(0.1%), SDS (0.1%) and Triton X-100 (0.1%) did not prevent the loss in activity at 80°C. Cysteine (10 mM) and PEG-1000 (10 mM) had marginal effect on thermostabilisation of xyloglucanase. Increase in the half-life from 7 to 20 min was observed after addition of glycine (2 M), β-mercaptoethanol (10 mM), PEG-8000 (10 mM) and glycerol (2 M). The addition of polyhydrophilic additives, mannitol and sorbitol (2 M) to the xyloglucanase solution at 80°C had the positive effect on thermostability with an increase in half-life from 7 to 30 min (Fig. 5).

The thermostabilizing effect of polyols was proportional to its molecular weight, which can be correlated to the number of hydroxyl groups per polyol molecule. Polyols have the ability to maintain solvophobic interactions and have the capability to form hydrogen bonds that play the key role in supporting the native conformation of the protein and aid in protein stabilization. The stabilizing effect of additives is not an absolute effect valid for all the enzymes; however, it depends on the nature of the enzyme, on its hydrophilic and hydrophobic character and on the degree of interaction with the additive.

**Table 4** Effect of additives on xyloglucanase activity

| Additives | Relative activity (%) |
|---|---|
| Control | 100 |
| β-mercaptoethanol (10 mM) | 182.34 |
| Cystein (10 mM) | 222.47 |
| Tween 80 (0.1%) | 71.49 |
| Glycerol (100 mM) | 94.84 |
| Mannitol (100 mM) | 95.18 |
| Sorbitol (100 mM) | 96.83 |

## Effect of additives and metal ions on xyloglucanase activity

The percent relative xyloglucanase activity was enhanced by 2.22 and 1.82 times after addition of cysteine and β-mercaptoethanol, respectively, at a concentration of 10 mM. The enzyme activity was not enhanced by addition of tween 80 (0.1%) and polyols (Table 4). Enhancement in xyloglucanase activity was observed with β-mercaptoethanol and cysteine. The enhanced activity may be due to the protection of sulfhydryl groups essential for enzyme activity from oxidation. An endoglucanase from *Aspergillus terreus* having high activity towards barley β-glucan and xyloglucan was positively modulated by β-mercaptoethanol (Nazir et al. 2009). Gupta and Gautam (1993); Cesar and Mrsa (1996) have reported an increase in α-glucosidase activity by 1.3% and xylanase activity by 1.48% with β-mercaptoethanol, respectively.

The relative xyloglucanase activity was increased by 30 and 40% by the addition of KCl and $ZnSO_4$, respectively, at concentration of 10 mM. Maximum inhibition of 75 and 88% was observed with $CuSO_4$ and $HgCl_2$. The addition of $CoCl_2$, $FeSO_4$, $NiCl_2$ and $CdCl_2$ in the concentrations of 0.1, 1 and 10 mM showed gradual decline in the xyloglucanase activity. There was negligible reduction in xyloglucanase activity after addition of NaCl, $Li_2SO_4$, $Pb(NO_3)_2$ and $MnSO_4$ in the reaction mixture (Table 5). Sinitsyana et al. (2010) have reported 20% enhancement in xyloglucanase activity in the presence of $Zn^{2+}$. Inactivation of xyloglucanase by $Cu^{2+}$ and $Hg^{2+}$ indicates the

presence of important thiol groups, carboxyl groups and histidine residues in the enzyme mechanism.

### Hydrolysis of glactoxyloglucan from TKP

Saccharification of galactoxyloglucan with thermostable xyloglucanase with respect to varying enzyme and substrate concentrations at different temperatures were pursued. A maximum hydrolysis of 57% was obtained in 6 and 12 h at 70 and 60°C, respectively, using thermostable xyloglucanase with an enzyme dosage of 40 U/ml and a substrate loading of 10 g/l (Table 6). At higher substrate concentration (25 g/l), a hydrolysis of 50% was obtained in 6 h at 70°C with an enzyme dosage of 20 U/ml as compared to 50°C wherein the same percentage of hydrolysis was achieved with 40 U/ml in 48 h (Menon et al. 2010b). The hydrolysis at higher temperature favours a reduction in process time and enzyme dosage. The role of thermostable xylanase in the hydrolysis of hemicellulosic substrates, favouring a reduction in process time and enzyme dosage was reported by Menon et al. (2010a).

**Table 5** Effect of metal ions on xyloglucanase activity

| Metals | Relative activity (%) | | |
|---|---|---|---|
| | 0.1 mM | 1 mM | 10 mM |
| Control | 100 | 100 | 100 |
| NaCl | 95 | 84 | 75 |
| $CoCl_2$ | 87 | 64 | 65 |
| $CuSO_4$ | 74 | 52 | 25 |
| $HgCl_2$ | 55 | 28 | 12 |
| $FeSO_4$ | 82 | 58 | 47 |
| $Li_2SO_4$ | 93 | 76 | 65 |
| KCl | 105 | 120 | 130 |
| $Pb(NO_3)_2$ | 91 | 72 | 59 |
| $NiCl_2$ | 80 | 69 | 44 |
| $ZnSO_4$ | 115 | 140 | 145 |
| $MnSO_4$ | 95 | 71 | 62 |
| $CdCl_2$ | 87 | 59 | 47 |

### Stability of xyloglucanase in commercial detergents and organic solvents

Xyloglucanase was stable in the presence of commercial detergents. The enzyme showed maximum stability in the presence of Ariel, having a residual activity of 91% after incubation at 50°C for 50 min. Xyloglucanase had 82, 84, 80 and 81% residual activities in the presence of Surf excel, Tide, Henko and Rin, respectively, under similar conditions (Fig. 6). Xyloglucanase was found to be stable in isopropanol and butanol for 25 min with residual activities of 68% and 70, respectively. The residual activities were in the range of 30–50% for other solvents (Table 7). Xyloglucanase was stable in the presence of commercial detergents such as Ariel, Surf excel and Tide, suggesting its application in detergent compositions especially for removing soils and spots.

## Conclusions

Xyloglucanases are important biocatalysts and are relatively less studied than other hydrolytic enzymes despite their potential applications in biotechnology. In the current context of biofuel production the hydrolysis of lignocellulose could be improved with the supplementation of xyloglucanase in an enzyme cocktail as their action increases the surface area of the substrate. The present study reports a thermostable xyloglucanase from an alkalothermophilic *Thermomonospora* sp. with a Mr of 144 kDa exhibiting specificity towards xyloglucan. This xyloglucanase is found to be stable in presence of detergents, indicating its potential as an additive to laundry

**Table 6** Enzymatic hydrolysis of GXG at 10 and 25 g/l substrate concentrations with *Thermomonospora* xyloglucanase at different temperatures

| Temperature (°C): | 40 | | | | 50 | | | | 60 | | | | 70 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Substrate concentration (g/l): | 10 | | 25 | | 10 | | 25 | | 10 | | 25 | | 10 | | 25 | |
| Enzyme dosage (U/ml): | 20 | 40 | 20 | 40 | 20 | 40 | 20 | 40 | 20 | 40 | 20 | 40 | 20 | 40 | 20 | 40 |
| Time (h) | Percent hydrolysis | | | | | | | | | | | | | | | |
| 3 | 10.20 | 12.09 | 8.69 | 10.35 | 20.22 | 29.62 | 17.54 | 24.09 | 28.69 | 34.67 | 25.23 | 30.25 | 38.47 | 42.84 | 29.20 | 35.96 |
| 6 | 12.50 | 18.69 | 10.66 | 15.92 | 22.77 | 30.19 | 20.16 | 30.22 | 40.59 | 42.81 | 37.25 | 41.69 | 55.21 | 57.41 | 49.85 | 51.33 |
| 12 | 19.36 | 26.38 | 15.28 | 23.58 | 23.14 | 32.05 | 22.47 | 34.82 | 55.24 | 57.89 | 48.65 | 50.36 | 55.39 | 57.33 | 50.05 | 52.54 |
| 24 | 22.64 | 32.25 | 20.67 | 28.34 | 27.51 | 39.62 | 25.61 | 40.33 | 55.50 | 57.25 | 49.25 | 52.67 | 55.58 | 57.68 | 50.58 | 52.12 |
| 48 | 28.92 | 40.66 | 24.69 | 33.69 | 38.24 | 55.68 | 32.05 | 49.81 | 55.89 | 57.66 | 49.81 | 52.33 | 55.97 | 57.92 | 50.66 | 52.85 |

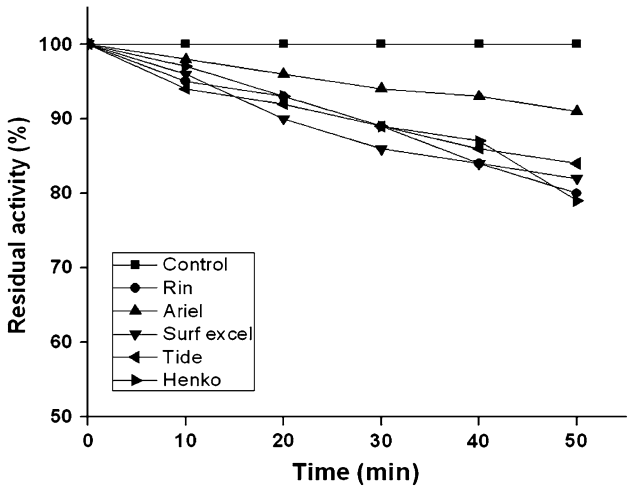


**Fig. 6** Stability of xyloglucanase in presence of various detergents. Xyloglucanase was incubated at 50°C in the presence of various detergents, and the samples were removed every 10 min for 1 h and the residual enzyme activity was determined under the standard assay conditions

**Table 7** Effect of organic solvents on stability of xyloglucanases at 50°C

| Solvent type | Solvent | Residual activity (%) | | |
|---|---|---|---|---|
| | | 0 min | 25 min | 50 min |
| Control | Nil | 100 | 28 | 6 |
| Water miscible | Acetone | 100 | 52 | 20 |
| | Ethanol | 100 | 30 | 8 |
| | Isopropanol | 100 | 68 | 32 |
| | Methanol | 100 | 59 | 27 |
| Water immiscible | Benzene | 100 | 44 | 18 |
| | Butanol | 100 | 70 | 39 |
| | Chloroform | 100 | 32 | 14 |
| | DMSO | 100 | 47 | 22 |
| | Hexane | 100 | 30 | 11 |

detergents. Exploitation of the thermostability of this xyloglucanase for hydrolysis of TKP is favoured since it would require a reduction in process time and enzyme dosage.

**Acknowledgments** MR acknowledges the financial support from CSIR Emeritus Scheme. MR is thankful to Dr Barry McCleary, Megazyme International Ireland Ltd for xyloglucan from tamarind seeds. VM acknowledge the senior research fellowship from CSIR Emeritus Scheme, Govt. of India.

## References

Anish TR (2007) Molecular characterization and applications of glycosidases from *Thermomonospora* sp. Ph D thesis, University of Pune

Benko Z, Siika-aho M, Viikari L, Reczey K (2008) Evaluation of role of xyloglucanase in the enzymatic hydrolysis of lignocellulosic substrates. Enzyme Microb Tech 43:109–114

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrisset B (2009) The Carbohydrate-active enZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37(database issue):D233–D238

Cesar T, Mrsa V (1996) Purification and properties of xylanase produced by *Thermomyces lanuginosus*. Enzyme Microb Tech 19:289–296

Cho Y, Wei G, Watkine S, Lee SR, Kim TR, Brady BattCA (1994) Thermostable variants of bovine $\beta$-lactoglobulin. Protein Eng 7:263–270

George SP, Ahmad A, Rao MB (2001) Studies on carboxymethyl cellulase produced by alkalothermophilic actinomycetes. Bioresour Technol 77:171–175

Gloster TM, Ibatullin FM, Macauley K, Eklo JM, Roberts S, Turkenburg JP, Bjørnvad ME, Jørgensen P, Danielsen S, Johansen KS, Borchert TV, Wilson KS, Brumer H, Davies GJ (2007) Characterization and three-dimensional structures of two distinct bacterial xyloglucanases from families GH5 and GH12*. J Biol Chem 282:19177–19189

Goyal P, Kumar V, Sharma P (2007) Carboxymethylation of tamarind kernel powder. Carbohydr Polym 69:251–255

Grishutin SG, Gusakov AV, Markov AV, Ustinov BB, Semenova MV, Sinitsyn AP (2004) Specific xyloglucanases as a new class of polysaccharide-degrading enzymes. Biochim Biophys Acta 1674:268–328

Gupta AK, Gautam SP (1993) Purification and properties of extracellular $\alpha$-glucosidase from the thermophilic fungus *Malbranchea sulfurea*. J Gen Microbiol 139:963–967

Hakamada Y, Arata S, Ohashi S (2011) Purification and characterization of xyloglucan specific glycosyl hydrolase from *Aspergillus oryzae* RIB 40. J Appl Glycosci 58:47–51

Hopwood DA, Bibb MJ, Chater KF, Kiersen T, Burton CJ, Kieser HM, Lydia DJ, Smith CP, Ward JM, Shrempf H (1985) In: Genetic manipulation of Streptomyces—a laboratory manual. The John Innes foundation Norwich, England

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17:1244–1245

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York

Leite RS, Gomes E, da Silva R (2007) Characterization and comparison of thermostability of purified $\beta$-glucosidases from a mesophilic *Aureobasidium pullulans* and a thermophilic *Thermoascus aurantiacus*. Process Biochem 42:1101–1106

Longo MA, Combes D (1999) Thermostability of modified enzymes-a detailed study. J Chem Technol Biotechnol 74:25–32

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275

Mandels M, Weber J (1969) The production of cellulases. Adv Chem 95:391–414

Marin E, Sanchez L, Perez MD, Puyol P, Calvo M (2003) Effect of heat treatment on bovine lactoperoxidase activity in skim milk: kinetic and thermodynamic analysis. J Food Sci 68:89–93

Martinez-Fleites C, Guerreiro CIPD, Baumann MJ, Taylor EJ et al (2006) Crystal structures of *Clostridium thermocellum* xyloglucanases, XGH74A, reveal the structural basis for xyloglucan recognition and degradation. J Biol Chem 281:24922–24933

Master ER, Zheng Y, Storms R, Tsang A, Powlowski J (2008) A xyloglucan-specific family 12 glycosyl hydrolase from

*Aspergillus niger*: recombinant expression, purification and characterization. Biochem J 411:161–170

Menon V, Prakash G, Prabhune A, Rao M (2010a) Biocatalytic approach for the utilization of hemicellulose for ethanol production from agricultural residue using thermostable xylanase and thermotolerant yeast. Biores Techno 101:5366–5373

Menon V, Prakash G, Rao M (2010b) Enzymatic hydrolysis and ethanol production using xyloglucanase and *Debaryomyces hansenii* from tamarind kernel powder: galactoxyloglucan predominant hemicellulose. J Biotech 148:233–239

Menon V, Prakash G, Rao M (2010c) Value added products from hemicelluloses: Biotechnological perspective. Glob J Biochem 1:36–67

Nazir A, Soni R, Saini HS, Manhas RK, Chadha BS (2009) Purification and characterization of an endo-glucanase from *Aspergillus terreus* highly active against barley $\beta$-glucan and xyloglucan. World J Microbiol Biotechnol 25:1189–1197

Pal A, Khanum F (2010) Characterizing and improving the thermostability of purified xylanase from *Aspergillus niger* DFR-5 grown on solid state medium. J Biochem Tech 2:203–209

Powlowski J, Mahajan Sonam, Schapira M, Master ER (2009) Substrate recognition and hydrolysis by a fungal xyloglucan-specific family 12 hydrolase. Carbohydr Res 344:1175–1179

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sinitsyana OA, Fedorova EA, Pravilinikov AG, Rozhkova AM, Skomarovsky AA, Matys VY, Bubnova TM, Okunev ON, Vintesky YP, Sinistyn AP (2010) Isolation and properties of xyloglucanases of *Penicillium* sp. Biochemistry 75:41–49

Wong DDWS, Chan VJ, McCormack AA, Batt SB (2010) A novel xyloglucan- specific endo-$\beta$-1, 4-glucanase: biochemical properties and inhibition studies. Appl Microbiol Biotechnol 86:1463–1471

Yaoi K, Mitsuishi Y (2004) Purification, characterization, cDNA cloning, and expression of a xyloglucan endoglucanase from *Geotrichum* sp. M128. FEBS Lett 560:45–50

Yaoi K, Nakai T, Hiyoshi A, Mitsuishi Y (2005) Cloning and characterization of two xyloglucanases from *Paenibacillus* sp. strain KM21. Appl Environ Microbial 71:7670–7678

Zhou Q, Rutland MW, Teeri TT, Brumer H (2007) Xyloglucan in cellulose modification. Cellulose 14:625–641