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Downstream processing of pectinase produced by *Aspergillus niger* in solid state cultivation and its application to fruit juices clarification

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Abstract

In this work, a protocol for the formulation of an enzyme concentrated product to be applied in fruit juice treatment is described. Downstream processing conditions for the recovery and concentration of pectinases produced by the new strain Aspergillus niger LB-02-SF in solid state cultivation were assessed. The solid-liquid ratio in the extraction step of pectinases recovery from the cultivated media was evaluated and the highest activity was obtained with a solid-liquid ratio of 1:10. The crude extract was concentrated by ultrafiltration and the total pectinase (TP) activity was 73.6-fold concentrated in relation to the crude extract, and a final TP titer of 663 U mL^{-1} was obtained with 73.7% of recovery yield. KCl and different glycerol concentrations were added to the concentrated extract and the stability of pectinases during the storage at 5°C for 59 weeks was tested. The formulation with 50% w/w glycerol was applied to the treatment of apple and grape juices and the results of these tests were statistically comparable to those obtained with two high-quality commercial preparations.

Keywords: solid-state cultivation; downstream processing; pectinase stability; pectinase formulation; juice clarification.

Pratical Application: The study characterizes the downstream processing and formulation of pectinases, which are not presented by the producing companies.

1 Introduction

Pectinases comprise a group of enzymes that hydrolyze pectic substances present in plant cells. Among the different applications, pectinases are widely used for depectinization of fruit juices (Jayani et al., 2005; Gummadi & Panda, 2003). Pectinases are mainly produced by filamentous fungi, especially by the genus *Aspergillus*, in submerged cultivation (SmC) or solid-state cultivation (SSC).

The recovery and concentration steps are important operations in enzyme production processing and may take into account the degree of purity needed for each particular application. Some commercial enzyme preparations consist essentially of concentrated culture broth plus additives to stabilize enzyme activity (European Commission, 2002). For SSC, one must consider that the produced metabolites are retained in a solid matrix and must be solubilized by solid-liquid extraction prior to the subsequent downstream operations. There is no consensus with regard to the ideal strategy to perform this extraction. In fact, different conditions for solid-liquid ratio, solvent, temperature and operation time are reported in the specialized literature (Rodríguez-Fernández et al., 2012; Díaz et al., 2007; Castilho et al., 2000).

In the next downstream step, the solubilized enzymes are subjected to a concentration procedure such as salt and solvent precipitation, two aqueous-phase separation or ultrafiltration, among others (Nakkeeran et al., 2010; Maciel et al., 2014). Ultrafiltration (UF) has been used as an attractive tool for many industrial separation processes and a very common application is in downstream processing for product concentration to remove a buffer or a solvent (Charcosset, 2006). UF-based downstream processing can be advantageous for enzyme separation, especially due to the low temperature of operation and the high separation factor.

In the present work, pectinases produced by SSC with *Aspergillus niger* LB-02-SF, a new strain isolated by Sandri et al. (2013), was used to develop an enzyme formulation to be used in the treatment of fruit juices. For this purpose, an experimental downstream processing protocol was defined, including conditions for the extraction from the solid media, further activated charcoal and microfiltration treatments to remove impurities and enzyme concentration by ultrafiltration. In the sequence, KCl and different concentrations of glycerol were evaluated as stabilizer additives for the enzyme preparations. Finally, the efficiency of the experimental formulation was tested in apple and grape juices treatment.

2 Materials and methods

2.1 Microorganism and cultivation conditions

The strain *A. niger* LB-02-SF, from the University of Caxias do Sul (Brazil) was propagated in glycerin agar medium and incubated at 30°C for 5 days. The sporulated cultures were stored at 4°C until the inoculation of culture media.

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The cultivation medium was defined by Fontana et al. (2005): wheat bran (Moinho Nordeste, Brazil), citric pectin (CP Kelco S.A, Brazil), glucose, salt nutrient solution and a volume of suspension of *A. niger* (1×10^7 spores/g) in distilled water to reach an initial moisture of 53%.

A. niger LB-02-SF cultivations were carried out in trays with 1460 g of medium and 4 cm of height, for 96 h, at 30°C. The trays were covered with a thin layer of gauze, which allowed gas and heat changes with the external environment by simple diffusion.

2.2 Extraction conditions

A. niger-cultivated solid media was homogenized and samples were suspended in distilled water (pH 4.0) in 250 mL Erlenmeyer flasks that were reciprocally agitated at 200 rpm (B. Braun Biotech model Certomat H, Germany). The extraction time of 15, 30, 60, 90 and 120 minutes was evaluated with a dry solid/liquid (S/L) ratio of 1/15, at 20°C. The effect of temperature on the extraction was assessed by incubating the samples in a S/L ratio of 1/15, at 20, 30 and 40°C for 60 minutes. S/L ratios of 1/5, 1/7.5, 1/10, 1/15, 1/30 and 1/45 were tested at the same volume of distilled water (30 mL) and different mass of solid, at 20°C for 60 minutes. The liquid enzymatic extract was separated from the residual solid mass by centrifugation (6,000 rpm for 10 min) and filtered through Whatman nº 1 filter paper.

2.3 Pre-treatment of crude extract with activated charcoal and microfiltration

The crude extract was pre-treated with activated charcoal at 5 g/L for 30 min. The mixture was centrifuged at 6,000 rpm for 10 min and the supernatant was filtered through Whatman No. 1 filter paper.

Microfiltration (MF) of the activated charcoal-treated enzyme extract was carried out in a hollow fiber polyimide membrane with 0.2 m^2 of area and pore size of 0.4 μm (PAM Membranas Seletivas, Brazil), at a pressure of 40 kPa.

2.4 Concentration of pectinases by ultrafiltration

Ultrafiltration operation was performed at 20°C using a polyethersulfone spiral wound membrane with 0.1 m^2 of area and a nominal molecular cut off of 10 kDa (Millipore, USA), at 100 kPa. The performance of UF process was evaluated by the activity concentration ratio (ACR), recovery yield (%) (Equation 1) and fold purification (Equation 2) as follows (Cheryan, 1998):

$$\text{Recovery yield (\%)} = \left(\frac{\text{Ac} \times \text{Vc}}{\text{Af} \times \text{Vf}}\right) \times 100 \quad (1)$$

$$\text{Fold purification} = \frac{\text{specific activity in the concentrate}}{\text{specific activity in the crude extract}} \quad (2)$$

where Vf, Vp and Vc are the volume of feed, permeate and concentrate (mL), Af, Ap and Ac are the enzyme activity of feed, permeate and concentrate (U mL^{-1}), respectively.

2.5 Formulation of the enzyme preparation

Glycerol (20, 30, 40 and 50% w/w) and KCl (2% w/w) were used as additives in the enzyme formulation. Thermostability of enzyme formulations and the control (without additives) were evaluated at 20, 30, 40, 50 and 60°C by 60 min. The study of shelf life of enzyme formulations and the control was performed during 59 weeks. The samples were stored at 5°C and TP activity was measured each 15 days.

2.6 Juice preparation and enzymatic treatment

Apple and grape fruits were heating at 35 and 45°C, respectively for 45 min and the juices were obtained pressing manually the pulps, filtered and finally enzymatic treated.

Commercial enzymes Pectinex Ultra SP-L and Novozym 33095 were compared with the experimental preparation in the clarification step. The dosage of 25 U of enzyme per 100 mL was indicated by the manufacture of commercial enzymes. Clarification was done for 45 min at 35°C and 45°C to the apple and grape juices, respectively. The juice clarity was determined by the sum of absorbances at 440 and 520 nm for apple juice and 420, 520 and 620 nm for grape juice (Rangana, 1977). The juice viscosity was measured at 30°C with a viscometer and the turbidity with a turbiditymeter at 23°C. Total phenols were determined by the Folin-Ciocalteu procedure (Singleton & Rossi, 1965).

2.7 Determination of pectinase activity and protein

Total pectinase (TP) activity was determined by measuring the decrease in viscosity of a citric pectin solution (Gainvors et al., 2000).

Protein was determined by the Bradford method (Bradford, 1976).

3 Results and discussion

3.1 Definition of solid / liquid extraction conditions

The downstream processing, firstly involved the extraction of enzymes from the solid media in order to achieve a solution with enzyme activities as high as possible. In these tests, total pectinase activity (TP) and protein concentration were used as evaluation parameters.

In standard conditions, no significant differences were observed when the time of extraction varied from 15 to 60 minutes. For all times, TP activity of 5.5 U ml^{-1} and protein concentration of 100 μg ml^{-1} were measured. This result could be important in an industrial point of view, because in a few minutes the extraction step could be completed.

When extraction temperatures of 20, 30 and 40°C were evaluated, a decrease of 42% in TP activity was observed at 40°C when compared to 20°C (Figure 1). Díaz et al. (2007), suggest that at higher temperatures the saturation of the solvent by a number of other compounds present in the media occurs, and that makes the enzyme extraction less efficient. However, no significant difference was noticed with respect to protein concentration at the different temperatures (Figure 1), and it is possible that a partial inactivation of enzyme occurred due to the combination of a higher temperature and the time of incubation.

Mohsen et al. (2009) [30] and Hendges et al. (2011), reported that pectinases produced by different strains of *A. niger* have shown to be almost completely stable at temperatures up to 30°C, but their activities have been reduced to approximately 60% and 80% of the initial, respectively, after exposed to a temperature of 40°C for 60 minutes.

The best results for TP activity recovery per gram of dry solid medium was obtained with an S/L ratios of 1/30 and 1/45 (Table 1). On the other hand, with S/L ratios of 1/5 and 1/7.5, TP recovery decreased significantly. The results can be explained by the fact that these tests were performed with 74% moist medium, although S/L ratios are expressed in dry medium basis. That means that approximately 3.8 g of moist medium had to be used to have 1.0 g of dry medium. As such, with S/L of 1/5 and 1/7.5, the volume of solvent was insufficient for enzyme extraction. When the TP extraction was evaluated taking into account enzyme units recovered per mL of solvent, the most concentrated activity solution was achieved with S/L of 1/10 ratio. Despite the fact that TP recovery with S/L of 1/10 was approximately 80% of that obtained with S/L of 1/45, this condition was chosen for downstream processing, because the greater the enzyme activity in the crude extract, the lower the initial volume to be concentrated and the time necessary to reach the desired activity of the concentrated extract. Rodríguez-Fernández et al. (2012), studied the extraction of polygalacturonase from solid medium and according to these authors, enzyme activity concentration is the best criterion to evaluate the extraction step due to the time and energy savings for the downstream process.

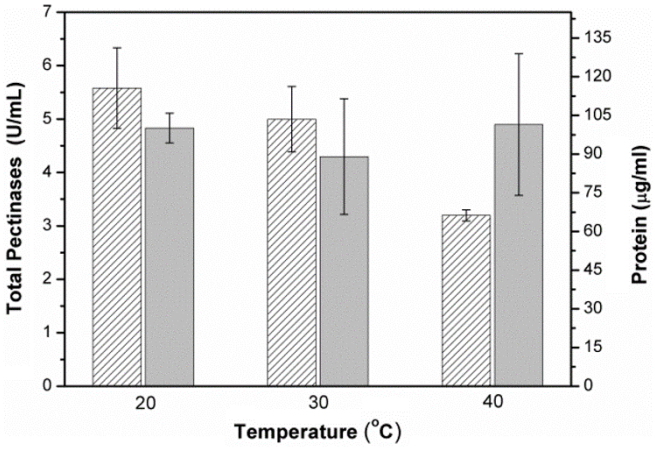

Figure 1. Effect of extraction temperature on total pectinase (TP) activity and protein from solid medium. Solvent, water pH 4.0; incubation time, 60 minutes; 1/15 solid/liquid ratio.

Table 1. Total pectinase (TP) activity recovery with different solid/liquid (S/L) ratios in the extraction step. Solvent, water pH 4.0; incubation time, 60 minutes; temperature, 20°C.

[a]S/L (g mL^{-1})	TP activity recovered (U g^{-1} of dry medium)	TP activity solubilized (U ml^{-1} solution)
1/45	132.6 ± 2.3	2.9 ± 0.05
1/30	130.2 ± 20.1	4.3 ± 0.7
1/15	114.3 ±14.3	7.6 ± 1
1/10	108.3 ± 10.8	10.8 ± 1.0
1/7.5	55.7 ± 3.2	7.4 ± 0.5
1/5	22.2 ± 2.3	4.4 ± 0.5

[a]S/L ratio is expressed in dry basis, considering an average moisture of 74% for the solid medium.

3.2 Evaluation of pre-treatments of crude pectinase extract

In order to remove pigments and some proteins produced during the SSC, the crude enzymatic extract was initially treated with activated charcoal (5 g L^{-1}). This previous treatment resulted in 72% and 14% of color and protein reduction, respectively and 18% of purification (Table 2) with no loss of TP activity. In a recent work (Dey et al., 2014), a high polygalacturonase purification (34.8 fold), with 69.8% of enzyme recovery, was described with 10 g mL^{-1} of activated charcoal powder. However, Nakkeeran et al. (2010), observed that the use of 5 g L^{-1} of activated charcoal resulted in the reduction of 83% of the color of a polygalacturonase preparation, with no significant activity loss.

The activated charcoal-treated enzyme extract was then submitted to microfiltration (MF) to retain impurities that could cause difficulties to the UF recovery step. According to Charcosset (2006), MF can remove polysaccharides, suspended solids, residual cell mass and other particulate debris from liquid suspensions. After MF pre-treatment, almost 94% of TP activity was recovered and a fold purification of 1.23 was obtained.

3.3 Assessment of ultrafiltration for the concentration of pectinases and other enzymes

In the UF, 30 L of enzyme solution was concentrated, and a volume concentration ratio (VCR) of 103.5 was reached. Figure 2a shows a flux decline of about 58% after 360 minutes of operation. According to Datta et al. (2009), flux decline in protein separation may be owed to the occurrence of a concentration polarization layer on the membrane surface that leads to increasing resistance to the flux. Rodríguez-Fernández et al. (2013), describe a similar behavior in the concentration of phytase by UF, the flux decline occurring as a consequence of fouling formation and viscosity increment of

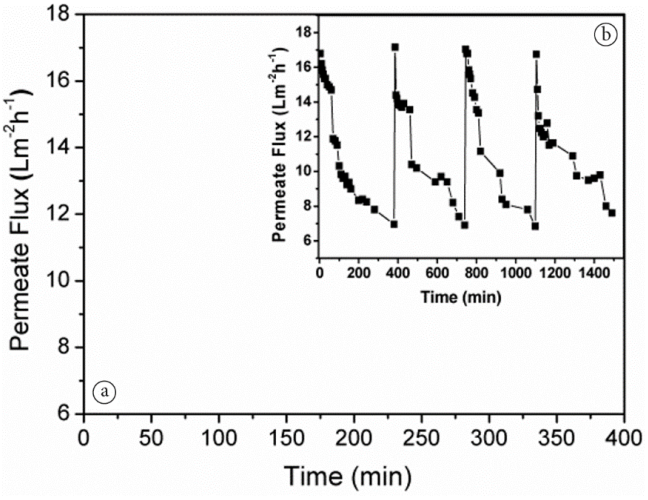

Figure 2. Ultrafiltration of enzymatic extract: (a) permeate flux behavior during 380 minutes; (b) permeate flux behavior after chemical cleaning.

the feed solution. On the other hand, the UF flux was completely re-established after each chemical cleaning (Figure 2b).

After UF step, TP activity was concentrated 73.6-fold, reaching a final enzyme titer of 663 UmL^{-1}, with a recovery yield of 73.7% (Table 2). Singh et al. (1999), using polysulphone membrane of 30 kDa to concentrate 100 L of pectinase extract, obtained an activity fold concentration of 50.7 and a recovery yield of 91%. According to Hwang & Sz (2011) and Powell & Timperman (2005), recovery losses are due to surface adsorption and concentration polarization effects on the membrane surface. In addition, recovery of each protein/enzyme is largely dependent on its molecular properties. Rodríguez-Fernández et al. (2013), reported the loss of phytase activity due to the action of shear forces in the UF membrane or conformational changes due to the interaction with membrane during filtration.

UF may be considered as a low resolution purification method in which rejection of molecules is determined by the molecular weight cut off of the membrane. Thus, in our test, enzymes and other proteins with molecular weight above 10 kDa were retained in the concentrate stream and this could explain the low fold purification attained (4.07). This result is in accordance with those of other works that reported values of 5.14 and 7.7 fold purification using membranes with 50 kDa and 10 kDa, respectively (Nakkeeran et al., 2008; Silva et al., 2007). However, the fold purification could be a relatively unimportant parameter for the evaluation of an enzyme recovery method, since the desirable purity degree of a preparation depends on the application. In the case of pectinases, high purity is relatively irrelevant if it is to be applied in the treatment of fruit juices, in which a multicomponent mixture of several enzymes is beneficial to this process. This is in accordance with the description of commercial preparations that consist in a broad spectrum of hydrolytic enzymes.

3.4 Formulation and stability of final enzyme product

Commercial enzyme formulations include additives to avoid protein unfolding and to preserve the stability of the enzyme product for several months or years. Glycerol is the most commonly employed additive in pectinolytic enzyme found in the market. Salts in low (<0.1M) or high (>0.1M) concentrations are also used to stabilize enzyme preparations (Weijers & Van't Riet, 1992). Thus, in the present work, glycerol was tested as stabilizing additive at different concentrations: 20%, 30%, 40% and 50% (w/w). Furthermore, 2% w/v KCl (0.34 M) was used in all formulations.

Thermostability is an important parameter to determine the application conditions of enzymes. As such, the thermostability of the different formulations was evaluated at 20, 30, 40, 50 and 60°C for 60 minutes. Under these conditions, the samples containing different glycerol concentrations presented similar behavior, with approximately the following residual TP activity: 100% at 20 and 30°C, 85% at 40°C, 65% at 50°C and 19% at 60°C. These results indicate that glycerol has no protective effect for pectinase activity, at least for the time of 60 minutes. The two commercial preparations used as references – Pectinex Ultra SP-L and Novozym 33095 – exhibited 36 and 51% of the initial TP activity after 60 minutes at 50°C. Ortega et al. (2004), observed, for the same test conditions, that the preparation Pectinase CCM retained 57% of its initial activity, whereas only 5% and 10% of the initial activities of Rapidase C80 and Pectinex 3XL, respectively, were preserved. These results confirmed that the experimental extract of *A. niger* LB-02-SF has potential to be applied in fruit juices treatment, which is usually carried out at temperatures from 30 to 50°C.

In the sequence, stability of enzyme formulations during storage was evaluated at 5°C for 59 weeks. In the control sample (0% glycerol), it was observed the occurrence of turbidity and precipitate formation, even in the initial weeks of storage, due to the growth of microorganisms as observed by optical microscopy. Otherwise, the samples with glycerol and KCl did not present turbidity or microbial contamination. After 38 weeks, the control sample presented a decrease in TP activity of 12% and a decline of 25% was observed in the last measured (Figure 3). The activities in all formulations remained close to 100% of the initial. In the 50% glycerol formulation, we observed an activity increase during the storage. Rodríguez-Fernández et al. (2013) described the same increase for phytase activity with 35% glycerol. The authors related the activation effect with the refolding of the peptide chains and the stabilization of the tertiary and quaternary structures as a result of the hydrogen bonds and other forces created between the enzyme and the glycerol. According to the literature, the enzyme-stabilizing effect of glycerol is due to the following reasons: i) the positive effect on medium water activity that reduces the possibility of microbial contamination (Iyer & Ananthanarayan, 2008) and ii) the preferential hydration of proteins that increases structural compaction and disfavor unfolding of proteins (Ó'Fágáin, 2003). Our results indicate that the use of 20% glycerol and 2% KCl would be enough to ensure the stability of enzyme formulation and so the UF-concentrated extract would be less diluted.

3.5 Enzymatic clarification of apple and grape juices

In juice clarification, the commercial enzymes and the experimental formulation (enzyme + glycerol 50% w/w), further referred as EF, were used in the same dosage. As depicted in Figure 4, apple and grape juice treatment with EF presented

Table 2. Overall results of different steps of recovery of total pectinase (TP) from *Aspergillus niger* LB-02-SF solid-state cultivation.

	Volume (L)	TP ($U\ mL^{-1}$)	Protein ($mg\ mL^{-1}$)	Specific activity ($U\ mg^{-1}$)	Recovery yield (%)	Fold activity concentration	Fold purification
Crude extract	31.1	9.0	0.33	27.6	-	-	-
Activated charcoal	30.7	9.2	0.28	32.6	100	1.01	1.2
Microfiltration	30.3	8.7	0.22	40.1	93.6	0.95	1.2
Ultrafiltration	0.293	663	4.1	163	73.7	76.2	4.1

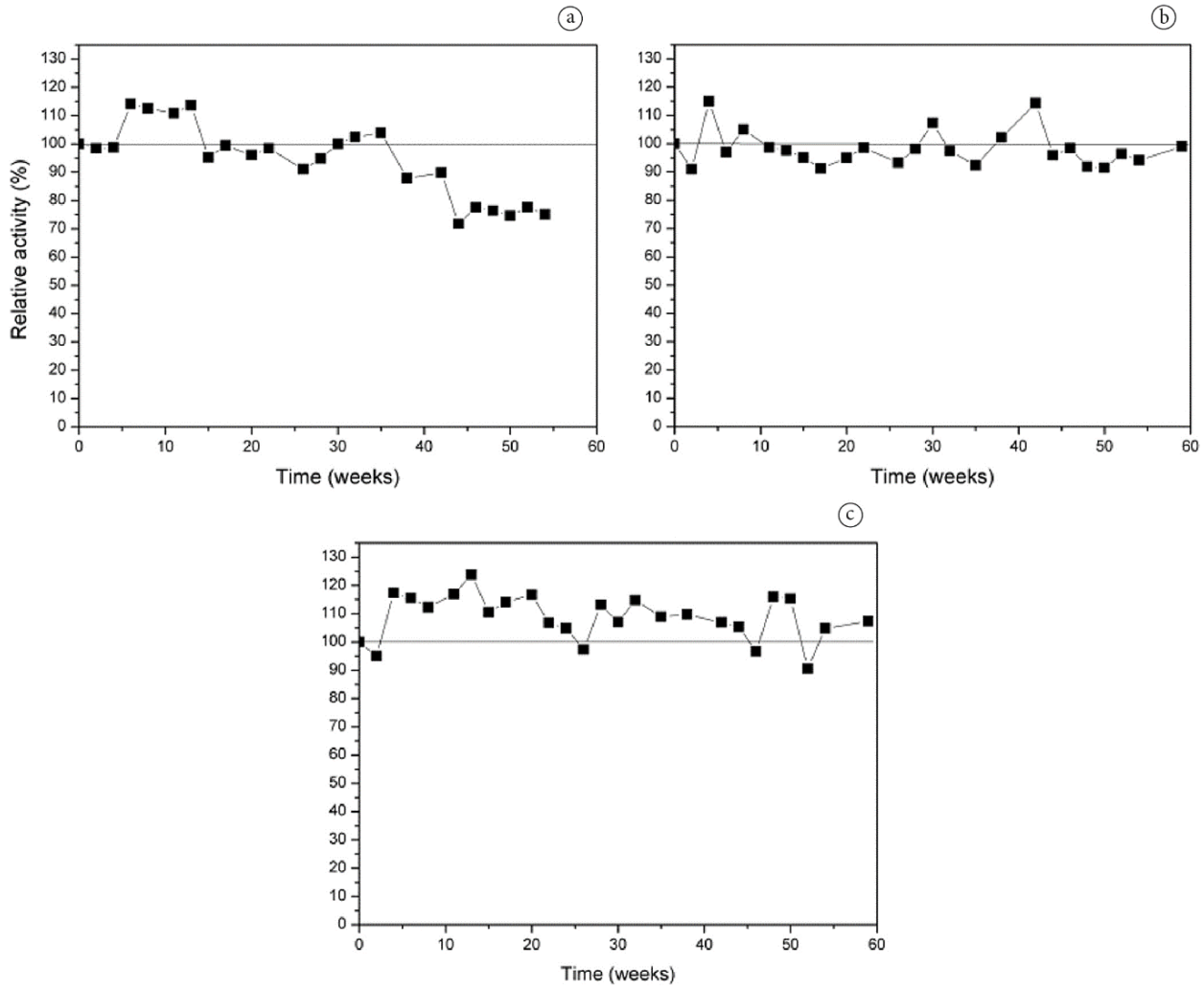

Figure 3. Preservation of pectinase activity during the long-term storage for control sample (a), 20% (b) and 50% (c) glycerol formulation at 5°C.

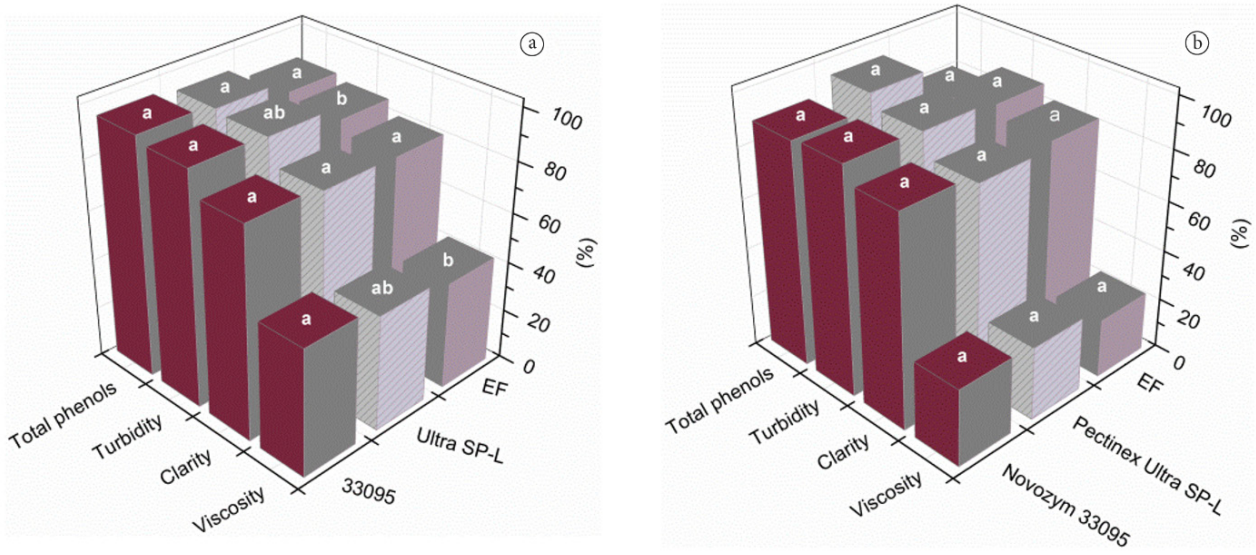

Figure 4. Effect of enzymatic treatment on preservation of total phenol content, decrease of turbidity and viscosity and increase of clarity of apple (a) and grape (b) juices. Commercial enzymes (Novozym 33095 and Pectinex Ultra SP-L) and experimental formulation (EF). Different letters (a-c) indicate significant differences ($p < 0.05$). The values correspond to the mean of three tests.

effects on turbidity, viscosity and clarity that were statistically similar to those measured with the commercial enzymes for both juices. According to Pinelo et al. (2010), the clarification of enzyme-treated fruit juices is due to the effect of pectinases on pectic substances which are degraded into small pectin fractions that tend to agglomerate and precipitate.

Total phenol content in both fruit juices was evaluated after the enzyme treatments (Figure 4). These substances are particularly important because of their great health-protecting capacity that relates to their antioxidant activity (Oszmiański et al., 2011). As can be seen, the use of EF did not significantly affect the level of total phenols compared to the commercial enzymes. The results reached with EF for both juices, confirmed the important role of enzymatic treatment in the hydrolysis of pectin-containing beverages.

4 Conclusions

The results obtained in bench scale in this work may be useful for scaling up the steps of recovery and concentration of enzymes produced by *A. niger* LB-02-SF in solid-state cultivation. The protocol proposed led to a downstream processing with high enzyme recovery in a few steps. The results of storage stability and performance in fruit juices treatment suggest a great potential for a future commercialization of the experimental enzyme formulation.

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