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Stabilization by multipoint covalent attachment of a biocatalyst with polygalacturonase activity used for juice clarification

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ABSTRACT

Derivatized-agarose supports are suitable for enzyme immobilization by different methods, taking advantage of different physical, chemical and biological conditions of the protein and the support. In this study, agarose particles were modified with MANAE, PEI and glyoxyl groups and evaluated to stabilize polygalacturonase from *Streptomyces halstedii* ATCC 10897. A new immobilized biocatalyst was developed using glyoxyl-agarose as support; it exhibited high performance in degrading polygalacturonic acid and releasing oligogalacturonides. Maximal enzyme activity was detected at 5 h of reaction using 0.05 g/mL of immobilized biocatalyst, which released 3 mg/mL of reducing sugars and allowed the highest product yield conversion and increased stability. These results are very favorable for pectin degradation with reusability up to 18 successive reactions (90 h) and application in juice clarification. Plum (4.7 °Bx) and grape (10.6 °Bx) juices were successfully clarified, increasing reducing sugars content and markedly decreasing turbidity and viscosity.

1. Introduction

Biologically, polygalacturonases (PG) are a class of enzymes with an important role in plant growth and phytochemistry processes because of its contribution in fruits softening through the hydrolytic action in α-(1,4) glycosidic bonds of pectin main backbone. Additionally, these enzymes are commercially important due to their continuously increasing demand in worldwide markets for application in the food industry for mash treatment, juice clarification and extraction of phytochemical compounds from plant tissues (Kashyap, Vohra, Chopra, & Tewari, 2001). However, these enzymes are commercialized in the soluble state, which requires the renovation of the biocatalyst for each reaction batch.

Immobilization is a powerful tool to improve enzyme properties such as stability, activity, specificity and selectivity. Moreover, the main purpose of enzyme immobilization is the recovery of the biocatalyst from the reaction medium and its reuse for extended periods under operation conditions (Cesar Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007).

Some techniques to achieve the immobilization of proteins have been widely used such as entrapment, cross-linking, adsorption or covalent attachment (Faber, 2000). In this sense, the attachment of enzymes on porous solid carriers (silica, agarose, glass) is a good strategy to improve the stability of the biocatalyst. Natural polymers, such as agarose particles with high porosity, reduced size and high surface area are an adequate carrier. Interactions with proteins can be directed by surface modifications, such as the attachment of aldehyde, polycationic and amino groups. So the derivatization of agarose with reactive groups such as glyoxyl, polyethylenimine (PEI) and monoaminoethyl-N-aminoethyl (MANAE) has been employed to immobilize proteins, resulting in high preservation of enzymatic activities.

Glyoxyl-agarose is composed of particles whose surface is activated with a large number of aldehyde groups with short spacer arms. The immobilization of proteins on this carrier occurs via multipoint covalent attachment through the region with higher density of primary amino groups, especially with ε-NH_2 from lysine residues (Cesar Mateo et al., 2007). Agarose coated with PEI groups of different molecular sizes can immobilize proteins through ionic interactions. This physicochemical immobilization is reversible, non-distorting and can proceed at variable ionic strength. Aminated agarose supports have been used to immobilize enzymes via multipunctual adsorptive interactions with protein carboxyl groups (Fernandez-Lafuente et al., 1992), providing good

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stability and no rigidification of the enzyme attached (Pessela et al., 2004).

The aim of this research was to develop an immobilized biocatalyst with PG activity produced by *Streptomyces halstedii* ATCC 10897, using agarose supports activated with different reactive groups for their application in fruit juice clarification. The enzyme activity of glyoxyl-agarose PG biocatalyst was enhanced by optimization of operational conditions such as time, protein concentration, protein loading and support amount. Finally, plum and grape juices were treated with effective results regarding turbidity, viscosity and reducing sugar content.

2. Materials and methods

2.1. Reagents and microorganism

Culture medium compounds were obtained from Britania S.A. (Argentina), polygalacturonic acid from citrus peel was purchased from Sigma S.A. (Brazil) and agarose gel CL-10B, from GE-Healthcare (Uppsala, Sweden). Bradford reagent was supplied by Bio-Rad Laboratories and other chemical compounds were purchased from Biopack S.A. (Argentina).

The microorganism *Streptomyces halstedii* ATCC 10897 is from the collection of Laboratorio de Investigaciones en Biotecnología Sustentable (LIBioS) of the National University of Quilmes (Argentina).

2.2. Streptomyces halstedii ATCC 10897 culture conditions

The strain was conserved in sterile glycerol 20% at −80 °C. The inoculum was grown for 16 h at 28 °C and 200 rpm in liquid medium with glucose (4 g/L), yeast extract (4 g/L) and malt extract (10 g/L). Then, the culture was inoculated in a previously designed induction medium containing soy peptone (15 g/L), NaCl (2.5 g/L), $MgSO_4$ (0.5 g/L) and KH_2PO_4 (1.0 g/L) at pH 8 in 1000 mL flasks for 12 h, as described by Ramírez-Tapias, Rivero, Britos, and Trelles (2015). Finally, cultures were centrifuged at 12,000*g* for 20 min using Beckman J2-MC to obtain cell-free supernatants.

2.3. Polygalacturonase purification

Cell-free supernatants were ultrafiltered up to 20× volume concentration. The Vivaflow Sartorius® cross flow polyethersulfone cassette system of 10 kDa MWCO was used at an operational flow rate of 10 L/h. Since the studied PG has a molecular weight of 48 kDa, the retained fraction was recovered and tested for enzymatic activity (Ramírez-Tapias et al., 2015). Protein content was quantified with Bradford reagent.

2.4. Agarose derivatization

Commercial agarose gel CL-10B has 100 μm of average bead size and is 10% cross-linked with 2,3-dibromopropanol. Agarose was activated to produce glyceryl-agarose in a suspension at pH 10 with glycidol. The particles were oxidized to obtain the glyoxyl-agarose support, as described by Guisan (2004). MANAE support was prepared based on the method by Fernandez-Lafuente et al. (1992). In brief, glyoxyl-agarose was suspended in 0.1 M bicarbonate buffer pH 10, and then 2 M ethylenediamine was added dropwise with continuous stirring; after 2 h the gel was reduced with solid $NaBH_4$. The aminated support was washed with distilled water. Ionic polymer 1.3 kDa PEI at 10% (w/v) was used to coat the glyoxyl-agarose support, as described by Mateo et al. (2007), and beads were obtained with highly reactive groups.

2.5. Enzyme immobilization

The ultrafiltered enzymatic solution was diluted to 1 mg/mL protein concentration using different buffers. Agarose supports were suspended in the enzyme solution at 0.1 g/mL. Glyoxyl-agarose was used in 100 mM glycine-NaOH buffer pH 10; for immobilization in PEI-agarose 30 mM phosphate buffer pH 7 was used, and MANAE-agarose was prepared in 5 mM phosphate buffer pH 7. Each suspension was incubated in an orbital shaker at 100 rpm for 3 h at 28 °C, and the percentage of immobilized protein was determined. Afterwards, solid $NaBH_4$ (1 mg/mL) was added to glyoxyl-agarose suspension and incubated for 30 min. Finally, biocatalysts were washed exhaustively with distilled water and used for the reactions.

2.6. Optimization of immobilization conditions

Different immobilization conditions were assayed by a complete two-factorial experimental design 5 × 3. Protein concentration was evaluated at values of 0.25, 0.50, 1.00, 1.50 and 2.00 mg/mL, and support amounts at 0.05, 0.10 and 0.20 g/mL. Two sequential stages of enzyme immobilization were carried out to maximize protein loading. The reduction reaction with $NaBH_4$ was done after the second immobilization process. The evaluated variables were immobilization percentage, D-galacturonic acid concentration and product yield. This experimental design with 15 runs was carried out in duplicate. Results were analyzed with Statgraphics Centurion XV.II.

2.7. Polygalacturonase reaction conditions

The PG assay was performed by reaction of biocatalyst suspension with 1% (w/v) polygalacturonic acid solution as substrate in 0.1 M glycine-NaOH buffer at pH 11 (1:1 vol ratio). Reaction kinetics with free and immobilized PG were evaluated in order to identify the time of maximum product yield, expressed as production of D-galacturonic acid (g) in relation to the protein content (g). The K_m and V_{max} parameters of Michaelis-Menten kinetics were determined using substrate solutions at concentrations of 0.25, 0.50, 1.00, 1.50, 2.00, 2.50 and 3.00% (w/v). Periodically, product conversion was measured, and results were fitted to Lineweaver-Burk linearization plot. PG activity was measured using a colorimetric method with 3′,5′-dinitrosalicylic (DNS) acid reagent (Miller, 1959) that quantifies the reducing sugars released during the incubation of reaction at 45 °C. D-galacturonic acid was used as standard.

2.8. Thermal and pH stability

Thermal stability of PG was analyzed, in terms of the residual activity, after exposure of the biocatalyst without substrate to temperatures between 40 and 70 °C, and aliquots were taken for the PG activity assay at regular intervals. The stability test at pH values between 3 and 11 was carried out by incubation of the biocatalyst in 0.1 M citrate-phosphate buffer (pH 3–8) and 0.1 M glycine-NaOH buffer (pH 9–11).

2.9. Reusability and storage stability

Reusability of immobilized PG on glyoxyl-agarose was evaluated using polygalacturonic acid 1% as standard reaction. After each cycle of 5 h, the used derivative was centrifuged for 15 s at 5000*g*, recovered and then washed twice with distilled water. Consecutive cycles of biocatalyst use were performed until enzyme inactivation.

Immobilized biocatalyst was stored at 4 °C in 5 mM phosphate buffer pH 7 for 145 days. Periodically, samples were taken, and enzymatic activity was evaluated. Storage stability was defined as the relative activity between the first and the successive reactions.

2.10. Juice preparation and clarification

Mature plums (*Prunus domestica*) and grapes (*Vitis vinifera*) were obtained from Mendoza region (Argentina). Juice preparation was carried out at 70% (w/v) with distilled water in an automatic blender for 3 min. Juice was filtered and then heated at 80 °C for 30 min for inactivation of intrinsic enzymes (Pedrolli, Monteiro, Gomes, & Carmona, 2009).

The clarification assays were performed for 4 h at 40 °C with 10 mg of glyoxyl-agarose biocatalyst per mL of juice. Subsequently, samples were centrifuged for 5 min at 1000g to separate the biocatalyst from the clarified juice. The clarity of the juice was determined by measuring the absorbance spectrum (400–700 nm) of each sample using as references juices without biocatalyst and juices clarified by filtration at 80 °C with vacuum system at 0.25 psi, 23.75 cm^2 filtration area and 2.5 μm particle retention. The clarification percentage was defined as the relation between the area under the recorded curve of enzymatic treated juices and the references. Reducing sugars released after the enzymatic treatment of the juice were determined using DNS acid reagent, and viscosity reduction was measured with a rheometer (AR-G2/TA Instrument) at 24 °C and 300 s^{-1} shear rate.

3. Results and discussion

3.1. Selection of derivatized-agarose support

Immobilization processes were carried out with three different derivatized-agarose supports: glyoxyl-, PEI- and MANAE-agarose and monitored for 180 min (Fig. 1). After 30 min, >60% of total protein was immobilized on each support, suggesting a fast reaction between the proteins and the different reactive groups on the support surface. Immobilization on glyoxyl- and MANAE-agarose maintained a constant fraction of protein attached to the support until the end of the process. Using PEI-agarose, protein immobilization decreased, so the process was not efficient enough. This fact could be explained as a reversible action of adsorptive interactions due to electrostatic linkages (Wu, He, Zhao, & Li, 2014). Nevertheless, both MANAE- and glyoxyl-agarose still had an immobilization percentage of proteins higher than 60% with stable bonds along the evaluated time.

It is widely known that immobilization could adversely affect enzyme activity, so it was necessary to prove that the heterogeneous biocatalysts retained their enzymatic activity. Therefore, immobilized PG biocatalysts were used for enzymatic reactions (Fig. 2a). The time course of polygalacturonic acid hydrolysis with each biocatalyst suggested that it proceeds since the start of the reaction, and the maximum product concentration was achieved at 4 h; further time showed no significant variations. Under these evaluated conditions, the best result was obtained using immobilized PG on glyoxyl-agarose, achieving 1.60 mg/mL as maximum concentration of D-galacturonic acid and a product yield of 3.0 g/g at 4 h (Fig. 2b). With PEI- and MANAE-agarose, 1.25 and 0.75 mg/mL of product, respectively, were obtained.

Each evaluated system of immobilization could have a unique behavior due to different conditions of the process, such as internal morphology of the support, its activation degree and conformational protein structure (Talbert & Goddard, 2012), so the mechanism of attachment may change the active site availability of enzyme, resulting in a different way of interaction with the substrate. Therefore, enzymatic activity comparisons among derivatized-agarose biocatalysts were useful to develop an immobilized biocatalyst with the highest PG activity to favor later applications in fruit processing. In this way, the PG active heterogeneous biocatalyst synthesized with glyoxyl-agarose showed better performance in both the percentage of protein immobilization and enzymatic activity, and was selected for further assays.

3.2. Development of PG active biocatalyst

After support selection, a two-factorial experimental design 5×3 was developed to define immobilization conditions using protein concentration and support amount as independent variables. The experimental data were statistically analyzed and modeled to identify the effects of the studied factors on response variables based on the correlation factors (R^2), which indicated that adjusted models account for more than 85% of the variation observed (Table 1).

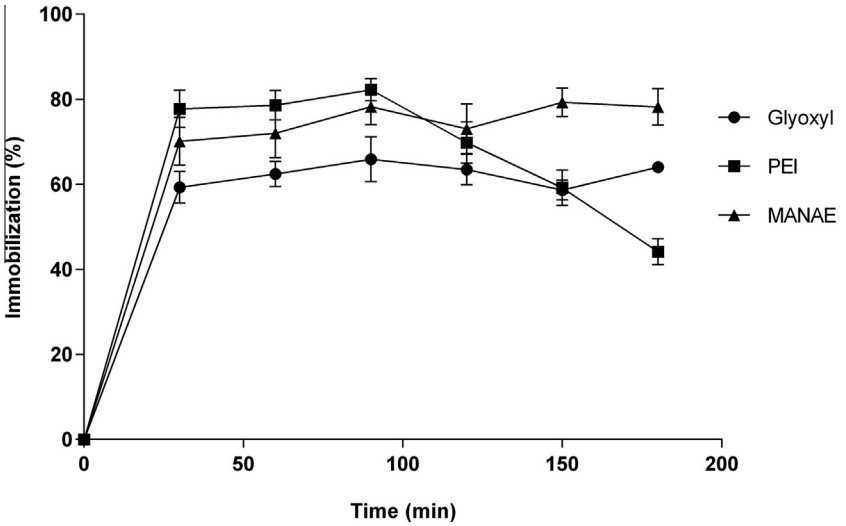

Fig. 1. Polygalacturonase immobilization on derivatized agarose supports. Immobilization processes were carried out at 28 °C and 100 rpm with an initial protein content of 1.00 mg/mL. Protein was measured in the soluble fraction at 30-min intervals.

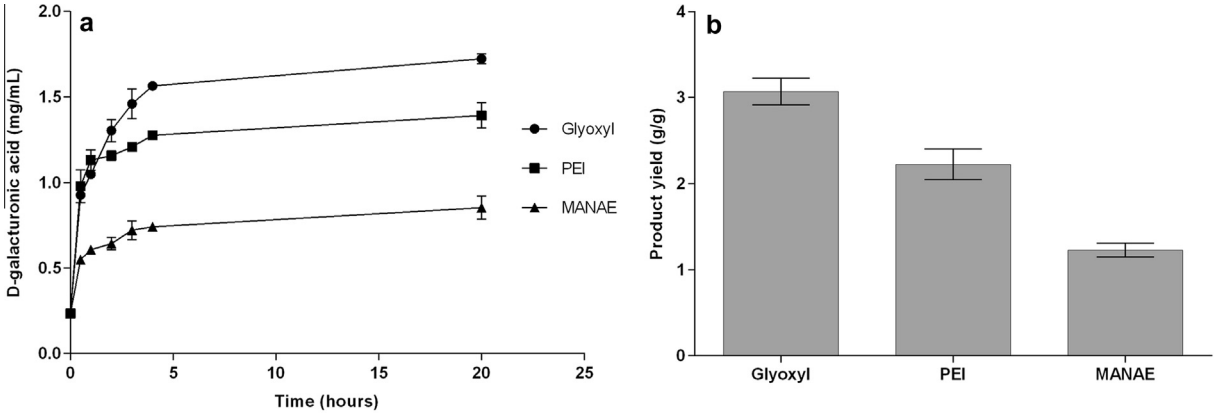

Fig. 2. Enzymatic reaction with immobilized biocatalyst. Time course of reaction (a) and product yield at 4 h (b). Hydrolysis reactions were carried out three times using polygalacturonic acid as substrate. Product yield is expressed in g of produced D-galacturonic acid per g of protein.

Table 1
Matrix of two-factorial experimental design 5 × 3 for PG immobilization varying protein concentration (A) and support amount (B) as studied factors.

	Run	Factors		Response variables[a]		
		Protein (A) mg/mL	Support (B) g/mL	Immobilization %	D-galacturonic mg/mL	Product yield[b] g/g
	1	0.25	0.05	0.402	0.537	8.777
	2	0.50	0.05	0.422	1.796	10.296
	3	1.00	0.05	0.460	2.282	9.714
	4	1.50	0.05	0.477	2.493	3.993
	5	2.00	0.05	0.508	2.170	2.186
	6	0.25	0.10	0.525	0.650	5.530
	7	0.50	0.10	0.572	0.959	3.356
	8	1.00	0.10	0.561	1.112	3.162
	9	1.50	0.10	0.570	1.313	1.501
	10	2.00	0.10	0.572	1.846	1.261
	11	0.25	0.20	0.473	0.700	5.355
	12	0.50	0.20	0.475	1.033	4.135
	13	1.00	0.20	0.510	1.738	4.003
	14	1.50	0.20	0.522	1.969	2.765
	15	2.00	0.20	0.533	1.938	1.584
P-value	Immobilization	0.000 (+)	0.000 (+)	AB = 0.209	AB = 0.718	AB = 0.016 (+)
	D-galacturonic	0.000 (+)	0.009 (−)	R^2 = 0.851	R^2 = 0.837	R^2 = 0.876
	Yield	0.000 (−)	0.000 (−)	Significance level 95%		

[a] Values are the mean of 2 replicates. Standard error was less than 10%.
[b] Product yield expressed as D-galacturonic acid production (g) relative to protein content (g).

Though protein concentration and support amount positively affected the percentage of protein immobilization (p-value = 0.000), this response varied between 40 and 60% in a narrow range. However, D-galacturonic acid concentration and product yield showed enhanced results compared to previous experiments (Fig. 2a). In this regard, the maximum D-galacturonic acid concentration was 2.393 mg/mL (run 4), a 1.5-fold increase, and the analysis of variance indicated that protein concentration showed a positive effect, as opposed to what happened with the support amount. In this experimental design, better product formation was obtained with high levels of protein and a lower support amount. On the other hand, product yield was higher when both factors were at their lowest levels because protein concentration and support amount had significant negative effects (p-value 0.000), but the interaction (AB) promotes this response. So, both factors must be at the lowest levels to achieve the best yields of product conversion.

Maximum product yields were obtained at runs 1, 2 and 3; these results suggest that the support amount of 0.05 g/mL exceeds by 3.3-fold the previous response (Fig. 2b) and protein concentration at 0.25, 0.5 and 1 mg/mL could also enhance product yields. In addition, protein loading on a support can affect its resistance to mass transfer due to spatial restrictions, which could be diminished using a low protein concentration (Talbert & Goddard, 2012).

Since product yield was higher with 0.05 g/mL of glyoxyl-agarose, it is important to improve the percentage of protein immobilization with the purpose of identifying a more efficient protein loading (milligrams of target protein bound per gram of support). In this sense, successive immobilizations stages were carried out with a fixed support amount (0.05 g/mL) and variable protein concentrations. Fig. 3a shows the protein attached to the support, reaching a total protein loading of 4.5, 10.0 and 16.8 mg/g with an initial soluble protein content of 0.25, 0.50 and 1.00 mg/mL, respectively.

The support capacity depends on the surface area, but protein-protein interactions could also occur with molecular diffusion within the particle pores. Even though, a binding capacity up to 16.8 mg/g was observed, the product yield remained at a mean value of 10 g/g without significant effects by variations in protein loading; so 4.5 mg/g (Fig. 3a) is preferable. It has been reported that the catalytic behavior of immobilized enzymes on porous microparticles depends on particle mobility and fits with the collision theory, where the reaction rate is a function of particle size, viscosity of medium and collision frequencies, which is

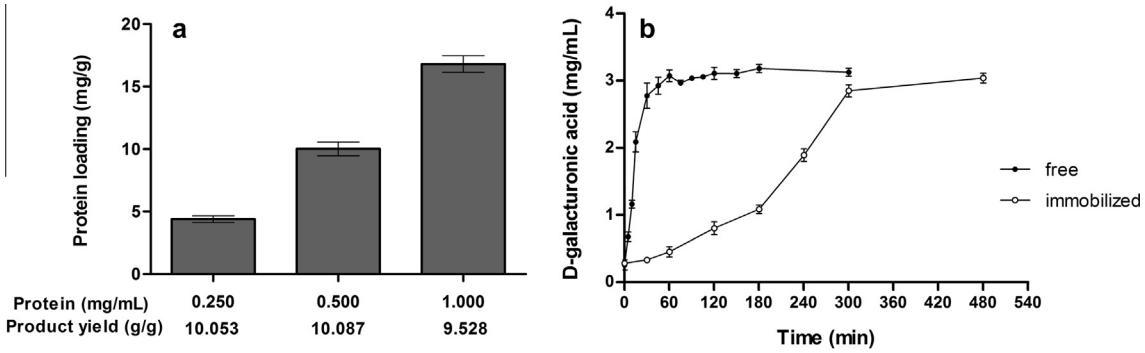

Fig. 3. Effect of protein loading of the glyoxyl-agarose biocatalyst on the enzymatic reaction. Immobilization by two successive processes and final product yield (a) and kinetics of polygalacturonic acid hydrolysis with soluble and immobilized PG (0.25 mg of protein per mL of reaction mixture).

favored by low moles of reactant (Jia, Zhu, & Wang, 2003). So, in this optimization process the lowest value of protein loading enhanced product yield and could be suitable for the mobility of particles and the catalytic performance of immobilized PG on glyoxyl-agarose. Therefore, the heterogeneous biocatalyst selected for degradation of pectic compounds was that prepared with 0.05 g/mL of glyoxyl-agarose by two independent and sequential immobilization processes for 3 h, with a protein concentration of 0.25 mg/mL to achieve a final protein loading of 4.5 mg/g and a product yield conversion of 10.1 g/g.

Some studies have reported experiences with different immobilized proteins on activated agarose (Fernandez-Lorente et al., 2015), but the heterogeneity of protein molecules and functionalized microparticles make it difficult to explain the structural findings. However, many efforts have been made to investigate immobilization systems with positive results, such as those reported for PG in the present work.

The implementation of immobilization techniques improves the stability of enzymes, but generally decreases the product yield. The maximum product conversion achieved did not differ significantly from 3 mg/mL of D-galacturonic acid, being a noteworthy result because the final product yield remained at 10 g/g with free and immobilized enzyme (Fig. 3b). Differences between reactions are based on kinetics; while in the reaction with free PG it took about 45 min to reach the maximum yield, immobilized PG hydrolyzed the same amount of substrate in 5 h. This behavior was due to diffusion limitations (Wu, He, Zhao, Qian, & Li, 2014). Michaelis-Menten parameters for immobilized enzyme were determined by Lineweaver–Burk graph, and results showed a maximum reaction rate (V_{max}) of 0.242 μmol/min and the K_m constant was 0.395 M.

3.3. Biocatalyst stability

The stability of a biocatalyst is defined as the time during which it retains 50% of its initial activity and could be affected by environmental conditions such as pH and temperature. The pH effects on immobilized PG were evaluated for 24 h at room temperature, and the results suggested that acidic conditions (pH 3–6) inactivate the enzyme, decreasing its activity up to 70%. On the contrary, neutral and alkaline environments (pH 7–11) did not affect the enzyme activity, probably due to the alkalophilic nature of the enzyme.

Regarding temperature conditions, immobilized PG on glyoxyl-agarose exhibited high stability for 12 h at 40 °C and then it dropped to 80% of relative activity at 24 h. At 50 °C the biocatalyst was stable for 6 h, which is a competitive result compared to some reports where immobilized bacterial PG enzymes by entrapment using agar-agar, calcium alginate and chitosan beads showed stability at 50 °C only for 2 h (Rehman, Nawaz, et al., 2014; Rehman, Aman, et al., 2014). Higher temperatures (60 and 70 °C) affected enzymatic activity drastically. Thus, the thermal stability behavior found in this study is applicable to fruit processing because the operating temperatures do not exceed 50 °C.

3.4. Storage and reusability assay

Storage at 4 °C was analyzed to ensure biocatalyst stability, and the immobilized derivative retained its activity for more than 145 days (85% relative activity). The reusability test of immobilized PG resulted in 18 successive reactions (90 h) with enzymatic activity. The results obtained in this study are competitive to be applied in fruit juice clarification due to the higher batch reusability compared to those which have reported up to 11 reuses (44 h) with PG entrapped into hydrogels and thermogels (Table 2).

3.5. Juice clarification

The process of clear juice preparation has two main stages: mechanical maceration, which includes chopping, grinding and pulping and pectin hydrolysis, which consists of filtration and fine clarification. In this regard, the catalytic performance of immobilized PG in juice clarification was evaluated with two different juices, grape and plum (Fig. 4). Single strength juices were characterized in terms of viscosity, turbidity, sugars content and soluble solids, which is within the values recommended by AIJN (European fruit juice association). After enzymatic treatment absorbance in the visible spectrum declined in clarified juices as immobilized PG degrades pectin molecules, reaching 55 and 61% of turbidity reduction for grape and plum juice, respectively. Simultaneously, the concentration of reducing sugars was higher than that of the initial natural juice due to the conversion of pectin into soluble oligogalacturonides, increasing by 16% for grape juice and 28% for plum juice reaching values of 810 mg/L and 350 mg/L, respectively. Additionally, a mean viscosity decrease of 95% was achieved, enhancing juice fluidity, stability and quality.

Some studies have demonstrated the feasibility of using fungal pectinases in soluble state for turbidity and viscosity reduction in

Table 2
Reusability of different immobilized pectinase biocatalysts.

Immobilized pectinase	Reuses	Operational time (h)	References
Agar-agar	4	0.7	Rehman et al. (2014)
Chitosan	10	2.5	Rehman et al. (2014)
Glyoxyl-agar	10	40	Li, Li, Wang, & Tain (2008)
Na-Alginate	10	44	Li et al. (2007)
Glyoxyl-agarose	18	90	This study [a]

[a] Reusability was evaluated with polygalacturonic acid as standard reaction.

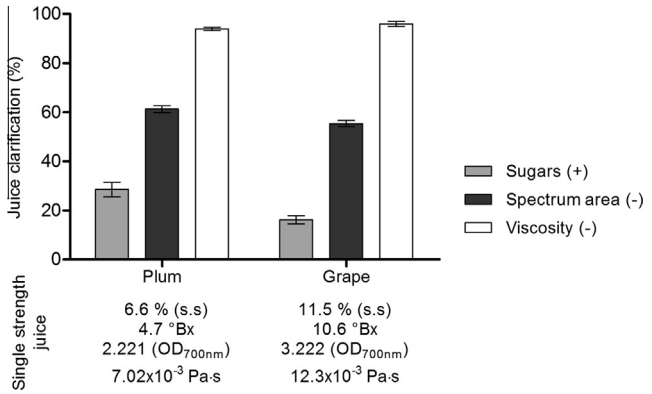

Fig. 4. Characterization of single strength fruit juices and application of immobilized PG biocatalyst in clarification of plum and grape juices. The clarification assays were carried out at 40 °C and 4 h with 10 mg of glyoxyl-agarose biocatalyst per mL of juice.

some juices such as blackcurrant, plum, apple, pineapple and tomato (Dey, Adak, Bhattacharya, & Banerjee, 2014; Mieszczakowska-Frac, Markowski, Zbrzeźniak, & Płocharski, 2012; Sandri, Fontana, Barfknecht, & da Silveira, 2011). Although different factors such as the variety of the fruit, enzyme sources, raw juice extraction process and enzymatic reaction conditions might be responsible for such a variation in the effects on clarification features, this research reports meaningful performance in the clarification process compared to those previously reported with the distinct advantage of reusability in several batches.

4. Conclusion

Stabilized biocatalyst with PG activity was used for the clarification of plum and grape juices and showed promising results for applications in the food industry. The findings displayed herein also demonstrated that different derivatizations of agarose are suitable for immobilization of PG from *Streptomyces halstedii* ATCC 10897 to degrade pectic compounds. A glyoxyl-agarose biocatalyst was developed by sequential stages in the immobilization process and resulted gave the highest activity and product yield conversion. Stability of immobilized PG under operating conditions was high even in alkaline conditions and was active for 90 h of process.

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