



Clarification of the pomegranate juice in a bioreactor packed by pectinase enzymes immobilized on the glass bead activated with polyaldehyde polysaccharides

Seyed Saeid Hosseini, Faramarz Khodaiyan^{*}, Seyed Mohammad Mousavi, Seyede Zahra Azimi

Bioprocessing and Biodetection Laboratory, Department of Food Science and Engineering, University of Tehran, Postal Code 31587-77871, Karaj, Iran

ARTICLE INFO

Keywords:

Pomegranate juice
Pectinase immobilization
Polyaldehyde polysaccharide
Continuous operation
Packed bed reactor

ABSTRACT

In this study, the glutaraldehyde, polyaldehyde pullulan and polyaldehyde kefiran were applied to immobilize the pectinase on the silanized glass bead and then a packed bed reactor (PBR) was used to continuous clarification process of the pomegranate juice. The results showed that the bioreactor packed with the enzymes immobilized by all three cross-linkers had the best performance at the temperature of 50 °C, a flow rate of 0.5 mL/min and treatment time of 42 min. After the clarification process, the turbidity of the pomegranate juice was significantly decreased from ~70.2 to ~17.4 NTU and the clarity of the samples was increased from ~81.15 to ~96.13%. The findings showed the clarification process had a significant effect on the pomegranate juice properties so that the total titratable acidity, reducing sugar content and lightness parameter were increased while pH, total soluble solids, viscosity, total phenol content and radical scavenging activity of the juice samples was decreased by this process. Finally, it must be stated that the bioreactor packed with polyaldehyde kefiran-immobilized pectinase had the best performance in the clarification of pomegranate juice due to the higher capacity of this cross-linker in the pectinase immobilization.

1. Introduction

Pectinase is a group of enzymes that can catalyze the hydrolysis reaction of pectin as one of the main structural polysaccharide presented in cell walls of all plants (Asgari, Labbafi, Khodaiyan, Kazemi, & Hosseini, 2020; Kazemi, Khodaiyan, Labbafi, & Hosseini, 2020). This group, which usually include three enzymes of pectin lyase, polygalacturonase and pectinesterase catalyzing the hydrolysis of pectin, polygalacturonic acid and the pectin ester bonds, has numerous industrial applications in the tea and coffee fermentation, animal feed production, agro-industrial waste treatment, fruit juice clarification, etc. (Amin, Bhatti, Bilal, & Asgher, 2017). Most of these processes can also be done by chemical methods, but the reason for preference of the enzymes is due to their economically, eco-friendly and high specificity, selectivity and activity in the mild conditions (Goh et al., 2012). However, despite these potential benefits, the use of free enzymes is also associated with limitations that the most important of them are low stability, the difficulty of recovery and lack of recyclability (Rehman, Aman, Zohra, & Qader, 2014). To overcome these limitations, enzyme immobilization has been widely considered by researchers as a strategy for the increase in catalyst

stability, operation control, recyclability and also the possibility of the continuous process by different bioreactors (Amin et al., 2017; Sheldon, 2007).

In the immobilization process, the selection of a suitable support and immobilization method is necessary to achieve high enzymatic process efficiency (Dal Magro et al., 2019). So far, various supports and protocols were used to immobilize different enzymes such as pectinase immobilization in agar-agar matrix or calcium alginate beads using entrapment technique (Rehman et al., 2013, 2014a), the covalent immobilization of pectinase in chitosan beads by formaldehyde and/or onto the macroporous polyacrylamide microspheres by glutaraldehyde (Lei & Jiang, 2011; Rehman, Nawaz, Aman, Baloch, & Qader, 2014) and the covalent immobilization of β -galactosidase, peroxidases and/or pectinase on functionalized glass beads by glutaraldehyde (Diano et al., 2008; Eskandarloo & Abbaspourrad, 2018; Gomez et al., 2006).

Among different support, glass beads are excellent due to their insolubility, stability and resistance to the harsh conditions in the continuous process (Hosseini, Khodaiyan, Mousavi, Azimi, & Gharaghani, 2020). To use the glass bead as a support, it is necessary to make changes in its surface so that the enzyme can be immobilized on it. For

^{*} Corresponding author.

E-mail address: khodaiyan@ut.ac.ir (F. Khodaiyan).

this purpose, in most studies, 3-aminopropyl triethoxysilane (3-APTES) was first used to form free amine groups on the glass surface, and then glutaraldehyde was applied to bind the enzyme on the functionalized glass beads. Although glutaraldehyde is a suitable cross-linker with high immobilization capacity, it is very toxic and therefore its industrial application can be disturbing. In previous studies, we used from the polyaldehyde pullulan and polyaldehyde kefiran (produced by the reaction between the mentioned polysaccharides and sodium periodate) as new safe cross-linkers for immobilization of pectinase enzymes on the functionalized glass beads and the obtained results showed that in addition to desirable performance, they were able to bind the more enzymes on the glass surface than glutaraldehyde (Hosseini, Khodaiyan, Mousavi, Azimi, & Gharaghani, 2020; Hosseini, Khodaiyan, Mousavi, Kennedy, & Azimi, 2020). However, in all our previous studies tests, pectin solution and batch system were used to examine the performance of the immobilized pectinase by the mentioned cross-linkers while the assurance is obtained when the mentioned system can lead to the clarification of a fruit juice in a continuous process which is highly preferred in relevant industries.

Pomegranate (*Punica granatum* L.), as a health-promoting fruit, is widely consumed in the form of fresh fruit and juice. The pomegranate juice is rich in valuable compounds that display functional and medicinal effects such as antioxidant, antimicrobial, antitumoral and antidiabetic properties (Viuda-Martos, Fernandez-Lopez, & Perez-Alvarez, 2010). The pomegranate peel is rich in pectin that enters the fruit juice during juicing and leads to create the undesirable turbidity and sediment in the product (Cerreti et al., 2017; Javanmarddakheli & Akbari, 2020). Therefore, the clarification is a necessary stage for the production of a consumer-friendly pomegranate juice (Aliasghari Aghdam, Mirsaeedghazi, Aboonajmi, & Kianmehr, 2018).

Considering the importance of the continuous process and also the use of safe cross-linkers for pectinase immobilization in the industrial clarification operation of fruit juice, in the current study, pectinase enzymes were first immobilized on the functionalized glass beads by glutaraldehyde and/or polyaldehyde polysaccharides (pullulan and kefiran) and then a packed bed reactor (PBR) filled with the mentioned glass beads was applied for the continuous clarification of the pomegranate juice. Also, the physicochemical, antioxidant and color properties of the pomegranate juice samples clarified by free and immobilized pectinases were compared with each other and with raw juice.

2. Materials and methods

2.1. Materials

The pullulan and kefiran were provided by Herbstreith & Fox KG (Germany) and Department of Food Science and Engineering, University of Tehran (Karaj, Iran), respectively. Glass beads with a diameter of 6 mm were obtained from Iran Beads Co. (Esfahan, Iran). Apple pectin (USP grade and DE of 72–76%) was supplied by Herbstreith & Fox KG (Germany). 3-aminopropyl triethoxysilane (3-APTES), nitric acid, 3,5-dinitrosalicylic acid (DNS), Sodium periodate, hydroxylamine hydrochloride, glutaraldehyde, D-(+)-galacturonic acid monohydrate, DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used in the current study were of analytical grade.

The Pectinex® Ultra Color enzyme with protein content of ~80 mg/mL (pectin lyase = ~96.77% of the total enzyme content and polygalacturonase = ~3.23% of the total enzyme content) was bought from Novozymes (Bagsvaerd, Denmark). The specific activity of the mentioned enzyme was 2.93 ± 0.13 unit/mg protein under the obtained optimum conditions (50 °C and pH 5).

2.2. Polyaldehyde polysaccharide production

In this section, sodium periodate was used to oxidize the pullulan and kefiran, and thereby the production of the polyaldehyde polysaccharide (Guo, Ge, Li, Mu, & Li, 2014). 100 mL of 5% (w/v) sodium periodate solution was added to 250 mL of 4% (w/v) polysaccharide solution and the pH was set to 5 using 1 M sulfuric acid. The oxidation reaction was performed for 3 h at ~40 °C in the dark condition. Afterward, the cold ethanol was added in a ratio of 2 to 1 (v/v) and the obtained mixture was stored at ~7 °C for 3 days. The obtained supernatant was separated by centrifuge at $10,000 \times g$ for 20 min, and after washing with distilled water and ethanol, it was freeze-dried for 1 day. The aldehyde content of oxidized pullulan and kefiran was 19.9 ± 1.3 and $23.6 \pm 0.9\%$ according to the method described by Kholiya, Chaudhary, Vadodariya, and Meena (2016).

2.3. Pectinase immobilization

The immobilization of pectinase on the glass beads by different cross-linkers was done by the method explained by Eskandarloo and Abbaspourrad (2018) with some modifications. This protocol was including four general stages:

- (I) Washing 23 cm^3 glass beads (~110 beads) using 5% nitric acid (85 °C, 120 min) and drying (110 °C, 24 h).
- (II) Immersing the glass beads in 10% (v/v) 3-APTES aqueous solution at pH adjusted by 1 N HCl (pH 4, 60 °C, 180 min) and drying (80 °C, 24 h).
- (III) Immersing the functionalized glass beads in 2.5% (v/v or w/v) of glutaraldehyde or polyaldehyde pullulan and/or polyaldehyde kefiran (23 °C, 2.5 h) and washing with distilled water.
- (IV) Immersing the active glass beads in enzyme solution diluted by sodium acetate buffer at a ratio of 1–4 v/v (pH 4, 23 °C, 5 h) and washing with the same buffer.

After immobilization, the protein content of glass beads was 4.51 ± 0.68 , 20.13 ± 0.98 and 57.75 ± 1.12 mg/ 23 cm^3 glass beads for enzymes immobilized by glutaraldehyde, polyaldehyde pullulan and polyaldehyde kefiran according to Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

Besides, the pectinase enzymes immobilized by glutaraldehyde, polyaldehyde pullulan and kefiran had the specific activities of 2.56 ± 0.12 , 2.21 ± 0.18 and 1.87 ± 0.24 unit/mg protein based on the DNS method described in the previous study (Hosseini, Khodaiyan, Mousavi, Kennedy, & Azimi, 2020). Note that one unit was the pectinase content required to produce 1 μmole of galacturonic acid per minute under the test conditions.

2.4. Pomegranate juice production

The pomegranates were obtained from a farm in Bootedeh village, Chamestan, Mazandaran, Iran. The fruits were washed, were cut in half and were pressed by a manual press machine, respectively. Afterward, the obtained raw juice was filtered through a Whatman paper and was stored in a refrigerator until use.

2.5. Packed bed reactor (PBR)

A jacketed glass cylinder (inner diameter 1.4 cm and length of 15 cm) packed with 23 cm^3 of enzyme-immobilized glass beads (~110 beads) was used to create a continuous process in which the buffered pectin solution (1% w/v, pH 3.4 by citrate buffer) or pomegranate juice (pH 3.35) was entered from the bottom and the hydrolyzed pectin solution or clarified juice was exited from the top while the water with a certain temperature was circulated in the outer layer. It should also be noted that the void volume (~10.65 cm^3) of PBR was calculated by subtracting

the actual volume of 23 cm³ glass beads (~12.43 cm³) from the total volume of PBR (~23.08 cm³).

2.6. Effect of clarification variables on the PBR efficiency

One factor at a time was applied to evaluate the effect of temperature, flow rate and treatment time on the performance of the bioreactor packed with the glass bead-immobilized enzymes. For this purpose, the investigated factor was varied, while the other effective factors were constant (for temperature: temperature of 30–90 °C and flow rate of 0.5 mL/min; for flow rate: flow rate of 0.1–10 mL/min and temperature of 50 °C; and for treatment time: time of 0–105 min, the temperature of 50 °C and flow rate of 0.5 mL/min). It should also be noted that the residence time in each flow rate can be easily calculated by dividing the void volume of bioreactor by the flow rate.

In all the experiments mentioned in this section, the pomegranate juice was considered as substrate and the juice turbidity (NTU), which was evaluated using a portable turbidimeter (WTW, 350 IR, Weilheim, Germany), was recorded as the response.

2.7. Volumetric productivity

In this section, the volumetric productivity of the pectinase enzyme, which is defined as mg/mL of the reducing sugar produced per min, was determined for both free and immobilized enzymes. In the batch system, 0.56 µL of pectinase solution and/or 23 cm³ of pectinase enzymes immobilized on the glass beads by glutaraldehyde or polyaldehyde polysaccharides was immersed in 10.65 mL of 1% (w/v) buffered pectin solution (pH of 3.4 ≈ pH of the tested juice) and the hydrolysis reaction was done at a temperature of 50 °C and treatment time of 21 min. In the continuous system, this parameter was determined according to the following equation (Singh, Saini, & Kennedy, 2011):

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

where *VP* is the volumetric productivity, *R* is the amount of the produced reducing sugar (mg/mL), *F* is the flow rate (mL/min) and the *V* is the void volume of the PBR (mL).

It should also be noted that reducing sugar in the above tests was measured based on the DNS method as reported by Miller (1959). For this purpose, 1 mL of DNS reagent was added to 1 mL of the reaction medium in a test tube and then the obtained solution was immediately heated for 5 min in boiling water and was cooled in an ice-water bath. After determining the absorbance at 540 nm, the reducing sugar content (mg/mL) was calculated by comparison of the obtained absorbance with the standard curve of D-galacturonic acid.

Table 1
The clarification treatments applied in this study.

Run	Treatment type	Abbreviation
1	Untreated pomegranate juice (control sample)	UPJ
2	Pomegranate juice clarified by the free pectinase	PPF
3	Pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a batch process	PIPG _b
4	Pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a continuous process	PIPG _c
5	Pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a batch process	PIPP _b
6	Pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a continuous process	PIPP _c
7	Pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefirin in a batch process	PIPK _b
8	Pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefirin in a continuous process	PIPK _c

2.8. Pomegranate juice clarification

As can be seen in Table 1, different batch and continuous treatments were applied to clarify the pomegranate juice under the optimal conditions previously achieved in this study. 56 µL free enzyme solution or 23 cm³ of the enzyme-immobilized glass beads were added to 10.65 mL pomegranate juice and the batch clarification process was continued at 50 °C for 42 min. On the other hand, and for continuous clarification, the pomegranate juice was clarified by a PBR at 50 °C for 42 min with a flow rate of 0.5 mL/min.

2.9. Untreated and treated pomegranate juice properties

2.9.1. Turbidity and clarity

The turbidity (NTU) of different samples was obtained using a turbidimeter (WTW, 350 IR, Weilheim, Germany) at room temperature. To determine the clarity, a spectrophotometer at 660 nm was used and the obtained data of the samples diluted with distilled water (five times) was reported as the transmittance percentage (%T). The following formula was used to calculate %T:

$$\%T = 10^{(2 - \text{Absorbance})} \quad (2)$$

2.9.2. pH and acidity

The pH of non-clarified and clarified juice samples was determined by a pH meter (GLP 22, Crison, Spain). Also, total titratable acidity (TTA) was evaluated by titration of juice samples with 0.1 N sodium hydroxide. In this experiment, the phenolphthalein reagent was applied as an indicator (Campos et al., 2016) and the obtained results were reported based on g citric acid per 100 mL pomegranate juice.

2.9.3. Total soluble solids (TSS) and reducing sugar content

The TSS (°Brix) was recorded by a refractometer (Abbe, Bellingham-Stanley, London, England) at ~23 °C. The reducing sugar content (mg/mL) of different juice samples was evaluated by the DNS method as described above.

2.9.4. Viscosity

The viscosity of different juice samples was measured by a viscometer (Brookfield Ametek, DV3TLVTJ0, USA) at a shear rate of 50 rpm and room temperature (Haghighatpanah et al., 2020).

2.9.5. Pectin presence test

This test was done by mixing the cold ethanol with different fruit juice sample in a ratio of 2:1 v/v and storing at refrigerator (~4 °C) for 24 h. After this time, the presence or absence of supernatant shows the presence or absence of pectin, respectively.

2.9.6. Total phenol content (TPC)

The TPC of fruit juice samples was measured by the Folin-Ciocalteu method (Kazemi, Khodaiyan, Hosseini, & Najari, 2019). For this purpose, 0.1 mL of fruit juice sample was mixed with 0.5 mL of Folin-Ciocalteu reagent solution (10% v/v) and 0.4 mL of sodium carbonate solution (10% w/v) and the obtained mixture was stored for 1 h in darkness. After recording the absorbance at 517 nm, the TPC was calculated by comparison of sample absorbance with the gallic acid standard curve (10–100 µg/mL). Therefore, the TPC was expressed as mg gallic acid equivalent per mL pomegranate juice sample (mg GAE/mL).

2.9.7. Antioxidant activity determination

In this section, ABTS and DPPH radical scavenging activity of the pomegranate juice were analyzed according to the method described by Kazemi et al. (2019) with some modification. At first, for preparation of ABTS solution, an ABTS solution (7.4 mM) was mixed with a potassium persulfate solution (2.6 mM) and the resulting mixture was stored in

darkness for 18 h and subsequently, its absorbance (at 734 nm) was adjusted to 0.7 ± 0.02 by diluting with distilled water. In the next step, 0.1 mL of the diluted pomegranate sample (50 times with distilled water) was added to 1.9 mL of the obtained ABTS solution and/or DPPH ethanolic solution (0.1 mM) and after vortexing, the obtained mixtures were stored for 45 min in darkness to complete the antiradical reaction. After recording the absorbance at 734 nm for ABTS radical scavenging activity (ABTS-RSA) and 517 nm for DPPH radical scavenging activity (DPPH-RSA), these antiradical activities were calculated as follows:

$$\begin{aligned} \text{ABTS - RSA (\%)} \\ \text{or} \\ \text{DPPH - RSA (\%)} \end{aligned} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100 \quad (3)$$

2.9.8. Color parameters

The parameters *L*, *a* and *b* were determined using a color reader (CHROMA METER, CR-400, Japan) at $\sim 23^\circ\text{C}$.

2.10. Statistical procedure

In the current study, to determine the existence of significant differences between the samples ($p < 0.05$), one-way ANOVA was applied using SPSS software v. 16. Each data was the mean of three replicates $\pm$ SD (standard deviation of the mean).

3. Results and discussion

3.1. Effect of temperature on the immobilized pectinase performance in the PBR

Fig. 1a showed the effect of temperature on the clarification of pomegranate juice using the pectinase immobilized by different cross-linkers in the PBR. In this experiment, the variable was the temperature ($30\text{--}90^\circ\text{C}$) and the response was fruit juice turbidity (NTU) while the flow rate was constant in 0.5 mL/min . The mentioned figure showed that in all temperatures, the turbidity of the juice clarified by polyaldehyde kefir-immobilized pectinase was lower than the enzymes immobilized by two other cross-linkers. These observations were possible due to the higher amount of the enzyme immobilization by polyaldehyde kefir as stated earlier (the protein content was 4.51 ± 0.68 , 20.13 ± 0.98 and $57.75 \pm 1.12\text{ mg/23 cm}^3$ glass beads for enzymes immobilized by glutaraldehyde, polyaldehyde pullulan and polyaldehyde kefir). Also, the obtained results indicated that in all three treatments, the turbidity was lowest at 50°C which this result is related to the optimal temperature of the immobilized pectinase enzymes because, in previous studies, it was shown that the maximum activity of the pectinase enzymes immobilized by the mentioned cross-linkers was obtained at 50°C (Hosseini, Khodaiyan, Mousavi, Azimi, & Gharaghani, 2020). Therefore, all other experiments were done at a temperature of 50°C .

3.2. Effect of flow rate on the immobilized pectinase performance in the PBR

Fig. 1b indicated the effect of different flow rates ($0.1\text{--}10\text{ mL/min}$) on the clarification of pomegranate juice while the temperature was considered constant at 50°C . As can be seen, the turbidity of juice samples was decreased when the flow rate was decreased from 10 (residence time of 1.065 min) to 0.1 (residence time of 106.5 min) mL/min. The decrease in turbidity with reducing the flow rate was probably related to an increase in the possibility of contact between the active site of enzymes and the pectin of fruit juice as a substrate (Ansari & Husain, 2010). However, as can be seen, no significant difference was observed for turbidity of juice samples in flow rates $0.1\text{--}0.5\text{ mL/min}$ and therefore the other tests on the fruit juice were performed at flow rate 0.5 mL/min (residence time of 21 min). Besides, the mentioned figure showed that

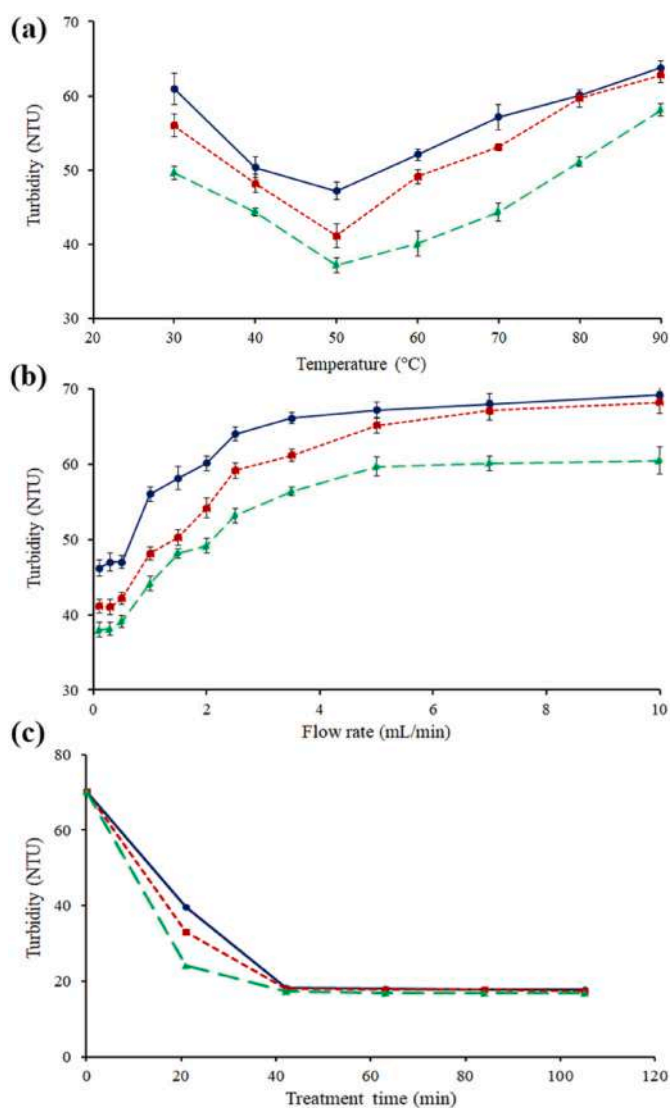


Fig. 1. (a) The effect of temperature on the packed bed reactor efficiency at flow rate of 0.5 mL/min . (b) The effect of flow rate on the packed bed reactor efficiency at temperature of 50°C . (c) The effect of treatment time on the packed bed reactor efficiency at temperature of 50°C and flow rate of 0.5 mL/min (Circle) bioreactor packed with pectinase enzymes immobilized by glutaraldehyde, (Square) bioreactor packed with pectinase enzymes immobilized by polyaldehyde pullulan and (Triangle) bioreactor packed with pectinase enzymes immobilized by polyaldehyde kefir.

the turbidity of the juice samples clarified by polyaldehyde kefir-immobilized pectinase enzymes was lower than that by pectinase immobilized using two other cross-linkers which was related to more immobilization of pectinase enzymes by the mentioned cross-linker as stated earlier.

3.3. Effect of treatment time on the immobilized pectinase performance in the PBR

Fig. 1c illustrated the influence of the treatment time on the turbidity of pomegranate juice samples. In this experiment, the treatment time was varied from 0 to 105 min while the temperature and flow rate were constant at 50°C and 0.5 mL/min , respectively. The obtained results indicated that the turbidity of juice samples was significantly decreased with increasing the treatment time from 0 to 42 min for the bioreactor packed with pectinase enzymes immobilized by all three cross-linkers. This observation may be due to an increase in contact time between

enzyme and substrate with increasing the treatment time (de Oliveira, Dias, da Silva, & Porto, 2018). However, the further increase in treatment time had no significant effect on this parameter and therefore the other experiments on the fruit juice samples were performed after 42 min clarification.

3.4. Volumetric productivity

In this step, the volumetric productivity of the free pectinase and the enzymes immobilized by different cross-linkers in batch and continuous processes was determined and the obtained data were shown in Table 2. As can be seen, the highest volumetric productivity was due to the pectinase immobilized by polyaldehyde kefirin (~ 0.085 and ~ 0.089 mg/mL.min for batch and continuous processes, respectively) and the pectinase enzymes immobilized by polyaldehyde pullulan and glutaraldehyde were in second and third places. The higher volumetric productivity for polyaldehyde kefirin-immobilized pectinase enzymes was probably due to the presence of more enzymes involved in the reaction when this cross-linker was applied to immobilization process as stated earlier (the protein content was 4.51 ± 0.68 , 20.13 ± 0.98 and 57.75 ± 1.12 mg/23 cm³ glass beads for enzymes immobilized by glutaraldehyde, polyaldehyde pullulan and polyaldehyde kefirin). Besides, the volumetric productivity of the polyaldehyde kefirin-immobilized pectinase enzymes in continuous operation was higher than that in the batch operation which may be related to the more contact between the active site of the immobilized pectinase and the pectin as the substrate in the continuous operation. Table 2 showed that in similar protein content (free enzymes and enzymes immobilized by glutaraldehyde), the volumetric productivity of free pectinase was more than the immobilized one which can be related to the negative effect of immobilization process on the enzyme activity as reported in other studies (Hosseini, Khodaiyan, Mousavi, Azimi, & Gharaghani, 2020; Lei & Jiang, 2011).

3.5. Effect of clarification on the pomegranate juice characterization

After clarification of the pomegranate by different treatments in optimal conditions (temperature of 50 °C and clarification time of 42 min for the batch operation and temperature of 50 °C, flow rate of 0.5 mL/min and clarification time of 42 min for the continuous operation), the different properties of the clarified samples were studied and the obtained data were compared with each other and with the raw juice sample.

3.5.1. Turbidity and clarity

As can be seen in Table 3 and Fig. 2, the turbidity of the clarified samples was significantly lower than the raw sample so that this

parameter was decreased from ~ 70.2 to ~ 17.4 after the clarification process. Also, as expected, the clarity was significantly increased from 81.15 to 96.13% with decreasing turbidity. Also, the obtained results (Table 3) clearly showed that the immobilization method used in this study (the use of polyaldehyde kefirin and polyaldehyde pullulan as cross-linker) had a very good performance in comparison with the conventional method (the use of glutaraldehyde as cross-linker). Besides, the resulting data indicated that the performance of the continuous operation was much better than the batch process. The obtained results were in line with the published data for blueberry juice and apple juice clarified using the enzymatic clarification process by Sandri, Lorenzoni, Fontana, and da Silveira (2013) and de Oliveira et al. (2018), respectively.

3.5.2. pH and acidity

Table 3 illustrated that the pH of the fruit juice samples was significantly decreased and the total titratable acidity (TTA) was increased after clarification. These observations were probably related to the pectin hydrolysis and liberation of galacturonic acids after enzymatic clarification (Sharma, Patel, & Sharma, 2014). Also, the obtained results were in agreement with the reported data for the pear juice and apple juice clarified using the enzymatic process by Joshi, Parmar, and Rana (2011) and Deng et al. (2019), respectively.

3.5.3. Total soluble solids (TSS) and reducing sugar content

The results of TSS measurement in Table 3 indicated that this factor was decreased by the clarification process which can be due to the deposition of solids compounds after the destruction of the network formed by the free and the immobilized pectinase enzymes. The findings were in accordance with the obtained results by Diano et al. (2008) for the apple juice treated with the immobilized pectinase. Also, Table 3 showed that the reducing sugar content was significantly increased after the pomegranate juice treatment by the free and immobilized pectinase which was possibly related to the liberation of the reducing sugars after the pectin hydrolysis.

3.5.4. Viscosity

Pectin is a polysaccharide with a high water holding capacity that can create a cohesive network structure in the fruit juice. Therefore, its hydrolysis can decrease this property, liberate the trapped water into the fruit juice medium and reduce the viscosity (Sin, Yusof, Sheikh Abdul Hamid, & Rahman, 2006). Table 3 showed that the clarification process decreased significantly the viscosity of the juice samples which was in line with the reported data by Diano et al. (2008) and de Oliveira et al. (2018) for the apple juice and umbu juice clarified using the immobilized pectinase, respectively.

3.5.5. Pectin presence test

Table 3 indicated that the pectin presence test was negative in all treated fruit juice samples while was positive in the raw sample. These results showed that the hydrolysis reaction of the pectin was complete by both the free enzyme and the pectinase immobilized by the different cross-linkers.

3.5.6. Total phenol content (TPC)

The results of TPC in Table 4 showed that this parameter was decreased after the enzymatic clarification process which was in agreement with the results obtained by Landbo, Pinelo, Vikbjerg, Let, and Meyer (2006) and Diano et al. (2008) for the enzymatic clarification of the black currant juice and the apple juice, respectively. Generally, the pectin hydrolysis can lead to the deposition of some compounds such as phenolics and thereby decrease their content in fruit juice. Also, the presence of secondary enzymatic activities can change the phenolic compounds profile which the result of both events is a negative effect on antioxidant properties as explained below (Pinelo, Arnous, & Meyer, 2006).

Table 2

The volumetric productivity of free and pectinase immobilized by different cross-linkers in the batch and continuous operations.

Treatments	Volumetric productivity (mg/mL.min)
FE	0.076 ± 0.001^c
GB	0.068 ± 0.002^d
GC	0.070 ± 0.003^d
PB	0.078 ± 0.002^c
PC	0.079 ± 0.003^c
KB	0.085 ± 0.002^b
KC	0.089 ± 0.001^a

FE: free pectinase; GB: pectinase immobilized by glutaraldehyde in the batch operation; GC: pectinase immobilized by glutaraldehyde in the continuous operation; PB: pectinase immobilized by polyaldehyde pullulan in the batch operation; PC: pectinase immobilized by polyaldehyde pullulan in the continuous operation; KB: pectinase immobilized by polyaldehyde kefirin in the batch operation; KC: pectinase immobilized by polyaldehyde kefirin in the continuous operation.

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

Table 3

Turbidity (NTU), Clarity (%T), pH, total titratable acidity (TTA; g citric acid/100 mL juice), total soluble solids (TSS; °Brix), reducing sugar content (RSC; mg/mL), viscosity (mPa.s) and pectin presence test of the untreated and treated pomegranate juice.

Parameters	Turbidity	Clarity	pH	TTA	TSS	RSC	Viscosity	Pectin test
UPJ	70.2 ± 0.3 ^a	81.15 ± 0.13 ^c	3.35 ± 0.01 ^a	1.08 ± 0.02 ^f	18.5 ± 0.0 ^a	56.31 ± 0.08 ^d	2.14 ± 0.08 ^a	Have
PFP	18.1 ± 0.3 ^b	92.13 ± 0.16 ^b	2.99 ± 0.02 ^d	1.24 ± 0.02 ^d	16.0 ± 0.0 ^d	61.27 ± 0.11 ^c	1.90 ± 0.04 ^b	None
PIPG _b	18.3 ± 0.1 ^b	92.07 ± 0.14 ^b	3.17 ± 0.04 ^b	1.14 ± 0.03 ^e	18.0 ± 0.0 ^b	61.00 ± 0.26 ^c	1.92 ± 0.07 ^b	None
PIPG _c	18.3 ± 0.2 ^b	92.11 ± 0.12 ^b	3.08 ± 0.01 ^c	1.17 ± 0.04 ^e	17.5 ± 0.0 ^c	61.21 ± 0.23 ^c	1.92 ± 0.06 ^b	None
PIPP _b	18.1 ± 0.2 ^b	92.11 ± 0.13 ^b	2.97 ± 0.03 ^d	1.22 ± 0.01 ^d	16.0 ± 0.0 ^d	62.98 ± 0.13 ^b	1.90 ± 0.07 ^b	None
PIPP _c	18.2 ± 0.1 ^b	92.14 ± 0.10 ^b	2.90 ± 0.02 ^e	1.29 ± 0.02 ^c	16.0 ± 0.0 ^d	63.13 ± 0.32 ^b	1.88 ± 0.04 ^b	None
PIPK _b	17.6 ± 0.3 ^c	96.21 ± 0.16 ^a	2.87 ± 0.01 ^f	1.37 ± 0.03 ^b	15.0 ± 0.0 ^e	67.21 ± 0.12 ^a	1.73 ± 0.05 ^c	None
PIPK _c	17.4 ± 0.2 ^c	96.13 ± 0.12 ^a	2.81 ± 0.02 ^g	1.43 ± 0.01 ^a	14.5 ± 0.0 ^f	67.14 ± 0.06 ^a	1.67 ± 0.06 ^c	None

UPJ: untreated pomegranate juice; PFP: pomegranate juice clarified by the free pectinase; PIPG_b: pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a batch process; PIPG_c: pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a continuous process; PIPP_b: pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a batch process; PIPP_c: pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a continuous process; PIPK_b: pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefirin in a batch process; PIPK_c: pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefirin in a continuous process.

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



Fig. 2. The effect of clarification process by the free and immobilized pectinase in the batch and continuous operations on the pomegranate juice. UPJ: untreated pomegranate juice; PFP: pomegranate juice clarified by the free pectinase; PIPG_b: pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a batch operation; PIPG_c: pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a continuous operation; PIPP_b: pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a batch operation; PIPP_c: pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a continuous operation; PIPK_b: pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefirin in a batch operation; PIPK_c: pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefirin in a continuous operation.

3.5.7. Antioxidant properties

To determine the antioxidant properties, ABTS radical scavenging activity (ABTS-RSC) and DPPH radical scavenging activity (DPPH-RSA) of the clarified juice samples were evaluated and the obtained data were compared with the raw sample (Table 4). As can be seen, the clarification process had a negative effect on these parameters which was probably related to the decrease phenolic compounds and also the deposition of some other antioxidants by pectin hydrolysis (de Oliveira et al., 2018; Su & Silva, 2006).

3.5.8. Determination of color parameters

To evaluate the color properties, three parameters of *L* (lightness), *a* (green-red value) and *b* (blue-yellow value) of the raw pomegranate juice and the clarified samples were measured. Table 4 showed that the lightness parameter was significantly increased after the clarification process which was possibly related to the removal of pectin as the most important cause of turbidity as previously reported by Sin, Yusof, Hamid, and Rahman (2006). Also, Table 4 showed that the clarification process led to a decrease in *a* value and an increase in *b* value meaning that this process decreased red color and increased the yellow color as can be seen in Fig. 2.

According to the results obtained from the clarified fruit juice analysis, it can be stated that the polyaldehyde kefirin-immobilized pectinase treatment had the greatest effect on the determined parameters which was related to the greater capacity of this cross-linker in enzyme immobilization and then the polyaldehyde pullulan-immobilized pectinase treatment was in second place. Also, the obtained results showed

that the continuous operation had more effect on the mentioned parameters than the batch operation which can be attributed to the better and more contact between the active site of the immobilized pectinase enzymes and the substrate in the continuous process.

4. Conclusion

In the current study, a common (glutaraldehyde) and two new cross-linkers (polyaldehyde pullulan and polyaldehyde kefirin) were used to immobilize the pectinase enzymes on the glass beads. After the immobilization process, a bioreactor packed with the glass bead-immobilized pectinase enzymes was applied to the pomegranate juice clarification. The optimum conditions for the continuous clarification process were obtained at the temperature of 50 °C, the flow rate of 0.5 mL/min and the treatment time of 42 min. The observations showed that the clarification led to a significant decrease in turbidity and a significant increase in clarity of the fruit juice samples. Also, the fruit juice properties were evaluated and the obtained results were compared with the raw sample. Generally, it was concluded that to clarify the fruit juice, the pectinase immobilization by the polyaldehyde polysaccharides especially polyaldehyde kefirin was much more efficient than by the glutaraldehyde as common cross-linker.

CRedit authorship contribution statement

Seyed Saeid Hosseini: Investigation, Methodology, Formal analysis, Writing - original draft. **Faramarz Khodaiyan:** Conceptualization,

Table 4

Total phenolic content (TPC; mg GAE/mL juice sample), antioxidant activity (ABTS radical scavenging activity (ABTS-RSA (%)) and DPPH radical scavenging activity (DPPH-RSA (%))) and color parameters of the untreated and treated pomegranate juice.

Parameters	TPC	Antioxidant activity		Color parameters		
		ABTS-RSA	DPPH-RSA	L	a	b
UPJ	2.48 ± 0.03 ^a	90.43 ± 0.14 ^a	91.37 ± 0.13 ^a	30.31 ± 0.11 ^f	16.12 ± 0.10 ^a	8.19 ± 0.10 ^d
PFP	2.14 ± 0.02 ^b	85.16 ± 0.10 ^c	85.27 ± 0.19 ^b	35.81 ± 0.09 ^d	15.26 ± 0.07 ^b	9.21 ± 0.14 ^c
PIPG _b	2.19 ± 0.06 ^b	86.32 ± 0.11 ^b	85.62 ± 0.17 ^b	34.98 ± 0.13 ^e	15.28 ± 0.14 ^b	9.15 ± 0.11 ^c
PIPG _c	2.13 ± 0.04 ^b	86.18 ± 0.13 ^b	85.47 ± 0.18 ^b	35.04 ± 0.16 ^e	15.22 ± 0.11 ^b	9.31 ± 0.10 ^c
PIPP _b	2.11 ± 0.06 ^b	85.32 ± 0.18 ^c	85.32 ± 0.15 ^b	37.08 ± 0.12 ^c	15.13 ± 0.13 ^b	9.31 ± 0.08 ^c
PIPP _c	2.00 ± 0.03 ^c	85.12 ± 0.10 ^c	84.28 ± 0.13 ^c	37.14 ± 0.10 ^c	15.10 ± 0.10 ^b	9.38 ± 0.13 ^c
PIPK _b	1.96 ± 0.06 ^c	83.27 ± 0.16 ^d	84.12 ± 0.09 ^c	40.41 ± 0.12 ^b	13.81 ± 0.18 ^c	9.85 ± 0.12 ^b
PIPK _c	1.93 ± 0.09 ^c	83.11 ± 0.08 ^d	83.14 ± 0.10 ^d	41.09 ± 0.08 ^a	13.41 ± 0.08 ^d	10.20 ± 0.16 ^a

UPJ: untreated pomegranate juice; PFP: pomegranate juice clarified by the free pectinase; PIPG_b: pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a batch process; PIPG_c: pomegranate juice clarified using the pectinase immobilized by glutaraldehyde in a continuous process; PIPP_b: pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a batch process; PIPP_c: pomegranate juice clarified using the pectinase immobilized by polyaldehyde pullulan in a continuous process; PIPK_b: pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefir in a batch process; PIPK_c: pomegranate juice clarified using the pectinase immobilized by polyaldehyde kefir in a continuous process.

^{a-f} Different letters in the same column indicate significant differences (p < 0.05).

Formal analysis, Project administration, Resources, Supervision. **Seyed Mohammad Mousavi**: Conceptualization, Validation, Visualization, Writing - review & editing. **Seyedeh Zahra Azimi**: Investigation, Methodology, Writing - review & editing.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication. Also, we confirm that the manuscript has been read and approved by all named authors and there are no other persons who satisfied the criteria for authorship but are not listed.

Acknowledgements

This study was financially supported by the Iran National Science Foundation (INSF), Iran [grant No. 97011331].

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