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α-L-Arabinofuranosidase from *Penicillium janczewskii*: Production with brewer's spent grain and orange waste

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The highest production of α-L-arabinofuranosidase by *Penicillium janczewskii* in medium with brewer's spent grain and orange waste was observed when cultivation was carried out in pH 5.0 at 25°C, for 8.5 days under shaking. The α-L-arabinofuranosidase present in the crude filtrate was optimally active at 60°C and pH 4.0; it was stable in a wide range of pH, maintaining 90% of the activity from 2.0 to 8.0. The enzyme was very stable at 40°C, maintaining 90% of the activity within 1 h. The estimated half-life at 50°C was 10 min, and at 60 and 70°C, it was 5 min. This enzyme was activated in the presence of Ba^{2+}, Ca^{2+}, Mn^{2+} and NH_4^+ while the ions Pb^{2+}, Mg^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+}, as well as PMSF, DTT, β-mercaptoethanol, EDTA and SDS inhibited it.

Key words: α-L-Arabinofuranosidase, *Penicillium janczewskii*, enzyme production, enzyme properties, industrial wastes.

INTRODUCTION

Arabinosyl residues are largely distributed in the side chains of some hemicelluloses, especially arabinoxylans. Usually, the presence of side chains can restrict the full enzymatic hydrolysis of hemicelluloses, preventing the complete degradation of these polymers (Numan and Bhosle, 2006; Peng et al., 2012).

Due to xylans heterogeneity and complexity, an enzymatic complex is required for their complete hydrolysis. The xylanolytic enzymes act cooperatively to degrade not only the main chain, but also the side chains of the polymer; this second group of enzymes is known as accessory or auxiliary enzymes (Polizeli et al., 2005). Among them, α-L-arabinofuranosidases (EC 3.2.1.55) are responsible to hydrolyze non-reducing ends of α-L-1,2-, α-L-1,3- and α-L-1,5-arabinofuranosyl residues of many branched oligo- and polysaccharides, especially those containing large amounts of arabinose, as arabinoxylans (found in cereal grain and softwood), arabinan and other polysaccharides containing L-arabinose (Saha, 2000; Numan and Bhosle, 2006; Seiboth and Metz, 2011).

The production of α-L-Arabinofuranosidases (ABF) can be observed in several microorganisms, as bacteria and fungi, and also in some plants (Lagaert et al., 2014). The production of theses enzymes by microorganisms is strongly influenced by the composition of the culture medium, mainly in relation to the carbon source. Agricultural and agro-industrial residues present potential to be used as raw material in the production of fuels and

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Abbreviations: ABF, α-L-arabinofuranosidase(s); **BSG,** brewer's spent grain; **OW,** orange waste.

chemical compounds in large scale, and their utilization to compose culture medium for microbial growth is a strategy both economical and environmentally interesting since these sources are usually available in large quantities at low cost.

Many fungi have been studied for the production of xylanolytic enzymes, and some *Penicillium* species are considered good ABF producers (Chávez et al., 2006). Among them, the production of this enzyme has been previously related in Penicillium sp. (Lee et al., 2011), *P. capsulatum* (Filho et al., 1996), *P. canescens* (Sinitsyna et al., 2003), *P. purpurogenum* (de Ioannes et al., 2000), *P. brasilianum* (Panagiotou et al., 2006), *P. funiculosum* (Guais et al., 2010) and *P. chrysogenum* ((Sakamoto and Thibault, 2001; Sakamoto and Kawasaki, 2003; Sakamoto et al., 2011).

The use of ABF individually or in addition to other hemicellulases represents a promising tool in biotechnological processes as in the production of medicines, for example, anti-glycemic and anti carcinogenic compounds, in the improvement of wine flavor, pre-treatment of cellulosic pulps, clarification of juices, production of animal feed with improved quality, bioethanol production, among others (Saha, 2000; Numan and Bhosle, 2006).

A *Penicillium janczewskii* Zaleski strain, isolated from soil of Brazilian Rainforest (São Paulo, Brazil) was characterized as a good xylanase producer (Tauk-Tornisielo et al., 2005), motivating studies about its xylanolytic system. A previous work about the production of xylanase, β-xylosidase and ABF showed that brewer's spent grain (BSG) and orange waste (OW) are good substrates for ABF production (Terrasan et al., 2010) by this fungal strain.

Some new knowledge about the biochemical properties of ABF is essential for the future application of the enzyme, of the xylanolytic complex or even of the fungus in biotechnological processes. Thus, the aim of this study was to evaluate the main conditions for optimized ABF production by *Penicillium janczweskii* in liquid medium with brewer's spent grain and orange waste, and also to study some biochemical properties of this extracellular crude enzyme.

MATERIALS AND METHODS

Microorganism

P. janczewskii was deposited in the Environmental Studies Center Collection, CEA/UNESP, São Paulo State, Brazil. It was maintained on Vogel solid medium (Vogel, 1956) with 1.5% (w/v) wheat bran at 4°C, and cultured periodically. The cultures were inoculated in the same medium with 1.5% (w/v) glucose and incubated for conidia production during 7 days at 28°C.

Growth conditions for enzyme production

Liquid cultures were prepared in Vogel medium supplemented with 2% (w/v) mixture of BSG and OW (1:1, w/w). Erlenmeyers flasks (125 ml) containing 25 ml of the medium were inoculated with one milliliter of a 1.10^7 conidia ml^{-1} suspension and incubated initially at 28°C for seven days under static conditions.

Effect of temperature and pH on enzyme production

The effect of temperature on enzyme production was verified by culturing the fungus at 20, 25, 30 and 35°C. The pH of the medium was adjusted to 6.5 and cultivation was carried out for seven days under static conditions. The effect of pH was verified by adjusting the pH of the medium to values ranging from 3.0 to 8.0, with 0.1 M HCl or NaOH. Cultivation was carried out at 25°C for seven days under static conditions.

Time-course of *P. janczewskii* growth and α-L-arabinofuranosidase production

Time-course of *P. janczewskii* growth and ABF production was carried out under static and under shaking (120 rpm) conditions pH 5.0 at 25°C. Fungal growth was estimated indirectly by the determination of intracellular protein content.

Preparation of crude enzyme

The crude filtrate was separated by vacuum filtration with Whatman No. 541 and used for extracellular enzyme activity and protein determinations. The mycelium was disrupted with sand and suspended in McIlvaine buffer pH 4.0; after centrifugation (9000×*g*, 20 min.), this supernatant was used for intracellular protein determination.

Enzyme and protein determinations

ABF activity was determined in a buffered reaction mixture containing 0.25% (w/v) *p*-nitrophenyl-α-L-arabinofuranoside (Sigma) and appropriately diluted enzyme solution. Initially, the reactions were carried out in McIlvaine buffer pH 4.0 at 50°C. The reaction was stopped by the addition of a saturated sodium tetraborate solution and the absorbance was measured at 405 nm (Kersters-Hilderson et al., 1982). One unit of activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min in the assay conditions. Specific activity was expressed as enzyme units per milligram of protein. The protein concentration was determined as described by Lowry et al. (1951), using bovine serum albumin as standard. Data represent the average and deviation of triplicates.

Biochemical characterization of crude α-L-arabinofuranosidase from *P. janczewskii*

Determination of optimum pH and temperature

Optimum pH was determined by assaying enzyme activity in pH ranging from 2.0 to 8.0 with different buffers, that is, 0.05 M Gly-HCl buffer for pH 2.0, 2.5 and 3.0, and McIlvaine buffer from pH 3.0 to 8.0 at 50°C. The optimum temperature was determined by assaying enzyme activity in temperatures from 30 to 90°C, with 5°C intervals, in McIlvaine buffer pH 4.0.

Stability in different pH and temperature

The stability in different pH was verified by measuring residual activity after incubation of the crude enzyme in different buffers in the pH range from 2.0 to 9.0 for 24 h at 4°C. The crude extract was

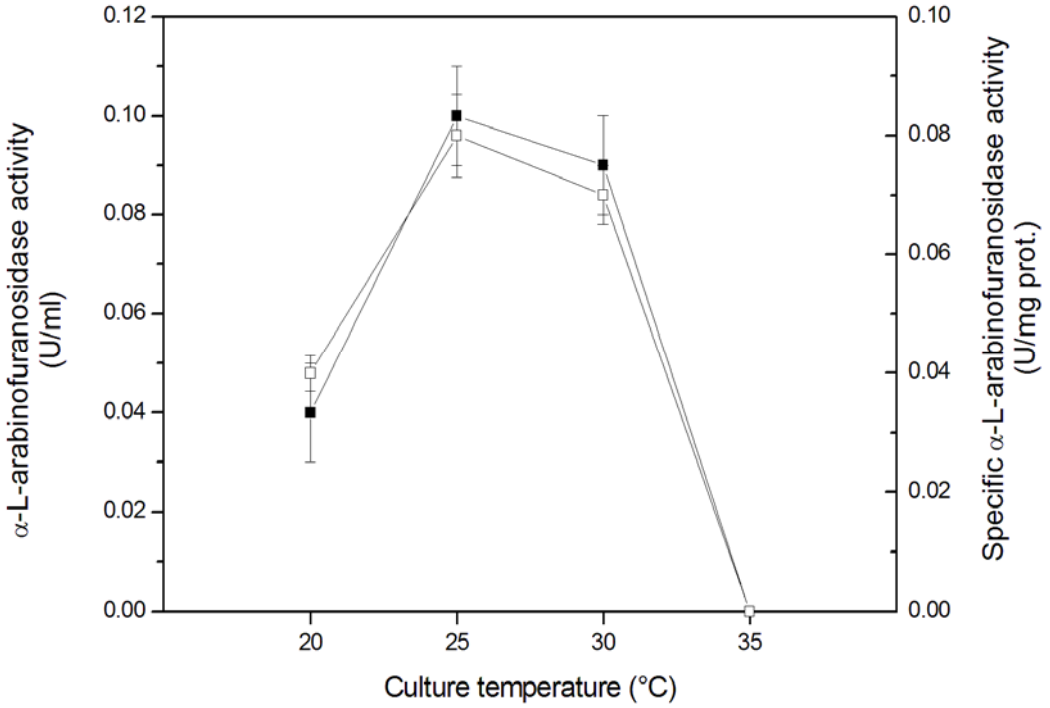

Figure 1. α-L-Arabinofuranosidase production by *P. janczewskii* in different temperatures. Growth conditions: Vogel liquid medium with brewer's spent grain and orange waste, pH 6.5, 7 days of cultivation under static conditions. ■, α-L-Arabinofuranosidase activity (U/ml); □, specific α-L-arabinofuranosidase activity (U/mg prot.).

incubated in 0.05 M Gly-HCl buffer for pH 2.0, 2.5 and 3.0, McIlvaine buffer from pH 3.0 to 8.0 and 0.05 M Tris-HCl buffer from pH 8.0 to 9.0. Thermal stability was evaluated by residual activity determination after incubation of the crude enzyme at 40, 50, 60 and 70°C for different periods. The half-life (T_{50}) of the enzyme was estimated for each temperature.

Influence of ions and other substances

The following substances were added to the reaction medium $CuCl_2$, $ZnSO_4$, $MnSO_4$, $BaCl_2$, $CaCl_2$, NH_4Cl, NaCl, $Pb(CH_3COO)_2$, $MgSO_4$, $CoCl_2$, $HgCl_2$, sodium citrate, SDS, EDTA, PMSF, DTT and β-mercaptoethanol, at final concentrations of 2 and 10 mM. The results were expressed in relation to the control (without any substance).

RESULTS AND DISCUSSION

Optimization of the α-L-arabinofuranosidase production by *P. janczewskii*

When *P. janczewskii* was cultivated in different temperatures (Figure 1), the highest ABF production was verified at 25°C (0.10 U/mL), intermediate values were observed at 20 and 30°C (0.04 and 0.09 U/mL, respectively), and no activity was verified at 35°C. The specific activity presented the same profile with the highest value observed at 25°C (0.08 U/mg prot.). The production of ABF by *P. brasilianum* and *P. purpurogenum* was also observed in intermediate temperatures (27-28°C). The enzymatic production by *P. brasilianum* also decreased at temperatures higher than 36°C (de Ioannes et al., 2000; Panagiotou et al., 2007). Some other *Penicillium* presented optimum temperature for ABF production slightly more elevated, at 30°C, such as *P. capsulatum*, *P. chrysogenum* and *P. funiculosum* (Filho et al., 1996; Guais et al., 2010; Sakamoto and Thibault, 2001).

In cultures carried out in different pH (Figure 2), the highest enzyme production was observed in cultures carried out at pH 5.0 (0.18 U/ml). At pH 4.0 and 6.0 cultures, the activity levels were also elevated (0.16 e 0.11 U/ml, respectively). The activity decreased at pH 3.0 and 7.0 (0.01 U/ml), and at pH 8.0, no activity was detected. The specific activity showed the same profile with highest value observed at pH 5.0 (0.13 U/mg prot.). The results are similar to others ABF from different species of *Penicillium*. The highest ABF production by *P. capsulatum* and *P. chrysogenum* was verified at pH 4.5 and 5.0, respectively (Filho et al., 1996; Sakamoto and Kawasaki, 2003); for both *P. brasilianum* and *P. funiculosum* it was observed at pH 6.0 (Panagiotou et al., 2006; Guais et al., 2010).

The time-course in static culture (Figure 3a) showed that enzyme production increased from the third to the sixth day (0.14 U/ml) of cultivation, in which was observed the peak of ABF production, and activity remained

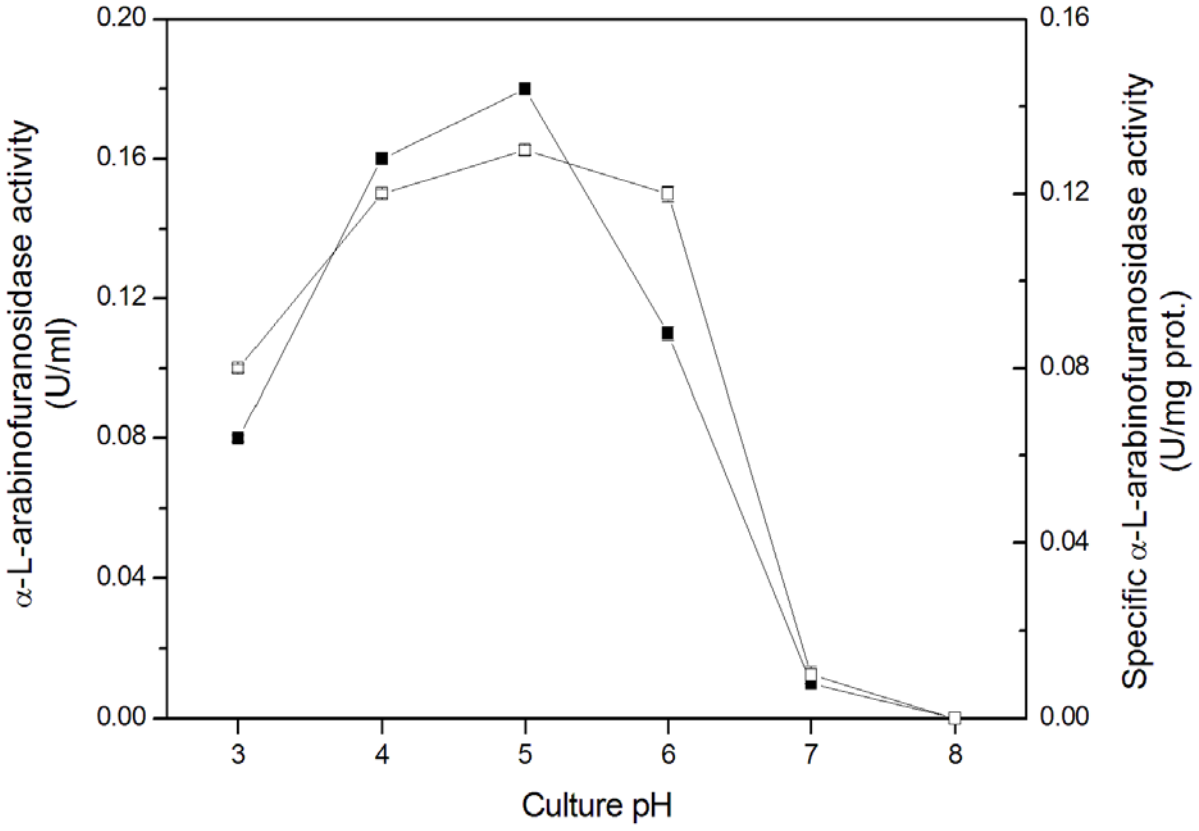

Figure 2. α-L-Arabinofuranosidase production by *P. janczewskii* in different pH. Growth conditions: Vogel liquid medium with brewer's spent grain and orange waste, at 25°C, for seven days under static conditions. ■, α-L-arabinofuranosidase activity (U/ml); □, specific α-L-arabinofuranosidase activity (U/mg prot.).

unchanged over the following days of cultivation. The specific activity presented similar profile but the highest value was observed at the seventh day (0.12 U/mg prot.) decreasing slightly until the tenth day (0.11 U/mg prot.).

In shaking condition (Figure 3b), the production increased from 3.5 days of cultivation and the peak of activity was observed at the tenth day (0.7 U/ml). At 8.5, 9.0 and 9.5 days-old cultures high activity levels were also observed, corresponding to 0.66, 0.64 and 0.62 U/ml respectively. The specific activities in this case were different after the eighth day, and the values at the seventh and tenth day were very similar (0.57 and 0.55 U/mg prot., respectively). A *Penicillium* strain (Rahman et al., 2003) and *Talaromyces thermophilus* (Guerfali et al., 2010) showed elevated ABF production around the seventh (0.5 U/ml) and sixth days (0.26 U/ml) of cultivation, respectively, when grown under shake condition.

The ABF production by *P. janczewskii* under shaking was much higher than that observed under static conditions. Some *Penicillium* species as *P. chrysogenum* and *P. brasilianum* (Panagiotou et al., 2006; Sakamoto and Thibault, 2001) present elevated ABF production in static condition while for *P. purpurogenum* and *P. funiculosum* was observed under agitation (de Ioannes et al., 2000; Guais et al., 2010).

The cultivation of *P. janczewskii* in static condition presented maximum growth (1.5 mg prot.) on the sixth day while under shake, the peak of fungal growth (4.17 mg prot.) was verified earlier at 3.5 day of cultivation (Figure 4). During the stationary growth phase, in static condition, it was observed that the filamentous mycelium covered the entire surface of the medium, while under agitation the mycelium formed pellets that were not immersed in the medium (not shown). Usually, static condition results in growth of a heterogenic filamentous mycelium, in which the superficial layers are in continuous contact with oxygen but with little interaction with the medium while the lower layers, are constantly in contact with the medium but with reduced oxygen availability. Depending on the intrinsic physiological characteristics of each microorganism, this situation can result in growth inhibition or in elevated production of secondary metabolites. Under agitation, a more homogeneous system can be achieved favoring the formation of mycelium pellets that in some cases is a prerequisite for the successful production of secondary metabolites or enzymes (Braun and Vecht-Lifshitz, 1991). This second situation apparently was the case of *P. janczewskii* in relation to ABF production.

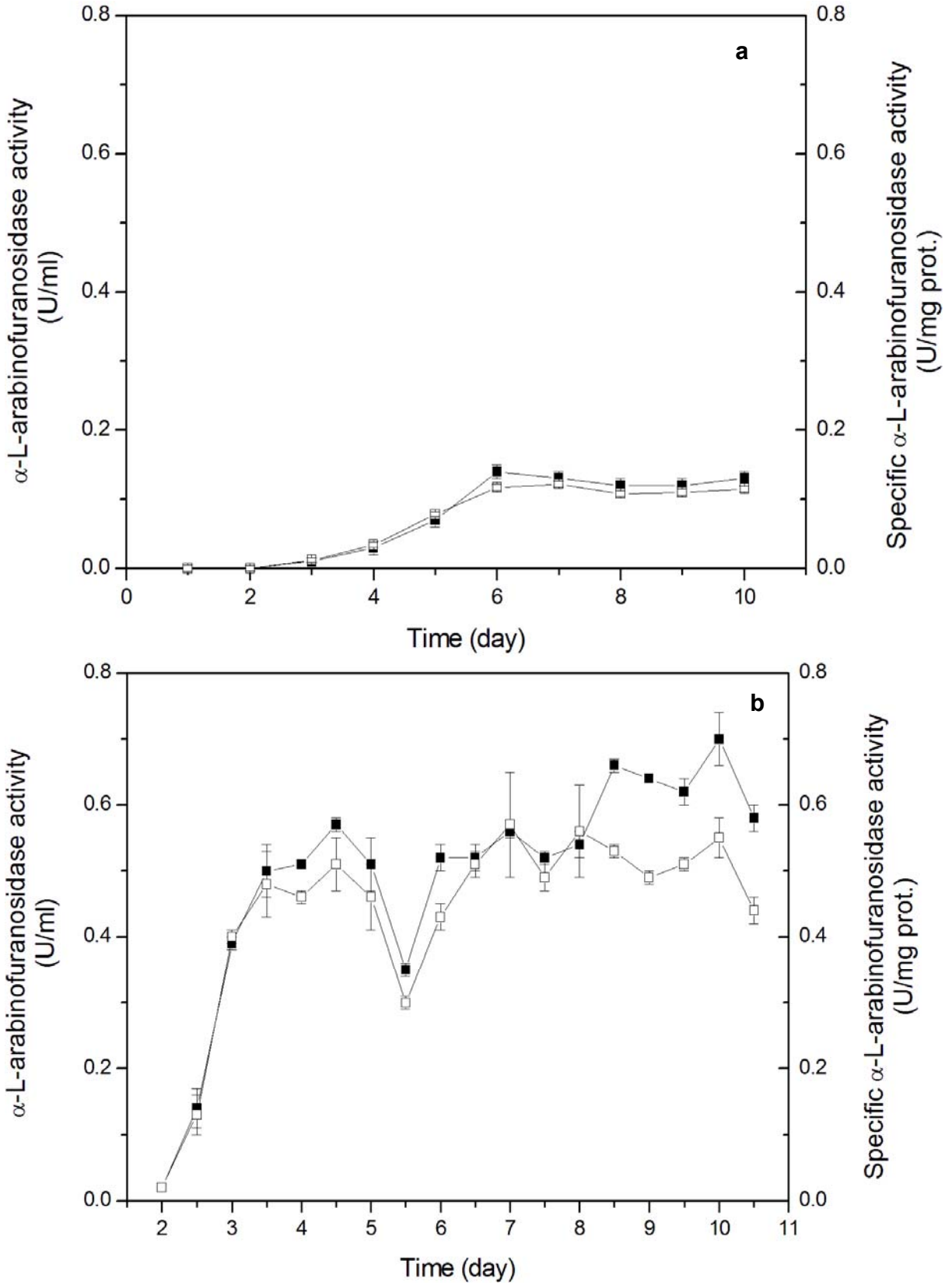

Figure 3. Time-course of α-L-arabinofuranosidase production by *P. janczewskii* in static **(a)** and shaking (120 rpm) **(b)** conditions. Growth conditions: Vogel liquid with medium brewer's spent grain and orange waste, pH 5.0, 25°C. ■, α-L-Arabinofuranosidase activity (U/ml); □, specific α-L-arabinofuranosidase activity (U/mg prot.).

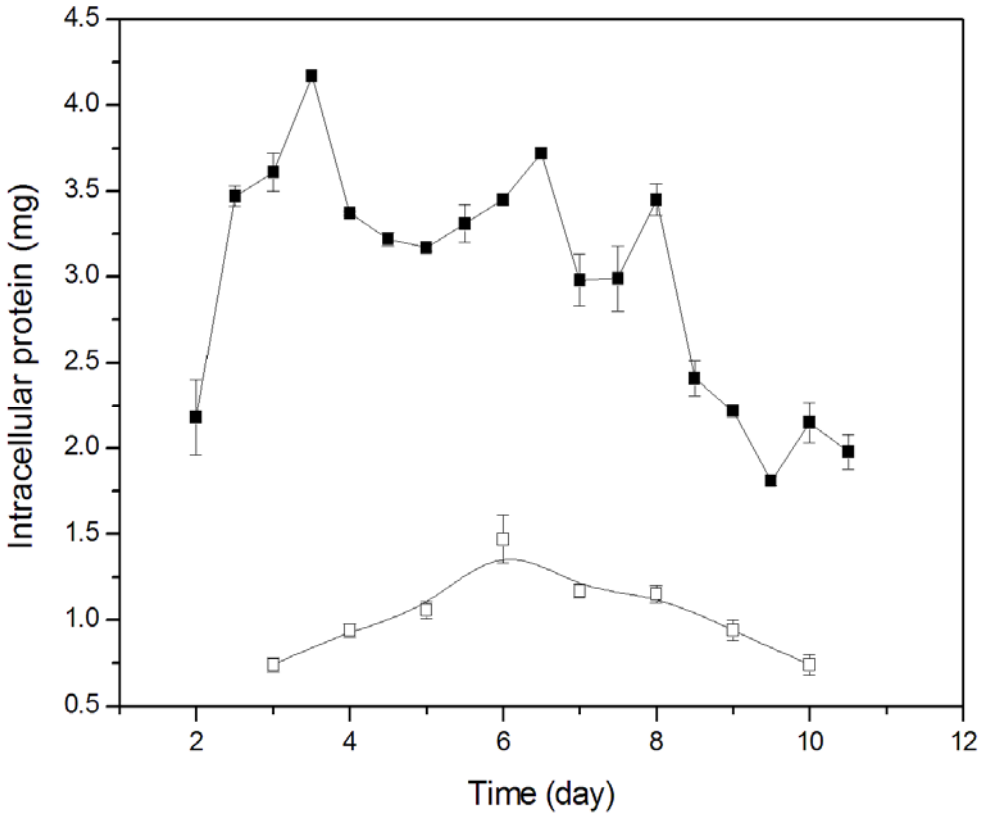

Figure 4. Time-course of *P. janczewskii* growing in static and shaking (120 rpm) conditions. Growth conditions: Vogel liquid medium with brewer's spent grain and orange waste, pH 5.0, 25°C. ■, Intracellular protein in shaking condition (mg); □, intracellular protein in static condition (mg).

Biochemical properties of α-L-arabinofuranosidase from *P. janczeswskii*

The ABF showed optimum activity in pH 4.0; in pH 3.5, the activity was similar to this and in pH 4.5, the activity was approximately 80% of the maximum observed. In pH 5.0, the activity strongly decreased since only 20% of the maximum was observed, and from this pH, the activity gradually decreased (Figure 5). Fungal ABF usually present optimum activity at acid pH as verified for those from *P. capsulatum*, *P. purpurogenum* and *P. chrysogenum* that also present optimum activity at pH around 4.0 (Filho et al., 1996; de Ioannes et al., 2000; Sakamoto and Kawasaki, 2003).

The optimum temperature was observed at 60°C; at 55°C, it was also elevated corresponding to 90% of the maximum activity, at 50 and 65°C, it decreased to 60% of the maximum. In other temperatures, the activity remained below 50% of the maximum and at 90°C almost no activity was observed (Figure 6). The ABF produced by several other mesophilic *Penicillium* strains usually present optimum activity in temperatures between 50 and 60°C. The ABF from Penicillium sp, *P. purpurogenum*, *P. chrysogenum* and *P. brasilianum* are optimally active at 50°C (Lee et al., 2011; Panagiotou et al., 2006; de Ioannes et al., 2000; Sakamoto and Kawasaki, 2003) and those from *P. capsulatum* and *P. funiculosum* at 60°C (Filho et al., 1996; Guais et al., 2010), as well as the one from *P. janczewskii*. Exceptionally, the optimum temperature for the *P. canescens* ABF is 70°C (Sinitsyna et al., 2003). The activity at elevated temperature is important for an application of this enzyme in processes that are carried in this condition, for example the biobleaching in pulp and paper industries in which pulp is present at high temperature, approximately 60°C (Beg et al., 2001).

The enzyme was stable over a wide pH range maintaining more than 90% of the activity from pH 2.0 to 9.0, but at pH 10.0, only 22% of the activity was observed (Figure 7). Usually, fungal ABF exhibit a wide range of pH stability, for example, *P. brasilianum* ABF maintains more than 80% of the activity at the pH range from 3.0 to 11.0 after incubation at 10°C for 6 h (Panagiotou et al., 2006). The ABF from *P. chrysogenum* retains more than 80% of the activity at a pH range from 3.0 to 8.0 after incubation at 30°C for 16 h (Sakamoto and Thibault, 2001). The wide range of pH stability of the *P. janczewskii* ABF is very interesting since it allows the enzyme to be applied in industrial processes carried out at various pH values.

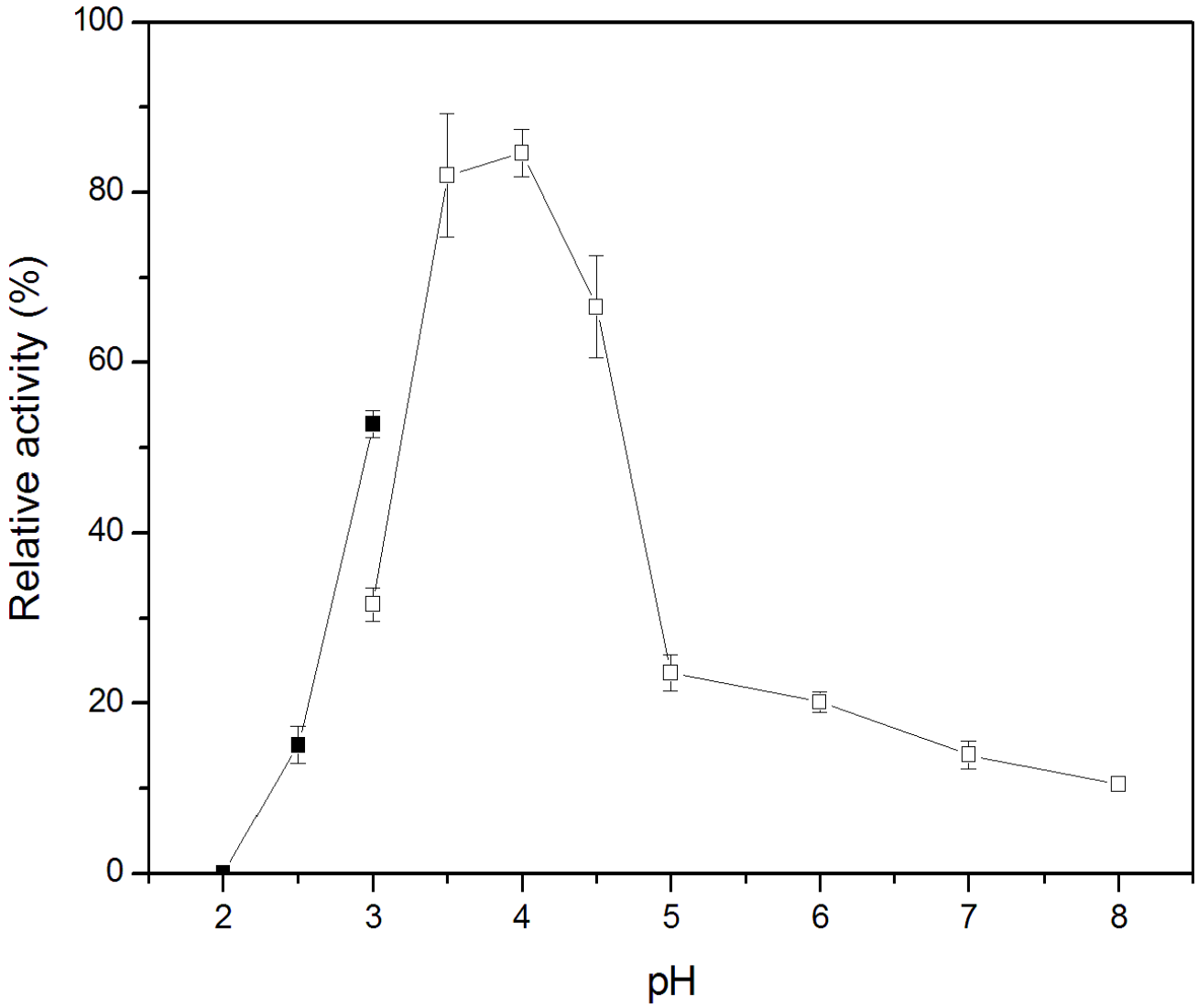

Figure 5. Influence of pH on α-L-arabinofuranosidase from *P. janczewskii*. Enzyme activity was assayed in 0.05 M Gly-HCl buffer (■) and McIlvaine buffer (□), at 50 °C.

The highest thermal stability of the enzyme was observed at 40°C maintaining more than 90% of the activity over a 1 h-period. At 50°C, the activity decreased to 52% of the initial after 10 min of incubation. The half-life values at 60 and 70°C were lower than 5 min (Figure 8). The purified ABF from *P. capsulatum* (Ara I) is very stable at 60°C and presents a half-life of 17.5 min at 70°C, while a second purified ABF from this fungus (Ara II) is less thermostable presenting a half-life of 8 min at 60°C (Filho et al., 1996). The *P. chrysogenum* ABF is not stable at 60°C; while at 50°C, more than 80% of the activity is retained after incubation for 1 h (Sakamoto and Kawasaki, 2003).

When the activity was assayed in the presence of different ions and substances (Table 1), the enzyme was slightly activated by Ba^{2+}, Ca^{2+}, Mn^{2+} and NH_4^+. On the other hand, Pb^{2+}, Mg^{2+} and Hg^{2+} negatively affected the enzyme, as well as Cu^{2+}, Zn^{2+} and Co^{2+} only at 10 mM. Mercury is known to interact with sulfhydryl groups of proteins changing and reducing its performance during catalysis. Similarly, the ions Cu^{2+} and Hg^{2+} also negatively affected the *P. chrysogenum* ABF while the other ions have no effect on the enzyme activity (Sakamoto and Thibault, 2001).

The activity was reduced by PMSF. This substance binds to serines and cysteines and it is commonly used to identify the presence of these amino acids in the active site of the enzymes, especially proteases. Usually, it is used to inhibit serine and cysteine proteases that can degrade the protein of interest after cell lysis (James, 1978). Thus, its negative effect on the ABF indicates the presence of these amino acids which should be important for catalysis mechanism and/or three-dimensional structure of this protein.

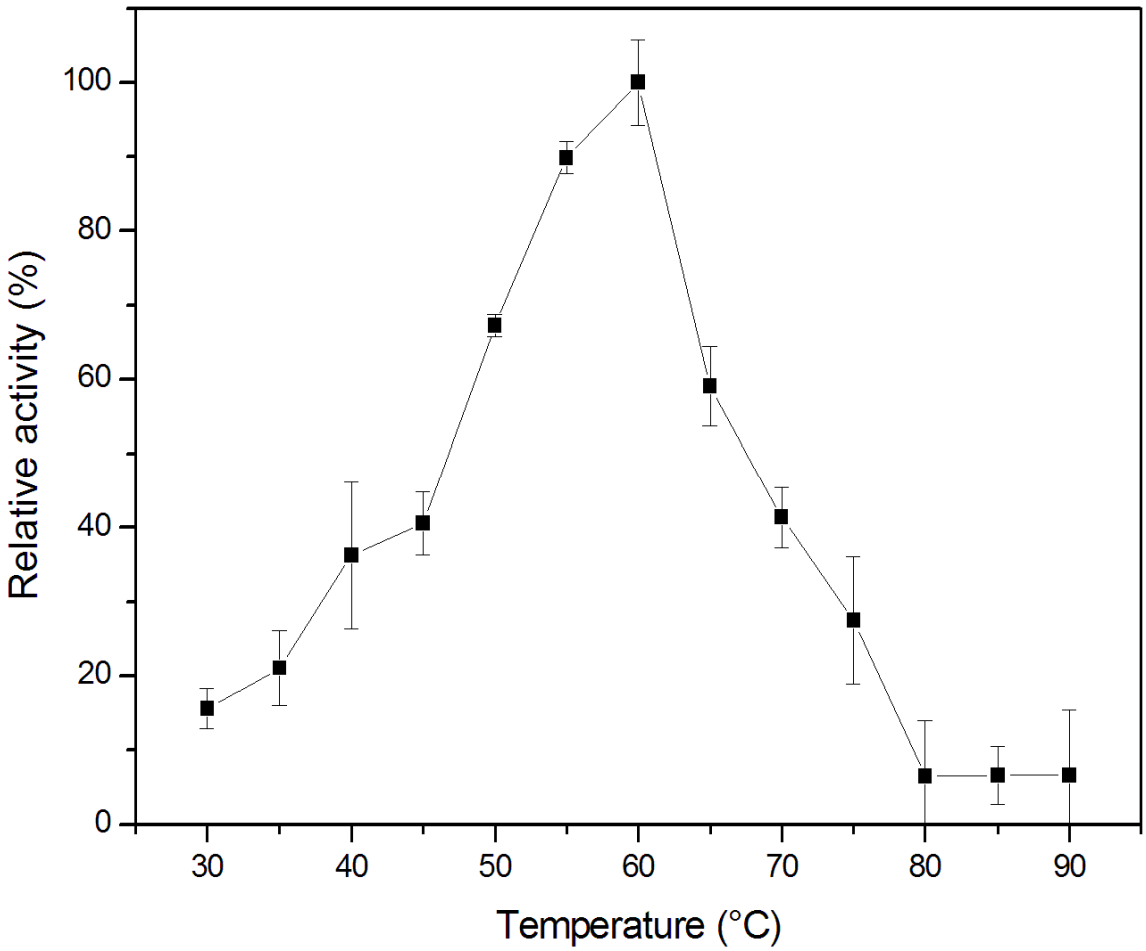

Figure 6. Effect of temperature on α-L-arabinofuranosidase from *P. janczewskii*., Enzyme activity assayed in McIlvaine buffer pH 4.0.

In different intensities, DTT and β-mercaptoethanol partially decreased the enzyme activity. These substances reduce disulfide bounds of proteins, and both are commonly used to prevent oxidation of sulfhydryl groups maintaining the enzyme activity. In this case, the decrease in the activity by these substances suggests that disulfide bridges should be important for maintaining the tertiary structure of the enzyme. This negative effect indicates indicate that ABF does not have cysteine but cystine, once it prevents S-H bounds of cysteine to be since these compounds promote breakage of disulfide bonds.

The addition of EDTA resulted in reduction of the enzyme activity suggesting that it requires a metallic ion as cofactor. The presence of SDS in both concentrations almost ceased the enzyme activity, indicating the importance of hydrophobic interactions for maintaining the three-dimensional structure of the protein.

Conclusions

P. janczewskii presented elevated production of α-L-arabinofuranosidase using brewer's spent grain and orange waste as substrates. Both residues are available in large scale and at low cost turning their use really interesting for industrial application. The selected culture conditions to increase the enzyme production were pH 6.5, at 25°C, for 8.5 days, being agitation (120 rpm) essential for elevated enzyme production by this fungal strain. This enzyme showed several properties interesting for future application in some industrial processes, since the optimum pH and temperature are 4.0 and 60°C, respectively. In the bleaching paper industry, for instance, the procedures are performed at temperatures higher than 50°C and pH values varying from 4.0 to 6.0. Besides, this enzyme presents great potential to be applied in biotechnological processes with other xylanolytic enzymes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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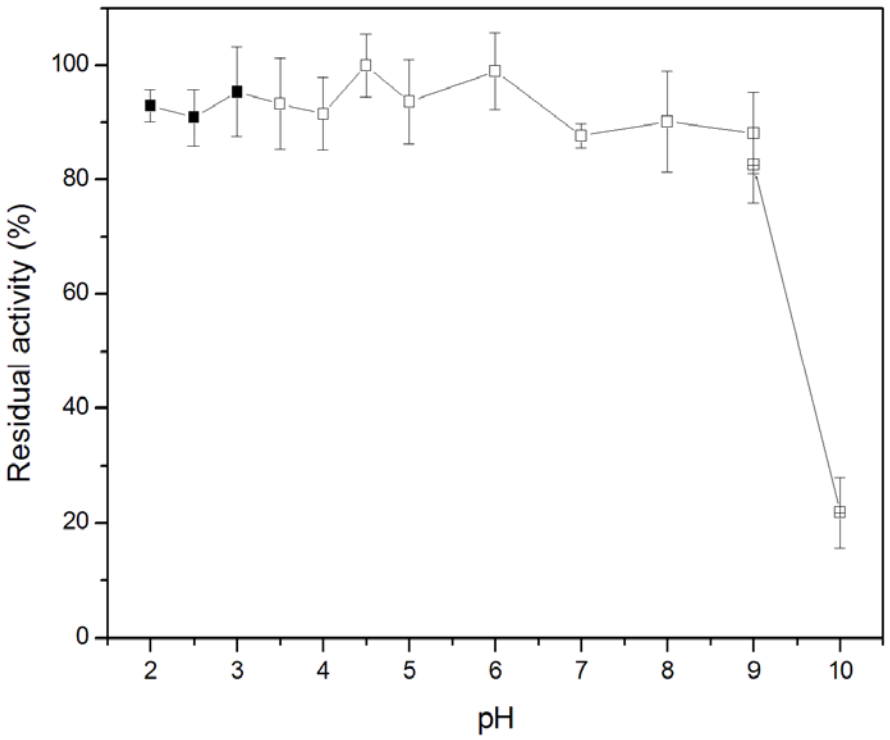

Figure 7. Stability in different pH of α-L-arabinofuranosidase from *P. janczewskii*. The residual activity was assayed in McIlvaine buffer pH 4.0 at 60°C after incubation in 0.05 M Gly-HCl (■), McIlvaine (□) and 0.05 M Tris-HCl (☒) buffers.

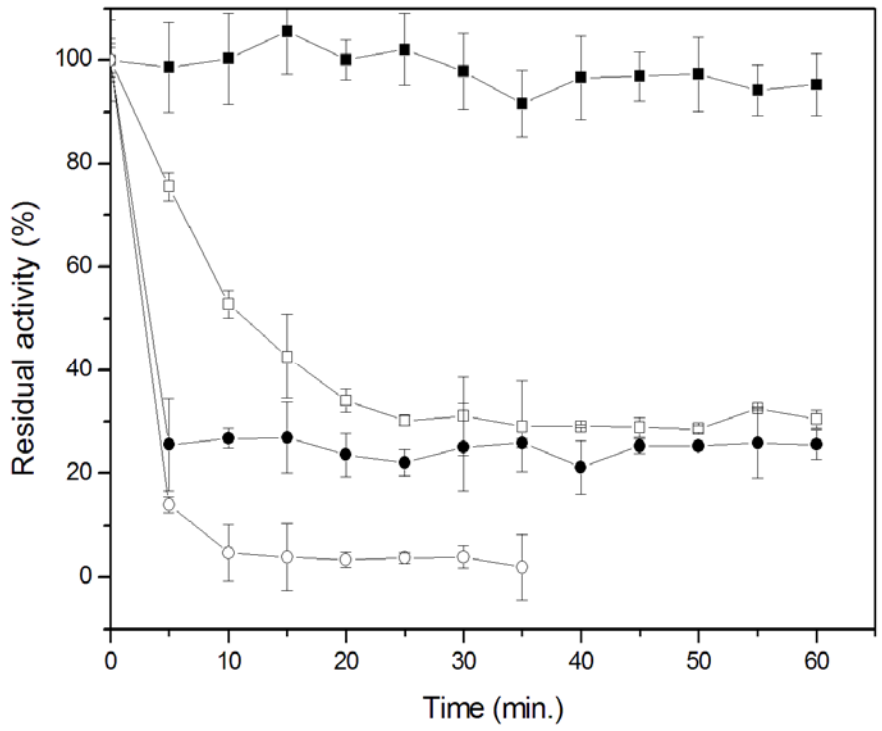

Figure 8. Thermal stability of α-L-arabinofuranosidase from *P. janczewskii*. Residual activity after incubation at 40 (■), 50 (□), 60 (●) and 70°C (○) assayed in McIlvaine buffer pH 4.0 at 60°C.

Table 1. Influence of ions and other substances on the α-L-arabinofuranosidase from *P. janczewskii*.

Substance	Relative Activity (%)	
	2 mM	10 mM
Control	100.0	100.0
$CuCl_2$	82.0 ± 0.4	8.2 ± 2.4
$ZnSO_4$	102.8 ± 3.0	77.0 ± 0.6
$MnSO_4$	111.5 ± 0.6	130.6 ± 11.6
$BaCl_2$	124.4 ± 1.1	112.5 ± 4.6
$CaCl_2$	120.6 ± 1.5	116.5 ± 3.7
NH_4Cl	114.4 ± 4.4	121.5 ± 5.0
NaCl	111.2 ± 5.3	110.8 ± 3.8
$Pb(CH_3COO)_2$	69.1 ± 4.5	47.7 ± 2.5
$MgSO_4$	61.8 ± 4.8	56.8 ± 11.2
$CoCl_2$	96.6 ± 0.2	33.7 ± 2.3
$HgCl_2$	44.6 ± 12.7	14.3 ± 21.9
Sodium Citrate	96.2 ± 0.5	106.7 ± 0.4
SDS	8.3 ± 16.7	5.1 ± 5.0
EDTA	68.5 ± 2.7	64.7 ± 2.2
PMSF	50.3 ± 0.6	22.0 ± 3.2
DTT	71.2 ± 3.6	62.5 ± 4.9
β-mercaptoethanol	57.2 ± 1.9	66.8 ± 2.6

The activity was assayed in McIlvaine buffer pH 4.0 at 60°C. Data are mean and standard deviation of triplicates.

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