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Partial purification and characterization of xylanases from *Aspergillus awamori* and *Aspergillus phoenicis*

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The aim of this study is to partially purify and characterize xylanases from two spoilage fungi *Aspergillus awamori* and *Aspergillus phoenicis*. Chromatography of xylanases from *A. awamori* and *A. phoenicis* on DEAE-Sepharose separated three (XynI, XynII and XynIII) and two (XynI and XynII) isoenzymes, respectively. Different relative activities percentage were delectated for *A. awamori* XynI and *A. phoenicis* XynI toward beachwood, oat spelts and birchwood xylans. The apparent K_m and V_{max} values for *A. awamorii* xynI and *A. phoenicis* xynI were 4.34 and 3.83 mg/ml, 0.28 and 0.38 µmol/ml, respectively. The acidic pH optimum has been reported for *A. awamori* XynI (pH 5.0) and *A. phoenicis* XynI (broad pH 4.5 to 5.5). The optimal temperatures of *A. awamorii* XynI and *A. phoenicis* XynI were 25 and 60°C, respectively. *A. awamori* XynI and *A. phoenicis* XynI were thermal stable up to 40 and 60°C, respectively. The effect of different metal cations on *A. awamori* XynI and *A. phoenicis* XynI showed partial inhibition and/or activation effects on the enzyme activity. *p*-HMB and sodium benzoate had only inhibitory effect on *A. phoenicis* XynI. Both benzoic and citric acids had moderate inhibitory effects on of *A. awamori* XynI and *A. phoenicis* XynI. The benzoic and citric acids were studied as antifungal compounds against *A. awamori* and *A. phoenicis* loaded on injured lemon and tomato, respectively.

Key words: *Aspergillus awamori*, *Aspergillus phoenicis*, xylanases, characterization.

INTRODUCTION

Xylans are hemicellulose compounds and are the second most abundant natural polysaccharide behind cellulose (Collins et al., 2005). These compounds are present in the cell wall and in the middle lamella of plant cells. Xylanolytic enzymes (O-glycoside hydrolases, EC 3.2.1.x) are a group of enzymes that are involved in the hydrolysis of xylans and arabinoxylan polymers. These enzymes include endo-1,4-β-xylanase, β-xylosidase, α-arabinofuranosidase and acetylxylan esterase (Biely, 1993). Xylanases hydrolyze 1,4-β-D-xylosidic linkages in xylan to produce xylo-oligosaccharide. Xylanases are widely distributed. They occur in both prokaryotes and eukaryotes (Dekker and Richards, 1976) and have been demonstrated in higher eukaryotes, including protozoa, insects, snails and germinating plant seeds (Taiz and

Honigman, 1976). Amongst the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases (Dekker, 1985). The xylanases have been reported mainly from bacteria (Sunna and Antranikian, 1997), fungi (Sunna and Antranikian, 1997), actinomycetes (Beg et al., 2000), and yeast (Liu et al., 1999). A wide variety of bacteria and fungi produce xylan-degrading enzymes, which they secrete into their immediate surroundings in order to break down the carbohydrate polymer xylan into shorter oligosaccharides which can then be used as an energy source by the microorganism. There are different types of xylanases varying in substrate specificities, primary sequences, folds and physicochemical properties (Colina et al., 2003).

Fungi in particular produce an abundance of extracellular pectinases and xylanases that are important factors for fungal spoilage (Miedes and Ester, 2004). In a previous paper (Al-Hindiet al., 2011), we isolated and

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identified two spoilage fungi *Aspergillus awamori* and *Aspergillus phoenicis* from lemon and tomato, respectively. The purpose of this paper is to study the characterization of xylanases from *A. awamori* and *A. phoenicis* for combating these fungi.

MATERIALS AND METHODS

Fruit spoilage fungi

A. awamori and *A. phoenicis* were isolated and identified from lemon and tomato as previously described (Al-Hindi et al., 2011).

Cultivation of fungi

A. awamori and *A. phoenicis* were inoculated under aseptic conditions in 250 ml Erlenmeyer flasks contained 5% lemon and tomato peels, respectively. The inoculated flasks were incubated at 28°C with shaking on a rotary incubator shaker at 150 rpm for 5 days. The cell-free broth was recovered by filtration using a polyamide tissue. The cell-free broth was subjected to dialysis against 20 mM Tris-HCl buffer, pH 7.2 over night. The dialyzate was centrifuged at 10,000 rpm for 12 min and the supernatant was designated as crude extract.

Partial purification of xylanases

Crude extracts from *A. awamori* and *A. phoenicis* were separately loaded on a DEAE- Sepharose CL-6B column (10 × 1.6 cm i.d.) equilibrated with 50 mM Tris-HCl buffer, pH 7.2. The enzyme was eluted with a stepwise gradient from 0.0 to 0.3 M NaCl in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. Protein fractions exhibiting enzyme activity were pooled.

Xylanase assay

Xylanase activity was assayed according to Miller (1959). The reaction mixture (0.5 ml) contained 1% xylan, 0.05 M sodium acetate buffer pH 5.5 and a suitable amount of enzyme. Assay was carried out at 37°C for 1 h. Then 0.5 ml dinitrosalicylic acid reagent was added and heated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 560 nm. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol of xylose per hour under standard assay conditions.

Protein determination

Protein was determined either by measuring the absorbance at 280 nm (Warburg and Christian, 1942) or by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Characterization of xylanase

K_m

The k_m value was determined from Lineweaver-Burk plot by using xylan concentrations from 2 to 6 mg/ml (Lineweaver and Burk, 1934).

Optimum pH

Enzyme activity was determined at various pH using 50 mM each of sodium acetate (pH 4.0 to 6.0) and Tris-HCl (6.5 to 8.5). The maximum activity was taken as 100% and percentage relative activity plotted against different pH values.

Optimum temperature

Enzyme activity was determined at a temperature range of 20 to 70°C. The maximum activity was taken as 100% and percentage relative activity were plotted against different temperatures.

Thermal stability

The enzyme was incubated at a temperature range of 20 to 70°C for 15 min prior to substrate addition. The percentage relative activity was plotted against different temperatures.

Effect of metal ions

The enzyme was incubated with 2 mM solution of Co^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Hg^{2+} for 15 min prior to substrate addition. The enzyme activity without metal ions was taken as 100% and percentage relative activity was determined in the presence of metal ions.

Effect of chemical compounds

Enzyme activity was determined in the presence of PMSF, *p*-HMB, β-mercaptoethanol, trypsin inhibitor, 1,10 phenanthroline, EDTA, sodium citrate and sodium oxalate, sodium benzoate, benzoic acid, gallic acid, tannic acid and citric acid at a concentration of 5 mM. The enzyme activity without chemical compound was taken as 100% and percentage relative activity was determined in the presence of chemical compound.

Effect of benzoic and citric acids on the growth of fruit spoilage fungi

Benzoic and citric acids were used at the concentration of 5 mM as antifungal compounds. The fruits were divided into four groups: 1- injured lemon and tomato without acid, 2- injured lemon and tomato with each acid, 3- injured lemon and tomato with *A. awamori* and *A. phoenicis*, respectively, 4 injured lemon and tomato with each acid and *A. awamori* and *A. phoenicis*, respectively. The development of spoilage fungi was observed after 3, 5 and 7 days intervals of incubation at 28°C.

RESULTS AND DISCUSSION

Partial purification of xylanases from *A. awamori* and *A. phoenicis*

Chromatography of xylanases from *A. awamori* and *A. phoenicis* on DEAE-Sepharose separated three (XynI, XynII and XynIII with specific activities 1287, 1558 and 812 units/mg protein, respectively) and two (XynI and XynII with specific activities 888 and 1165 units/mg protein,
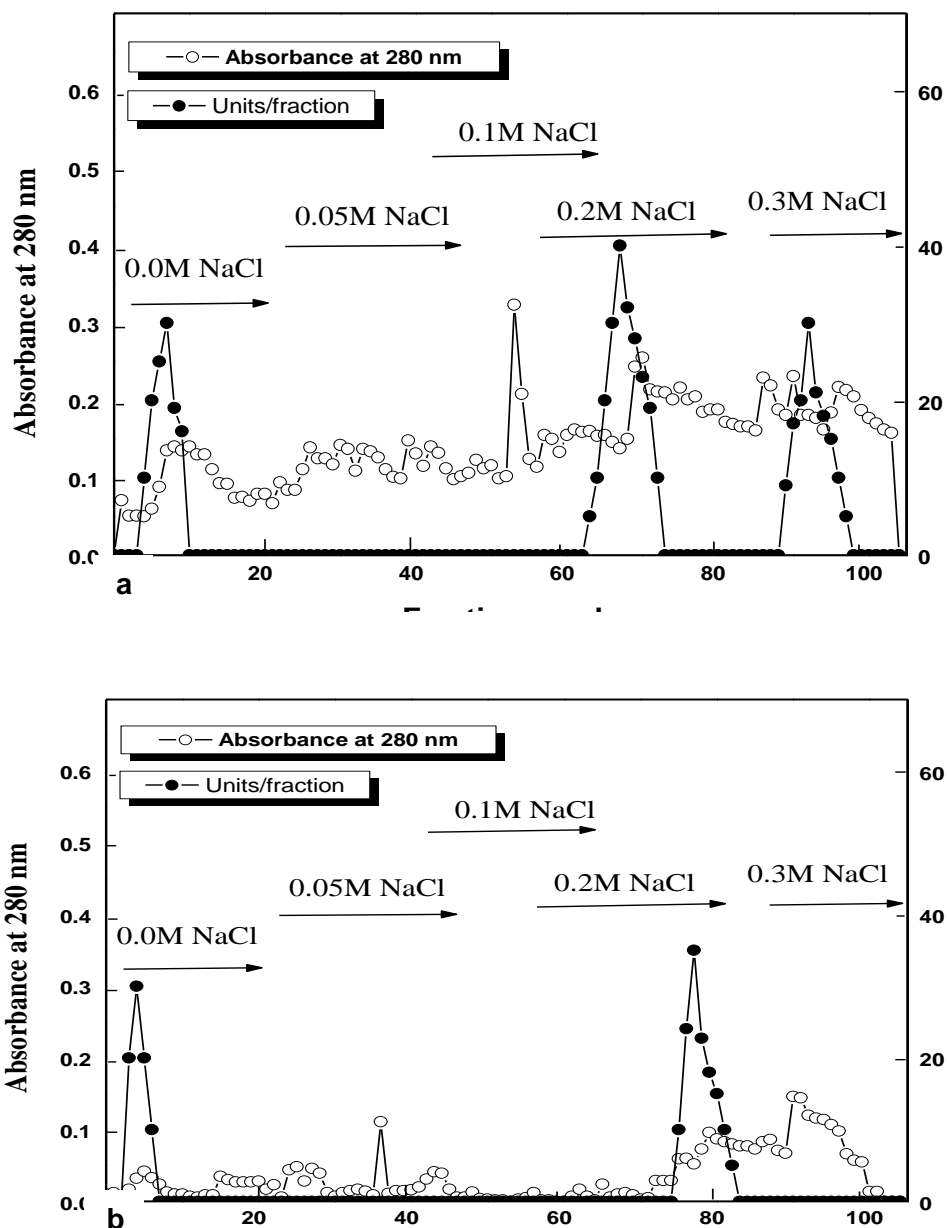
Figure 1. A typical elution profile for the chromatography of xylanase from (a) *A. awamori* and (b) *A. phoenicis* cell-free broths on DEAE-Sepharose column (10 × 1.6 cm i.d.) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.

respectively) isoenzymes, respectively (Figure 1, Table 1). Various numbers of xylanse isoenzymes with different specific activities were also separated from *Aspergillus niger* SCTCC 400264 (xynA1 and xynB with specific activities 16.58 and 1201.7 units/mg protein, respectively) (Yi et al., 2010), *A. ficuum* AF-98 (specific activity 288.7 units/mg protein) (Lu et al., 2008), *A. awamori* 2B.361U2/1 (specific activity 490 units/mg protein) (Teixeira et al., 2010) and *A. niger* GS1 (specific activity

522 units/mg protein) (Amaro-Reyes et al., 2011).

Characterization of *A. awamori* XynI and *A. phoenicis* XynI

With regard to substrate specificity, different sources of xylans have been tried as substrates. The enzyme acti-vity with xylan from beachwood was regarded as 100%

Table 1. Partial purification of xylanases from *A. awamori* and *A. phoenicis*.

Purification steps	Total units	Total protein (mg)	Specific activity (unit/mg protein)	Fold purification	Recovery (%)
Cell–free broths: *A. awamori*	873	0.91	959	1.00	100
A. phoenicis	245	0.96	255	1.00	100
DEAE-Sepharose: *A. awamori*					
XynI	103	0.08	1287	1.34	11.7
XynII	226	0.145	1558	1.62	25.8
XynIII	147	0.181	812	0.86	16.8
A. phoenicis					
XynI	80	0.09	888	3.48	32.6
XynII	120	0.103	1165	4.56	48.9

One unit of enzyme activity was defined as the amount of enzyme which produced one µmol xylose per h under standard assay conditions.

Table 2. Substrate specificity of xylanase isoenzymes from *A. awamori* and *A. phoenicis*.

Source of xylan	Relative activity (%)	
	A. awamori XynI	*A. phoenicis* XynI
Beachwood	100	100
Oat spelts	136	121
Birchwood	71	69

Each value represents the average of two experiments.

100% activity. Different relative activities percentage were delectated for *A. awamori* XynI (136% Oat spelts and 71% Birchwood) and *A. phoenicis* XynI (121% Oat spelts and 69% Birchwood) (Table 2). Little information has been reported on the substrate specificity for fungal xylanases. *Fusarium oxysporum* xylanase retained 75 and 65% of its activity by using oat spelts xylan and birchwood xylan compared with beachwood xylan, respectively (Christakopoulos et al., 1996). The substrate specificity using birchwood, beechwood and oat-spelt xylans was studied for xylanase from *Chaetomium* sp. CQ31 (Jiang et al., 2010).

The apparent K_m and V_{max} values for *A. awamorii* xynI and *A. phoenicis* xynI were 4.34 and 3.83 mg/ml, 0.28 and 0.38 µmol/ml, respectively (Figure 2). Two extracellular xylanases produced by the thermo tolerant fungus *Aspergillus caespitosus*, both xylanases had the K_m and V_{max} values of 2.5 mg/ml and 1679 units/mg protein (xylI) and 3.9 mg/ml and 113 U/mg protein (xylIII) (Sandrim et al., 2005). The K_m and V_{max} values were found to be 3.85 mg/ml and 570 µmol/mg/min of xylanase from *Aspergillus foetidus* (Shah and Dutta, 2005). The K_m values of two xylanases from *Penicillium sclerotiorum* using birchwood xylan and oat spelt xylan were 6.5 and 2.6 mg/ml for xylanase I, respectively, whereas the K_m values of xylanase II for these substrates were 26.61 and 23.45 mg/ml (Knob and Carmona, 2010).

The pH optimum was the very important physical parameter influencing the activity of enzymes. In this study, the acidic pH optimum has been reported for *A. awamori* XynI (pH 5.0) and *A. phoenicis* XynI (broad pH 4.5 to 5.5) (Figure 3). Similar acidic pH optima were reported for xylanases from *A. terreus* (pH 6.0) (Chidi et al., 2008), Marasmius sp. (pH 6.0) (Ratanachomsri et al., 2006), *Trichoderma viride* (pH 5.0) (Simoes et al., 2009), *Sporotrichum thermophile* (pH 4.0) (Kaur and Satyanarayana, 2004) and *Aspergillus niger* GS1 (pH 5.5) (Amaro-Reyes et al., 2011). The alkaline pH optima were reported for xylanases from *Penicillium citrinum* (pH 7.49) (Ghoshal et al., 2011) and *A. niger* (pH 7.5) (Coral et al., 2002).

The best temperature range for the production of xylanase by fungi is usually from 20 to 30°C (Rahman et al., 2003). However, the optimal temperatures of *A. awamorii* XynI and *A. phoenicis* XynI were 25 and 60°C, respectively (Figure 4). These optimal temperatures similar to those observed for xylanases from *Penicillium citrinum* (29°C) (Ghoshal et al., 2011), *A. ficuum* (45°C) (Lu et al., 2008), *A. awamorii* (60°C) (Lemos et al., 2000), *A. nidulans* (50 and 55°C) (Reis et al., 2003) and *A. oryzae* (60°C) (Kitamoto et al., 1999).

A. awamori XynI and *A. phoenicis* XynI were thermal stable up to 40 and 60°C, respectively (Figure 5). Similar thermal stability was reported for xylanases from *A. awamorii* (35 and 50°C) (Lemos et al., 2000), *Talaromyces thermophilus* (50°C) (Maalej et al., 2008),
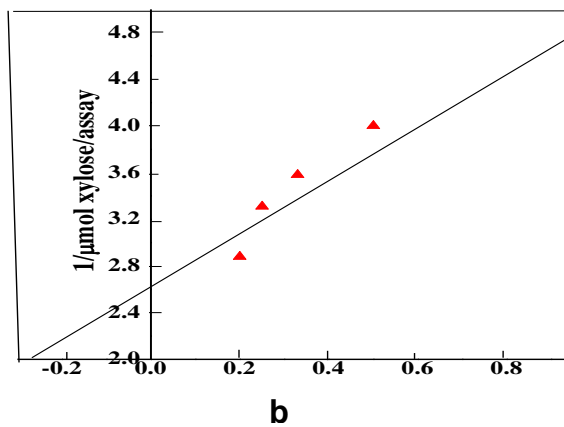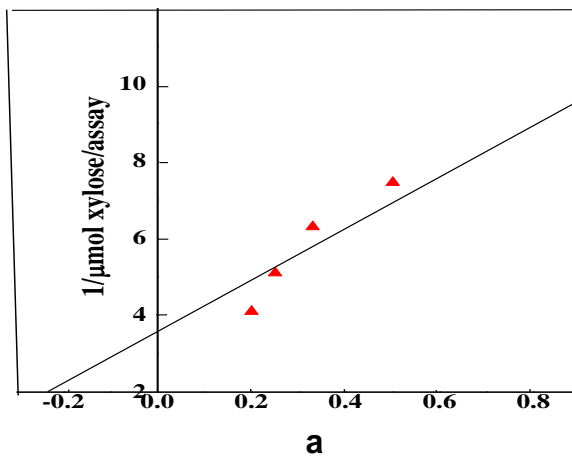
Figure 2. Lineweaver-Burk plot relating (a) *A. awamori* XynI and (b) *A. phoenicis* XynI reaction velocities to xylan as substarte concentrations. The reaction mixture contained in 0.5 ml: 50 mM sodium acetate buffer, pH 5.5 suitable amount of enzyme and concentrations of xylan ranging from 1 to 5 mg. Each point represents the average of two experiments.
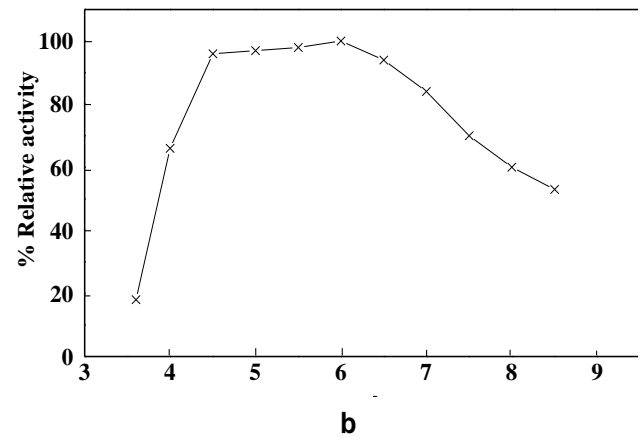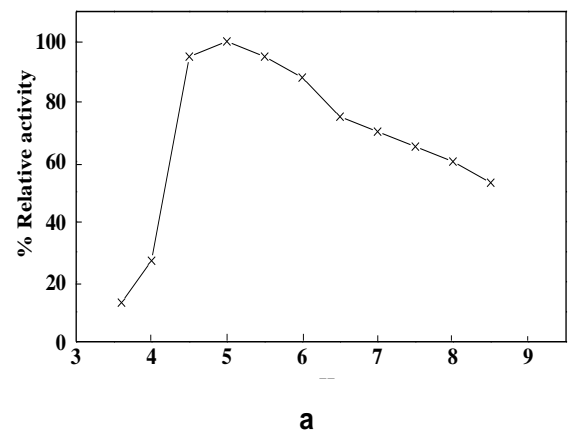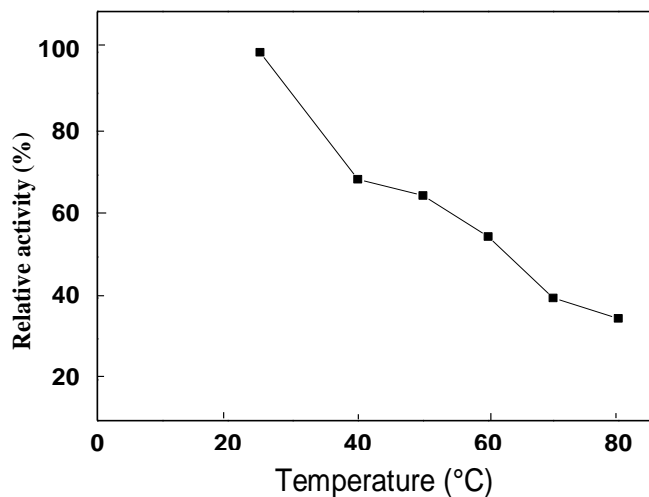

Figure 3. pH optimum of (a) *A. awamori* XynI and (b) *A. phoenicis* XynI. The reaction mixture contained in 0.5 ml: 2.5 mg xylan, suitable amount of enzyme and 50 mM sodium acetate buffer (pH 3.6 to 6.0), 50 mM Trsi-HCl buffer (6.5 to 8.5). Each point represents the average of two experiments.

Trichoderma resei QM 9414 (45°C) (Dekker, 1993) and *Thermomyces lanuginosus* (60°C) (Cesar and Mirsa, 1996). The effect of different metal cations such as Zn^{2+}, Pb^{2+}, Ca^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} at 2 mM on *A. awamori* XynI and *A. phoenicis* XynI showed partial inhibition and/or activation effects on the enzyme activity (Table 3). For *A. awamori* XynI, the partially inhibitory effect were detected by Hg^{2+} (19% inhibition) and Cu^{2+} (24% inhibition), while the activation effect were detected by Ca^{2+}, Co^{2+} and Ni^{2+} with enhancement in activity by 61, 32 and 51%, respectively. For *A. phoenicis* XynI, the effectiveness of metal cations as partially inhibitory effects were in the order of $Co^{2+} < Pb^{2+} < Cu^{2+} < Hg^{2+}$ with 10, 31, 70 and 88% inhibition, respectively. Xylanase of *Talaromyces thermophilus* was activated by Co^{2+}, and Cu^{2+}, and inhibited by Hg^{2+} Ba^{2+}, and Mn^{2+} (Maalej et al.,

2008). In *A. foetidus*, the xylanase activity was found to be enhanced by Co^{2+} and significantly inhibited by Hg^{2+} and Cu^{2+} (Shah and Dutta, 2005).

The effect of different compounds such as β-mercaptoethanol, PMSF, trypsin inhibitor, *p*-HMB, 1,10 phenanthroline, benzoic acid, gallic acid, tannic acid, EDTA, sodium benzoate, sodium citrate, sodium oxalate and citric acid at 5 mM on the activity of *A. awamori* XynI and *A. phoenicis* XynI was studied (Table 4). The most of compounds had no significant effect on the enzyme activity. Similarly, EDTA, PMSF, and 1,10-phenanthroline had no effect on the *A. niger* gr xylanase activity (Naganagouda et al., 2009). While mercaptoethanol had no significant effect on the two isoenzymes, the xylanase activity in *Thermomyces lanuginosus* was found to be en-hanced by β-mercaptoethanol (Cesar and Mirsa, 1996).
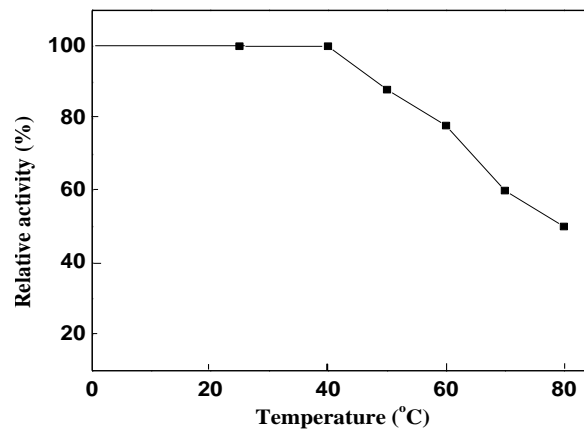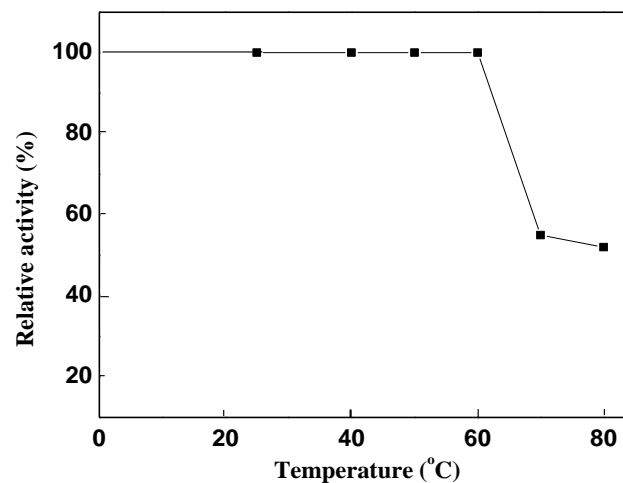
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(a)
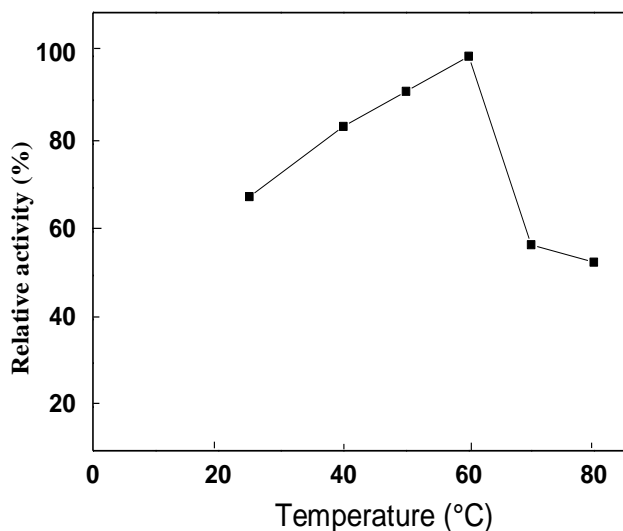
b

Figure 4. Temperature optimum of (a) *A. awamori* XynI and (b) *A. phoenicis* XynI. The enzyme activity was measured at various temperatures using the standard assay method as previously described. Each point represents the average of two experiments.

(b)

Figure 5. Effect of temperature on the thermal stability of (a) *A. awamori* XynI and (b) *A. phoenicis* XynI. The reaction mixture contained in 0.5 ml: 50 mM sodium acetate buffer, pH 5.5 and suitable amount of enzyme. The reaction mixture was preincubated at various temperatures for 30 min prior to substrate addition, followed by cooling in an ice bath. The enzyme activity was measured using the standard assay method as previously described. Activity at zero time was taken as 100% activity. Each point represents the average of two experiments.

p-HMB had inhibitory effect on *A. phoenicis* XynI (66% inhibition). Similarly, *p*-HMB` completely inhibited *T. lanuginosus* xylanase (Cesar and Mirsa, 1996). Sodium benzoate caused partial inhibitory effect on *A. phoenicis* XynI (28% inhibition). Benzoic and citric acids had moderate inhibitory effects on of *A. awamori* XynI and *A. phoenicis* XynI. A ligned of 3-ethoxy salicylidene amino benzoic acid (ETSAN) was tested against *A. niger* and *F. oxysporum* xylanases and the results has shown a lesser activity (Mounika et al., 2010).

Effect of benzoic and citric acids on the growth of fruit spoilage fungi

From the above results, the benzoic and citric acids were studied as antifungal compounds against *A. awamori* loaded on injured lemon and *A. phoenicis* loaded on tomato (Figures 6 and 7). Comparing the effects of the two treatments, citric acid was found to be more effective against fungal activity than benzoic acid. The reduction of

Table 3. Effect of metal cations on xylanase isoenzymes from *A. awamori* and *A. phoenicis*.

Metal cations	Relative activity (%)	
	A. awamori XynI	*A. phoenicis* XynI
Zn^{2+}	102	106
Pb^{2+}	105	69
Ca^{2+}	161	94
Hg^{2+}	81	12
Cu^{2+}	76	30
Co^{2+}	132	90
Ni^{2+}	151	95

Each value represents the average of two experiments.

Table 4. Effect of chemical compounds at 5 mM concentration on xylanase isoenzymes *A. awamori* and *A. phoenicis*.

| Chemical compound	Relative activity (%)	
	A. awamori XynI	*A. phoenicis* XynI
β-Mercaptoethanol	110	99
PMSF	95	105
Trypsin inhibitor	98	93
p-HMB	92	34
1,10 Phenanthroline	90	99
Benzoic acid	44	67
Gallic acid	100	101
Tannic acid	95	87
EDTA	89	109
Sodium benzoate	102	72
Sodium citrate	102	111
Sodium oxalate	111	95
Citric acid	55	52

The xylanase activity was measured in the presence of compounds listed. The activity without added compound was taken as 100% activity. Each value represents the average of two experiments. PMSF, phenylmethylsulfonyl fluoride; *p*-HMB, *p*-hydroxymercuribenzoic acid; EDTA, ethylenediamine tetraacetic acid.
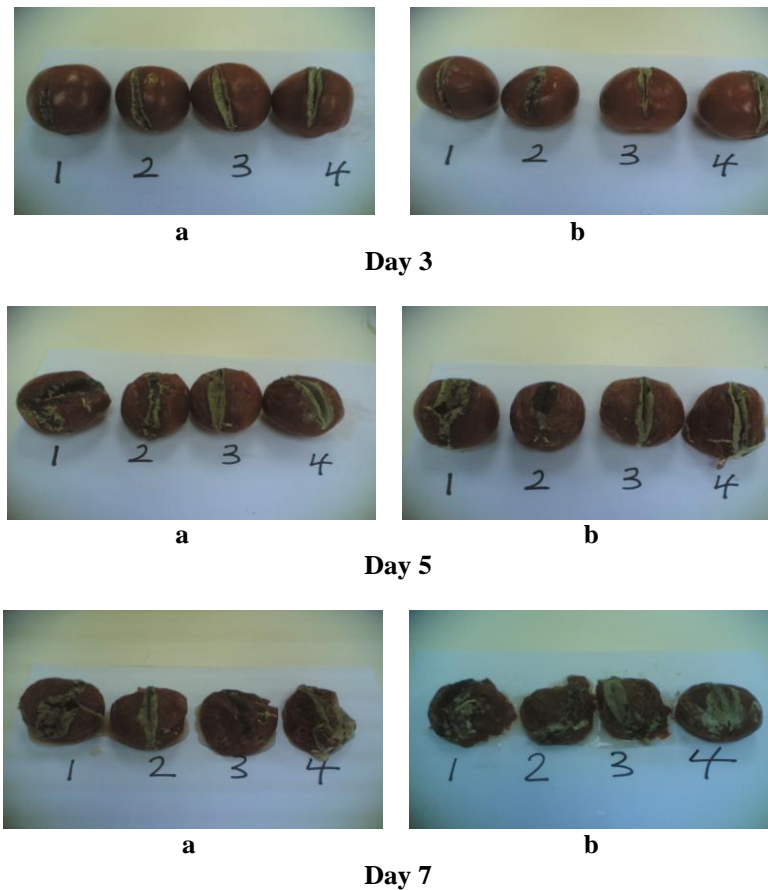

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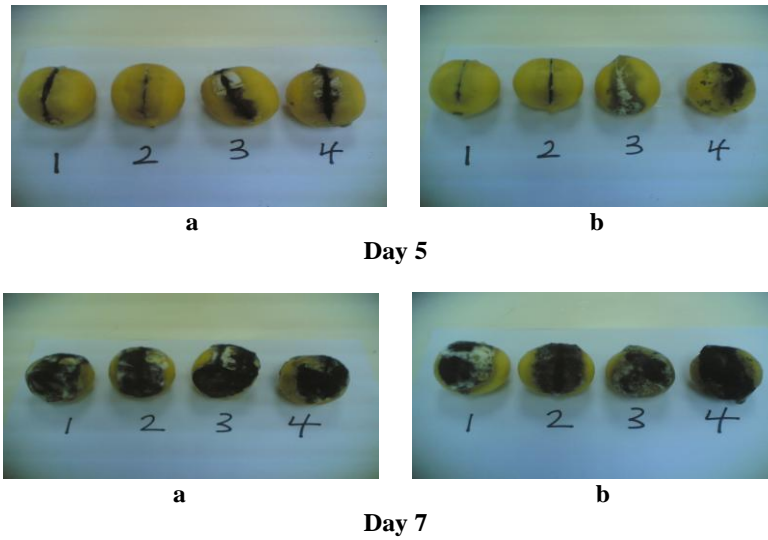
Figure 6. Effect of (a) benzoic acid and (b) citric acid on the development of *A. awamori* from lemon on days 3, 5 and 7 of incubation at 28°C. 1- injured fruits without acid, 2- injured fruits with acid, 3- injured fruits with fungi, (4) injured fruits with acid and fungi.

Figure 7. Effect of (a) benzoic acid and (b) citric acid on the development of *A. phoenicis* from tomato on days 3, 5 and 7 of incubation at 28°C. 1- injured fruits without acid, 2- injured fruits with acid, 3- injured fruits with fungi, (4) injured fruits with acid and fungi.

fungal spoilage in hard peel fruits such as lemon treated with citric acid was highest as compared with the same fruits treated with benzoic acid. However, the two acids had the same effect on fungal spoilage of soft peel fruits such as tomato. Fruits treated with acids only as compared with other treatments showed more resistant to fungal spoilage development and less infection up to day 3 for soft peel fruits, and up to day 5 for hard peel fruits. Both acids showed their ability to prevent the development of fungal spoilage and to prolong the validity of fruits for time period and to enhance the shelf life of fruits. Also, the observation indicated that the formation of fungal spoilage in soft peel fruits is much faster than in hard peel fruits.

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