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PII: S1878-8181(18)31020-X

DOI: https://doi.org/10.1016/j.bcab.2019.101091

Article Number: 101091

Reference: BCAB 101091

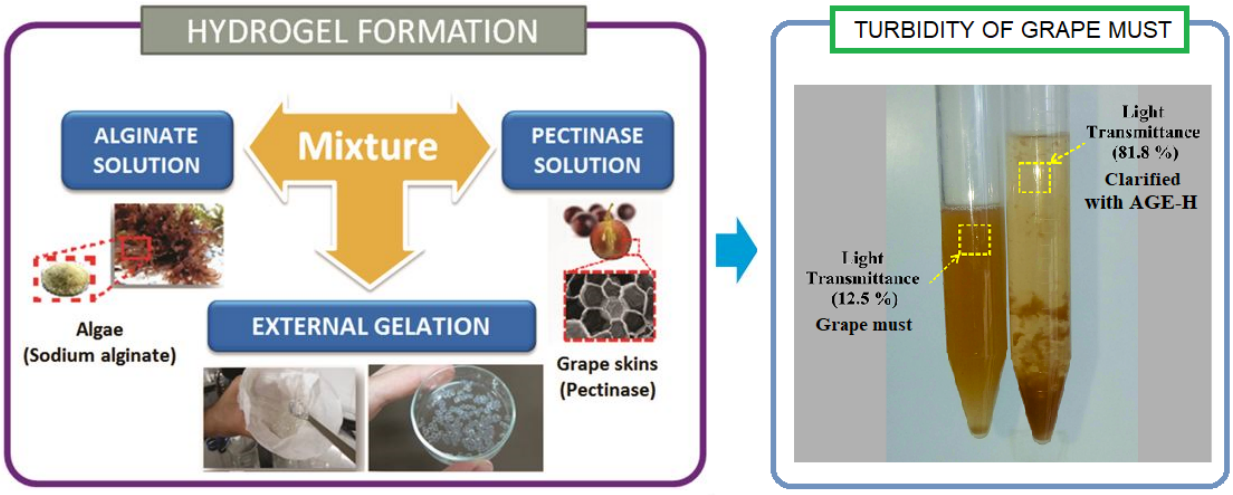
To appear in: *Biocatalysis and Agricultural Biotechnology*

Received Date: 26 December 2018

Revised Date: 8 March 2019

Accepted Date: 9 March 2019

Please cite this article as: Martín, Marí.C., López, O.V., Ciolino, André.E., Morata, V.I., Villar, M.A., Ninago, M.D., Immobilization of enological pectinase in calcium alginate hydrogels: A potential biocatalyst for winemaking, *Biocatalysis and Agricultural Biotechnology* (2019), doi: https://doi.org/10.1016/j.bcab.2019.101091.



Immobilization of enological pectinase in calcium alginate hydrogels: a potential biocatalyst for winemaking

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Abstract

A biocatalyst was obtained by immobilizing an enological commercial pectinase within calcium alginate hydrogels using an entrapment technique, and its catalytic activity was evaluated during different storage conditions. Hydrogel beads were stored at 4ºC in three different ways: (i) wet, in citrate buffer solution (pH 3.8); (ii) dehydrated by using a vacuum stove; and (iii) freeze-dried. Biocatalyst surface and their internal morphology were characterized by Scanning Electron Microscopy and a good enzyme distribution throughout alginate matrix was observed. Fourier Transform Infrared Spectroscopy results confirmed the presence of absorption bands associated with amino groups present in enzymes. Immobilization procedure did not modify the optimal pH and temperature (pH = 4.0 and 20 ºC) for pectinase activity, comparing to free enzyme. Entrapped pectinase showed activity until six reaction cycles with 40 % residual activity. Storage stability studies demonstrated that wet entrapped pectinase retained its initial enzymatic activity up to 11 weeks, whereas that lyophilized hydrogels retained its original activity after 8 months of storage. These results suggest that immobilized pectinase may be successfully exploited in various industrial applications, with special concern in grape juice clarification process. Thus, the turbidity of grape must decreased significantly using the immobilized pectinase during 150 min at 20 ºC. This biocatalyst could be easily removed after clarification process and it can be reused, minimizing production economic costs in wine industry.

Keywords: Calcium alginate hydrogels, pectinase immobilization, biocatalyst, winemaking.

1. Introduction

Biotechnology, together with the development of new materials, has become an economic factor that generates significant annual incomes. Enzymes are eco-friendly catalysts widely employed in many food industrial processes, such as winemaking. From the pre-fermentation stage, through fermentation, post-fermentation and aging, enzymes catalyze various biotransformation reactions (Claus, 2017). Many of these enzymes originate from the grapes itself, the grape's indigenous microflora and the microorganisms present during winemaking. Since the endogenous enzymes of grapes, yeasts, and other microorganisms present in musts and wines are often neither efficient nor sufficient to effectively catalyze the corresponding reactions, commercial enzymes are widely used as supplements (Mojsoc, 2013). Among commercial enzymes, pectinases have a considerable influence on both the sensory and technological properties of wines (Merín et al., 2015). Particularly, cold-active acidic pectinases are potentially relevant to achieve wines with better aromatic profiles due to low temperature fermentation can increase the production and retention of volatile compounds (Martín and Morata de Ambrosini, 2014). In addition, pectinases can help to improve the clarification and filtration process, releasing more color and flavor compounds contained in grape skin, and making more effective the liberation of phenolic compounds (Belda et al., 2016). Pectinases (E.C.3.2.1.15) are a heterogeneous group of enzymes that catalyze pectin hydrolysis by breaking glycosidic linkage of galacturonic acid, decreasing beverages viscosity, which is responsible to cause their turbidity and undesirable cloudiness (Ridley et al., 2001). Even though pectinases are frequently used in soluble form, enzymes in this state are often unable to meet the industrial requirement due to their short-term operational stability and the difficulty for their recovery and reuse (Bibi et al., 2015;

Sheldon and van Pelt, 2013). In accordance with Lira de Oliveira et al. (2018), enzymes immobilization is a good alternative to overcome these limitations. These authors stressed that this procedure presents several advantages, among which are: i) confinement or attachment of the enzyme in a defined space region while retaining its catalytic activity, ii) exploitation of its activity repeatedly or continuously, iii) enhancement of its stability, under either storage or operational conditions, iv) easy separation from the product, and v) minimization of product contamination. Immobilization is a process to confine or localize the enzyme within/onto a carrier and retained its activity for continuous uses. Different methods have been used for enzymes immobilization, which can be categorized into three types such as binding of enzyme to a carrier, enzymes crosslinking, and entrapment or encapsulation of enzymes within polymers (Rehman et al., 2016). In this sense, Dal Magro et al., (2016) synthetized a combined cross-linked enzyme aggregates based on pectinases–cellulases, using glutaraldehyde (GA) as crosslinking agent. These authors proposed these crosslinked enzymes for application in the clarification of grape juice and reported that they presented around 2.4 times more thermal stability than the free enzyme, being reusable with total conversion of substrate to product for 4 cycles. It is important to highlight that GA used in the synthesis of these enzymes, has potential acute health effects and is corrosive to metals, so its manipulation should be careful. Entrapment technique is one of the immobilization methods that physically restricts enzymes within a confined polymer space or networks made from different synthetic and natural polymers such as poly(acrylamide), nylon, ion-exchange resins, agar, and alginate, among others (Lei and Jiang, 2011; Rehman et al., 2015; Kumar et al., 2017). Each has its own advantages and disadvantages (Li et al., 2008). The synthetic polymers such as poly(acrylonitrile) (Godjevargova and Gabrovska,

2003) and nylon (Mohy, 2016), could be used for enzyme immobilization, but on contrary to the natural macromolecules (Krajewska, 2004), they have some disadvantages as the imperfect biocompatibility and hydrophobicity. Another option is polyvinyl alcohol (PVA) since its lattice structure of sponge, characterized by very dense porosity and a high specific pore volume, is recommended to be used for enzymes entrapment (Esawy et al., 2013). Rehman et al., (2014) immobilized a pectinase within 3 % agar-agar matrix using entrapment method, reporting that entrapped pectinase showed activity until 10th cycle and maintain 69 % activity even after third cycle.

Among natural polymers, alginate is a natural anionic polysaccharide derived from marine algae, which can form thermally stable and biocompatible hydrogel in the presence of calcium cations (Andriani et al., 2012; Lencina et al., 2015). The main advantage of this technique is the simplicity through which mechanically stable, non-toxic, and cheap spherical particles can be obtained (Flores-Maltos et al., 2011). Entrapment within insoluble calcium alginate beads has been shown to be an effective approach due to their biocompatibility (non-toxic nature), low cost, and effective particle size (Gülay and Şanli-Mohamed, 2012; Rehman et al., 2016; Sandoval-Castilla et al., 2010). Abdel Wahab et al., (2018) reported that pectinase immobilized on grafted alginate-agar gel beads retained 100 % and 56 % of its activity for three and nine successive cycles, respectively. However, the preparation of these beads involved not only GA but also polyethyleneimine (PEI) which have undesirable effects on health, so un-reacted PEI and GA should be well removed after beads synthesis. Accordingly, it is important to use calcium alginate to immobilize enzymes such as pectinase, avoiding the use of any additional reactive with adverse effects.

In the present study, a non-toxic and low cost biocatalyst was obtained by immobilizing a

commercial enological pectinase within insoluble calcium alginate beads, and its catalytic activity was studied. Biocatalyst beads were submitted to dehydration and to freeze-drying in order to maintain their biological activity. Beads were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM), in addition, they mechanical behavior was study by Texture Profile Analysis (TPA). It was also evaluated the effect of pH and temperature on catalytic properties of the immobilized enzyme, and their storage stability under refrigeration and reusability in term of recycling efficiency, as well as the effect of the entrapped pectinase on grape must clarification.

2. Materials and methods

2.1. Materials

Sodium alginate (Fluka, Switzerland, Nº 71238), with a weight average molar mass of 231,500 g mol^{-1} and a mannuronic/guluronic ratio (M/G) of 0.79 measured by ^{1}H-NMR (Gomez et al., 2007) was employed to prepare beads. Extrazyme® pectinase (Institut Oenologique de Champagne, Épernay, France) used to developed the biocatalyst is a highly concentrated maceration and extraction enzyme for enological use. Pectin from citrus peel (degree of esterification 60 %, Fluka, 76280), calcium chloride (Sigma Aldrich), and other chemical reagents used in this research were of analytical grade. Dinitrosalicylic acid (DNS) solution at 1 % (w/v) was prepared as follow: 2-hydroxy-3,5-dinitrobenzoic acid was dissolved in deionized water and subsequently a sodium hydroxide solution (50 ºC, under stirring) and a sodium-potassium tartrate salt were added. Final solution was diluted in deionized water, filtered and stored at room temperature (Miller, 1959).

2.2. Obtaining and preservation of biocatalyst beads

Hydrogel beads were prepared by external gelation from aqueous solutions of sodium alginate and pectinase onto $CaCl_2$ solution. Pectinase suspensions (0.3 % w/v) were added to sodium alginate solutions (4.0 % w/v) in a 1:1 (v/v) ratio at 4 ºC in a water bath. Pectinase-alginate mixture was added drop-wise with a hypodermic syringe into cold sterile $CaCl_2$ solution (2.5 % w/v), under mechanical stirring. Obtained beads (~3.0 mm diameter) were hardened by keeping them in the same $CaCl_2$ solution during 4 hours at 4 ºC.

Finally, the hydrogel so obtained were submitted to exhaustive rinsing with distilled water in order to remove the unentrapped pectinase. After this procedure, samples were stored in three different ways: (i) wet hydrogel beads were maintained in citrate buffer solution (50 mM, pH 3.8) at 4 ºC; (ii) some beads were dehydrated by using a vacuum stove at 30 ºC until constant weight; and (iii) hydrogels were freeze-dried at -36 °C and 0.022 mmHg during 8 hours using a Rificor S.H lyophilizer. Alginate hydrogel and alginate/enzyme hydrogel beads were labeled as AG-# and AGE-#, in which # represents the storing state o beads: hydrated (H), dehydrated (D), and lyophilized (L).

2.3. Swelling of hydrogels

Swelling behavior of hydrogels (AG, AGE-D, AGE-L) was studied by determining their equilibrium degree of swelling (Lencina et al., 2015). Experiments were performed by immersing hydrogels in a temperature-controlled water bath. At different immersion times, weight of swollen hydrogels was measured, after surface water was wiped off carefully with an absorbent paper. All assays were performed by triplicate and average values were reported. Equilibrium swelling ratio (SR) was calculated according to Eq. (1).

$$SR = \frac{W_s - W_d}{W_d} \quad (1)$$

where W_s is the weight of gels in the swollen state and W_d is the weight of gels in the dry state.

2.4. Characterization of biocatalyst beads

Internal morphology of beads was characterized by SEM. Dehydrated and lyophilized beads were previously cryo-fractured in liquid nitrogen, then mounted on bronze stubs, and coated with a gold layer by using an argon plasma metallizer (sputter coater PELCO 91000). This study was performed in an LEO 40XVP Scanning Electron Microscope (Jena, Germany), operated at 10 kV. In addition, Energy-Dispersive X-ray (EDX, Model DX-4) with UTW window and Standarless Quantification Method was used to analyze element compositions of the biocatalyst.

FTIR spectra of calcium alginate, pectinase and alginate/pectinase beads were obtained using a Nicolet® FTIR 520 spectrometer. Dehydrated and lyophilized samples were triturated as fine powder, mixed with KBr (Sigma Aldrich) (1.0 % w/w), and compressed into transparent discs in a hydraulic press. Spectra were recorded at 4 cm^{-1} resolution over 4000 - 400 cm^{-1} range, by using an accumulation of 64 scans and dried air as background.

Alginate (AG) and alginate/pectinase beads (AGE-H, AGE-D, and AGE-L) were submitted to a TPA by using a Texture Analyzer model *TA-XT2i* (Stable Micro Systems, UK), equipped with a 5 kg load cell. In the case of dehydrated and lyophilized samples, they were previously swelled in buffer-citrate solution at 4 °C during 30 minutes. Automated detection of probe contact with beads was carried out with a force of 0.005 N. Samples

178 were tested at a constant crosshead speed of 0.5 mm s^{-1}, at room temperature, by applying
179 two compression cycles to 30 % of deformation, and by using a steel cylinder probe
180 (diameter 35 mm). Textural properties such as hardness, cohesiveness, springiness, and
181 resilience (ratio between the areas under the compression and decompression curves) were
182 calculated from textural profile, obtained by using the Texture Expert Software for
183 Windows (Sandoval-Castilla et al., 2010).

184 ***2.5. Enzymatic activity and kinetics parameters***

185 Pectinolytic activity was assayed by measuring the amount of reducing sugars released
186 from a pectin dispersion using 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) and
187 galacturonic acid as standard (Sigma Aldrich, USA). 0.05 mL free enzyme aqueous
188 solution (0.3 % w/v) was mixed with 0.45 mL substrate (0.25 % w/v citric pectin in 50 mM
189 citrate buffer, pH 3.8) and incubated at 50 ºC during 15 min. In the case of immobilized
190 enzyme, 0.1 g of alginate/pectinase beads (AGE-H, AGE-D, and AGE-L) were used instead
191 of free enzyme solution. Then, DNS solution (1 % w/v, 0.50 mL) was added, by keeping
192 tubes in a boiling water bath during 10 min and absorbance was measured at 530 nm,
193 against blank reagent. One unit of pectinase activity is defined as "the amount of enzyme
194 required to release 1 μmol of galacturonic acid per minute under standard assay conditions".
195 The effect of temperature and pH on the pectinolytic activity of free (E) and immobilized
196 (AGE-H) enzyme was also studied. In order to evaluate the influence of temperature,
197 different assays were carried out by using incubation temperatures from 20 to 60 ºC, while
198 keeping constant substrate concentration and pH. On the other hand, the effect of pH was
199 studied by using different buffer solutions with pH ranging from 3.0 to 8.0, at constant
200 temperature and substrate concentration. Buffer solutions of citric/citrate (50 mM, pH 3.0

to 6.0) and phosphate (80 mM, pH 7.0 and 8.0) were employed for this analysis.

Kinetic parameters (K_m and V_{max}) for both free and immobilized pectinases were calculated from the Lineweaver-Burk equation using computed linear regression calculations, under conditions given above at different substrate concentrations in the range of 0.1-1.25 % (w/v).

2.6. Reusability and storage stability studies

Reusability of immobilized pectinase was studied by repeatedly re-using a defined amount of AGE-H hydrogel beads in pectin hydrolysis reaction. Beads (0.1 g) were mixed with 0.45 mL pectin substrate, prepared as it was previously described, and incubated at 50 ºC. After 15 min reaction time, beads were washed with deionized water to remove any residual substrate and their activity was tested with fresh substrate solution. This process was carried out for 6 consecutive cycles. Activity was determined after each cycle in terms of residual activity, by considering 100 % activity for the first cycle.

Finally, in order to evaluate the stability of immobilized pectinase, all samples were stored under refrigeration conditions (4ºC) for 8 months. Wet beads (AGE-H) were stored in 50 mM citrate buffer solution at pH 3.8 and 4 °C, while dehydrated (AGE-D) and lyophilized (AGE-L) beads were kept in a hermetic container at 4 ºC. Periodically, AGE-H, AGE-D and AGE-L samples were taken and their residual enzymatic activity was measured by following the spectrophotometric method previously described.

2.7. Application of immobilized pectinase for raw grape must clarification

Raw white grape must, *Vitis vinifera* L. cv. Torrontés was donated by a local cellar. The appropriate amount of immobilized enzymes (or equivalent amount of free enzyme) was mixed with 10 mL raw grape must and treated for 150 min at 20 ºC. After enzymatic treatment, clarity of the must was evaluated. The percent of transmittance was considered a measure of must clarity and it was determined at 650 nm, with distilled water as a reference. Additionally, the color of the clarified grape must was measured at 420 nm and a pectin test was conducted on the supernatant with ethanol acidified (5 % v/v HCl) to evaluate remnant pectin. All experiments were repeated three times.

2.8. Statistical analysis

The data are the average of three replications and were submitted to analysis of variance (one-way ANOVA) and comparison of mean values was performed by Fisher's least significant difference test, conducted at a significance level $p < 0.05$.

3. Results and discussion

3.1. Characterization of biocatalyst beads

Surface and internal morphology of dehydrated and lyophilized beads were studied by SEM (Figure 1). As it can be seen, drying techniques influenced AG beads size and shape, being the air-dried samples smaller than freeze-dried ones (Figure 1a and Figure 1c). Besides, alginate dehydrated beads without pectinase exhibited smooth surfaces and a homogeneous internal structure without pores and cracks (Figure 1a), similarly to morphologies reported by Jain and Datta (2016). On the other hand, freeze-dried beads (Figure 1c) had no time to shrink, so the areas of the former ice crystals were transformed into cavities cell-like structures. These results can be attributed to the fast sublimation of frozen water within the

matrix. In this sense, Iliescu et al., (2014) reported a similar behavior and stressed that high porous structures will probably exhibit better swelling capacity. SEM images of beads containing pectinase, revealed a considerable extent of irregularity and the presence of agglomerates (Figures 1b and 1d). Rehman et al., (2013) reported analogous changes in surface topologies of air-dried samples and the presence of particles or agglomerates after enzyme´s immobilization. These authors stressed that these changes can be attributed to the entrapment of different molecules of enzyme onto the porous surface of polymers. In addition, similar results were reported for freeze-dried calcium alginate beads, with the appearance of particles or agglomerates within beads cavities (Dai et al., 2018; Fernandez-Arrojo et al., 2013; Ma et al., 2017; Peng et al., 2016).

Commercial enzyme and beads were also characterized by EDX. In Figure 2 are shown the spectra corresponding to free pectinase (E) and lyophilized beads (AG-L and AGE-L). Elemental analysis revealed that commercial enzyme is mainly composed by carbon (C) and oxygen (O), while AG-L beads showed signals of C, O, calcium (Ca), and chlorine (Cl). Obtained weight percent of these elements were similar to those reported in the literature (Gülay and Şanli-Mohamed, 2012; Sen et al., 2016). For AGE-L beads, it was observed a reduction of ~ 34 % in the amount of Ca and the appearance of a sodium (Na) signal. This result could be attributed to the enzyme hindering effect of alginate gelation process by lowering ions exchange.

As complementary characterization, FTIR test were performed for free pectinase, calcium alginate and alginate entrapped pectinase. The results are shown in Figure 3. Neat pectinase (E) spectrum displayed a stretching vibration peak at 1650 cm^{-1} associated to amine group

(Dai et al., 2018; Seenuvasan et al., 2014). At 3430 cm^{-1} and 627 cm^{-1} appeared signals associated to N-H and C-N stretching vibrations, respectively (Mahesh et al., 2016). For calcium alginate (AG-L), a broad band at 3300 cm^{-1} corresponding to stretch vibration of hydroxyl groups was detected. Besides, the vibration of C-H bond appeared at 2930 cm^{-1} as well as two strong peaks at 1590 cm^{-1} and 1410 cm^{-1} attributed to the asymmetric and symmetric stretching of carboxyl groups. Furthermore, a signal observed at 1030 cm^{-1} (corresponding to symmetric and asymmetric vibration bands of C-O-C bonds typical of the polysaccharide rings) was also detected (Nastaj et al., 2016).

On the other hand, for immobilized pectinase (AGE-L), despite the absorption bands already assigned, a strong and acute signal at 1720 cm^{-1} was observed. Sojitra et al., (2017), stressed that between 1700 - 1600 cm^{-1} it was found a spectral region associate to protein's structural components, particularly amide-I bands, that can be associated with the secondary structure of immobilized enzyme.

Concerning swelling behavior of dehydrated and lyophilized beads, regardless the presence of the enzyme, they presented swelling ratio values of 1.33 and 3.33 times, respectively. The main difference in beads swelling capacity could be derived from the dehydration process. The highest swelling of lyophilized samples could be associated to the very porous morphology which improves the diffusion of water molecules within alginate structure (Iliescu et al., 2014).

Figure 4 shows textural properties of calcium alginate and alginate/pectinase beads. Dehydrated beads showed higher hardness values (AG-D: 8.44 ± 0.82 N) than hydrated (AG-H: 0.32 ± 0.05 N) and lyophilized beads (AG-L: 0.17 ± 0.02), after swelling in a buffer citrate solution at 4 °C. Besides, alginate/pectinase beads exhibited significantly

lower hardness, comparing to hydrogels without pectinase. A 55 % hardness reduction was observed for AGE-D when compared to AG-D samples. On the other hand, for hydrated and lyophilized samples, entrapped enzyme caused a hardness reduction of around 30 %. This behavior is in accordance with the results obtained from EDX analysis, where a lower ions exchange could provide hydrogels with low cross-linking degree. Regarding to springiness, cohesiveness, and resilience properties, they did not change in comparison to beads without pectinase.

3.2. Properties of free and immobilized pectinase

3.2.1. Optimal reaction pH and temperature

Effect of media pH on enzymatic activity of alginate entrapped pectinase was studied by using different buffer solutions, with pH values ranged from 3.0 to 8.0, at constant temperature (50 ºC) and 0.25 w/w substrate concentration. Figure 5a shows activity-pH profiles of the free enzyme (E) and an immobilized pectinase (AGE-H). As it can be observed, in both cases pectinolytic activity was affected by the reaction medium acidity reaching a maximum activity at pH 4, which was considered as the optimum acidity within the assayed range. In this sense, AGE-H beads exhibited an activity of 71 % at pH = 3. This behavior could be associated with the strong electrostatic interactions between carboxyl groups of alginate chains that occur on higher acid conditions, hindering the pectin substrate diffusion inside the beads. In addition, Shin et al., (2004) and Elnashar et al., (2010) reported similar behavior in calcium-alginate beads stored under acidic conditions (pH < 3) showing a bead size decrease and a less swelling capacity. On the other hand, AGE-H samples presented higher activity at pH 5 and 6 compared to free enzyme.

These might be due to microenvironment of calcium alginate matrix that contributes to the structural and conformational stability of pectinase. Dai et al., (2018) reported optimal pH value for free and immobilized enzymes between 4.0 and 5.0, respectively. These authors stressed that immobilized pectinase had a wider active pH range when compared to the free enzyme, retaining over 80 % of the original activity up to pH = 5. This indicates that immobilized enzyme is more resistant towards pH changes compared to the free enzyme. Similar results and behavior for immobilized pectinases were already reported by Abdel Wahab et al., (2018).

Temperature effect on the activity of free (E) and immobilized pectinase (AGE-H) was carried out by assaying different incubation temperature, from 20 to 60 ºC, at a constant pH value (3.8) and 0.25 w/w substrate concentration. As it can be observed in Figure 5b, both samples followed a similar behavior, reaching a maximum activity at 50 ºC, which was considered the optimum temperature within the assayed range. No changes in enzymatic activity at optimum temperature (50 ºC) were observed by entrapping pectinase within calcium alginate matrix. On the other hand, a slightly increment on activity of AGE-H was observed at 20 ºC. In this sense, several authors stressed that enzymes stability as well as their activity are improved when they are entrapped onto microspheres (Demir et al., 2001; Fernandez-Arrojo et al., 2013; Nawaz et al., 2015).

From the obtained results, it can be observed that the alginate entrapped pectinase shows good catalytic efficiency in conditions nearby to those of winemaking (pH: 3.6-4.0 and temperature: 20-30 °C), similar to free pectinase (Figure 5). Particularly, as reported in previous works (Martín and Morata de Ambrosini, 2014), cold-active pectinases are potentially relevant in the fruit juice clarification and wine industry. Pectin causes turbidity

and undesired solid suspension in grape musts and clarification process often utilizes pectinases with activity at low temperature to degrade and remove pectin. Thereby, the proposed biocatalysts could be used during grape must clarification process, resulting more efficient than the free enzyme.

3.2.2. Kinetic parameters

The Michaelis-Menten constant (K_m and V_{max}) was obtained from Lineweaver-Burk plots (Figure 6). For free enzyme K_m and V_{max} were determined as 5.45 mg/mL and 0.628 μmol/min, while in the case of immobilized enzyme K_m and V_{max} were determined as 5.61 mg/mL and 0.584 μmol/min, respectively. K_m values for both free and immobilized pectinase were similar, which signifies that immobilized enzymes had the same affinity for its substrate than the free forms. This indicate that no negative effect of immobilization there was in terms of increased steric hindrance of the active site, or the loss of enzyme flexibility necessary for substrate binding, as reported other authors (Dai et al., 2018; Sojitra et al., 2017). However, the V_{max} value of the immobilized pectinase was slightly lower than that of the free enzyme, indicating that the rate of hydrolysis of respective substrate was reduced after immobilization. This might be due to higher difficulty in the diffusion of substrate to reach active site of enzyme after immobilization, or also due to higher mass transfer resistance to substrate/enzyme imposed by the support on the macromolecular substrate (Nadar and Rathod, 2016).

3.2.3. Reusability and storage stability of biocatalyst beads

Reusability of immobilized enzyme influences the financial feasibility of bioprocess and

356 justifies its analysis (Goradia et al., 2006). Assays were carried out at 50 ºC and pH 3.8 in
357 batch reactions and results of residual activity after each cycle are shown in Figure 7. After
358 the first cycle, catalytic activity of immobilized pectinase decreased nearby 25 %, and
359 retained almost 37 % of its initial activity after six cycles. This loss of activity might be due
360 to pectinase leakage from calcium alginate beads due to washing, or conformational
361 changes by repeated uses, as it was already reported by Rehman et al. (2013). Obtained
362 values in this study are in good agreement with those reported for pectinases entrapped into
363 calcium alginate beads under the same conditions (Rehman et al., 2013). Nevertheless, they
364 are lower than those obtained by using covalently bonding pectinase through multipoint
365 attachment on activated supports (Kumar et al., 2017). As an outcome, immobilized
366 enzyme could be used for wine or fruit juice clarification processes, as well as pre-
367 treatment even when they presented less activity; whereas the free enzyme can only be used
368 one time.

369 Storage stability is one of the key factors for any industrial enzyme. An enzyme with
370 excellent storage capacity without losses its biocatalytic efficiency is also desirable (Romo-
371 Sánchez et al., 2014). In order to evaluate the effect of storage conditions on the pectinase,
372 (E) free enzyme solution and (AGE-H) wet beads were stored in 50 mM citrate buffer (pH
373 3.8) at refrigerate conditions at 4 °C. Figure 8 shows that immobilized pectinase was found
374 to be stable and retained its initial activity for at least 11 weeks of storage under mentioned
375 conditions. On the contrary, free pectinase only retained about 72 % of its original activity
376 up to 5 weeks of storage. After these periods of time, pectinase preservability was lost due
377 to the appearance of microbial contamination in the storing buffer solution of both samples.
378 This improved stability of the immobilized enzyme could be due to the neutralization of

charged residues by interaction with the matrix, better physical contacts or structural rigidness or higher stabilization of the enzyme (Bhushan et al., 2015; Demir et al., 2001; Fernandez-Arrojo et al., 2013; Landarani-Isfahani et al., 2015; Nawaz et al., 2015; Seenuvasan et al., 2014).

On the other hand, freeze-drying process has been widely employed to maintain biological activities over a long period of time (Nakagawa et al., 2013). Consequently, alginate/pectinase beads were dehydrated and lyophilized. Resulting material was kept in a refrigerator at 4 °C and its enzymatic activity was periodically tested. The results indicated that lyophilized hydrogels (AGE-L) retained their initial activity up to 8 months, whereas dehydrated beads (AGE-D) retained only 30 % of the initial activity for the same time (Figure 9). Hence, freeze-drying procedure could be used to prepare alginate/pectinase biocatalysts for better stability and preservation.

3.3. Clarification of grape must

Pectin substances are responsible for the consistency, turbidity and appearance of fruit juice. In wine industry, the presence of these substances in grape must causes an increase in their viscosity, prolonging and hindering the processes of filtration and clarification. Therefore, raw grape must was treated by adding 0.3 % (w/v) free enzyme, or the same concentration of immobilized enzyme (0.3 g/mL), and transmittance percent and color at 420 nm was measured after 150 min at 20 ºC. Additionally, pectin test was conducted on supernatant after enzymatic treatments with ethanol acidified (5 % v/v HCl in ethanol) to evaluate remnant pectin. It was proved that the immobilized pectinase could effectively decompose the pectin in grape musts. The formation of pectin flocs facilitated the production of a clear

supernatant with the removal of colloidal part of the must (Figure 10).

Table 1 summarizes the properties of grape must before and after pectinase clarification process. Enzyme treated grape musts had higher light transmittance and smaller color value than untreated must. These properties resulted better with the immobilized pectinase than with using free enzyme. As it can be seen, grape musts treated with free and immobilized enzyme exhibited a light transmittance values 5.4 and 6.5 times higher than untreated must, respectively, while the immobilized pectinase showed an increment of 21% in clarification efficiency compared to free enzyme. In addition, a significantly smaller color value was found for the entrapped enzyme than to free enzyme. Remnant pectin was not detected in enzyme treated grape musts.

4. Conclusions

An enological pectinase was successfully immobilized within calcium alginate hydrogels by using entrapment technique. Morphological characterization evidenced the presence of particles and agglomerates of the enzyme throughout alginate matrix, on the surface as well as inside the matrix. FTIR analysis confirmed the presence of absorption bands associated with the amino groups present in pectinase. Regarding to enzymatic activity, the immobilization procedure did not influence the optimal pH and temperature values for pectinase activity when compared to the free enzyme. The lyophilized immobilized pectinase kept their original activity after 8 months of storage. The activity of entrapped pectinase was retained after 6 reaction cycles, with 37 % of residual activity. In addition, grape must turbidity decreased rapidly in the presence of the immobilized pectinase, being more effective than the free enzyme. According to these results, the proposed methodology is a convenient alternative to obtain new biocatalysts for wine industry purposes.

Acknowledgement

Authors wish to thank to the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), the Universidad Nacional del Sur (UNS, Argentina), the Fondo para la Investigación Científica y Tecnológica (FONCyT, Argentina) [Grant PICT-2016-0181] and, the Universidad Nacional de Cuyo (UNCuyo, Argentina), [Grant SECTYP/L018] for the financial support given to perform this research.

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610 **Figure Captions**

611 **Figure 1**: SEM images of (a) dehydrated and (c) lyophilized beads without enzyme; and (b)
612 dehydrated and (d) lyophilized beads with 0.3 % (m/v) pectinase.

613 **Figure 2:** EDX spectra of commercial pectinase (E), calcium alginate lyophilized beads
614 (AG-L) and alginate/pectinase lyophilized beads (AGE-L).

615 **Figure 3:** FTIR analysis of commercial pectinase (E), calcium alginate lyophilized beads
616 (AG-L) and alginate/pectinase lyophilized beads (AGE-L).

617 **Figure 4:** First compression cycle of TPA essay. (a) Dehydrated, (b) hydrated and (c)
618 lyophilized alginate and alginate/pectinase beads. Photos show the shape and size of
619 alginate/pectinase beads.

620 **Figure 5:** Effect of (a) pH and (b) temperature on the enzymatic activity of (○) free
621 pectinase and (◆) immobilized pectinase.

622 **Figure 6:** Lineweaver-Burk curves of (○) free pectinase and (◆) immobilized pectinase.

623 **Figure 7:** Reusability of immobilized pectinase (AGE-H) in batch reactions.

624 **Figure 8.** Storage stability of free pectinase (E) and immobilized pectinase (AGE-H).

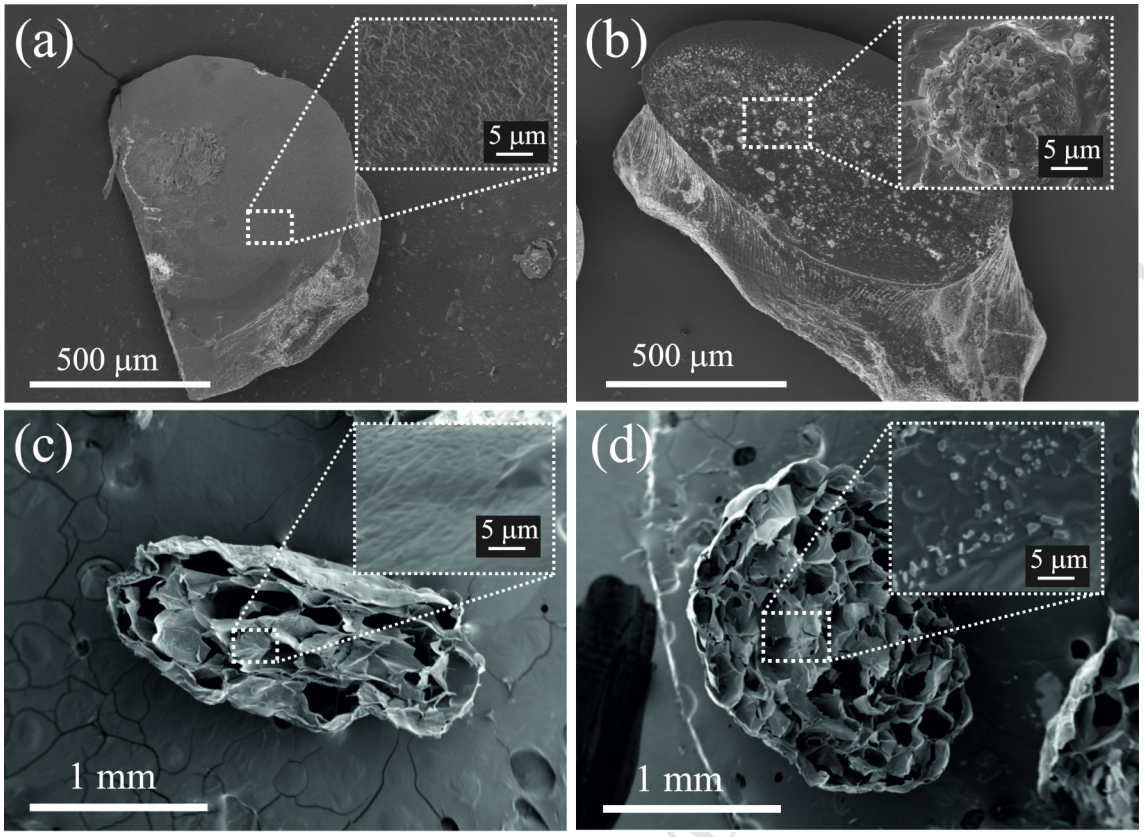
625 **Figure 9.** Effect of storage on the enzymatic activity of dehydrated (AGE-D) and
626 lyophilized (AGE-L) alginate/pectinase beads.

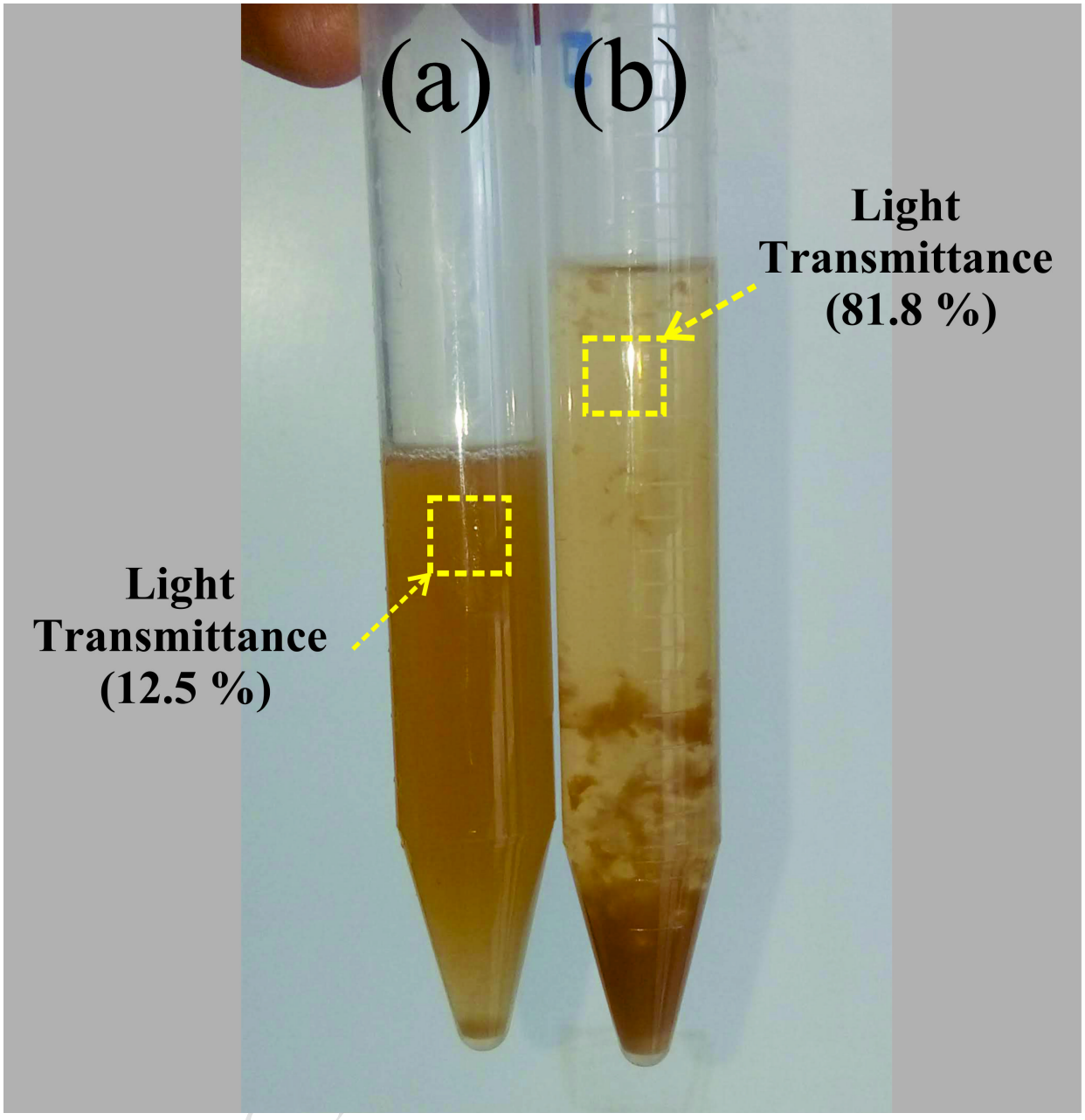
627 **Figure 10.** Photograph of grape must (a) before and (b) after clarification process with the
628 (AGE-H) immobilized pectinase.

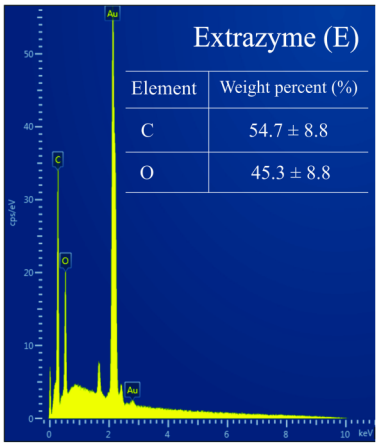
Table 1. Properties of grape must before and after pectinase clarification

Properties	Untreated grape must	Grape must after pectinase treatment	
		Free enzyme	Immobilized enzyme
Light transmittance (%) at 650 nm	12.5 ± 0.1[a]	67.2 ± 0.1[b]	81.8 ± 0.8[c]
Color at 420 nm	1.135 ± 0.191[a]	0.619 ± 0.015[b]	0.429 ± 0.033[c]
Pectin	(+)	(-)	(-)

The values are the average of three determinations ± standard deviation. Different letters in the same line indicate significant differences ($p<0.05$) between the treatments.

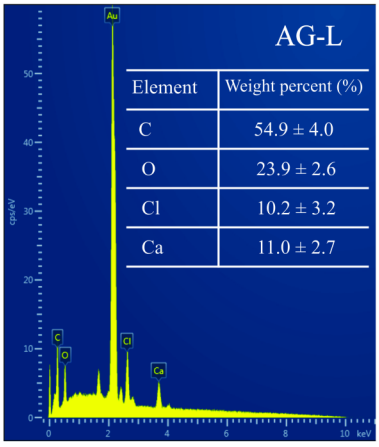






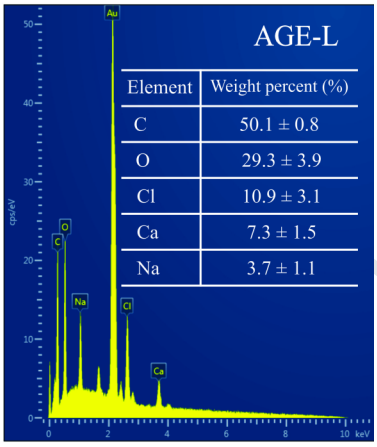
Extrazyme (E)

Element	Weight percent (%)
C	54.7 ± 8.8
O	45.3 ± 8.8



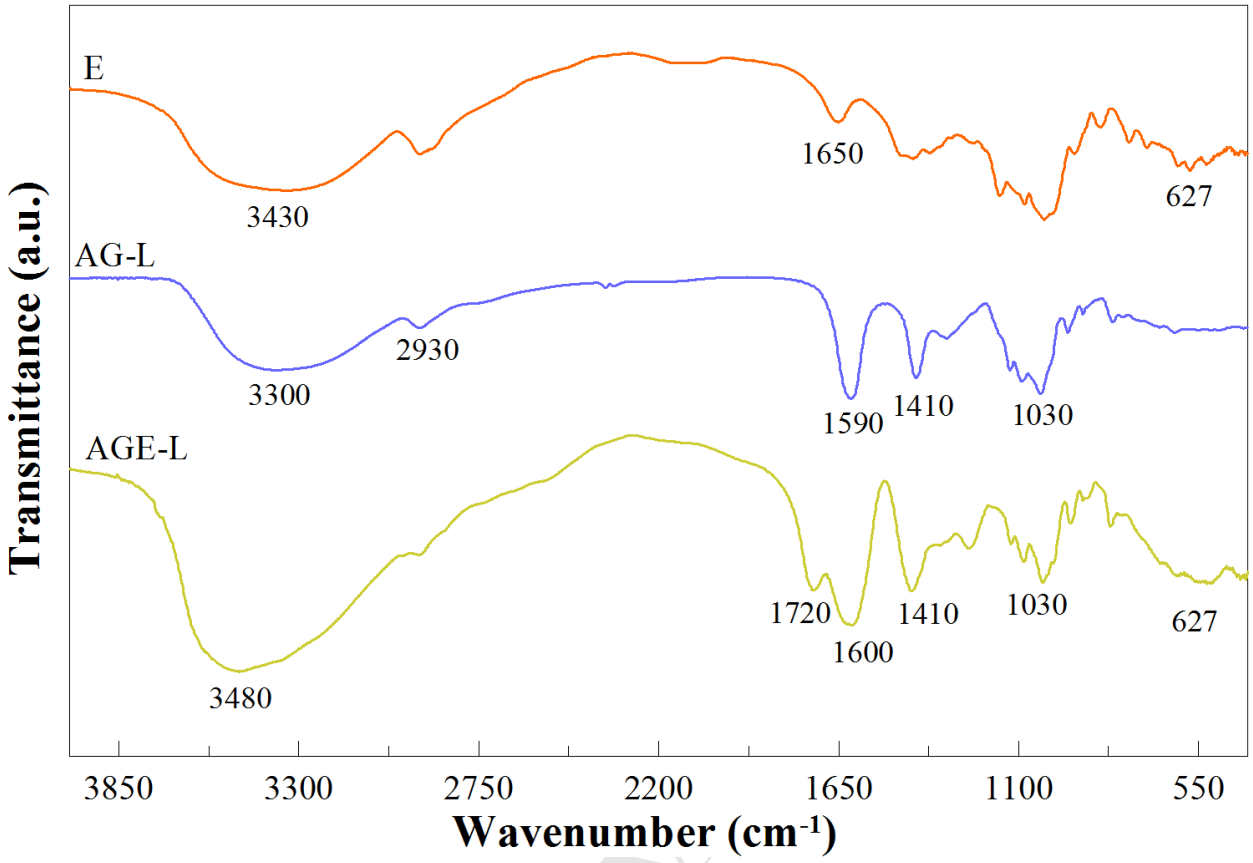
AG-L

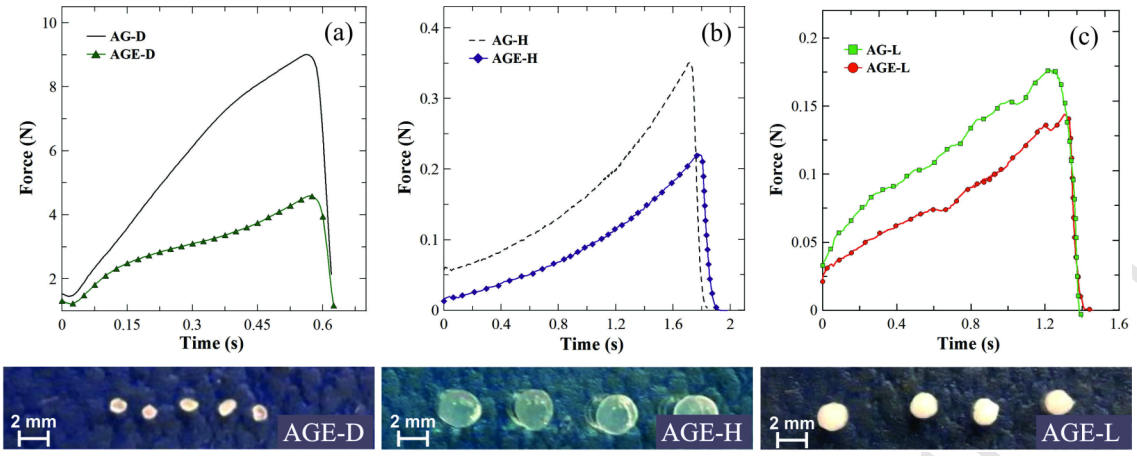
Element	Weight percent (%)
C	54.9 ± 4.0
O	23.9 ± 2.6
Cl	10.2 ± 3.2
Ca	11.0 ± 2.7

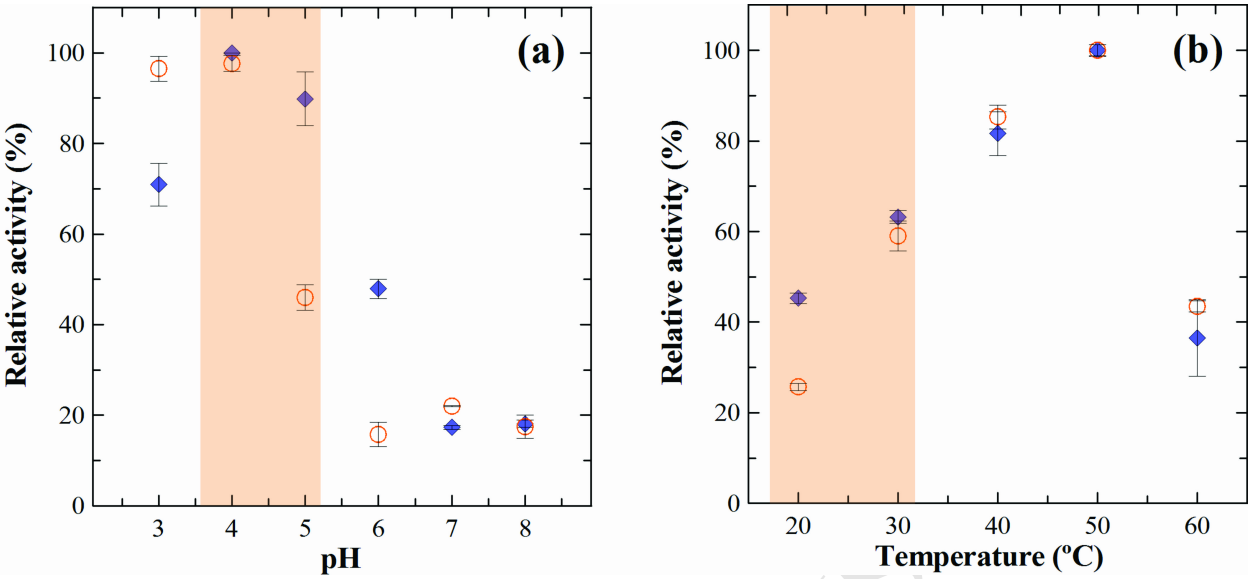


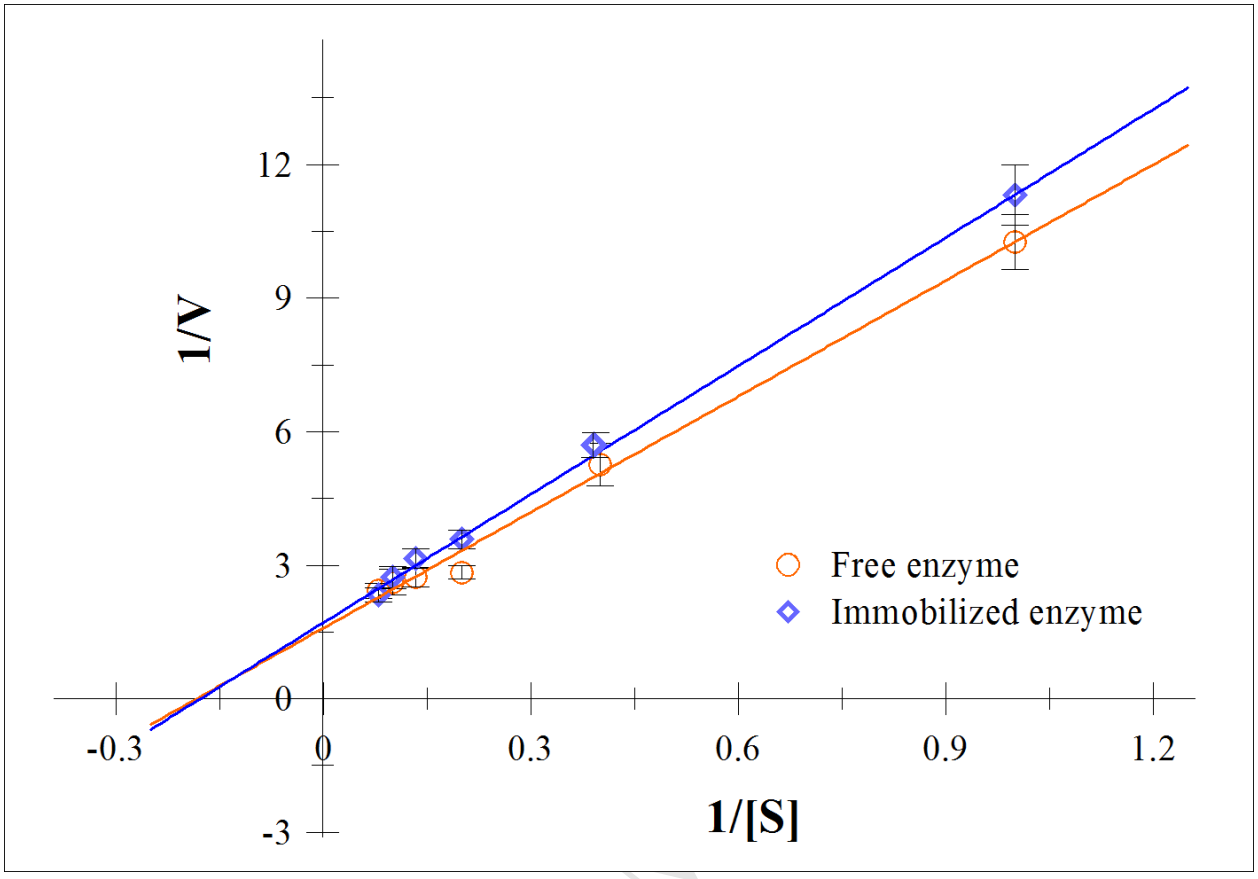
AGE-L

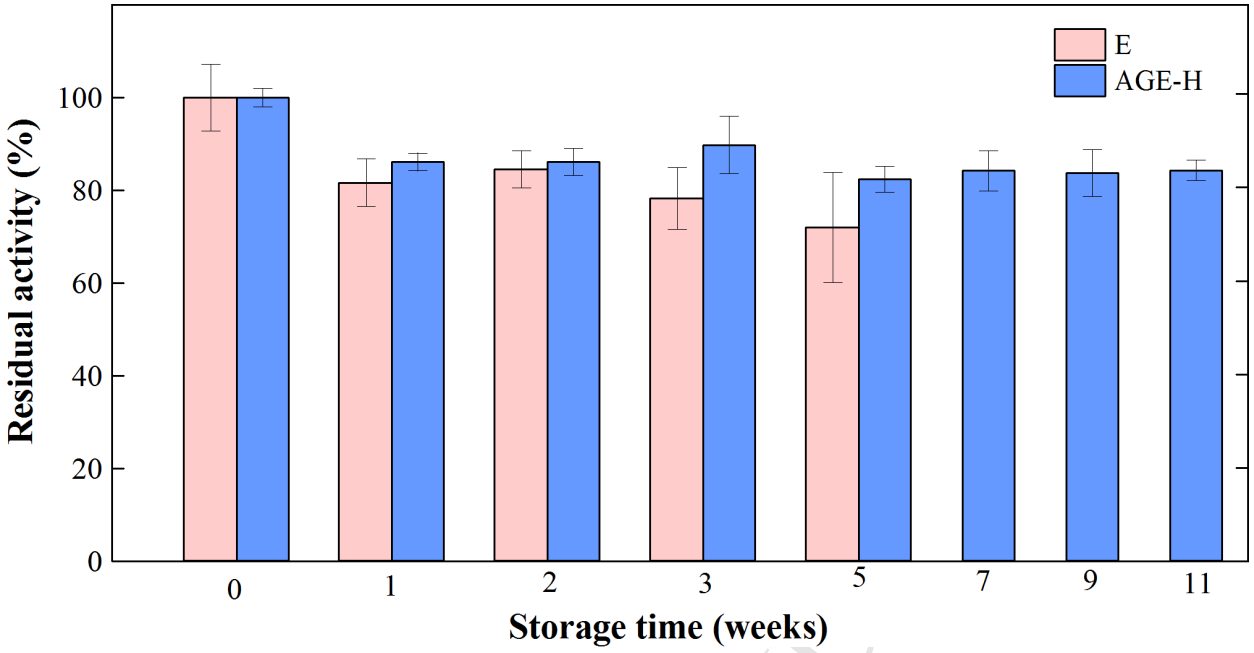
Element	Weight percent (%)
C	50.1 ± 0.8
O	29.3 ± 3.9
Cl	10.9 ± 3.1
Ca	7.3 ± 1.5
Na	3.7 ± 1.1

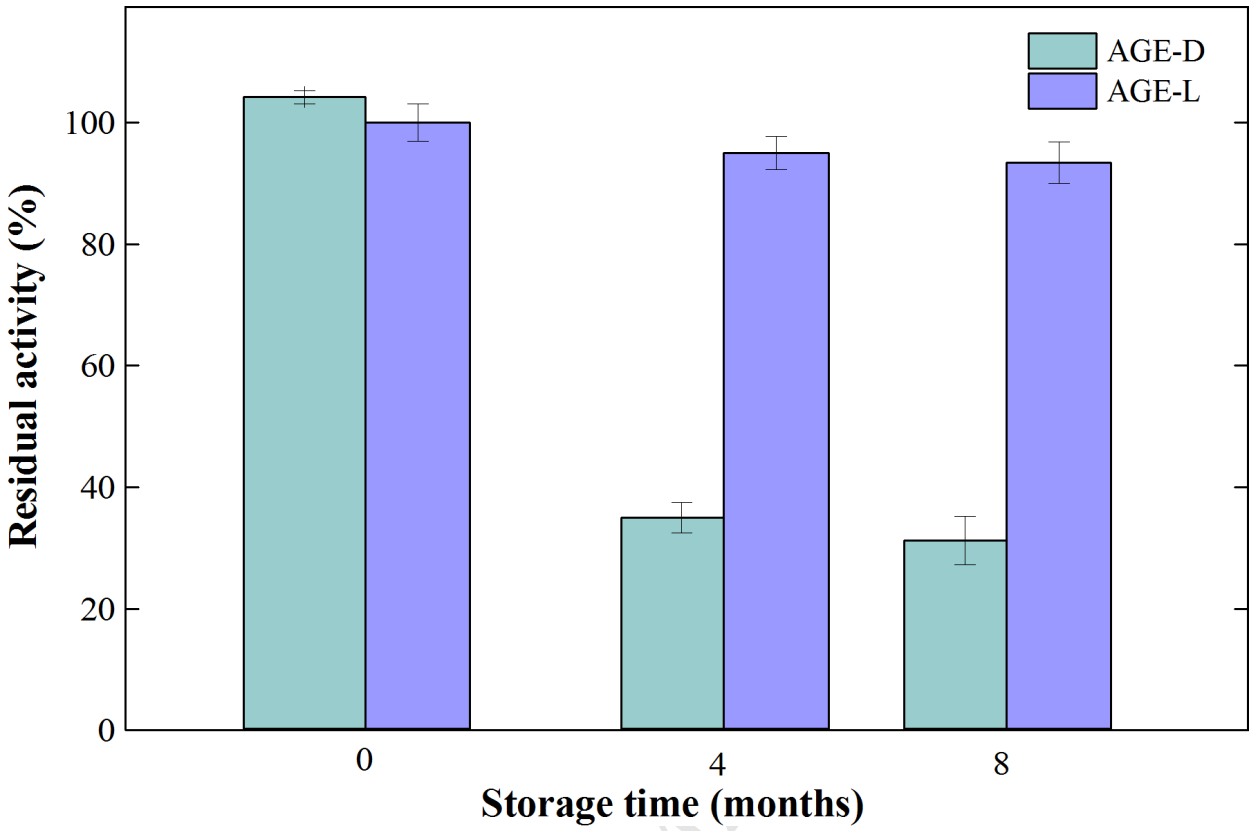


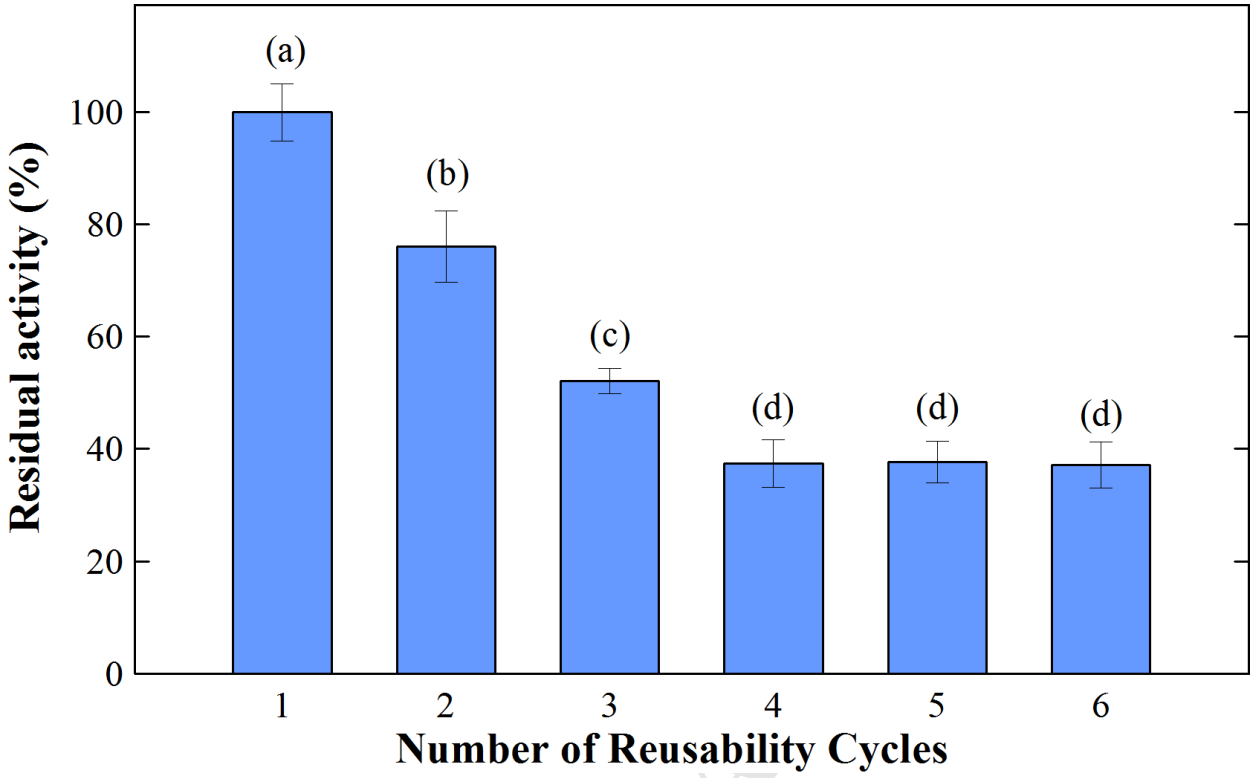












Highlights

Pectinase was effectively immobilized on calcium alginate beads via entrapment.

SEM and FTIR analysis evidenced the presence of pectinase throughout alginate matrix.

Immobilization did not modify optimal pH and temperature of free pectinase activity.

Immobilized pectinase showed activity even until six times of recycling.

The grape must turbidity decreased significantly using the immobilized pectinase.