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Production, Purification and Characterization of Hemicellulose from *Penicillium janthinellum* 3CHP

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Abstract

Penicillium janthinellum 3CHP, a mesophilic fungal strain, isolated from the pine forest of Simla hill was found to produce an array of hemicelluloses when grown in urea medium with sorghum straw as the carbon source. For purification these enzymes, were subjected to ion exchange chromatography. The active fractions pertaining to á-arabinofuranosidase obtained from it were pooled and concentrated using PM-10 (10 KDa cut off) fitted into an Amicon cell and further purified by gel filtration chromatography. Active fractions which exhibited high á-arabinofuranosidase activity in gel filtration chromatography were again pooled, concentrated and analyzed by SDS PAGE and zymogram development using L-umbelliferyl arabinofuranoside as substrate. Two isoforms of á-arabinofuranosidase were observed with molecular weight of approximately 60 and 80 KDa respectively. á-arabinofuranosidase, was further characterized with respect to various parameters such as temperature, pH, effect of various metal ions and chemicals, substrate specificity, and thermo-stability.

Key words: α-L-arabinofuranosidase, gel filtration chromatography, ion-exchange chromatography, zymogram

Introduction

Lignocellulose is a complex polymeric material composed of 40-60% cellulose, 20-40% hemicelluloses and 15-25% lignin (Spano *et al.* 1980). Hemicelluloses are more heterogeneous and the second most abundant organic structure which refers to a group of homo- and hetero- polymers consisting of xylopyranose, mannopyranose, glucopyranose and galactopyranose main chains with a number of substituents (Jeffries 1994). Xylan and glucomannan are the two main types of hemicellulose (Viikari *et al.* 1992). Xylan is a complex and major heteropolysaccharide constituent of hemicellulose which is present in the form of arabinoglucuronoxylan in softwood and grasses, and as *O*-acetyl-4-*O*-methylglucurono-β-D-xylan in hardwood (Laine 2005). This heteropolymer containing a backbone of β-1,4 linked xylopyranosyl units is substituted with L-arabinosyl, acetyl, feruloyl, *p*-coumaroyl and glucoronyl residues (Howard *et al.* 2003). The frequency and the composition of the branches in isolated xylans are dependent upon their source and method of extraction (Wong *et al*. 1988). Glucomannan and galactomannan, the major hemicellulolytic components of softwoods, consist of a β-1,4-linked D-mannose backbone that is substituted with α-1,6-linked D-galactose.While galactoglucomannan also contains â-1,4-linked D-glucose residues (Ronald & Jaap 2001).

Due to the heterogeneity and complex chemical nature of hemicellulose, its hydrolysis into simpler constituents (monomers, dimers or oligomers) requires the action of a wide spectrum of enzyme with diverse catalytic specificaties and modes of action Endo-1,4-âxylanase cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the subtrate.A variety of microorganisms are reported to produce endoxylanases that can degrade β-1,4 xylan in a random fashion, yielding a series of linear and branched oligosachharide fragments (Kormelink *et al.* 1993, Bhat & Hazlewood 2001). Exo-1,4-β-D-xylosidase catalyzes the hydrolysis of xylooligosaccharides by removing successive D-xylose residues from the non-reducing end. α-L-Arabinofuranosidase hydrolyzes the terminal, non-reducing arabinofuranosyl groups of arabinans, arabino-xylans, and arabinogalactans. α-D-glucuronidase is required for the hydrolysis of

α-1,2-glycosidic linkages between xylose and D-glucuronic acid. Esterases break the bonds of xylose to acetic acid (xylan acetyl esterase), arabinose side chain residues to ferulic acid (feruloyl esterase), arabinose side chain residues to *p*-coumaric acid (*p*-coumaroyl esterase). Cleavage of acetyl, feruloyl and *p*-coumaroyl groups from the xylan are helpful in removal of lignin. Hemicellulases have various applications from biotechnological and industrial point of view (Nigam 2011). Potential applications of hemicellulases include biobleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification, converting lignocellulosic substances to feed-stock and fuels, as dietary supplement to treat poor digestion and pharmaceuticals etc. Interestingly, the hemicellulases required for each of the above processes are of different characteristics and therefore, the choice of hemicellulase and the producing microorganisms could differ. Nevertheless, few of the microbial strains produce versatile enzymes that can perform optimally well for different process applications. Recently, the interest generated on functional foods and possible use of xylooligosaccharides as pre-biotics is another area where the role of hemicellulases needs to be evaluated (Bhat 1998). Because of these applications, hemicellulases possess tremendous commercial potential and need to isolate new sources of hemicellulases and their characterization is the thrust arena that should be continued with interest. Hemicellulases are produced by many bacteria and fungi. From an industrial point of view, filamentous fungi are interesting producers of these enzymes. Among various filamentous fungi, *Penicillium* sp. have also been exploited for the production of hemicellulases. According to Chavez *et al.* (2004), Penicillia constitute a rich source of enzymes for the biodegradation of hemicelluloses. In the current study, we have produced α-L-arabinofuranosidase from *P. janthinellum*, which has been characterized with regard to various parameters.

Methodology

Culture of the fungus

A mesophilic fungal strain *Penicillium janthinellum* 3CHP was used in the experiment. Its culture was grown at 30°C on PDA medium. A spore suspension from a five day old slants of the culture was prepared in 6.0 ml of sterilized water blank containing Tween 80 (0.1% w/v) as surfactant.

Enzyme production

Sorghum straw was cut into small pieces, dried in an oven at 50°C and ground to approximately 2mm size. Solid state fermentation (SSF) was carried out in Erlenmeyer flasks (250 ml) that contained sorghum straw (5g) as carbon source and 15 ml of urea medium of the following composition (%w/v); $(NH_4)_2SO_4$ 1.0; Urea 0.3; KH_2PO_4 0.3; $MgSO_4$ 0.05; $CaCl_2$ 0.05; K_2HPO_4 0.05. The medium was sterilized at 15 psi for 15 minutes at 121°C. Prior to sterilization, the pH of the medium was adjusted to 7.0. Urea, being labile to heat, was separately cold-sterilized using membrane filter of size 0.2 µm. The production medium was inoculated with 2 ml of spore suspension prepared as above, and incubated in water-saturated atmosphere at atmosphere at 30°C for 5 days. The enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks and the contents were mixed thoroughly with the help of sterile glass rod. Then it was kept under shaking conditions on a rotary shaker (ORBITEK) at 140 rpm for 30 min. The fermented slurry was filtered through muslin cloth and centrifuged at 10,000 rpm for 10 min to remove the debris. Resultant clear supernatant was added with EGTA-EDTA mix (each @ 1 mM) and PMSF (phenyl methyl sulphonyl fluoride, 0.2 M) to inhibit protease activity and sodium azide (0.02 % w/v) to avoid further contamination. The enzyme extract was then stored in sterilized vials at -20°C and it was used for enzyme assay whenever necessary.

α-L-arabinofuranosidase assay

α-L- arabinofuranosidase activity of the crude extract as well as fractions obtained from ion-exchange and gel filtration chromatography was determined in the following way.

The substrate *p*NP-α-arabinofuranoside (3mM) was used to assay α-L- arabinofuranosidase (EC 3.2.1. 55). The reaction mixture containing 25 µl of enzyme, 25 µl of substrate solution and 50 µl of sodium acetate buffer (50 mM, pH 5.0) were incubated at 50°C for 30 minutes in dark. The reaction was terminated using 100 µl of NaOH glycine buffer (400 mM, pH 10.8) and the developed colour was read at 405 nm using ELISA reader (MULTISKAN; Labsystems).

$$\text{Activity} = \frac{\text{Optical Density(OD)} \times \text{Dilution Factor} \times 45.432}{104.3}$$

Where OD= Mean of reaction mixtures-(substrate blank + enzyme blank)

In case of active fractions obtained after ion-exchange chromatography and gel filtration chromatography, α-L- arabinofuranosidase activity was determined in the same way except that the reaction mixture was incubated at 50°C in dark for an hour.

Estimation of protein

The protein in the fractions eluted from ion exchange chromatography as well as gel filtration chromatography was determined by protein-dye binding method given by Lowry.

Purification of hemicellulases

Desalting

Enzyme extract was concentrated ten times using PM-10 membrane (10 K Da cut off) fitted into an Amicon cell. Further washing was given to it with the help of sterilized milli Q water to remove any salts present in the enzyme extract.

Ion-exchange chromatography

Enzyme extract (70 mg) was loaded onto source 15 Q column and was eluted under isocratic conditions followed by linear gradient of 1 M NaCl in 0.020 M Tris HCl at a flow rate of 0.5 ml per minute. All the fractions (5 ml each) were analyzed for α-L-arabinofuranosidase activity as well as for protein. Active fractions corresponding to the maximum α-L-arabinofuranosidase obtained during NaCl gradient elution were pooled, desalted and concentrated with 50 mM sodium acetate buffer pH 5 having 0.15 M NaCl , using PM-10 membrane (10 K Da cut off) fitted into an Amicon cell and concentrated enzyme was subjected to gel filtration chromatography for further purification of the enzyme.

Gel filtration chromatography

One ml of enzyme extract was applied to the Sephacryl HR 200 (Sigma) matrix column and eluted with two bed volumes of sodium acetate buffer (50 mM, pH 5.0) containing NaCl (0.15 M, pH 5.0) at a flow rate of 0.2 ml per minute. The active fractions collected (2ml each) were assayed for α-L-arabinofuranosidase activity as well as for protein and they were further pooled, concentrated and used for SDS-PAGE and zymogram analysis and for the characterization of the enzyme.

Molecular weight determination by SDS-PAGE

The homogeneity and molecular weight of the enzyme was determined by using 12% SDS-PAGE gel (Laemmli, 1970) using Bio-Rad Mini-Protein II electrophoresis unit. Protein bands in the gel were visualized by silver staining method (Blum *et al.* 1987).

Zymogram analysis

Zymogram analysis of the hemicellulases obtained in SDS PAGE was performed using modified method of Taylor *et al*. (2006). SDS-PAGE gels were further renatured by incubating them in refolding buffer [20 mM *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid (pH 6.8), 2.5% Triton X-100, 2mM Dithiothreitol (DTT), and 2.5 mM $CaCl_2$] for 1 hour at room temperature and held at 4°C overnight in fresh refolding buffer. The gels were thoroughly washed 5-6 times in distilled water and overlaid with the substrate solution prepared by dissolving 1.8 mg of the substrate, á-L-umbelliferyl arabinofuranoside, in DMSO (1 ml) and 1 ml of sodium acetate buffer (50 mM, pH 5.0). The bands were observed using UV-transillluminator (Bio- Rad).

Characterization of the enzyme

The active fractions obtained after gel filtration chromatography corresponding to α-L-arabinofuranosidase which gave purified bands on SDS-PAGE and zymogram analysis were further characterized for their temperature and pH optima, effect of metal ions and chemicals, various substrate specificities and thermostability.

Results and Discussion

In the present study, production and purification of hemicellulases was carried out from *P. janthinellum* 3CHP. One of the hemicelluloses, α-L arabinofuranosidase, was purified and characterized for its different parameters.

Ion-exchange chromatography

The results below (Fig. 1) show the elution profile of α-L-arabinofuranosidase activity. The chromatogram shows that α-L-arabinofuranosidase eluted as two distinct peaks (AF I and AF II) under isocratic and NaCl gradient conditions, respectively. Fractions showing maximum α-L-arabinofuranosidase activities were pooled, concentrated and used for further purification by gel filtration chromatography.

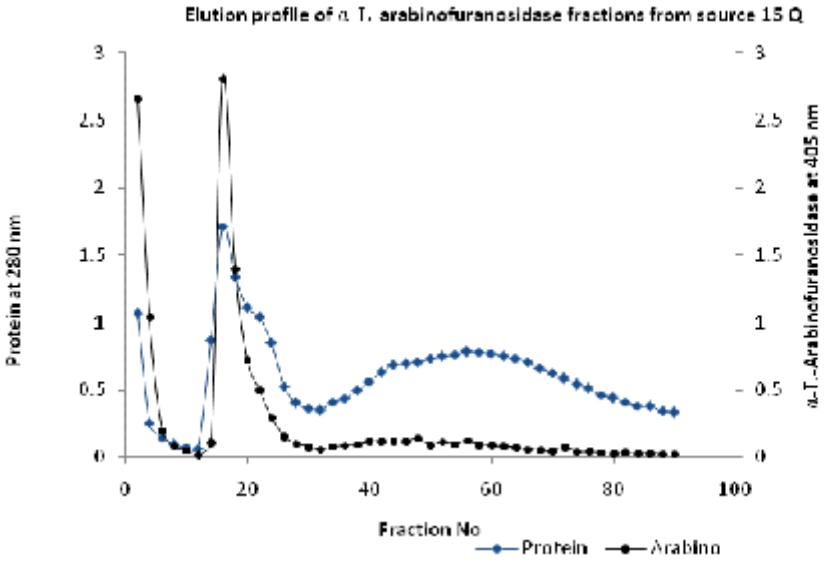

Fig.1. Elution profile for α-L-arabinofuranosidase active fractions from ion-exchange chromatography

Gel filtration chromatography

Fig. 2 shows different chromatograms depicting elution profiles for α-L-arabinofuranosidase along with its respective protein in 112 different fractions obtained after gel filtration chromatography. The fractions exhibiting high α-L-arabinofuranosidase activity were subjected to SDS-PAGE analysis to assess the production of arabinofuranosidase. The fractions from 26 to 48 were found to contain purified α-L-arabinofuranosidase enzyme (Fig. 2). Fig. 3(a) shows the SDS-PAGE of the concentrated active enzyme fractions (α-L-arabinofuranosidase) after gel filtration chromatography and Fig. 3(b) shows the activity staining of the renatured gel using α-L-umbelliferyl arabinofuranoside as substrate. The results show that these fractions contained two α-L-arabinofuranosidase isoforms on SDS-PAGE. The bands showed fluorescence when reacted with α-L-umbelliferyl arabinofuranoside indicating the presence of α-L-arabinofuranosidase activity in these bands. The protein bands were of approximately 63 and 80 KDa respectively.

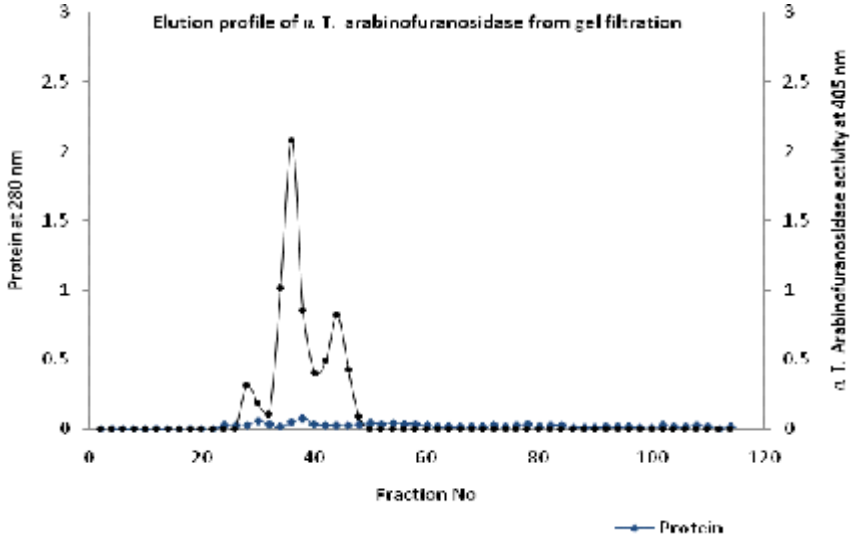

Fig.2. Elution profile for α-L-arabinofuranosidase active fractions from gel filtration chromatography

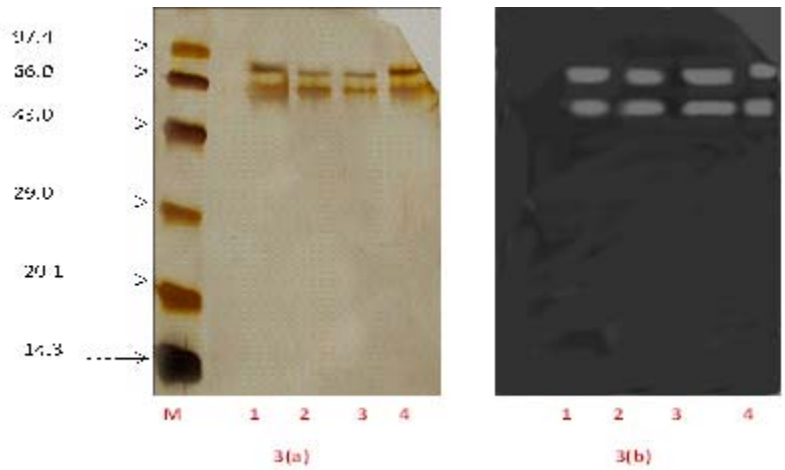

Fig. 3(a). SDS–PAGE of á-L-arabinofuranosidase

Fig. 3(b). Zymogram to detect á-L-arabinofuranosidase

Characterization of purified α-L-arabinofuranosidase

After having α-L-arabinofuranosidase purified, an attempt was made to thoroughly characterize this enzyme with respect to various parameters.

Effect of temperature

Fig.4 shows the effect of different temperatures on α-L-arabinofuranosidase activity. It was observed that the activity increased when the temperature increased from 20°C to 50°C and decreased steadily thereafter. The enzyme though showed appreciable activity at 80°C but total loss of activity was observed at 90°C. Optimum temperature for the enzyme was also found to be 50°C for *P. purpurogenum* (Ioannes *et al*. 2000) and *P*. sp. LYG0704 (Lee *et al.* 2011).

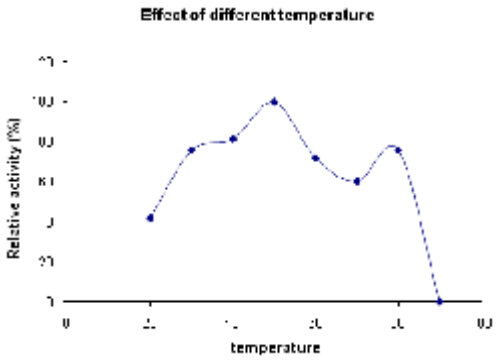

Fig. 4. Effect of different temperatures on α-L-arabinofuranosidase

Effect of pH

Results in Fig. 5 decipher the effect of different pH on á-L-arabinofuranosidase activity. The enzyme was found to be completely inactive at pH 3.0 and 4.0. The enzyme activity was found to be optimum between pH 6.0 and 7.0. The activity declined beyond pH 7.0. Gilead and Shoham (1995) reported that α-L-arabinofuranosidase activity was maximum in *Bacillus stearothermophilus* at pH 5. 5-6.0 where as the optimum pH for the same enzyme was found to be 4.0 in *P. purpurogenum* (Ioannes *et al.* 2000). Yan *et al.* (2012) reported optimum pH for α-L-arabinofuranosidase from *Chaetomium* sp. was 5.0

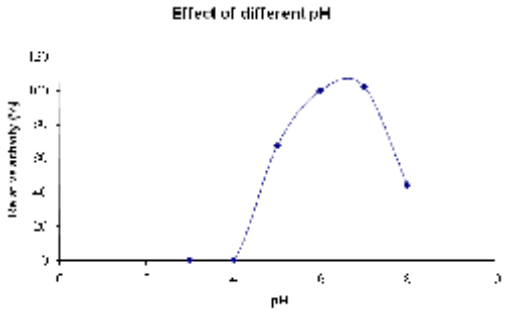

Fig. 5. Effect of different pH on á-L-arabinofuranosidase

Effect of metal ions and chemicals

The effect of various metal ions and chemicals (5mM) is elucidated in Fig. 6. Various metal ions and chemicals (5mM) were found to positively modulate the activity of α-L-arabinofuranosidase. Presence of metal ions Mg, Mn, K, Zn, Cu, Fe as well as reducing agent DTT also positively influenced the activities of the enzyme. In addition, reducing agent mercaptoethanol as well as

chelating agent EDTA also showed positive influence on the enzyme activity. Higher activity of the enzyme in presence of mercaptoethanol and DTT indicates the presence of thiol groups in their active site. Increased enzyme activity in presence of reducing agents like mercaptoethanol and DTT may be due to suitable changes in conformation of protein (Badhan *et al*. 2007).

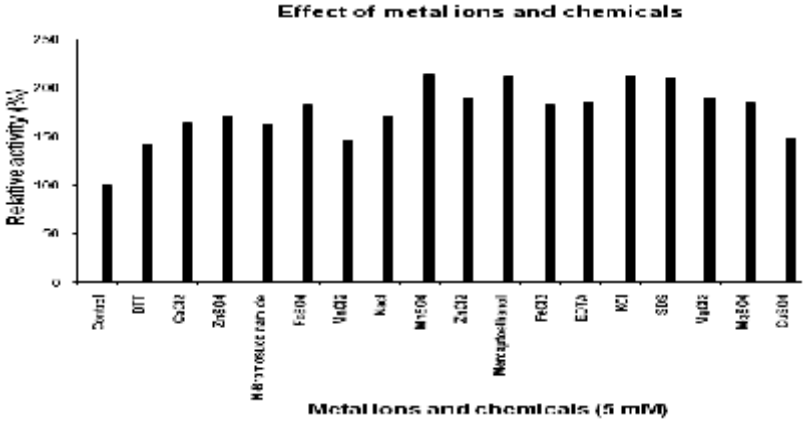

Fig.6. Effect of different metal ions and chemicals (5mM) on α-L- arabinofuranosidase

Substrate specificity

Table 1. reveals the effect of various *p*NP-substituted substrates on α-L-arabinofuranosidase activity. Except for *p*NP-α-L-arabinofuranoside, the enzyme showed low activity against other *p*NP-substituted substrates. The enzyme did not exhibit any activity with *p*NP-Palmitate and *p*NP-Galactopyaranoside and showed negligible activity with *p*NP-Mannopyranoside. The effect of *p*NP-Lactopyranoside and *p*NP-Cellobioside was almost equal on the enzyme activity though the effect was quite low compared to *p*NP-α-L-arabinofuranoside. This is in commensurate with the data obtained by Gilead and Shoham (1995) who reported that α-L- arabinofuranosidase from *Bacillus stearothermophilus* T-6 did not show any activity with *p*NP-Palmitate, *p*NP-Galactopyaranoside and *p*NP-Mannopyranoside.

Table1. Substrate specificities of α-L-arabinofuranosidase

Substrates	Relative activity (%)
*p*NP-α-L-arabinofuranoside	100
*p*NP-β-xylopyranoside	1.87
*p*NP-Lactopyranoside	15.69
*p*NP-Cellobioside	15.30
*p*NP-Glucopyranoside	4.14
*p*NP-Mannopyranoside	1.28
*p*NP-Galactopyranoside	0.59
*p*NP-Acetate	18.49
*p*NP-Myristate	4.93
*p*NP-Butyrate	14.88
*p*NP-Stearate	8.88
*p*NP-Palmitate	0.19

Thermostability

The results in Fig. 7 and 8 show that the enzyme, α-L-arabinofuranosidase, was apparently stable at 40°C and 50°C at pH 7.0 and showed no loss of activity even after 90 minutes of incubation. The enzyme activity was lost to some extent after longer incubation. The loss of activity was comparatively more at pH 6.0 as compared to pH 7.0. This result is similar to the data reported previously by Singh *et al.* (2000) on *Thermomyces lanuginosus.* They had reported that the enzyme α-L-arabinofuranosidase from *T. lanuginosus* was quite stable at 50°C at pH 5.0.

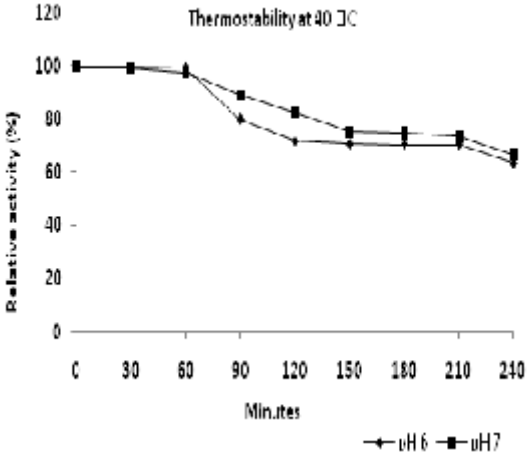

Fig. 7. Thermostability of α-L-arabinofuranosidase at 40°C, pH 6.0 and pH7.0

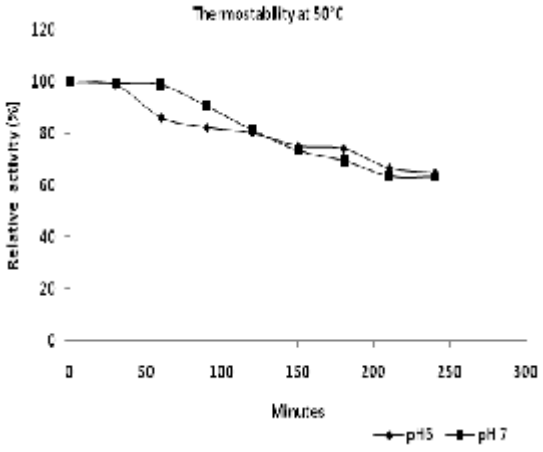

Fig. 8. Thermostability of α-L-arabinofuranosidase at 50°C, pH 6.0 and pH7.0

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