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Efficient Hydrolysis of Anthocyanins by *Malbranchea pulchella* β-glucosidases Immobilized on MANAE-agarose and ConA-sepharose Supports

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ABSTRACT

β-glucosidases (BGLs) are enzymes that typically hydrolyze short chain oligosaccharides. However, some BGLs can hydrolyze anthocyanins and may be apply in the clarification process from different food industry, especially grape juice and wine. In this work, Malbranchea pulchella BGLs were immobilize on different supports and the derivatives were apply in grape juice and red wine clarification. BGL-MANAE and BGL-ConA were stable at all pH tested, keeping up to 70% activity after 24 h incubation. BGL-MANAE and BGL-ConA showed relative activity of 60% and 100%, respectively, at 50 °C after 24 hours incubation. Both derivatives were efficiently reuse 20 times and were significantly stable in the presence of 0.05 M and 0.1 M glucose and 5%, 10% and 15% ethanol, keeping up 70 % activity after 24 hours of incubation. The derivatives were effective in red wine and grape juice clarifications. The BGL-MANAE derivatives were 8.4% and 24.6% more efficient than BGL-ConA clarification on concentrate and diluted wines, respectively. Resembling, BGL-MANAE derivatives were 15% and 24.6% more efficient than BGL-ConA clarification of diluted and concentrate juices, respectively. Therefore, the biocatalysts produced in this work, especially BGL-MANAE, can be efficiently applied in grape juice and wine industry to obtain lighter products.

Keywords: *Malbranchea pulchella* β-glucosidases, Enzyme immobilization, clarification process, grape juice and wine industry.

INTRODUCTION

β-glucosidases (BGLs) are enzymes which normally catalyze β-1,4-linked, β-1,3 and β-1,6 hydrolysis from the non-reducing end of small chain oligosaccharides (Bhatia *et al*., 2002). In addition, these enzymes can hydrolyze anthocyanins, which are important coloring agents found in plant foods (Spain *et al*., 2000). In grapes juice and red wine, anthocyanins are the main pigments present (Sanchez-Torres *et al.*, 1998; Liu, 2002). Thus, one possible BGLs application is in the white wine production, with reduced red color, or in the rosé wine production, from red grape varieties, where the maceration process, can be extended and generates an excess of color which may be removed by enzymatic treatment (Sanchez-Torres *et al*., 1998). In the industry, particularly the food trade, the enzymatic catalysis is increasing on use and the enzyme immobilization can be a key tool, since this plaything allows for catalyst reuse, along with cost reduction and process control gain (Mateo *et al*., 2007;

Rodrigues *et al.*, 2013). Furthermore, the enzyme immobilization can improve properties such as activity, stability, selectivity and decreased inhibition by the product formed (Mateo *et al*., 2007). In this context, the main goal of this work was the *M. pulchella* BGLs immobilization in different supports and their application in red wine and grape juice clarification.

MATERIAL AND METHODS

Toward to achieve the main goal, the first step was to produce the enzymes. Initially, the crude extract was produced through cultivation on Lummy medium (0.4% yeast extract, 0.05% Na_2HPO_4, and 1.8% citric acid [w/v], supplemented with 0.6% cellobiose and 4 % sugarcane bagasse triturated [w/v]). In this medium, 1.0 x 10^6 *M. pulchella* spores/mL were inoculate on 1 mL of suspension and left grown for 72 hours, at 40°C and 180 rpm. The enzymatic activity was determined by p-nitrophenyl-β-D-glucopyranoside (pNPG) hydrolysis, measured at 410 nm. The assay was initiated with 15 µL of suspension (supernatant plus derivative) added to 10 µL of McIlvaine buffer pH 6.0 and 25 µL of pNPG (4 mM in H_2O), and incubated for 5 minutes at 50°C, for 5 minutes. Then, the protein concentration of the enzyme extract was measure using Coomassie Brilliant Blue G-250 according to the Bradford method (Bradford, 1976). The absorbance was measure at 595 nm and protein corresponded as mg of protein per mL (mg/mL).

After enzyme production and measure of the protein concentration, the next step was the *M. pulchella* BGLs immobilization on MANAE-agarose and Con A-Sepharose ™ 4B. In this perspective, all immobilization experiments were carried out using BGLs crude extracts, which were previously precipitated with 70% ammonium sulfate, suspended in 1 mL of 25 mM Tris-HCl plus 100 mM NaCl, and eluted on Bio-Gel P-100 (20 cmx2 cm). The fractions that showed BGL activity were put together in a pool and dialyzed in Milli-Q water at 4°C. Then, the extract was equilibrated in 10 mM sodium phosphate buffer, pH 7.0.

As control, BrCN-Sepharose (Amersham Biosciences) was first activated according to the manufacturer proceedings. For the immobilization process, 1 g BrCN-Sepharose activated on 10 mL of BGLs crude extract was added. This suspension was subject to gentle and constant stirring on a mechanical stirrer rolls for 20 minutes, at 4°C, pH 7.0 (Mateo *et al*., 2005). Subsequently, the sample was filtered, washed, and incubated with 1 M ethanolamine solution, pH 8.0, for 1 hour. Then, the derivative was filter and washed with distilled water.

The immobilization was carried out in MANAE-agarose ionic support, previously prepared, and affinity support Con A-Sepharose ™ 4B. Each 1 g of support was added 10 mL of BGLs extract in 10 mM sodium phosphate buffer, pH 7.0. The suspensions were kept at 4°C under gentle agitation in roller stirrer. Once enzyme adsorbed, the derivatives were washed with distilled water, filtered and stored at 4°C. Posteriorly, the characterization of derivatives was carried out in order to assess the enzyme stability pH, heat-inactivation, inhibition by glucose and ethanol, and reuse.

Homemade grape juice and red wine (Portugal) were use in the experiments. The anthocyanins hydrolysis using BGLs immobilized on MANAE-agarose and in Concanavalin A was verified. The tests were carried out on pure juice, diluted juice in water (1:1), pure red wine and red wine diluted with water (1:1). They were added 50 mg of derivative in aliquots of 2 mL of juice and wine (diluted and concentrated). The suspensions (more derivatives juice/wine) were maintained at 50°C and 1000 rpm for 6 hours. Staining was measure by spectrophotometric method at 520 nm.

RESULTS AND DISCUSSION

The BGLs were 100% immobilize on MANAE-agarose and Concanavalin A-Sepharose but 50% on BrCN-Sepharose. The BGL-MANAE presented a hyperactivation correspondent to10.03-times and 2.74-times for BGL-ConA, compared to free enzyme.

In the assay to test the pH stability for enzymatic activity, the derivative BGL-BrCN control showed less activity relative or equal to 30% at pH 2.0, 3.0 and 4.0. The activity was 35% and 50% at pH 5.0 and 6.0, in 24 hours of incubation. However, BGL-MANAE and BGL-ConA showed equal or higher relative activity than 70% at all pH tested after 24 hours, where 100% was the highest activity achieved for each derivative.

In the assay to test temperature stability for enzyme activity, the derivative BGL-BrCN showed relative activity of about 60% at 40°C, 40% at 50°C and 20% at 60°C, after 24 hours. On the other hand, BGL-MANAE showed relative activity of 80% at 40°C, 60% at 50°C and 20% at 60°C. BGL-ConA showed 100% activity at temperatures 40°C, 50°C and 60°C in 24 hours of incubation, where 100% was the highest activity achieved for each. In the reuse assay, BGL-MANAE and BGL-ConA were used for up to 20-times with 50% and 75% of the initial relative activity, respectively. The highest activity achieved was considered as 100%.

BGL-MANAE showed no inhibition by 0.05 M and 0.1 M glucose concentrations. Moreover, the assays with the derivatives plus 0.25 M, 0.5 M, and 1 M glucose, have shown more than 50% residual activity after 24 hours. On the other hand, BGL-ConA has shown higher activity or equal to 70% of the original activity in all tested glucose concentrations, after 24 hours.

BGL-MANAE remained over 90% of the initial activity in the presence of 5% and 10% ethanol. Besides, the activity this derivate remained over 80% in the presence of 15% ethanol, in 24 hours. In addition, the BGL ConA-derived remained over 80% of the initial activity at all ethanol concentrations tested, after 24 hours. These results are promising and demonstrate the importance of immobilization, as compared to the control.

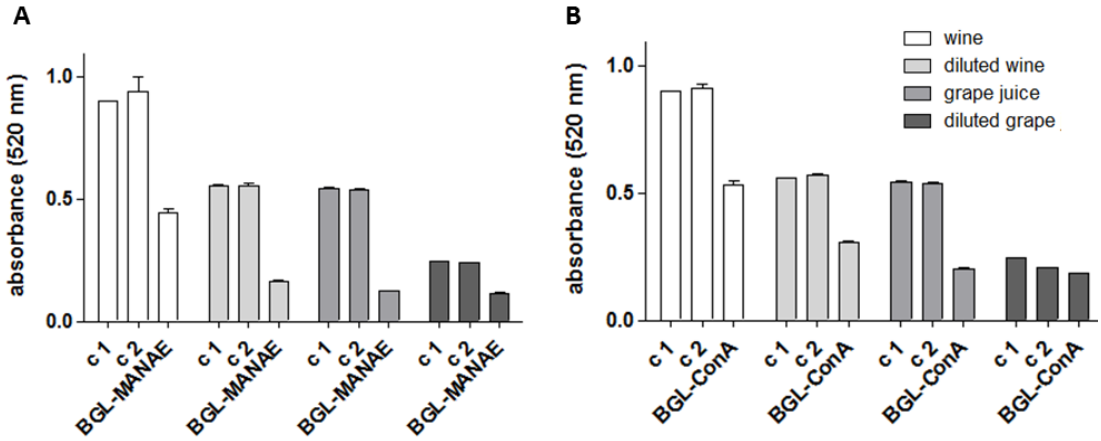

Figure 1. Red wine and grape juice clarification by M. *pulchella* BGLs on MANAE-agarose (A) and ConA-Sepharose (B). The control (c1) is the juice and wine incubated in the catalytic absence; control 2 (c2) is the juice and wine incubated in the presence of 50 mg of MANAE-agarose and ConA-sepharose, enzyme free; BGL-

MANAE and BGL-ConA correspond to the juice and wine incubated in the presence of 50 mg of each derivative. The samples were maintained at 50°C, 1000 rpm, for 6 h.

Therefore, the derivatives have shown to be stable on pH acid and high temperatures. Thus, the derivatives were evaluated concerning the ability to clear red wine and grape juice. Both derivatives were effective in red wine and grape juice clarification (Figure 1). However, the BGL-MANAE derivative was 8.4% and 24.6% more efficient than BGL-ConA clarification of wine concentrate and diluted (1:1) and 15% and 36% more efficient in clarification of juice concentrate and diluted (1:1).

CONCLUSIONS

As far as we know, this is the first work that had use immobilized BGLs from a thermophilic fungi applied on grape juice and red wine clarification. *M. pulchella* BGLs immobilized on MANAE-agarose and Concanavalin A-Sepharose came up with useful biochemical characteristics for food industry application, especially through their ability to be stable on pH acid and high temperatures over long periods. Besides, they can be reuse up to 20-times without significant loss of efficiency, making their use economically more feasible for the industry. BGL-MANAE was more efficient than the BGL-ConA in red wine and grape juice clarification and, therefore, it appears as a promising biocatalyst to use in the anthocyanins hydrolysis and, consequently, in the production of white wines with reduced red color and rosé wines from several grapes varieties.

REFERENCES

BRADFORD, M. M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry, v. 72, n. 1-2, p. 248-254, 1976.

LIU, S.-Q. Malolactic fermentation in wine–beyond deacidification. Journal Applied of Microbiology, v. 92, n. 4, p. 589-601, 2002.

MATEO, C., PALOMO, J. M., FERNANDEZ-LORENTE, G., GUISAN, J. M., FERNANDEZ-LAFUENTE, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microbial and Technology,40(6), 1451-1463.

RODRIGUES, R. C., ORTIZ, C., BERENGUER-MURCIA, A., TORRES, R., FERNÁNDEZ-LAFUENTE, R. (2013). Modifying enzyme activity and selectivity by immobilization. Chemical Society Reviews, 42(15), 6290-6307.

SÁNCHEZ-TORRES, P.; GONZÁLEZ-CANDELAS, L.; RAMÓN, D. Heterologous expression of a *Candida molischiana* anthocyanin-β-glucosidase in a wine yeast strain. Journal Agricultural Food Chemistry, v. 46, p. 354-360, 1998.

SPAGNA, G., BARBAGALLO, R. N., PIFFERI, P. G., BLANCO, R. M., GUISAN, J. M. (2000). Stabilization of a β-glucosidase from *Aspergillus niger* by binding to an amine agarose gel. Journal of Molecular Catalysis B: Enzymatic, 11(2), 63-69.

BHATIA, Y.; MISHRA, S.; BISARIA, V. S. Microbial β-glucosidases: cloning, properties, and applications. Critical Review Biotechnology, v. 22, n. 4, p. 375-407, 2002.