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Combi-CLEAs from Pectinases and Cellulases for Grape Juice Clarification

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ABSTRACT

This work aims to prepare combined cross-linked enzyme aggregates (combi-CLEAs) of pectinases-cellulases for grape juice clarification. For combi-CLEAs preparation, it was used the Rohapect 10L preparation, 110 mM of glutaraldehyde and 2 h of reaction time. Bovine serum albumin (BSA) was used as feeder and improved the volumetric activity, recovered activity and thermal stability. Combi-CLEAs-BSA prepared using 0.4 mg.mL^{-1} of enzyme mixture and 2.4 mg.mL^{-1} of BSA presented an activity of 14 U.mL^{-1}, 18 % of recovered activity and 3-times more thermal stability compared to soluble enzymes. The combi-CLEAs and combi-CLEAs-BSA were tested in repeated batches, being reused for 4 and 6 cycles, respectively, keeping 100 % of the initial activity. The combi-CLEAs and combi-CLEAs-BSA appear to be suitable alternatives of immobilized biocatalyst for the clarification of grape juices.

Keywords: pectinase; cellulase; cross-linked enzyme aggregates; bovine serum albumin; grape juice clarification.

INTRODUCTION

In the processing of grape juice, pressing the fruit leads to a disruption of the cell walls, releasing the internal juice. This method may generate some problems such as excessive viscosity and turbidity by the formation of insoluble particles which may hinder processing and decrease the quality of the juice (Kashyap et al., 2001). This turbidity can be found in fruit juices in different degrees, mainly due to the presence of polysaccharides. In order to overcome these problems, depolymerizing enzymes are extensively applied in fruit juice processing, since they promote the hydrolysis of such compounds, improving filtration, clarification and stabilization (Pinelo et al., 2010; Sandri et al., 2011).

Cross-linked enzyme aggregates (CLEAs) are prepared by precipitating the target enzyme with the addition of specific precipitant agents such as organic solvents, non-ionic polymers or inorganic salts followed by cross-linking with a bifunctional reagent (Garcia-Galan et al., 2011). Thus, it can be also prepared the named combi-CLEAs by co-precipitation of two or more different enzymes. This may be a solution in the case of cascade reactions or if the catalyst must attack several substrates by different enzymes (Cruz et al., 2012).

Based on these aspects, the objective of the present work was to prepare combi-CLEAs of pectinases-cellulases for the application in the grape juice clarification. The effect of the enzyme and BSA concentrations on recovered activity were also investigated. Finally, the soluble enzymes, combi-CLEAs and combi-CLEAs-BSA were characterized and their

enzymatic activities, thermal stability, operational stability and juice clarification were compared.

MATERIALS E METHODS

Raw grape juice, after pressing and without any treatment, was kindly donated by Vitivinícola Jolimont (Canela, RS, Brazil). Rohapect 10L was acquired from Amazon group (Brazil). All other reagents and solvents were of analytical grade.

The enzymatic activities of cellulase (CE) and total pectinase (PE), as well as polygalacturonase (PG), pectinlyase (PL) and pectin methyl esterase (PME) activities, were performed as described by Dal Magro et al.(2016).

For the combi-CLEA preparation, Rohapect 10L was diluted in sodium citrate buffer (50 mM, pH 4.8). Then, 0.1 mL of the diluted enzyme was added to 0.9 mL of isopropyl alcohol (precipitant agent) with 110 mM of glutaraldehyde (cross-linking agent), and homogenized in a roller mixer for 2 h at room temperature. After, the combi-CLEA was recovered by centrifugation (3000 × *g*, 5 min), the supernatant was removed and the combi-CLEA were washed 3-times with sodium citrate buffer (50 mM, pH 4.8) to ensure the elimination of all free enzyme and residual glutaraldehyde. Finally, the combi-CLEA were suspended in 1 mL of sodium citrate buffer (50 mM, pH 4.8), analyzed and stored at 4 ºC.

The thermal stability was performed by incubating the enzymes in sodium citrate buffer (50 mM, pH 4.8) at 50 ºC. Periodically, samples were withdrawn and the total pectinase activity was measured.

Operational stability was carried out by pectin hydrolysis. 2 U of total pectinase was added to 1.0 mL of substrate (1 $g.L^{-1}$ of pectin) prepared in sodium citrate buffer (50 mM, pH 4.8), and incubated at 37 ºC during 15 min, under agitation. Combi-CLEA and combi-CLEA-BSA were recovered from the reaction mixture by centrifugation (3000 × *g*, 5 min) and subsequently washed with sodium citrate buffer (50 mM, pH 4.8), before addition of fresh substrate for a new cycle. The concentration of product formed after each cycle was determined by the DNS method according to Miller (1959).

RESULTS E DISCUSSION

In order to improve the enzymatic activity of combi-CLEAs, experiments were performed varying the concentration of Rohapect 10L extract (0.1 to 1.0 $mg.mL^{-1}$ of protein) and BSA (0.2 to 5.0 $mg.mL^{-1}$) for combi-CLEAs preparation.

As can be seen in Fig. 1-a, the enzymatic activity of combi-CLEAs increased with the amount of enzyme up to 0.4 $mg.mL^{-1}$. Higher concentrations did not lead to an increase in expressed enzyme activity, the recovered activity decreased when larger amount of enzyme was used. Then, in order to obtain a CLEA with good enzymatic activity and high recovered activity, the enzyme concentration of 0.4 $mg.mL^{-1}$ was selected.

Regarding to BSA, analyzing the results of Fig. 1-b, it can be seen that BSA concentration had a positive effect up to 2.4 $mg.mL^{-1}$. The increase of the combi-CLEAs-BSA activity compared to combi-CLEAs activity can be explained probably by a change in the morphology of the combi-CLEAs promoted by BSA. The BSA provides less compact aggregates with pores having larger diameters, facilitating the access of the substrate to active sites of enzymes, therefore the diffusion problems can be reduced (Shah et al., 2006).

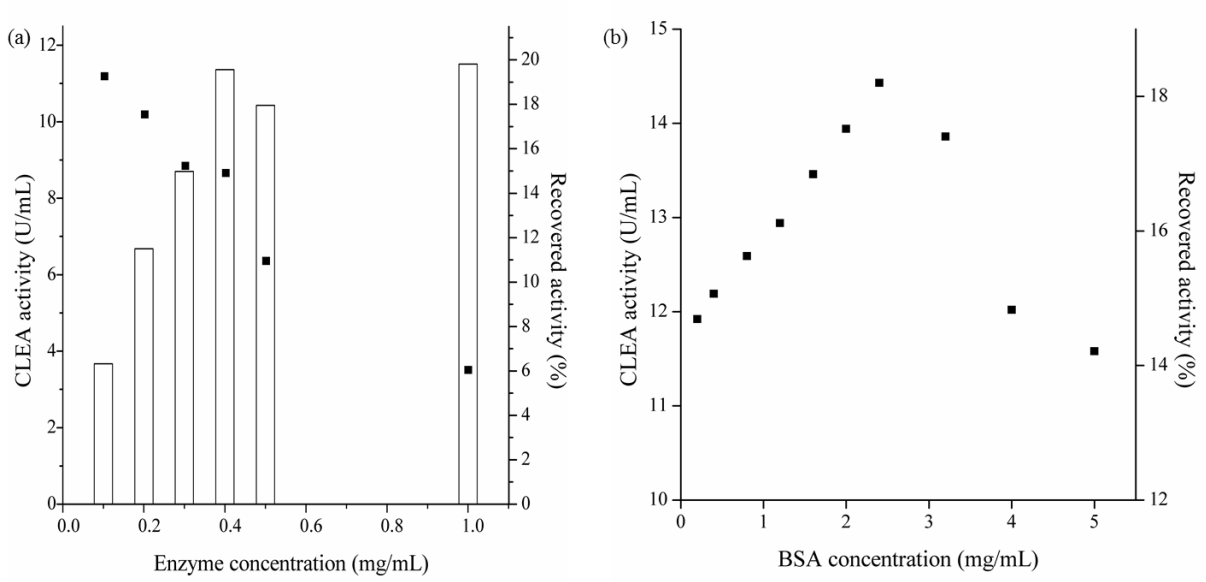

Figure 1. Effect of the enzyme concentration (a) in combi-CLEAs activity (bars) and recovered activity (points), and of the BSA (b) in combi-CLEAs and recovered activity.

The combi-CLEAs and combi-CLEAs-BSA were characterized according to their residual activity of PE, PG, PL, PME and CE (Table 1). For all enzymatic activities analyzed, the combi-CLEAs-BSA presented higher values than combi-CLEAs.

Table 1. Enzymatic activities of the soluble enzyme, combi-CLEAs and combi-CLEAs-BSA

Treatments	**Enzymatic activities** ($U.mL^{-1}$)				
	PE	**PG**	**PL**	**PME**	**CE**
Soluble enzyme	79.01	90.75	13.75	49.00	8.60
Combi-CLEA	11.72	5.49	0.96	2.13	0.58
Combi-CLEA-BSA	14.31	9.80	1.75	6.73	2.15

The thermal stability at 50 ºC indicated a decreased enzyme activity along the time due to thermal inactivation (Fig, 2-a). The thermal constant inactivation rate (k) of the soluble enzyme was 0.0158 min^{-1} and $t_{1/2}$ was 43.87 min. For the combi-CLEAs and combi-CLEAs-BSA, a significant improvement in the thermal stability was observed, since k decreased (0.0068 and 0.0050 min, respectively) and $t_{1/2}$ increased (103.45 and 133.30 min, respectively), providing stabilization factors of 2.36 and 3.04, respectively for the combi-CLEAs and combi-CLEAs-BSA. In the combi-CLEAs, the multipoint interactions between glutaraldehyde and proteins (enzymes and BSA) provide a confinement effect for protein configuration, which increase the enzyme stability (Shah et al., 2006).

The operational stability is one of the main reasons of enzyme immobilization. The results presented in Fig. 2-b showed that the biocatalysts prepared could be reused during several cycles. In total, 12 cycles of hydrolysis were conducted, and until the fourth cycle, the combi-CLEAs presented 100 % of the initial activity. The combi-CLEAs-BSA could be reused during 6 cycles with 100 % of the initial activity. After these cycles, the combi-CLEAs and combi-CLEAs-BSA progressively decreased their enzymatic activities. At the end of the 12 cycles, combi-CLEAs and combi-CLEAs-BSA still presented 30 % and 46 % of initial activity, respectively.

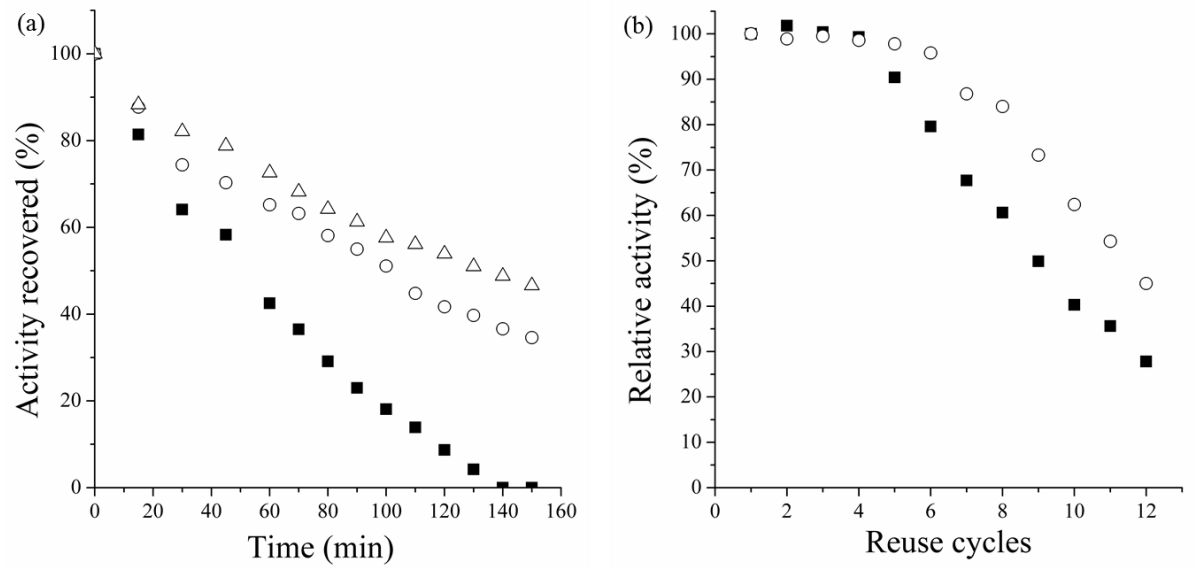

Figure 2. Thermal inactivation kinetics (a) of soluble enzyme (■), combi-CLEAs (○) and combi-CLEAs-BSA (△) at 50 °C and reusability (b) of combi-CLEAs (■) and combi-CLEAs-BSA (○) for pectin hydrolysis.

CONCLUSIONS

Combi-CLEAs of pectinases and cellulases were prepared for grape juice clarification. The use of BSA as proteic feeder seems to be a good option to obtain an improved cross-linking. When combi-CLEAs were produced with BSA, higher enzymatic activities were achieved, providing 3.04-times more thermal stability than the soluble enzyme. Combi-CLEAs-BSA can be reused for 6 cycles with total conversion of substrate to product, and at the end of 12 cycles, the biocatalyst was still able to convert 46 % of substrate.

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