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High xylanase production by *Trichoderma viride* using pineapple peel as substrate and its apllication in pulp biobleaching

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Xylanases are hydrolases which depolymerize the xylan components present in plants cell wall. Commercial applications for these enzymes include its use in the pulp bleaching, food and animal feed industries, among others. Recently, there is a great interest on the exploitation of agro-industrial wastes as low-cost raw materials for value-added compounds production, as xylanolytic enzymes for industrial applications. This is the first report about the xylanase production using pineapple peel as substrate. The xylanase production by *Trichoderma viride* was optimized and the enzymes produced were biochemically characterized. Additionally, the effect of these enzymes on pulp biobleaching process was evaluated. High xylanase production was obtained with pineapple peel at 2% concentration, for seven days, in stationary cultivation at 28°C, in pH 7.5. Xylanases were more active at 50°C, pH 6.0-6.5 remaining stable at pH 5.0-5.5. *T. viride* xylanase was stable at 40°C, showing the half-life ($T_{1/2}$) value of 255 min. The enzyme was remarkably stimulated by Mg^{2+} and Zn^{2+}, while Pb^{3+} and Hg^{2+} were strong inhibitors of the xylanase activity. This work shows the ability of the filamentous fungus *T. viride* to produce high levels of xylanases using pineapple peel as substrate, an inexpensive and abundant agro-industrial waste. Therefore, the reduction on kappa number achieved in this investigation revealed the application potential of these enzymes in biobleaching process.

Key words: Agro-industrial wastes, filamentous fungi, submerged fermentation, pulp pretreatment, xylanolytic enzymes.

INTRODUCTION

After cellulose, xylan is the most abundant polysaccharide present in wood, agricultural and several agro-industrial wastes. This complex heteropolysaccharide consists of a main chain of 1,4-β-D-xylose monomers which is partially acetylated and substituted in different degrees by a variety of side chains, mainly single α-D-glucuronosyl and α-L-arabinosyl units (Collins et al., 2005; Wakiyama et al., 2008). Due to its structural

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complexity, several hydrolases are required for complete degradation of xylan. The main enzymes involved in xylan degradation are the endo-β-(1,4)-xylanases. These enzymes cleave the internal β-(1→4) bonds in the xylan backbone at non-modified residues, yielding different chain length substituted xylooligosacharides (Zhang et al., 2007; Sharma and Kumar, 2013).

Recently, xylanases have been extensively studied due to their industrial applications (Buthelezi et al., 2011). An example is the bioconversion of lignocellulosic residues in their constituent sugars. These enzymes have attracted attention due to their role in the production of xylose which can be converted into bioethanol and xylitol (Laxmi et al., 2008).

Other industrial applications include the improvement of digestibility of animal feedstock, clarification of wines and juices and the baking of rye to improve the bread volume and crumb structure (Romanowska et al., 2006; Khandeparker and Numan, 2008; Gupta and Kar, 2009; Albert et al., 2011). In the last years, the use of xylanases as bleaching agents of wood kraft pulps has been considered the main industrial application of xylanolytic enzymes. Many studies have demonstrated that the pulp treatment with xylanases promotes the release of lignin from the pulp, thereby reducing the chlorine required in this process (Khandeparker and Numan, 2008; Gupta and Kar, 2009; Albert et al., 2011).

The agro-industrial residues are composed of lignocellulosic material and are generated in large quantities in agribusiness, food, wood, pulp and paper industries, among others. These wastes contain many high-value constituents, such as carbohydrates and fibers.

Also, current trends emphasize that agro-industrial wastes have potential for microbial enzymes production. However, these potentially valuable materials are often disposed in the environment without an adequate treatment, increasing environmental damage (Dashtban et al., 2009).

The global market for enzymes is expected to reach 8.0 billion in 2015 (Li et al., 2012). However, the production cost of xylanases is the major factor limiting its use, requiring the development of low cost production systems (Buthelezi et al., 2011). According to Widjaja et al. (2009) and Ali et al. (2012), the cost of the industrial enzymes can be decreased by the establishment of the optimal conditions for its production, including the use of agro-industrial wastes as substrates.

Pineapple waste is one of the agro-industrial residues which represent a serious environmental pollution (Rani and Nand, 2004). The peel is rich in cellulose, hemicellulose and other carbohydrates with a substrate potential generation of methane by anaerobic digestion. Brazil is the second largest pineapple producer in the world, reaching a volume of 2.318.120 tons (FAOSTAT, 2013). Thus, a large amount of pineapple peel waste is generated over the years. However, the pineapple peel waste has received little attention and its use is limited mainly in animal feed (Tran, 2006; Paengkoum et al., 2013). *Trichoderma viride* is an excellent biocontrol agent (Reena et al., 2013), cellulolytic microorganism (Mojsov, 2010) and xylanase producer (Goyal et al., 2008; Simões et al., 2009).

The aims of this study were to produce xylanases from a *T. viride* strain on agro-industrial wastes and bio-chemically characterize these enzymes. Additionally, the potential application of *T. viride* xylanases in pulp bleaching process was evaluated.

MATERIALS AND METHODS

Fungal strain and culture conditions

T. viride J40 was isolated from the Atlantic forest soil on Ecologic Station Juréia-Itatins, located in São Paulo State, Brazil. The strain belongs to the culture collection of the Environmental Studies Center - CES/UNESP, Brazil.

The fungus was propagated on Vogel's solid medium (Vogel, 1956) containing 1.5% (m/v) glucose and 1.5% (m/v) agar, at 28°C, during 7 days for conidia production. After, the strain was maintained at 4°C. Submerged fermentation was prepared in 125 ml flasks containing 25 ml of the Vogel's medium, pH 6.5, supplemented with 1% carbon source and inoculated with 1.0 ml conidia suspension (7×10^7 conidia/mL) to each flask. The cultures were incubated at different conditions for the xylanase production optimization. After incubation, cultures were filtered by vacuum through filter paper Whatman number 1. The crude filtrate was assayed for extracellular activity and total secreted protein. All cultures were carried out in duplicate to verify the reproducibility and the results are presented through mean values.

Enzyme assay

For xylanase assay, 20 µL of the crude filtrate, 480 µL of the McIlvaine buffer pH 6.5 and 750 µL of 1% birchwood (*Betula* spp.) xylan solution prepared in the same buffer were incubated for 5 min at 50°C. After pre-determined periods, the reaction was stopped by the addition of 250 µL of 3,5-dinitrosalicylic acid. The reducing sugars liberated were measured according to Miller (1959), using xylose as standard. For this, the sample was heated at 100°C for 5 min and allowed to cool to room temperature in ice bath. After that, 2.5 mL of distilled water was added and the absorbance was determined in spectrophotometer at 540 nm. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugar per min, under assay conditions. Specific activities were expressed as enzyme units per milligram of secreted protein. All determinations were developed in triplicate and the results were presented as mean values.

Total protein determination

In order to determine the fungal growth, the intracellular protein was measured. The mycelium was frozen and macerated with sand in McIlvaine buffer pH 6.5. The slurry was centrifuged at 3.900 x *g* and the supernatant was used as an intracellular protein source. As previously mentioned, the crude filtrate was used as a source of extracellular protein. In both cases, the total protein concentration was determined by modified Bradford method (Sedmak and Grossberg, 1977), using bovine serum albumin (BSA) as standard.

For this, the sample appropriately diluted was mixed with the Bradford reagent. The samples were then incubated at room temperature for 5 min. After that, the absorbance was determined in spectrophotometer at 595 nm. All determinations were developed in triplicate and the results were presented as mean values.

Culture conditions for xylanase production

Agro-industrial wastes

The agricultural wastes wheat bran (*Triticum* spp.), apple peel (*Malus* spp.), brewer's spent grain (*Hordeum vulgare*), passion fruit peel (*Passiflora edulis*), orange peel (*Citrus sinensis*), rice peel (*Oryza sativa*), soybean peel (*Glycine max*) and pineapple peel (*Ananas comosus*) were obtained locally. The residues were prepared by exhaustive washing with distilled water, dried at 80°C for 24-48 h and milled (35 mesh).

Enzyme production on different carbon sources

In order to verify xylanase induction, the Vogel's liquid medium was supplemented with various dried substrates as sole carbon source at a concentration of 1% (w/v). The inoculated flasks were incubated at 28°C under stationary condition, for five days. Xylanase activity was determined as described previously. After the selection of the best agro-waste for xylanase production, some concentrations of this carbon source were evaluated from 0.5 to 3.0% (w/v).

Effect of incubation period, initial pH and temperature on xylanase production

The incubation period influence on xylanase production was evaluated in stationary culture for 9 days. The effect of initial pH on the enzyme production was evaluated from 3.0 to 9.0 and the cultivation temperature influence was verified from 20 to 30°C. The initial pH values were adjusted by the addition of 1.0 M sodium hydroxide or hydrochloric acid solutions.

Enzyme characterization

Optimum pH and temperature xylanase activity

The optimum temperature was determined by measuring the activity at temperatures ranging from 25 to 75°C in McIlvaine buffer pH 6.5. In order to establish the optimum pH of the enzyme, the relative activity was determined in different pH values, using McIlvaine buffer pH 3.0-8.0, at the optimum temperature.

Xylanase thermostability and pH stability

The thermal stability was measured by incubating the crude filtrate for different periods at 40, 45 and 50°C. Following incubation, the enzyme solution was frozen and the remaining activity was determined. For pH stability assays, the culture filtrate was diluted (1:2 v/v) in McIlvaine buffer for pH range from 3.0-8.0. The samples were incubated at 4°C for 24 h. After this period, the residual xylanase activity was assayed under optimal conditions.

Effect of ions and other substances on xylanase activity

The enzymatic activity was measured in presence of different ions ($BaCl_2$, $MgSO_4$, $ZnSO_4$, NH_4Cl, $Co(NO_3)_2$, $MnSO_4$, $CuSO_4$, $CaCl_2$, sodium citrate, NaCl, $Pb(CH_3COO)_2$ and $HgCl_2$ and substances [glycerol, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol] at 2 and 10 mM concentrations. The enzyme assay was performed in optimal conditions and the relative activities were expressed as a percentage against the control.

Optimization of kraft pulp pretreatment with *T. viride* xylanases

Xylanases produced by *T. viride* under optimized submerged fermentation conditions were used for biobleaching of *Eucalyptus grandis* kraft pulp in order to evaluate its potential use in this process. The optimization of kraft pulp pretreatment was carried out in completely randomized block design with different enzyme dosages, temperature, reaction time and pH as individual treatments, in triplicate. For this, the oxygen pre-bleached pulp at 10% (w/v) consistency was pretreated with *T. viride* crude extract in polyethylene bags, with a xylanase dosing of 10 U/g oven dried pulp, at 50°C, in pH 6.0, for 60 min, except the variable parameter. An untreated pulp sample was also incubated simultaneously under these conditions and used as control.

After the treatment, the cellulose pulps were filtered on a Büchner funnel, rinsed with 200 mL of distilled water and used for pulp properties determination. The optimum pretreatment conditions were determined by measuring kappa number and viscosity parameters. These procedures were conducted according to the standard methods of Technical Association of the Pulp and Paper Industry (TAPPI test methods, 1996). The data were submitted to one-way analysis of variance and compared through the Tukey test, using the Statistical Analysis Software (SAS).

RESULTS AND DISCUSSION

Influence of the carbon source and its concentration on the xylanase production

The use of agro-industrial wastes as carbon source in the growth medium is an alternative to reduce the costs and increase the enzyme production. Thereby, the xylanase production by *T. viride* using different agro-industrial wastes was investigated (Table 1). The carbon source that induced the highest enzymatic activity and fungal growth was pineapple peel, corresponding to 35.12 U/mL and 20.38 mg protein, respectively. *T. viride* was also able to produce high levels of xylanase in the presence of wheat bran and brewer's spent grain, corresponding to 29.39 and 28.64 U/ml. At the present date, only cellulose production using pineapple peel as substrate was described in the literature (Folakemi et al., 2008; Saravanan et al., 2013). Meenakshi et al. (2008) found maize straw as the best inducer for xylanase production by other *T. viride* strain, reaching 6.24 U/ml, whereas sugarcane bagasse and wheat bran were established as the best substrates for xylanase production by distinct *Trichoderma reesei* strains (Irfan and Syed, 2012; Kar et al., 2013).

Rice peel provided minimal fungal growth, with intermediate levels of xylanase produced (9.95 U/ml). No significant levels of xylanase were observed with orange peel when compared to the other lignocellulosic

Table 1. Influence of agro-industrial wastes on xylanase production by *T. viride*.

Carbon source (1% w/v)	Intracellular protein (mg)	Enzymatic activity (U/mL)	Specific activity (U/mg extracellular protein)
Wheat bran	13.05 ± 0.89	29.39 ± 1.63	31.48 ± 2.78
Apple peel	22.56 ± 2.52	14.27 ± 1.14	13.51 ± 1.62
Brewer's spent grain	8.25 ± 2.15	28.64 ± 2.26	38.13 ± 6.65
Passion fruit peel	20.04 ± 1.09	11.08 ± 1.14	14.32 ± 0.89
Orange peel	9.75 ± 0.61	1.53 ± 0.15	2.06 ± 0.21
Pineapple peel	20.38 ± 1.58	35.12 ± 1.27	24.79 ± 1.32
Rice peel	1.53 ± 0.02	9.95 ± 0.59	33.52 ± 5.85
Soybean peel	2.89 ± 0.12	15.21 ± 1.02	12.74 ± 0.85

Average and standard deviation of two cultures.

Table 2. Effect of pineapple peel concentration on xylanase production by *T. viride*.

Carbon source (% w/v)	Intracellular protein (mg)	Enzymatic activity (U/mL)	Specific activity (U/mg extracellular protein)
0.5	7.75 ± 0.39	10.68 ± 0.11	21.83 ± 3.04
1.0	20.38 ± 1.58	35.12 ± 1.27	24.79 ± 1.32
1.5	19.57 ± 0.64	45.36 ± 1.57	27.88 ± 2.06
2.0	35.35 ± 1.73	48.05 ± 1.89	23.56 ± 1.2
2.5	14.27 ± 0.17	36.47 ± 2.71	14.5 ± 0.78
3.0	8.49 ± 0.89	31.77 ± 1.78	19.01 ± 1.27

Average and standard deviation of two cultures.

materials, although *T. viride* has shown a good development in this substrate. The differences verified on xylanases levels produced with various agro industrial wastes are possibly related on distinct composition and the accessibility of the substrates. Considering the high xylanase production obtained with pineapple peel, this waste was selected for the subsequent optimization experiments.

The most efficient concentration of pineapple peel to induce *T. viride* xylanase production and fungal growth was 2% (w/v), with 48.05 U/ml and 35.35 mg protein, respectively (Table 2). High xylanase levels were produced by *Trichoderma atroviride* when 3.0% (w/v) of untreated sugarcane bagasse was used as substrate, after 3-4 days of fermentation (Grigorevski-Lima et al., 2013). Sanghvi et al. (2010) found that an increase in concentration of substrate lead to a decrease in xylanase activity produced by *Trichoderma harzianum.* According to Gupta et al. (2009), a high substrate concentration let to increased viscosity, which influenced medium components and oxygen transfer.

Effects of culture conditions on xylanase production

Culture conditions are essential for the successful production of an enzyme. For this reason, optimization of these parameters is important for a process improvement. In stationary culture, with pineapple peel as carbon source, the highest extracellular xylanase production as units per volume and specific activity were obtained in 7-day-old cultures, corresponding to 54.15 U/mL and 37.33 U/mg protein, respectively (Figure 1a). The maximal fungal growth measured by the intracellular protein concentration was also observed in 7^{th} day, corresponding to 56.85 mg of protein (not shown). Likewise, Kar et al. (2006) verified that xylanase production by *T. reesei* SAF3 exhibited a similar increase following the biomass peak. In addition, Norazlina et al. (2013) achieved a gradual increase of xylanase levels produced by *Trichoderma* sp. from the second day to the sixth day of the fermentation process.

Sanghvi et al. (2010) observed that the xylanase production by *T. harzianum* with wheat straw increases up to 12 days of cultivation. Generally, a further increase in incubation time resulted in a decrease in enzyme production. The decline in xylanase production observed in the present study may be due to the susceptible portion of xylan molecules that are rapidly digested and only the crystalline portions remains (Jing-Min et al., 1998).

Temperature and pH are important environmental

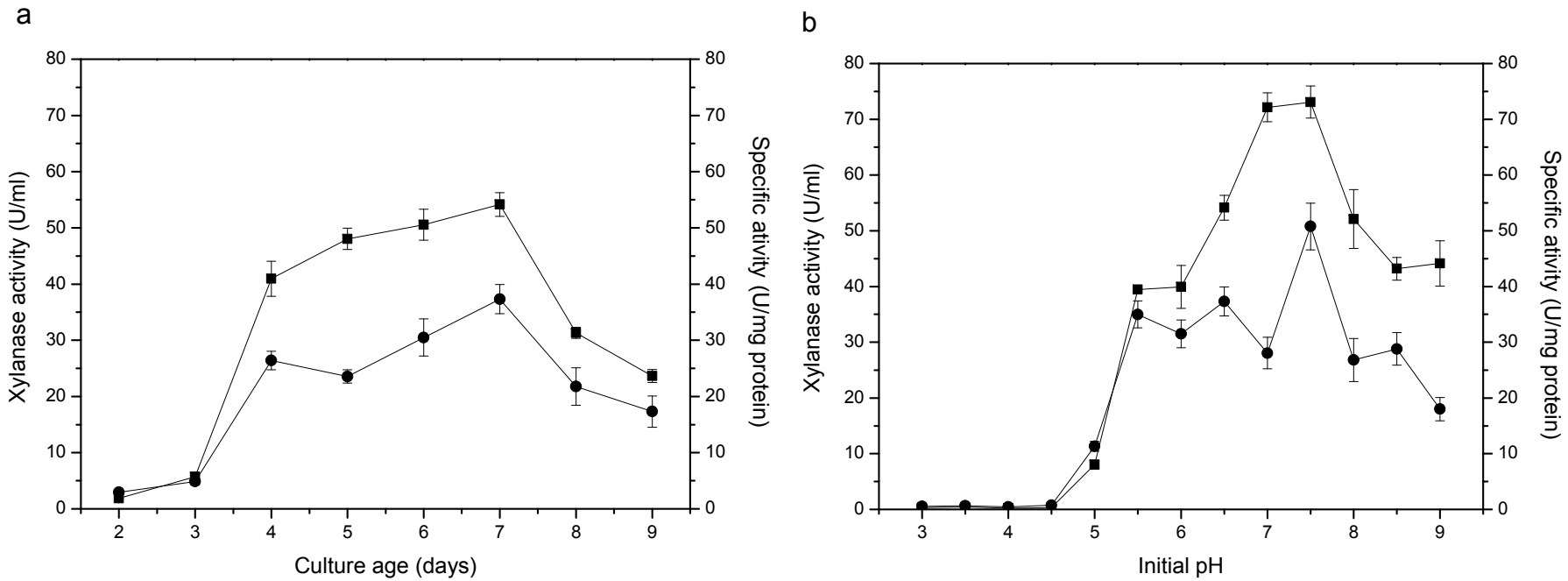

Figure 1. Time-course (a) and initial pH (b) effect on xylanase production by *T. viride*. (■) xylanase activity (U/mL); (●) specific xylanase activity (U/mg of protein). Culture conditions: Vogel medium with pineapple peel 2% (w/v), at 28°C. **(a)** pH 6.5; **(b)** stationary condition for seven days.

parameters that determine growth rates of microorganisms and significantly affect the level of xylanases produced. The influence of pH on *T. viride* xylanase production is showed in Figure 1b. Xylanase production was verified in all pH evaluated. The highest activity in units per volume was observed in pH from 7.0 to 7.5 (72.15 and 73.09 U/ml, respectively). Similarly, the maximal fungal development was verified in the same pH range, corresponding to 59.46 mg protein and 58.03 mg protein (not shown). The xylanase production at neutral pH values has been reported previously in *T. reesei* cultivated on the xylan based media (Bailey et al., 1993; Xiong et al., 2004). On the other hand, the optimum pH value for the growth and xylanase production by *Trichoderma* sp. T-1 and T-2 were found to be 5.5 and 5.7, respectively (Mohan et al., 2011). According to Bailey et al. (1993), the optimal pH medium for xylanase production depends not only on the fungal strain considered, but also on the nature of the carbon source in the cultivation medium.

The effect of temperature on xylanase production by *T. viride* is presented in Table 3. The maximum value of xylanase production and highest specific activity were verified at 28°C, corresponding to 73.09 U/mL and 50.78 U/mg protein. It is remarkable that the optimum temperature for the fungal growth and xylanase production correspond to the environmental temperature which the fungus was initially isolated. Meenakshi et al. (2008) achieved the maximum xylanase production by *T. viride* at 25°C, whereas *Trichoderma* sp. showed enhanced xylanase production at 30°C (Pang et al., 2006).

Significantly higher levels of xylanase were obtained after optimization in this study, corresponding to 73.09 U/mL. The production levels of xylanase verified in this work are higher than many reported in the literature with other agro-industrial wastes. The titers of xylanase produced by a *T. virde* strain was grown in a medium containing maize straw corresponding to

Table 3. Effect of different temperatures on xylanase production by *T. viride*.

Temperature (°C)	Intracellular protein (mg)	Enzymatic activity (U/mL)	Specific activity (U/mg extracellular protein)
20	21.57 ± 0.61	36.62 ± 2.05	14.10 ± 1.09
25	28.43 ± 0.51	37. 25 ± 2.83	16.85 ± 2.20
28	58.02 ± 2.35	73.09 ± 2.87	50.78 ±3.25
30	50.64 ± 3.76	49.46 ± 0.98	31.08 ± 1.24

Average and standard deviation of two cultures.

4.6 U/mL (Goyal et al., 2008). *T. harzianum* presented maximum xylanase production of 26.5 U/mL, upon induction with melon peel (Seyis and Aksoz, 2005). On the other hand, when *T. atroviride* was cultivated in sugarcane bagasse, a xylanase titer of 61.3 U/mL was observed (Grigorevski-Lima et al., 2013). Pineapple peel constitutes a renewable resource and can serve as an abundant and inexpensive carbon source. As a result, the use of the above-mentioned waste in the production of xylanase by *T. viride* would decrease the cost of production in an environmentally sound manner.

The crude filtrate from *T. viride* produced with pineapple peel obtained under optimized conditions was evaluated for the presence of cellulolytic enzymes. It was cellulase-free as it exhibited negligible cellulase activity, suggesting that the production of cellulolytic and xylanolytic enzymes is under separate regulators control (Biely et al., 1993). Similarly, no detectable cellulase activity was noted during whole cultivation period of *T. reesei* on xylan containing enriched medium (Kar et al., 2006) and when this fungal specie was cultivated in presence of rice straw (Soroor et al., 2013). However, according to Goswami and Pathak (2013), fungal xylanases generally are associated with cellulases. Thus, the absence of cellulase activity in the crude extract of *T. viride* is an important feature, which enables its application in industrial process which cellulase activity is undesirable, such as pulp, paper and textile industries.

Biochemical properties of *T. viride* xylanase

Xylanase from *T. viride* showed maximal activity at 50°C (Figure 2a). This temperature has been reported as optimal for xylanases from many fungal species, including *Trichoderma* spp. (Irfan and Syed, 2012; Lopes et al., 2013). Conversely, the optimal temperature for *T. harzianum* xylanases was 60°C (Seyis and Aksoz, 2005; Ahmed et al., 2012).

T. viride xylanase exhibited optimal activity at pH 6.0-6.5 (Figure 2b). Most xylanases presents optimal activity in pH between 5.0 and 7.0 (Madlala et al., 2001) and among the acidophilic xylanases, majority of them showed high activity only under slight acid conditions, as verified in this study. Irfan and Syed (2012) determined pH 5.0 as optimal for xylanases produced by *T. viride.* The same was observed for *T. harzianum* xylanases (Ahmed et al., 2012).

The enzyme stability remains a critical aspect for its biotechnological applications. For this reason, thermal and pH stability of xylanase produced by *T. viride* were investigated. The enzymatic preparation was incubated without substrate at 40, 45 and 50°C. *T. viride* xylanase was stable at 40°C, showing the half-life ($T_{1/2}$) value of 255 min (Figure 3a). This enzyme is more thermostable that other fungal xylanases, such as those from *Trichoderma longibrachiatum* strains (Chen et al., 1997; Medeiros et al., 2003).

The xylanase produced by *T. viride* was stable in acid conditions (Figure 3b). High stability (93.88 and 85.25%) was observed in pH 5.0 and 5.5, while low residual activity (less than 50%) was observed at pH 3.0, 7.5 and 8.0. In the range of pH 6.5-7.0, around 50% of its initial activity was maintained. Usually, microbial xylanases are stable over a wide pH range (3-10) (Kulkarni et al., 1999). Irfan and Syed (2012) verified that xylanases produced by another *T. viride* strain showed pH stability in the range 4.0-70, whereas *T. reesei* xylanase showed stability at pH values of 3.0 to 7.5 (Huang et al., 2013). The biochemical properties exhibited by *T. viride* xylanase such as optimum activity in slight acid conditions and pH stability are attractive for some industrial applications, such as in pulp and paper industry, in which optimal activity at pH 6.0 is required (Polizeli et al., 2005; Ahmed et al., 2012).

In order to verify the effect of substances on xylanase activity, the crude filtrate was incubated in the presence of several metallic ions and chemical reagents, at 2 and 10 mM concentrations (Table 4). *T. viride* xylanase was remarkably stimulated when incubated with Mg^{2+} and Zn^{2+}. The ion Mg^{2+} also activated the xylanase from another *T. viride* strain (Irfan and Syed, 2012), whereas *Trichoderma* sp. xylanase was activated by Zn^{2+} (Sathiyavathi and Parvatham, 2013). The slight activation of *T. viride* xylanases observed in the presence of Ba^{2+} and NH_4^+ may be explained by the enzymatic structure stabilization by these ions. The requirement of monovalent cations for activities of a number of different enzymes has been reported in the literature. However, NH_4^+ activation is a property not common to all previously described *Trichoderma* spp. xylanases.

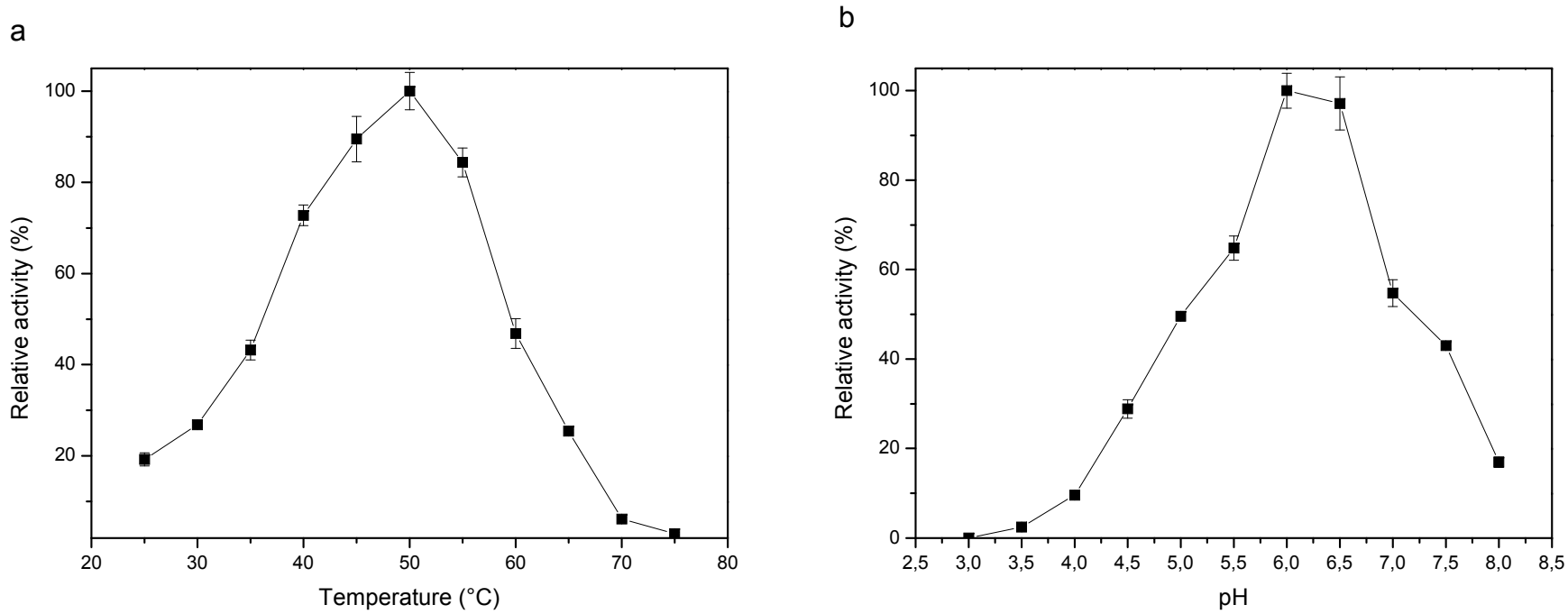

Figure 2. Influence of temperature (a) and pH (b) on xylanase activity from *T. viride*. Culture condition: Vogel medium with 2% pineapple peel (w/v) under stationary condition for 7 days, pH 7.5 at 28°C. **(a)** Xylanase activity was assayed with McIlvaine buffer pH 6.0 and **(b)** with McIlvaine buffer from pH 3.0 to 8.0, at 50°C.

T. viride xylanase showed enhanced activity in the presence of the reducing agent β-mercaptoethanol. This fact can be explained by preventing the oxidation of sulfidryl groups by this thiol group-protecting agent. Medeiros et al. (2003), Franco et al. (2004) and Soroor et al. (2013) also verified the involvement of cysteine residues in the maintenance of enzyme active conformation in *T. longibrachiatum*, *T. harzianum* and *T. reesei* xylanases, respectively.

Cu^{2+} and Hg^{2+} were strong inhibitors of xylanase activity, while Ca^{2+}, sodium citrate, Na^{+}, glycerol and Pb^{3+} had a moderate inhibitory effect. Similarly, *T. reesei* xylanases were inhibited by Cu^{2+} and Hg^{2+} (Soroor et al., 2013). The inhibition by Hg^{2+} seems to be a general property of xylanases, indicating the presence of thiol groups of cysteine residues in enzyme active sites or around them. Cu^{2+} ions are known to catalyze auto-oxidation of cysteines, which leads to the formation of intramolecular and intermolecular disulphide bridges (Vieille and Ziekus, 2001). The inhibitory effect of Cu^{2+} could be explained by the presence of sulfhydryl group in the catalytic center of these enzymes.

T. viride xylanase was also inhibited by SDS and EDTA, indicating that hydrophobic interactions are important for tertiary structure maintenance and metals ions are required for its action, respectively. Likewise, the xylanases from another *T. viride* strain also were inhibited by EDTA, exhibiting 26% of initial xylanase activity at 10 mM concentration (Irfan and Syed, 2012). In contrast, EDTA at a 5 mM concentration has a weak inhibitory effect on *T. reesei* xylanases (Soroor et al., 2013).

Kraft pulp biobleching using *T. viride* xylanases

In recent years, the use of xylanase in pulp

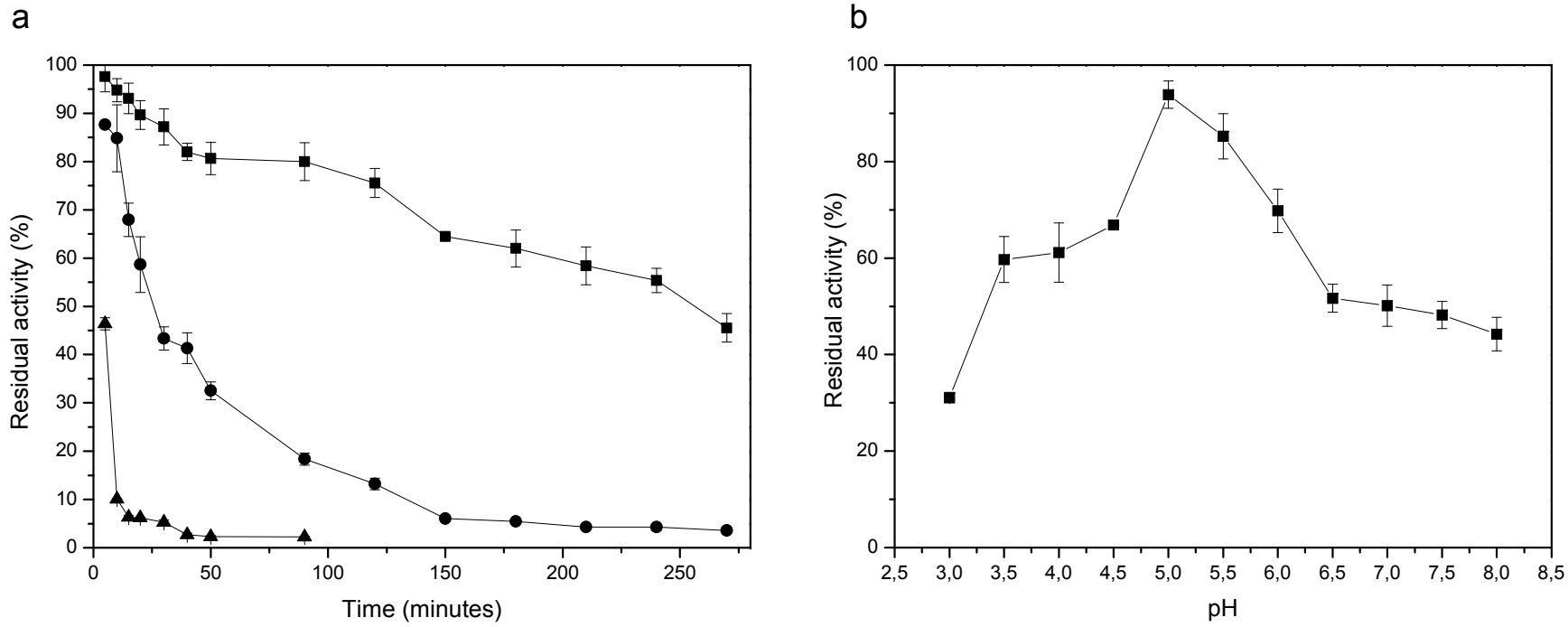

Figure 3. Thermal (a) and pH (b) stability of xylanase activity from *T. viride.* **(a)** The enzymatic preparation was incubated at (■) 40, (●) 45 and (▲) 50 without substrate. **(b)** The enzymatic preparation was incubated without substrate with McIlvaine buffer from pH 3.0 to 8.0 at 4°C for 24 h. In both assays, the residual xylanase activity was assayed with McIlvaine buffer, pH 6.0 at 50°C.

pretreatment has been given special attention as it may reduce the chlorine compounds consumption by up to 30%, so that a 15-20% reduction in organochlorines in the effluents can be achieved (Polizeli et al., 2005). For this reason, the application of *T. viride* crude extract in kraft pulp biobleaching was investigated and optimized. During optimization, the pulp properties kappa number and viscosity were determined. A decrease in kappa number and viscosity maintenance would indicate a better efficiency of the xylanase treatment.

The effect on enzyme dosage on the pretreatment process was investigated in pH 6.0, at 50°C, for 60 min. The data in Table 5 reveal that the least mean kappa number was verified with enzyme dosage of 10 U/g dried pulp (12.99), followed by 15 (13.02) and 20 U/g dried pulp (13.01). However, these values are not statically different. Similarly, Nagar et al. (2013) did not found any differences in kappa number between treatments using 10, 12.5 and 15 U/g dried pulp. These results are in accordance with several studies that established an enzyme dosage of 10 U/g dried pulp as optimum for pretreatment of hardwood kraft pulp (Dhiman et al., 2009; Garg et al., 2011). Conversely, the best performance of *T. longibrachiatum* and *Trichoderma* sp. xylanase preparations were obtained with an enzyme dosage of 25 and 40 U/g dried pulp, respectively (Medeiros et al., 2007; Sathiyavathi and Parvatham, 2013).

The influence of temperature on the pulp pretreatment was investigated with enzyme dosage of 10 U/g dried pulp, pH 6.0, for 60 min (Table 5). The highest decrease in resulting kappa number was observed at 50-55°C. Many studies have reported temperatures in the range of 50-60°C as optimum for pulp bleaching (Nagar et al.,

Table 4. Effect of different substances on xylanase from *T. viride*.

Substance	Xylanase activity (%)	
	Concentration	
	2 mM	10 Mm
Control	100	100
$CuSO_4$	90.53 ± 3.31	19.96 ± 1.57
$ZnSO_4$	104.86 ± 4.39	140.25 ± 1.69
$MnSO_4$	111.53 ± 3.61	80.03 ± 2.25
$BaCl_2$	100.44 ± 4.13	108.48 ± 1.89
$CaCl_2$	77.11 ± 4.17	65.4 ± 2.15
NH_4Cl	103.56 ± 2.25	106.81 ± 4.49
NaCl	74.52 ± 2.84	70.74 ± 3.61
SDS	86.07 ± 0.82	65.3 ± 1.25
$MgSO_4$	133.02 ± 1.38	114.11 ± 2.75
Sodium citrate	74.35 ± 4.23	69.02 ± 4.88
$Co(NO_3)_2$	106.54 ± 0.78	92.94 ± 2.25
$HgCl_2$	11.75 ± 0.44	0
$Pb(CH_3COO)_2$	78.34 ± 4.52	58.34 ± 3.21
EDTA	68.04 ± 2.63	66.16 ± 0
β-mercaptoethanol	131.74 ± 1.84	148.61 ± 2.78

Table 5. Pretreatment optimization of kraft pulp with *T. viride* xylanases.

Enzyme dose (U/g dried pulp)	Kappa number	CTS (%)
Control	16.67^{a}	19.22^{a}
5	14.60^{b}	19.20^{a}
10	12.99^{c}	19.19^{a}
15	13.02^{c}	19.23^{a}
20	13.01^{c}	19.17^{a}
Temperature (°C)		
45	14.60^{a}	19.22^{a}
50	12.99^{c}	19.19^{a}
55	13.27^{bc}	19.17^{a}
60	14.16^{ab}	19.23^{a}
Time (min)		
0	16.63^{a}	19.23^{a}
60	12.99^{b}	19.19^{a}
120	11.93^{c}	19.18^{a}
180	11.91^{c}	19.18^{a}
pH		
5.5	13.46^{a}	19.23^{a}
6.0	12.99^{ab}	19.19^{a}
6.5	11.81^{c}	19.17^{a}
7.0	12.09^{bc}	19.19^{a}

2013; Dhiman et al., 2009; Garg et al., 2011). Savitha et al. (2009) reported that 60°C was the optimum temperature for attaining maximum kappa number reduction of waste paper pulp with the purified xylanase from *T. harzianum*.

The effect of xylanase pretreatment time is also shown

in Table 5. It was investigated with enzyme dosage of 10 U/g dried pulp, pH 6.0, at 50°C. The maximum efficiency of the xylanases in this process was achieved after 120 min of incubation, corresponding to a reduction in mean kappa number from 16.63 to 11.93. It can also verify that a longer period of incubation did not enhance the pretreatment efficiency, significantly. Likewise, some authors have been determined 120 min as the biobleaching optimal incubation time (Sandrim et al., 2005; Ko et al., 2011). Sathiyavathi and Parvatham (2013) verified that the kappa number was reduced significantly with increase in reaction time up to 3 h when *Trichoderma* sp. crude extract was used, whereas other researcher have verified maximal efficiency after 180 min of incubation (Shindu et al., 2006; Kiddinamoorthy et al., 2008; Kumar et al., 2009).

The pH influence on pulp treatment was evaluated with an enzyme dosage of 10 U/g dried pulp, at 50°C, for 60 min of incubation (Table 5). Greater bleaching efficiency was verified at pH values of 6.5-7.0, corresponding a kappa number of 12. These results are in accordance with other studies in literature, which related the optimum pH in the range of 6.0-8.0 (Sandrim et al., 2005; Ko et al., 2011; Sangui et al., 2009).

In addition, it can be observed in all treatments that the pulp viscosity was not significantly modified (Table 5), indicating the maintenance of pulp integrity, due to absence of cellulolytic enzymes in crude filtrate, as previously mentioned. This is a crucial aspect to take into account in biobleaching process, since cellulases could damage the fibers, resulting in loss of strength and performance (Terrasan et al., 2013).

The data obtained revealed that the kraft pulp pre-treatment was more efficient when conducted at 50°C, in pH 6.5, for 120 min, with an enzyme dosage of 10 U/g dried pulp. Under these conditions, the kappa number was reduced to 10.12 (6.55 points), which corresponds a kappa efficiency of 39.29. The results obtained in the treatment with *T. viride* enzymes was higher than those achieved with other fungal xylanases, which usually show reductions in kappa number between 0.9 and 5.07 units (Sandrim et al., 2005; Medeiros et al., 2007; Sangui et al., 2009; Terrasan et al., 2013; Guimaraes et al., 2013).

Conclusions

T. viride J40 strain was able to produce high levels of xylanase using pineapple peel as substrate. The *T. viride* xylanase showed optimal activity at 50°C and pH 6.0-6.5 and it was able to act and exhibited stability in moderate temperatures and acid pH values. The use of pineapple peel as carbon source can decrease the costs for the enzyme production and avoids environmental problems of the inappropriate disposition of this waste. Therefore, the reduction on kappa number achieved in this present investigation revealed the application potential of *T. viride* xylanases in biobleaching process.

Conflict of interests

The authors have not declared any conflict of interest.

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