



Continuous clarification of grape juice using a packed bed bioreactor including pectinase enzyme immobilized on glass beads

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ABSTRACT

A continuous clarification of grape juice was undertaken in a packed bed reactor (PBR) using pectinase enzymes immobilized on glass beads. The enzyme immobilization process decreased the specific activity and the volumetric productivity from 3 ± 0.2 to 2.7 ± 0.1 units mg^{-1} and from ~ 0.085 to ~ 0.070 $\text{mg mL}^{-1} \text{min}^{-1}$, respectively. Also, it increased the optimum pH from 5.0 to 5.5, but did not influence the optimum temperature (50°C). The best conditions for clarification of the grape juice were obtained at a flow rate of 0.5 mL min^{-1} and a treatment time of 42 min. After clarification, pH, total soluble solids, turbidity, total phenolic content, antiradical activity and viscosity of grape juice were decreased, and on the other hand, total titratable acidity and the color parameter L were increased. Generally, this study showed that the continuous operation in a PBR including glass bead immobilized enzyme can be a potential alternative for the batch system used in juice industries.

1. Introduction

Grapes (*Vitis vinifera*) are one of the most popular fruits in the world, and are usually consumed as fresh fruit, juice, wine or marmalade (Ghafoor et al., 2009). Grape juice is rich in flavonols, anthocyanins, procyanidins and phenolic acids and therefore has positive effects on human health as anti-cancer and anti-inflammatory agents (Capanoglu et al., 2013; da Silva Haas et al., 2019). However, the major problems in the industrial production of juice is its turbidity and the formation of sediment during storage (Tapre & Jain, 2014). The cause of these problems is mainly a hydrocolloid called pectin and so removing it is needed to produce a customer-friendly product.

Pectin is a plant polysaccharide composed of galacturonic acid chains and used as an emulsifier, thickener and stabilizer agent in the food and drug industries (Mosayebi et al., 2018; Pasandide et al., 2018). Despite these unique properties, the presence of this polysaccharide in many fruit juice samples has been problematic as stated earlier, and the best way to remove pectin during the clarification step remains an issue. (Sharma et al., 2014). Enzymatic clarification of different fruit juice samples has a high potential and could be a cost-effective method (Lettera et al., 2016; Salim et al., 2018). Pectinase is the most important enzyme used for fruit juice clarification. This enzyme reduces cloudiness and improves the quality of fruit juice by hydrolysis of pectic substances (Sojitra et al., 2017). However, the native form of the enzyme has

limitations such as high cost, low stability and problems related to its recovery and reusability. Enzyme immobilization on a suitable carrier can overcome some of the limitations and defects (Chauhan et al., 2015; dos Santos et al., 2019; Wang et al., 2016; Yu et al., 2018) and lead to a continuous process. The enzyme immobilization has been done using different methods of which covalent immobilization, adsorption and entrapment on/in the supports are the main ones (Ko et al., 2018; Rehman, Nawaz, et al., 2014; Sheldon, 2007). For instance, Amin et al. (2017) immobilized the fungal exo-polygalacturonase on sodium alginate support by covalent (using glutaraldehyde as a cross-linker) and/or adsorption methods. Rehman, Aman, et al. (2014) applied the entrapment method for pectinase immobilization in an agar-agar matrix. Lei and Jiang (2011) used glutaraldehyde for covalent immobilization of pectinase onto macroporous polyacrylamide microspheres. The covalent immobilization method seemed to have better results with less enzyme release. The choice of a suitable support such as glass beads with high performance, easy availability, high stability and low cost are important benefits (Eskandarloo & Abbaspourrad, 2018).

Although the enzyme immobilization is a necessary condition for a continuous enzymatic process, it is impossible to do without the use of a bioreactor. A packed bed reactor (PBR), is a simple and inexpensive reactor, which includes one or more tubes filled with a catalyst, in which the reaction mixture flows continuously with controlled conditions (de Oliveira et al., 2018). Continuous reactors have the advantage of ease of

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operation, better control of the reaction conditions and product quality, high production performance and low operating costs (Nakkharat & Haltrich, 2007).

The aim of this study was the immobilization of a pectinase enzyme on glass beads using a covalent method and the development of a continuous process for clarifying grape juice in a PBR. Pectinase was immobilized on functionalized glass beads using glutaraldehyde and then the optimum conditions of enzyme activity were determined. In the next step, a bioreactor after obtaining optimum conditions was used to clarify grape juice.

2. Material and methods

2.1. Materials

Apple pectin (United States Pharmacopeia (USP) grade and degree of esterification) (DE) of 72–76% was supplied by Herbreith & Fox KG (Nuremberg, Bavaria, Germany). Pectinase enzyme (Pectinex® Ultra Color, with a protein content of 80 mg mL⁻¹) with optimum temperature and pH of 50 °C and 5, which had been determined in a previous study (Hosseini et al., 2020), was obtained from Novozymes (Bagsvaerd, Denmark). Glass beads (6 mm diameter) were purchased from the Iran Beads Co. (Esfahan, Iran). Nitric acid, hydrochloric acid, acetic acid, 3-aminopropyl triethoxysilane (3-APTES), glutaraldehyde, sodium acetate, 3,5-dinitrosalicylic acid (DNS), D-galacturonic acid monohydrate and gallic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade and acquired from Merck Chemical Co. (Darmstadt, Hesse, Germany).

2.2. Activation of glass beads

The glass beads were treated with 3-APTES to create the free amine groups and then were immersed in glutaraldehyde to create the free aldehyde groups according to the following steps (Eskandarloo & Abbaspourrad, 2018; Hosseini et al., 2020):

The glass beads (~110 beads = 20 cm³) were immersed in 5% nitric acid at 90 °C for 60 min to remove surface contaminants, and then were washed with distilled water and were placed in an oven (Model BM55E, Fan Azma Gostar Co., Tehran, Iran) at 110 °C for 24 h. The dried beads were immersed in 10% (v/v) 3-APTES solution at 60 °C for 3 h at pH 4, washed with distilled water and placed in an oven (80 °C, 24 h). The silanized glass beads were immersed in 2.5% (v/v) glutaraldehyde at 25 °C for 1 h and washed with distilled water.

2.3. Enzyme immobilization

The pectinase immobilization was done using the method described by Hosseini et al. (2020). Functionalized glass beads (20 cm³) were soaked in the enzyme solution diluted with 0.1 M sodium acetate buffer (1:4 v/v (2 mL enzyme solution with protein concentration of 80 mg mL⁻¹ + 8 mL buffer), pH 4) at ~25 °C for 5 h. After washing with buffer, they were stored at 4 °C until use for a maximum of 4 wk. The protein content of the glass beads was measured using the Lowry method by protein difference in the enzyme solution before and after glass beads treatment (Lowry et al., 1951) using the equation below:

$$\text{Protein content (BSA E)} = (\text{mg} / 10 \text{ glass beads}) = C_i - (C_R + C_W) \quad (1)$$

where C_i = amount of protein in the initial solution, C_R = amount of protein in the solution remaining after immobilization and C_W = amount of protein in the wash solution. The protein content of 20 cm³ glass beads was 4.51 mg. Bovine serum albumin (BSA) was used to prepare the calibration curve assuming the BSA was 100% pure and reporting the results as BSA equivalents (E).

2.4. Evaluation of enzyme activity

The evaluation of enzyme activity was done with a DNS method (de Oliveira et al., 2018). The apple pectin and D-galacturonic acid (10–1000 µg mL⁻¹) were considered as substrate and standard, respectively. Also, one unit (U) of the enzyme activity was defined as the amount of enzyme required to generate 1×10^{-6} mol of galacturonic acid min⁻¹ with the experimental conditions and the activity of the free and immobilized pectinase was determined according to their specific activity (U/mg protein).

Enzyme solution (56 µL which included, 4.51 mg (BSA E) of enzyme) was added to 10 mL of 1% (w/v) pectin solution prepared in 0.1 M sodium acetate buffer (pH 5) and the hydrolysis reaction was done for 15 min at 50 °C. The reaction medium (1 mL) was mixed with 1 mL of DNS reagent. The solution was immediately placed in a Ben Marie (Behsan Laboratory, Tehran, Iran) (100 °C, 5 min) and then was cooled in an ice-water bath. Distilled water (3 mL) was added and its absorbance (Model SP-UV500DB, Spectrum Co., Cologne, North Rhine-Westphalia, Germany) was determined at 540 nm. For the immobilized pectinase, 20 cm³ of the glass beads immobilized enzyme was used instead of free enzyme and the reaction was carried out at pH 5.5, 50 °C, 15 min.

2.5. Packed bed reactor apparatus

A double-walled glass cylinder with an inner diameter of 14 mm and a length of 150 mm was used as the PBR. In the inner chamber, 20 cm³ of the enzyme-containing glass beads were packed and in the outer chamber, water flowed to regulate temperature. The buffered pectin solution or grape juice (as described in the preparation of fruit juices section), as substrates were pumped from the bottom of the system and the product exited from the top. The total PBR volume was 23.1 cm³ and the actual volume of 20 cm³ of glass beads was 12.4 cm³ and thereby the void volume was 10.7 cm³ based on equation (2). (Singh et al., 2011):

$$V_v = V_1 - V_2 \quad (2)$$

where V_v = the void volume, V_1 = the total PBR volume and V_2 = actual volume of 20 cm³ glass beads calculated from their individual volume.

2.6. Effect of different flow rates on the enzyme activity

The flow rate was varied from 0.1 to 10 mL min⁻¹, while other factors including pH of the buffered pectin solution (5.5) and the process temperature (50 °C) were constant. Also, the residence time of the substrate solution in the column, as a flow rate dependent factor, was determined based on equation (3). (Singh et al., 2011):

$$\text{Residence time (min)} = \frac{V}{F} \quad (3)$$

where F = flow rate (mL min⁻¹) and V = the reactor volume (mL).

2.7. Effect of pH and temperature on the enzyme activity

The effects of pH and temperature were measured using a factor at a time design. For the selection of the optimum pH, temperature and flow rate were constant at 50 °C and 0.5 mL min⁻¹, while pH of the pectin solution was varied from 2 to 8 using different buffers (KCl–HCl (pH 2), acetate (pH 3–5.5) and KH₂PO₄–NaOH (pH 6–8)). For temperature, pH and flow rate were constant at 5.5 and 0.5 mL min⁻¹, while the temperature was varied (30–90 °C) (Panesar et al., 2011).

2.8. Volumetric productivity of the free and immobilized pectinase

To determine the volumetric productivity of the free enzyme and the immobilized pectinase in a batch mode, 56 µL of enzyme solution or 20 cm³ of the enzyme-containing glass beads were added to the buffered

pectin solution (1% (w/v)) and the hydrolysis reaction was done with optimum conditions (pH 5 and at 50 °C for free pectinase, pH 5.5 at 50 °C for immobilized pectinase in a batch process and flow rate of 0.5 mL min⁻¹, pH of 5.5 and temperature of 50 °C for immobilized pectinase in the continuous process). The resulting data were reported based on the mg of the reducing sugar mg mL⁻¹ min⁻¹ of the reaction medium min⁻¹. On the other hand, the volumetric productivity of the enzyme-containing glass beads packed in the PBR was calculated using equation (4) (Singh et al., 2011):

$$\text{Volumetric productivity (mg mL}^{-1} \text{ min}^{-1}) = R \times \frac{F}{V} \quad (4)$$

where R = the reducing sugars produced (mg mL⁻¹), V = the reactor volume (mL) and F = flow rate (mL min⁻¹).

2.9. Reusability of immobilized pectinase in the batch operation

The reusability of the glass bead immobilized pectinase was determined by doing repeated cycles of pectin hydrolysis reaction in batch mode. In each cycle, 20 cm³ glass beads immobilized pectinase was added to 10 mL of 1% (w/v) pectin solution (pH 5.5 using a 0.1 M sodium acetate buffer) and the hydrolysis reaction was done for 15 min at 50 °C. After each cycle, the glass beads were washed with the sodium acetate buffer (pH 4) and immersed again in the fresh substrate. The immobilized pectinase activity in each cycle was determined using the DNS method as mentioned earlier.

2.10. Clarification of grape juice in the packed bed reactor

2.10.1. Preparation of fruit juices

Three kg of seedless white grapes (*Vitis vinifera* L. CV. Asgari, Zanjan, Iran) were obtained from a local market in Tehran, Iran in July. The grapes were ripe, had a sweet taste and a thin skin. The grape fruits were washed with tap water and then the grape juice was obtained using a household juicer (Model SJ 3000 W, Braun, Kronberg, Hesse, Germany). To separate insoluble compounds, the resulting juice was filtered using Whatman No. 1 filter paper (Maidstone, Kent, England).

2.10.2. Fruit juice clarification

The juice clarification was done in the PBR at a flow rate 0.5 mL min⁻¹, at 50 °C and different treatment times to achieve the maximum clarity of the grape juice. The juice samples were cooled in an ice-water bath and were centrifuged at 10000×g (Model Universal 320 centrifuge, Hettich Centrifuge, Andreas Hettich GmbH & Co. KG, Tuttlingen, Baden-Württemberg, Germany) at room temperature for 15 min and, the degree of clarification of the fruit juices was determined at 625 nm (de Oliveira et al., 2018; Diano et al., 2008). After determining the best treatment time, the grape juice was clarified using three methods: (1) free enzyme; (2) immobilized enzyme in a batch process and (3) immobilized enzyme in a continuous system. Using optimal conditions, the grape juice clarification process was done using the three methods mentioned. The clarified samples were cooled in an ice-water bath and were centrifuged (at 10000×g, room temperature, 15 min).

The physicochemical properties of the juices clarified using the methods above were compared to untreated sample using the following tests:

The pH of fruit juices was determined using a pH meter (GLP 22, Crison, Barcelona, Spain). Total titratable acidity (TTA) of fruit juice samples was measured using the titration of samples with sodium hydroxide (0.1 N, Merck) in the presence of phenolphthalein (Merck) as an indicator and the results were reported based on g tartaric acid/100 mL juice (Campos et al., 2016). Total soluble solids (TSS) of samples were determined using a refractometer (Abbe, Bellingham-Stanley, London, England) at ~25 °C and the resulting data was reported as °Brix. Turbidity (NTU) was measured using a portable turbidimeter (Model

350 IR, Xylem Analytics Germany Sales GmbH & Co. KG, Munich, Bavaria, Germany).

The total phenol content (TPC) was determined using the Folin-Ciocalteu method. Juice or standard (0.5 mL) (GA, Sigma) was mixed with 2.5 mL of Folin-Ciocalteu reagent (Merck) (10% v/v) and 2 mL of sodium carbonate solution (Merck) (10% w/v). The mixtures were incubated for 1 h at 25 °C and, their absorbance measured at 750 nm. The results were expressed as mg GA mL⁻¹ juice sample (Kazemi et al., 2020). Antioxidant activity of the juice samples was measured using a DPPH (1,1-diphenyl-2-picrylhydrazyl) method as described by Kazemi et al. (2020). Briefly, 1 mL of juice sample was mixed with 4 mL of ethanolic DPPH solution (0.1 mM). After 30 min incubation in the dark, the absorbance was measured at 517 nm using the UV-Vis spectrophotometer. DPPH radical scavenging activity (DPPH-RS; %) was calculated according to the equation below:

$$\text{DPPH-RS (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (5)$$

where A_{control} = the absorbance of the control (water + DPPH) and A_{sample} = the absorbance of the sample (juice + DPPH).

The viscosity of juice samples was measured using a programmable viscometer (Model DV3TLVTJ0, Brookfield Ametek, Amherst, MA, USA) at a shear rate of 50 rpm at room temperature. The color parameters of L, a and b were determined using a color reader (Model CR-400, CHROMA METER, Konica Minolta Co., Tokyo, Japan). The pectin test was done by adding three volumes of ethanol to the samples and storing at 4 °C for 24 h. If pectin compounds were seen in the juice, such as a cloud, this suggested that the sample had pectin and the test is positive (Deng et al., 2019).

2.11. Statistical analysis

Data were statistically analyzed using one-way analyses of variance (ANOVA) with the Statistical Package for the Social Sciences (SPSS) version 16 (IBM software, New York, NY, USA). The significant differences were evaluated using the Duncan test at the 5% level.

3. Results and discussion

3.1. Influence of different flow rates with the immobilized pectinase activity

To evaluate the effect of flow rate (0.1–10 mL min⁻¹) on the immobilized pectinase activity, the reducing sugar production in the buffered pectin solution (1 mg mL⁻¹, pH 5.5, 50 °C) by the immobilized pectinase was studied in the continuous flow PBR. Fig. 1 shows that the production of reducing sugar was increased with a decrease in flow rate

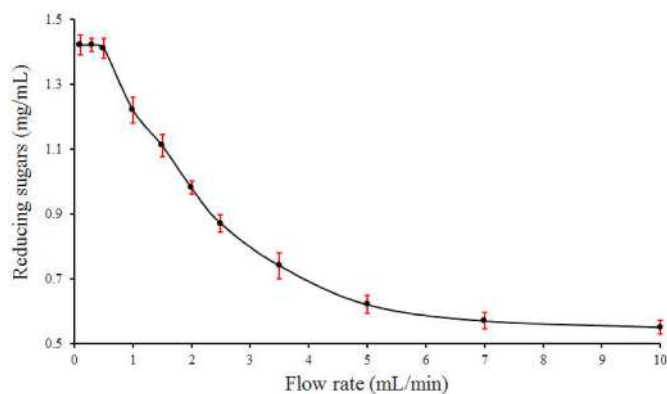


Fig. 1. The effect of flow rate on the production of reducing sugars in the buffered pectin solution (1 mg/mL, pH 5.5, 50 °C) using the PBR charged with glass bead-immobilized pectinase.

from 10 to 0.1 mL min⁻¹. This behavior was probably related to an increase in residence time (Ansari & Husain, 2010). However, as can be seen, with increasing the flow rate from 0.1 to 0.5 mL min⁻¹, the production efficiency did not change significantly and therefore, subsequent tests were done at a flow rate of 0.5 mL min⁻¹ (residence time of 21 min). However, in different studies, the various optimum flow rates were reported. For example, de Oliveira et al. (2018) reported that the best flow rate for a PBR charged with pectinase immobilized onto alginate beads was 10 mL min⁻¹. In another study, it was observed that the best flow rate for depectinization in a PBR with immobilized pectinase on polyacrylonitrile beads was 23 mL min⁻¹ (Diano et al., 2008). Also, the investigation of the effect of flow rate on the immobilized pullulanase activity indicated that by increasing the flow rate, the hydrolysis of pullulan was reduced (Singh et al., 2011). These results showed that bead size, reactor volume, support type and immobilization method had significant effects on the optimum flow rate of immobilized pectinase enzymes.

3.2. Influence of pH on the immobilized pectinase activity in the PBR

The effect of pH on the activity of the glass bead-immobilized pectinase in PBR was determined based on relative activity. Fig. 2A shows that the best pH for the immobilized pectinase was 5.5, while this factor

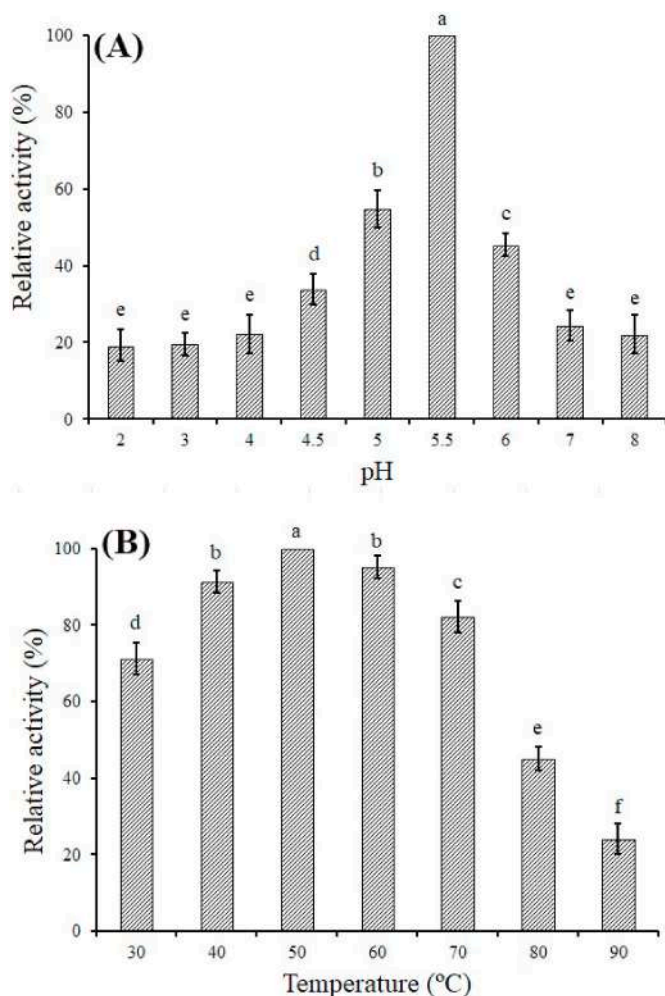


Fig. 2. The effect of pH and temperature on the immobilized pectinase activity. For pH, the target factor was varied, while temperature and flow rate were fixed at 50 °C and 0.5 mL min⁻¹ and for temperature, pH and flow rate were constant at 5.5 and 0.5 mL min⁻¹ a-f Different letters indicate significant differences ($p < 0.05$).

for the free enzyme was 5.0 (Hosseini et al., 2020). This difference in optimum pH was probably due to the charged surface of the glass beads and enzyme producing a charged microenvironment, which might affect the nature of the enzymes and thereby change the optimum pH of the immobilized enzyme (de Oliveira et al., 2018). Previous studies showed that the type of enzyme, the process conditions and the immobilization method can affect the optimum pH of enzymes (Rehman, Aman, et al., 2014). In a similar study, Diano et al. (2008) reported that the optimum pH was shifted from 5.6 for the free pectinases to 6.2 for the pectinases covalently immobilized on polyacrylonitrile beads. In another study, Li et al. (2007) reported that the optimum pH of the pectinase enzyme shifted from 3 to 3.5 after the immobilization process whereas, in another study, this factor did not change after enzyme immobilization on calcium alginate beads (Rehman et al., 2013). The study of the effect of pH on free and immobilized β -galactosidase on collagen showed that the optimal pH value for the free enzyme was 5.0, whereas this value for immobilized biocatalyst was 6.0 (Gennari et al., 2018).

3.3. Influence of temperature on the immobilized pectinase activity in the PBR

The temperature effects on the relative activity of the immobilized pectinase packed in PBR are shown in Fig. 2B. The temperature of 50 °C was the best and it was selected as the optimum temperature for further tests. No significant differences were found between the optimum temperature of the free and immobilized pectinase (Hosseini et al., 2020). The effect of this factor on the activity of various enzymes in the form of free or immobilized has been measured. Dal Magro et al. (2018) reported that the optimum temperature of the pectinase enzyme was shifted from 50 to 60 °C after immobilization on the glutaraldehyde-activated magnetite. In another study, the maximum activity of free pectinase was obtained at 45 °C but this parameter for immobilized enzyme on alginate beads was at 50 °C (de Oliveira et al., 2018). However, in another study, the optimum temperature of the pectinase enzyme did not change after immobilization in chitosan beads (Rehman, Nawaz, et al., 2014).

3.4. Specific activity of the free and immobilized pectinase

The specific activity of the free and immobilized pectinase was determined at optimal reaction conditions (temperature of 50 °C, pH of 5.0 for free enzyme and pH of 5.5 for immobilized pectinase). This parameter for the free and immobilized pectinase was 3.0 ± 0.2 and 2.7 ± 0.1 units mg⁻¹ respectively. The specific activity of the free enzyme was higher than the immobilized pectinase, which was possibly due to a decrease in substrate access to the active site of the immobilized enzyme (Hosseini et al., 2020). The results were higher than that of the immobilized pectinase on macroporous polyacrylamide microspheres (~ 1.4 unit mg⁻¹) (Lei & Jiang, 2011). In another study, the specific activity for the free and immobilized pectinase on silica-coated chitosan particles was reported as 1.7 and 1.0 unit mg⁻¹ which was lower than the current results (Lei & Bi, 2007).

3.5. The volumetric productivity of the free and the immobilized enzyme

The results for the volumetric productivity of the free and the immobilized pectinase for the batch and continuous operations are given in Table 1. Although the volumetric productivity of the free enzyme was significantly higher than that of the immobilized enzyme, no significant difference was observed between the batch and continuous operations. The increase or decrease in volumetric productivity is dependent on the type of enzyme and the immobilization method. For example, while this parameter for free pectinase was higher than immobilized form in the present study, Warmerdam et al. (2014) reported that the volumetric productivity of the immobilized galactosidase on Eupergit C 250 was higher than the free enzyme.

Table 1

The volumetric productivity of free and immobilized pectinase with batch and continuous operations.

Sample	Volumetric productivity (mg mL ⁻¹ min ⁻¹)
Free pectinase	0.08 ± 0.00 ^a
Immobilized pectinase (batch)	0.07 ± 0.00 ^b
Immobilized pectinase (continuous)	0.07 ± 0.00 ^b

^a Different letters in the same column indicate significant differences ($p < 0.05$).

^b Different letters in the same column indicate significant differences ($p < 0.05$).

3.6. Reusability of the immobilized pectinase

Fig. 3 shows the reusability of the immobilized pectinase in a batch operation. After 3 cycles, no significant decrease was observed in enzyme activity. Also, the glass bead immobilized pectinase maintained ~75, ~68 and ~59% of its initial activity after 7, 10 and 15 cycles. The results indicated that the glass bead immobilized pectinase had better reusability than the pectinase immobilized on the alginate beads using an entrapment method because de Oliveira et al. (2018) reported that the alginate beads immobilized pectinase lost ~70% of its activity after 8 cycles. Andriani et al. (2012) showed that the cellulose immobilized on calcium alginate using an entrapment method lost ~90% of its initial activity after 4 cycles. This difference is due to the immobilization method used because the previous studies reported that the reusability with the covalent immobilization was more than with the entrapment method (Hosseini et al., 2020).

3.7. Clarification of grape juice

To determine the best treatment time, clarification using free and immobilized pectinase was done for different times with optimum conditions and the results are shown in Fig. 4. This figure shows that the maximum clarity of the juice treated with the free enzyme and the immobilized pectinase in the PBR was achieved after 42 min and a further increase in process time had no significant effect on this parameter and therefore, it was selected as the best process time and the rest of the tests were done at optimum conditions on the grape juice clarified after the mentioned period. In several studies, the treatment time was considered as an important factor for the clarification of juice samples and the results were different depending on the type of juice, the immobilization method and treatment conditions. Chauhan et al. (2015) reported that maximum clarification of pineapple juice using immobilized pectinase on celite was obtained after 1 h. Also, an optimum recirculation time of 131 and 41 min was reported for apple juice clarified using the pectinase immobilized on polyacrylonitrile beads in a

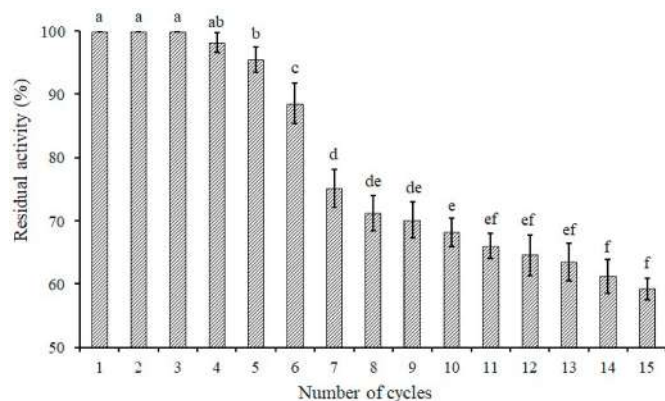


Fig. 3. Reusability of the immobilized pectinase on the glass beads with a batch operation. ^{a-f} Different letters indicate significant differences ($p < 0.05$).

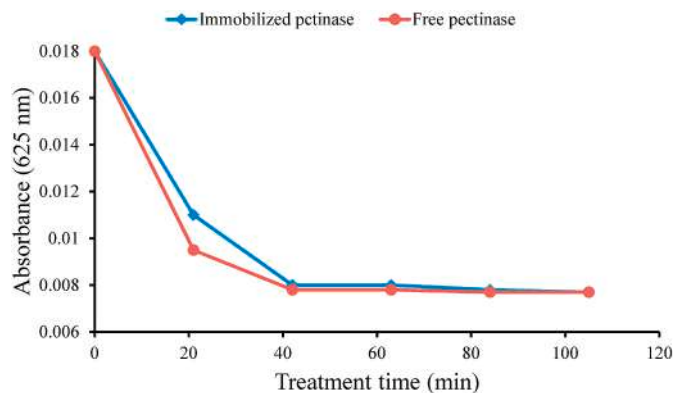


Fig. 4. The effect of treatment time on the clarification of the grape juice using the free enzyme and glass bead-immobilized pectinase packed in a PBR.

PBR and a fluidized reactor, respectively (Diano et al., 2008).

3.8. Physicochemical properties of grape juice samples

With the best conditions for free and immobilized pectinase at 50 °C for 42 min in batch and continuous at a flow rate 0.5 mL min⁻¹, grape juice was treated and compared with untreated samples.

Table 2 shows that the pH of juice samples decreased with clarification with the free and immobilized enzyme, while total titrable acidity (TTA) was increased. No significant difference was observed for juice samples clarified using the different enzymatic treatments. The results were consistent with data reported by Joshi et al. (2011) and de Oliveira et al. (2018) for pear and apple juice clarified using soluble pectinase and immobilized pectinase on alginate beads, respectively.

The use of free and immobilized pectinase leads to the drop of grape juice TSS as shown in Table 2. The results indicated that the differences between samples were significant. This decrease in TSS was probably related to the deposition of suspended compounds with pectin hydrolysis (Laorko et al., 2010). The same behavior was also observed for the apple juice clarified with immobilized pectinase on polyacrylonitrile beads (Diano et al., 2008).

Depending on the type of juice, turbidity can be a positive or a negative feature. For example, clarification of apple and grape juice is necessary while in orange juice, a little turbidity is a desirable feature. According to Sharma et al. (2014), pectin and other plant cell wall substances are the main cause of turbidity and therefore enzymatic hydrolysis of them may be necessary. Fig. 5 shows that the clarification

Table 2

pH, total titrable acidity (TTA; g tartaric acid/100 mL juice), total soluble solids (TSS; °Brix), turbidity (NTU), total phenolic content (TPC; mg GAE/mL juice sample), DPPH radical scavenging activity (DPPH-RS; %), viscosity (mPa.s), color parameters (L, a and b) and pectin test of the grape juice.

Parameter	Untreated juice	Clarified juice		
		FP ^A	IP (batch) ^B	IP (continuous) ^C
pH	3.62 ± 0.02 ^a	3.51 ± 0.01 ^b	3.54 ± 0.03 ^b	3.53 ± 0.02 ^b
TTA	0.2 ± 0.0 ^b	0.4 ± 0.0 ^a	0.3 ± 0.0 ^a	0.4 ± 0.0 ^a
TSS	10.0 ± 0.0 ^a	8.3 ± 0.0 ^c	9.1 ± 0.0 ^b	9.1 ± 0.0 ^b
Turbidity	46 ± 1 ^a	14 ± 1 ^d	28 ± 1 ^b	21 ± 2 ^c
TPC	1.5 ± 0.0 ^a	1.3 ± 0.0 ^b	1.4 ± 0.0 ^b	1.4 ± 0.0 ^b
DPPH-RS	94 ± 2 ^a	81 ± 2 ^b	85 ± 3 ^b	83 ± 1 ^b
Viscosity	1.4 ± 0.0 ^a	1.2 ± 0.0 ^b	1.2 ± 0.0 ^b	1.2 ± 0.0 ^b
L	20 ± 1 ^a	28 ± 1 ^b	26 ± 1 ^b	28 ± 1 ^b
a	-0.0 ± 0.5 ^b	1.6 ± 0.2 ^a	1.3 ± 0.4 ^a	1.5 ± 0.7 ^a
b	7.2 ± 0.1 ^a	6.5 ± 0.1 ^b	6.6 ± 0.2 ^b	6.6 ± 0.3 ^b
Pectin	Have	None	None	None

^{a-d} Different letters in the same row indicate significant differences ($p < 0.05$).

A: free pectinase; B: immobilized pectinase in batch operation; C: immobilized pectinase in continuous operation.

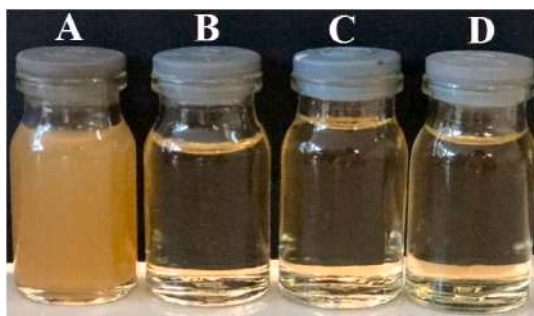


Fig. 5. The untreated (A) and the treated grape juice using free (B) and immobilized pectinase in batch (C) and continuous (D) systems.

of grape juice led to a decrease in turbidity. Table 2 shows that the lowest turbidity of treated samples was due to the grape juice clarified using the free enzyme. This factor for samples treated using the immobilized pectinase was acceptable and comparable with the free enzyme (Fig. 5). These observations were also consistent with reports of Sin et al. (2006), Chatterjee et al. (2004) and Pinelo et al. (2010) for different fruit juice samples.

The results of the TPC analysis in Table 2 shows that this parameter decreased after the clarification process. This can be related to the deposition of some phenolic compounds along with the suspended compounds after pectin hydrolysis. With decreasing TPC, the DPPH radical scavenging activity also decreased which was possibly attributed to a direct relationship of TPC with this property (Su & Silva, 2006). No significant difference was observed between the grape juice samples treated with different clarification methods. These results were consistent with those of previous studies (de Oliveira et al., 2018; Landbo et al., 2006; Sreenath & Santhanam, 1992).

Viscosity is an important parameter in fruit juice production and pectic materials and other soluble polysaccharides are the reason for their high viscosity. The pectin hydrolysis leads to a decrease in the water holding capacity of this polysaccharide and thereby an increase in free water and a decrease in viscosity (de Oliveira et al., 2018). Table 2 shows that the viscosity of the grape juice samples was reduced with clarification with free and immobilized pectinase in the batch and continues operations. A decrease in viscosity of fruit juice samples with the enzymatic clarification process was previously reported (Diano et al., 2008; Lee et al., 2006).

The color parameters of juice samples are one of the important parameters for sensory evaluation. *L* indicates the amount of lightness and should be as high as possible for clear juice (Abdullah et al., 2007). In Table 2, this parameter was increased after the clarification process of samples due to the removal of suspended substances (Sin et al., 2006). No significant difference was observed between the treated samples using free and immobilized enzymes showing the high efficiency of the immobilization process. Clarification led to an increase and a decrease in the *a* and *b* parameters, respectively. de Oliveira et al. (2018) observed similar results for apple juice clarified using immobilized pectinase with alginate beads in a PBR.

Table 2 shows that after clarification of juice samples with free and immobilized pectinase, the pectin test was negative, meaning that pectic polysaccharides were completely hydrolyzed. This showed that both the batch and continuous methods using free and immobilized pectinase had an acceptable potential for use in the clarification of grape juice (Deng et al., 2019).

4. Conclusion

The pectinase enzymes were efficiently immobilized on the functionalized glass beads using glutaraldehyde and then the optimum temperature and pH of the immobilized pectinase were compared with

the native one. A PBR charged with the glass beads immobilized pectinase was used to clarify grape juice. The results showed that the possible maximum clarification in PBR was obtained at a flow rate of 0.5 mL min^{-1} after 42 min. The investigation of the physicochemical properties of grape juice samples treated using free and immobilized pectinase in comparison with the untreated one showed that the system might be an alternative for commercial juice production.

CRediT authorship contribution statement

Seyedeh Zahra Azimi: Investigation, Methodology, Writing - original draft, Formal analysis. **Seyed Saeid Hosseini:** Investigation, Methodology, Formal analysis, Writing - review & editing. **Faramarz Khodaiyan:** Conceptualization, Supervision, Validation, Writing - review & editing.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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References

- Abdullah, A. L., Sulaiman, N. M., Aroua, M. K., & Noor, M. M. M. (2007). Response surface optimization of conditions for clarification of carambola fruit juice using a commercial enzyme. *Journal of Food Engineering*, 81(1), 65–71.
- Amin, F., Bhatti, H. N., Bilal, M., & Asgher, M. (2017). Improvement of activity, thermostability and fruit juice clarification characteristics of fungal exo-polygalacturonase. *International Journal of Biological Macromolecules*, 95, 974–984.
- Andriani, D., Sunwoo, C., Ryu, H. W., Prasetya, B., & Park, D. H. (2012). Immobilization of cellulase from newly isolated strain *Bacillus subtilis* TD6 using calcium alginate as a support material. *Bioprocess and Biosystems Engineering*, 35(1–2), 29–33.
- Ansari, S. A., & Husain, Q. (2010). Lactose hydrolysis by β galactosidase immobilized on concanavalin A-cellulose in batch and continuous mode. *Journal of Molecular Catalysis B: Biocatalytic*, 63(1–2), 68–74.
- Campos, P. R., Modenes, A. N., Espinoza-Quinones, F. R., Trigueros, D. E., Barros, S. T., & Pereira, N. C. (2016). Improvement on the concentrated grape juice physico-chemical characteristics by an enzymatic treatment and membrane separation processes. *Anais da Academia Brasileira de Ciências*, 88(1), 423–436.
- Capanoglu, E., de Vos, R. C., Hall, R. D., Boyacioglu, D., & Beekwilder, J. (2013). Changes in polyphenol content during production of grape juice concentrate. *Food Chemistry*, 139(1–4), 521–526.
- Chatterjee, S., Chatterjee, S., Chatterjee, B. P., & Guha, A. K. (2004). Clarification of fruit juice with chitosan. *Process Biochemistry*, 39(12), 2229–2232.
- Chauhan, S., Vohra, A., Lakhanpal, A., & Gupta, R. (2015). Immobilization of commercial pectinase (polygalacturonase) on celite and its application in juice clarification. *Journal of Food Processing and Preservation*, 39(6), 2135–2141.
- Dal Magro, L., Silveira, V. C., de Menezes, E. W., Benvenuti, E. V., Nicolodi, S., Hertz, P. F., Klein, M. P., & Rodrigues, R. C. (2018). Magnetic biocatalysts of pectinase and cellulase: Synthesis and characterization of two preparations for application in grape juice clarification. *International Journal of Biological Macromolecules*, 115, 35–44.
- Deng, Z., Wang, F., Zhou, B., Li, J., Li, B., & Liang, H. (2019). Immobilization of pectinases into calcium alginate microspheres for fruit juice application. *Food Hydrocolloids*, 89, 691–699.
- Diano, N., Grimaldi, T., Bianco, M., Rossi, S., Gabrovska, K., Yordanova, G., & Bencivenga, U. (2008). Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors. *Journal of Agricultural and Food Chemistry*, 56(23), 11471–11477.
- Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β -galactosidase immobilized on functionalized glass beads. *Food Chemistry*, 251, 115–124.
- Gennari, A., Mobayed, F. H., Volpato, G., & de Souza, C. F. V. (2018). Chelation by collagen in the immobilization of *Aspergillus oryzae* β -galactosidase: A potential biocatalyst to hydrolyze lactose by batch processes. *International Journal of Biological Macromolecules*, 109, 303–310.
- Ghafoor, K., Choi, Y. H., Jeon, J. Y., & Jo, I. H. (2009). Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from

- grape (*Vitis vinifera*) seeds. *Journal of Agricultural and Food Chemistry*, 57(11), 4988–4994.
- Hosseini, S. S., Khodaiyan, F., Mousavi, S. M. E., Azimi, S. Z., & Gharaghani, M. (2020). Immobilization of pectinase on the glass bead using polyaldehyde kefir as a new safe cross-linker and its effect on the activity and kinetic parameters. *Food Chemistry*, 309, 125777.
- Joshi, V. K., Parmar, M., & Rana, N. (2011). Purification and characterization of pectinase produced from apple pomace and evaluation of its efficacy in fruit juice extraction and clarification. *Indian Journal of Natural Products and Resources*, 2(2), 189–197.
- Kazemi, M., Khodaiyan, F., Labbafi, M., & Hosseini, S. S. (2020). Ultrasonic and heating extraction of pistachio by-product pectin: Physicochemical, structural characterization and functional measurement. *Journal of Food Measurement and Characterization*, 14(2), 679–693.
- Ko, C. Y., Liu, J. M., Chen, K. I., Hsieh, C. W., Chu, Y. L., & Cheng, K. C. (2018). Lactose-free milk preparation by immobilized lactase in glass microsphere bed reactor. *Food Biophysics*, 13(4), 353–361.
- Landbo, A. K. R., Pinelo, M., Vikbjerg, A. F., Let, M. B., & Meyer, A. S. (2006). Protease-assisted clarification of black currant juice: Synergy with other clarifying agents and effects on the phenol content. *Journal of Agricultural and Food Chemistry*, 54(18), 6554–6563.
- Laorko, A., Li, Z., Tongchitpakdee, S., Chantachum, S., & Youravong, W. (2010). Effect of membrane property and operating conditions on phytochemical properties and permeate flux during clarification of pineapple juice. *Journal of Food Engineering*, 100(3), 514–521.
- Lee, W. C., Yusof, S., Hamid, N. S. A., & Baharin, B. S. (2006). Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). *Journal of Food Engineering*, 73(1), 55–63.
- Lei, Z., & Bi, S. (2007). The silica-coated chitosan particle from a layer-by-layer approach for pectinase immobilization. *Enzyme and Microbial Technology*, 40(5), 1442–1447.
- Lei, Z., & Jiang, Q. (2011). Synthesis and properties of immobilized pectinase onto the macroporous polyacrylamide microspheres. *Journal of Agricultural and Food Chemistry*, 59(6), 2592–2599.
- Lettera, V., Pezzella, C., Cicatiello, P., Piscitelli, A., Giacobelli, V. G., Galano, E., & Sannia, G. (2016). Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification. *Food Chemistry*, 196, 1272–1278.
- Li, T., Wang, N., Li, S., Zhao, Q., Guo, M., & Zhang, C. (2007). Optimization of covalent immobilization of pectinase on sodium alginate support. *Biotechnology Letters*, 29(9), 1413–1416.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265–275.
- Mosayebi, V., & Tabatabaei Yazdi, F. (2018). Optimization of microwave assisted extraction (MAE) of pectin from black mulberry. *Journal of Food and Bioprocess Engineering*, 1(1), 57–66.
- Nakharat, P., & Haltrich, D. (2007). β -Galactosidase from *Talaromyces thermophilus* immobilized onto Eupergit C for production of galacto-oligosaccharides during lactose hydrolysis in batch and packed-bed reactor. *World Journal of Microbiology and Biotechnology*, 23(6), 759–764.
- de Oliveira, R. L., Dias, J. L., da Silva, O. S., & Porto, T. S. (2018). Immobilization of pectinase from *Aspergillus aculeatus* in alginate beads and clarification of apple and umbu juices in a packed bed reactor. *Food and Bioprocess Processing*, 109, 9–18.
- Panesar, R., Panesar, P. S., Singh, R. S., & Kennedy, J. F. (2011). Hydrolysis of milk lactose in a packed bed reactor system using immobilized yeast cells. *Journal of Chemical Technology and Biotechnology*, 86(1), 42–46.
- Pasandide, B., Khodaiyan, F., Mousavi, Z., & Hosseini, S. S. (2018). A Box-Behnken experimental design for microwave assisted extraction optimization of pectin from citron peel. *Journal of Food and Bioprocess Engineering*, 1(2), 149–156.
- Pinelo, M., Zeuner, B., & Meyer, A. S. (2010). Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. *Food and Bioprocess Processing*, 88(2–3), 259–265.
- Rehman, H. U., Aman, A., Silipo, A., Qader, S. A. U., Molinaro, A., & Ansari, A. (2013). Degradation of complex carbohydrate: Immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 using calcium alginate as a support. *Food Chemistry*, 139(1–4), 1081–1086.
- Rehman, H. U., Aman, A., Zohra, R. R., & Qader, S. A. U. (2014). Immobilization of pectin degrading enzyme from *Bacillus licheniformis* KIBGE IB-21 using agar-agar as a support. *Carbohydrate Polymers*, 102, 622–626.
- Rehman, H. U., Nawaz, M. A., Aman, A., Baloch, A. H., & Qader, S. A. U. (2014). Immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 on chitosan beads for continuous degradation of pectin polymers. *Biocatalysis and Agricultural Biotechnology*, 3(4), 282–287.
- Salim, D., Anwar, Z., Zafar, M., Anjum, A., Bhatti, K. H., & Irshad, M. (2018). Pectinolytic cocktail: Induced yield and its exploitation for lignocellulosic materials saccharification and fruit juice clarification. *Food Bioscience*, 22, 154–164.
- dos Santos, J. P., Dittgen, C. L., El Halal, S. L. M., & Vanier, N. L. (2019). Catalytic efficiency, structure, and recycling behavior of electrospun polyvinyl alcohol-xylanase fibers cross-linked by glutaraldehyde. *Food Biophysics*, 15, 155–161.
- Sharma, H. P., Patel, H., & Sharma, S. (2014). Enzymatic extraction and clarification of juice from various fruits - a review. *Trends Post Harvest Technol*, 2(1).
- Sheldon, R. A. (2007). Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis & Catalysis*, 349(8–9), 1289–1307.
- da Silva Haas, I. C., Toaldo, I. M., Gomes, T. M., Luna, A. S., de Gois, J. S., & Bordignon-Luiz, M. T. (2019). Polyphenolic profile, macro and microelements in bioaccessible fractions of grape juice sediment using *in vitro* gastrointestinal simulation. *Food Bioscience*, 27, 66–74.
- Singh, R. S., Saini, G. K., & Kennedy, J. F. (2011). Continuous hydrolysis of pullulan using covalently immobilized pullulanase in a packed bed reactor. *Carbohydrate Polymers*, 83(2), 672–675.
- Sin, H. N., Yusof, S., Hamid, N. S. A., & Rahman, R. A. (2006). Optimization of enzymatic clarification of sapodilla juice using response surface methodology. *Journal of Food Engineering*, 73(4), 313–319.
- Sojitra, U. V., Nadar, S. S., & Rathod, V. K. (2017). Immobilization of pectinase onto chitosan magnetic nanoparticles by macromolecular cross-linker. *Carbohydrate Polymers*, 157, 677–685.
- Sreenath, H. K., & Santhanam, K. (1992). The use of commercial enzymes in white grape juice clarification. *Journal of Fermentation and Bioengineering*, 73(3), 241–243.
- Su, M. S., & Silva, J. L. (2006). Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (*Vaccinium ashei*) by-products as affected by fermentation. *Food Chemistry*, 97(3), 447–451.
- Tapre, A. R., & Jain, R. K. (2014). Pectinases: Enzymes for fruit processing industry. *International Food Research Journal*, 21(2).
- Wang, M., Hua, X., Yang, R., & Shen, Q. (2016). Immobilization of cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus* on commercial resin Duolite A568. *Food Bioscience*, 14, 47–53.
- Warmerdam, A., Benjamins, E., de Leeuw, T. F., Broekhuis, T. A., Boom, R. M., & Janssen, A. E. (2014). Galacto-oligosaccharide production with immobilized β -galactosidase in a packed-bed reactor vs. free β -galactosidase in a batch reactor. *Food and Bioprocess Processing*, 92(4), 383–392.
- Yu, D., Zhang, X., Zou, D., Wang, T., Liu, T., Wang, L., & Jiang, L. (2018). Immobilized CALB catalyzed transesterification of soybean oil and phytosterol. *Food Biophysics*, 13(2), 208–215.