

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

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ABSTRACT

For the first time, polyaldehyde kefir was applied to immobilize the pectinase on the glass bead and the finding was compared with free enzyme. Polyaldehyde kefir, produced by periodate oxidation of kefir, showed an aldehyde content of $23.6 \pm 0.9\%$ that was confirmed by FTIR. The results showed although the optimum temperature (50°C) was not changed by immobilization, the optimum pH was shifted from 5.0 to 5.5. In optimal conditions, the specific activity of the free and immobilized pectinase was 2.93 ± 0.13 and 1.87 ± 0.24 unit/mg, respectively. Also, the immobilized pectinase had a higher thermal and pH stability than free enzyme. K_m and V_{max} of the immobilized pectinase were higher and lower than the free enzyme, respectively. In addition, SEM and AFM images illustrated a completely non-uniform surface after enzyme immobilization on the glass bead, which seemed to be related to the polyaldehyde kefir strands.

1. Introduction

Pectinase is the generic name for a group of the enzymes that lead to the degradation of pectin as a complex heteropolysaccharide present in cell wall and middle lamella of all land plants (Hosseini, Khodaiyan, Kazemi, & Najari, 2019; Kazemi, Khodaiyan, & Hosseini, 2019a). Pectinase has various applications in food industries especially in fruit juices clarification due to the ability of this enzyme to hydrolyze pectin, because this polysaccharide can contribute to the creation of the turbidity and undesirable cloudiness in the bottle during storage (Rehman et al., 2013; Rehman, Aman, Zohra, & Qader, 2014). However, the application of the native enzyme is associated with limitations such as instability, high cost and difficult to recovery after the enzymatic process, which can be minimized with the immobilization process (Rehman, Aman, et al., 2014; Sheldon, 2007). So far, the different methods were used for pectinase immobilization including adsorption, entrapment and covalent binding (de Oliveira, Dias, da Silva, & Porto, 2018). Among them, covalent bonding is a highly efficient method with the lower probability of the leaching out of enzyme from carrier and thereby, long-term process stability (Lei & Jiang, 2011; Rehman, Nawaz, Aman, Baloch, & Qader, 2014). However, the use of suitable support and immobilization protocol in the method is necessary for final success.

Glass beads are very good supports for the different enzymes

because of their stability, insolubility with high capacity to bind enzyme and no negative effect on enzyme action (Gomez et al., 2006; Kahraman, Bayramoğlu, Kayaman-Apohan, & Güngör, 2007). To use the glass beads, their surface are first activated by amino groups of various molecules especially 3-aminopropyl triethoxysilane (3-APTES) and then glutaraldehyde is commonly used as a cross-linker to connect the enzymes to the active surfaces of glass beads (Eskandarloo & Abbaspourrad, 2018; Kahraman et al., 2007). Glutaraldehyde is an excellent cross-linker and at the same time very toxic and therefore an attempt to replace it with a new safe, yet efficient cross-linker is very attractive and reasonable.

Kefir is a microbial polysaccharide of kefir grain flora including approximately equal amount of glucose and galactose, which are attached to each other with different glycosidic bonds (Shahabi-Ghahfarrokhi, Khodaiyan, Mousavi, & Yousefi, 2015; Zolfi, Khodaiyan, Mousavi, & Hashemi, 2014). Theoretically, with creating aldehyde groups in this polysaccharide and producing poly-aldehyde kefir by partial oxidation, it can be used as a safe and efficient cross-linker (see to Fig. 1). Sodium periodate is one of the compounds extensively used for this purpose. In oxidation process by this compound, IO_4^- ion cleaves the C_2-C_3 bond in the monosaccharide units of kefir and forms free aldehyde groups (Börjesson, Larsson, Westman, & Ström, 2018; Guo, Ge, Li, Mu, & Li, 2014; Li, Wu, Mu, & Lin, 2011).

Therefore in this paper, pectinase enzyme was covalently

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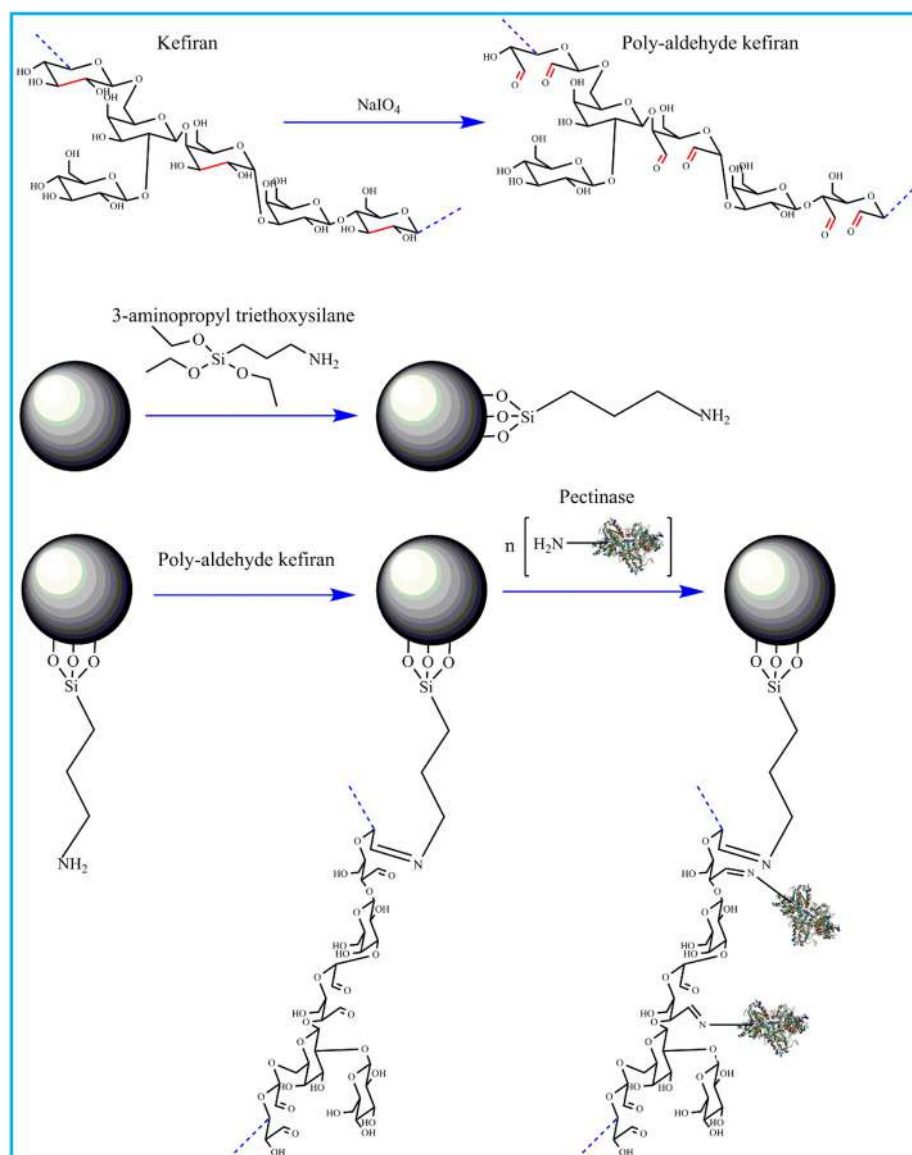


Fig. 1. The mechanism of polyaldehyde kefir production and pectinase immobilization on the glass bead.

immobilized on functionalized glass beads (glass bead + 3-APTES) by poly-aldehyde kefir as a new cross-linker. Also, the immobilized enzyme properties such as pH and temperature profile, the thermal and pH stability, and kinetic parameters were investigated and compared with free enzyme.

2. Materials and methods

2.1. Materials and chemicals

Kefiran exopolysaccharide was provided by Bioprocessing and Biodetection Lab., University of Tehran, Iran. Apple pectin (USP grade and DE of 72–76%) was supplied by Herbstreith & Fox KG (Germany). Pectinase enzyme (Pectinex® Ultra Color, protein concentration = ~80 mg/ml) was purchased from Novozymes (Bagsvaerd, Denmark). Glass beads (diameter of 6 mm) were obtained from Iran Beads Co. (Esfahan, Iran). Also, sodium periodate, sulfuric acid, hydroxylamine hydrochloride, sodium hydroxide, nitric acid, hydrochloric acid, (3-Aminopropyl) triethoxysilane (3-APTES), sodium acetate, acetic acid, 3,5-dinitrosalicylic acid (DNS) and D-(+)-galacturonic acid monohydrate were achieved from Sigma Chemical Co.

(St. Louis, MO, USA). The other chemicals were of analytical grade.

2.2. Production of poly-aldehyde kefir

The poly-aldehyde kefir was produced by the oxidation reaction with sodium periodate (Guo et al., 2014). For this purpose, 50 mL of sodium periodate aqueous solution (0.05 g/mL) was added to 125 mL of kefir aqueous solution (4 %w/v) under stirring and then pH was adjusted to 5 by 1 M H_2SO_4 solution. The reaction was done at 40 °C in the dark for 3 h and then the oxidized kefir (poly-aldehyde kefir) was precipitated by adding 96% ethanol (2:1 v/v) and storing at 7 °C for 72 h. The supernatant was eventually separated by centrifuging ($10,000 \times g$, 20 min), was washed with distilled water and 96% ethanol (three times) and was freeze-dried (1 day).

The aldehyde content of the obtained poly-aldehyde kefir was determined according to the method reported by Kholiya, Chaudhary, Vadodariya, and Meena (2016). 20 mL of 0.05 g/mL aqueous hydroxylamine hydrochloride solution in pH of 5 was added to 25 mL of 0.05 g/mL aqueous poly-aldehyde kefir solution in same pH and stirred at 40 °C for 4 h (pH adjustment was done by sodium hydroxide solution). In the next stage, the obtained mixture was titrated with

1 mol/L sodium hydroxide (V_1). Also, the aqueous kefir solution was used as blank and the volume of the consumed sodium hydroxide was considered as V_2 . The aldehyde content (AIC) of the poly-aldehyde kefir was calculated by the equation below:

$$\text{AIC (\%)} = \frac{(V_1 - V_2) \times C_{\text{NaOH}}}{1000 \times m/M} \times 100 \quad (1)$$

where C_{NaOH} was 1 mol/L, m was the dry weight (g) of kefir and M was approximately the molecular weight of repeating unit in poly-aldehyde kefir.

Also, FTIR spectra of the native and poly-aldehyde kefirans were obtained by a spectrometer (Perkin Elmer Co., MA, USA) using the KBr disk method in the resolution and the wavenumber range of 4 and 4000–400 cm^{-1} , respectively (Hamidi, Kennedy, Khodaiyan, Mousavi, & Hosseini, 2019).

2.3. Pectinase immobilization on the glass bead by poly-aldehyde kefir

Based on Fig. 1, the immobilization procedure was as follows (Bóddalo et al., 1991; Gomez et al., 2006):

About 210 g glass beads were cleaned with 5% nitric acid aqueous solution (85 °C, 120 min). After washing with the distilled water and drying in an oven (110 °C, 24 h), the glass beads were immersed in 10 % v/v of 3-APTES aqueous solution at 60 °C for 3 h (pH was adjusted with 6 N HCl to 4), were washed with distilled water and were dried (80 °C, 24 h), respectively.

In the next stage, the treated glass beads were immersed in 2.5 %w/v of kefir aqueous solution for 2 h at ~25 °C. After washing with distilled water, the glass beads were stirred in pectinase enzyme solution (5 h, ~25 °C) prepared by diluting the Pectinex® Ultra Color with sodium acetate buffer solution (1:4 v/v) at pH of 4. Eventually, pectinase-immobilized glass beads were washed with the sodium acetate buffer solution (pH of 4) and were stored at 4 °C until use.

To evaluate the adequacy of the immobilization method, the protein content of glass beads was calculated by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using the equation below:

$$\text{Protein content (mg/10 glass beads)} = C_i - (C_R + C_W) \quad (2)$$

where C_i , C_R and C_W were the amount of protein in: the initial solution, the solution remained after immobilization process and the washing solution.

Based on the above equation, the protein content of the glass beads was 5.25 ± 0.86 mg/10 glass beads after immobilization process.

2.4. Determination of the free and immobilized pectinase activity

The activity of the free and immobilized pectinase was determined according to de Oliveira et al. (2018) by DNS method. One unit of the enzyme activity was defined as the amount of enzyme required to form 1 μmole of galacturonic acid per min under the experiment conditions of this study. It should also be noted that the pectinase activity was reported based on specific activity (units/mg protein). Also, apple pectin solution was used as substrate and D-(+)-galacturonic acid monohydrate was applied as standard to draw a calibration curve.

1 mL of 1 mg/ml of free pectinase was stirred in 2 mL of 1 %w/v apple pectin solution prepared in 0.1 M acetate buffer (pH 5.0) at 50 °C for 15 min. After this time, 1 mL of the reaction medium was transferred to a test tube containing 1 mL of DNS, was heated in a boiling water bath (5 min) and was cooled in an ice-water bath, respectively. After adding 3 mL of distilled water, the absorbance of the sample was recorded in 540 nm. The activity of the immobilized pectinase was evaluated in a similar way to the above, with the difference that 10 glass beads attached to enzyme were substituted for the free pectinase.

2.5. Effect of immobilization on the activity and stability of pectinase

The influence of pH on the free and immobilized pectinase activity was investigated by carrying out the enzymatic hydrolysis reaction in different buffers with pH of 2–8 (50 °C, 15 min). pH stability of the free and immobilized pectinase was determined by immersing the glass beads in different buffer solutions (pH 2–8) for 30 min and then performing the enzymatic hydrolysis reaction for evaluation of residual activity at pH of 5 for the free enzymes and pH of 5.5 for the immobilized enzymes.

The effect of temperature on the activity of the free and immobilized pectinase was examined by doing the reaction at different temperatures (30–90 °C) in pH of 5 for free enzymes and pH of 5.5 for the immobilized enzymes and reaction time of 15 min for both cases. On the other hand, thermal stability of enzymes was determined by pre-incubating the free and immobilized pectinase at different temperatures (30–60 °C) and then performing the reaction at 50 °C for determination of the residual activity of the enzymes.

Also, in this section, the reusability of the immobilized pectinase, as a factor of its operational stability, was investigated by repeatedly re-using the beads in the reaction medium. After the end of each stage, the glass beads were separated, were washed by the sodium acetate buffer (pH 4) and were reintroduced in the fresh substrate, respectively. In each cycle, the substrate, temperature and reaction time were 2 mL of 1 %w/v apple pectin solution prepared in 0.1 M acetate buffer (pH 5.5), 50 °C and 15 min, respectively. It should also be noted that the enzymatic hydrolysis reaction was done in accordance with Section 2.4.

2.6. Determination of kinetic parameters for free and immobilized enzyme

The Michaelis–Menten plot was used to calculate the kinetic parameters as following equation:

$$V = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \quad (3)$$

where V_{max} was the maximum reaction rate, $[S]$ was pectin concentration in the reaction medium (2–20 mg/mL) and K_m was the Michaelis–Menten constant. Like the previous section, here, too, the reaction conditions were in accordance with Section 2.4.

2.7. Surface characterization

Before and after pectinase immobilization on the glass beads, the surface morphology and the surface topography were investigated by a scanning electron microscope (SEM, Vega3, Tescan Co. Ltd., Brno, Czech Republic) at the accelerating voltage of 15 KV and an atomic force microscope (AFM, NT-MDT, Russia) at room temperature, respectively.

3. Results and discussion

3.1. Synthesis of the polyaldehyde kefir

Sodium periodate oxidation is a well-known industrial method used in the production of the modified starch and cellulose (Guo et al., 2014). In this method, periodate ions attack on the monosaccharides in the polysaccharide structure and cleave the carbon-carbon bond and produce ring-opened structure with free aldehyde groups that can be used as cross-linker to immobilize the enzyme on the different surfaces (Kholiya et al., 2016; Mu, Guo, Li, Lin, & Li, 2012). During this process, a different number of aldehyde groups is produced depending on the type of polysaccharide, sodium periodate concentration and the reaction conditions (Börjesson et al., 2018; Guo et al., 2014). However, there is not always a completely direct relationship between the amount of the consumed ions and the aldehyde content, which is more related to the formation of hemiacetal through the reaction between free

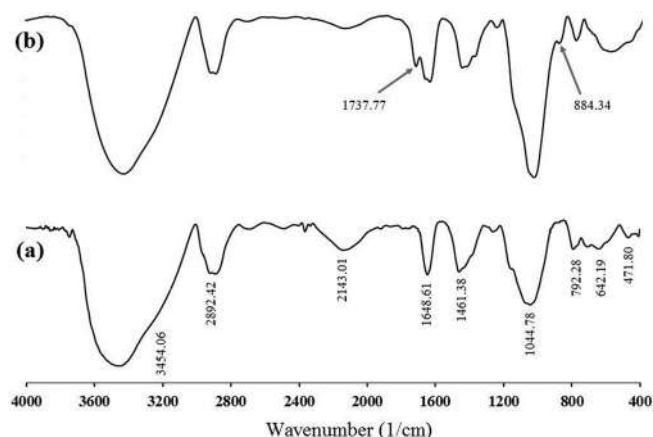


Fig. 2. FTIR spectra of (a) unmodified kefir and (b) polyaldehyde kefir.

aldehyde groups and adjacent hydroxyl groups (Gupta, Tummalapalli, Deopura, & Alam, 2013; Kim, Wada, & Kuga, 2004). In present study, this oxidation method was applied to form the free aldehyde groups on the kefiran exopolysaccharide. After the end of the reaction, the aldehyde content of the polyaldehyde kefir was $23.6 \pm 0.9\%$. Also, the formation of the aldehyde groups in the kefiran structure was confirmed by FTIR analysis.

Fig. 2 showed the FTIR spectra of the unmodified kefir and polyaldehyde kefir. In the polyaldehyde kefir (Fig. 2b), two new peaks were observed at $\sim 1737\text{ cm}^{-1}$ due to the aldehyde carbonyl group (Li et al., 2011) and at $\sim 884\text{ cm}^{-1}$ due to the hemiacetal bonds formation (Kholiya et al., 2016). These signals confirm the formation of the aldehyde group on the kefiran structure. Other absorbance peaks in both spectra are related to the kefiran structure. The broad peak between 3000 and 3500 cm^{-1} is assigned to OH stretching in polysaccharide structure (Kazemi, Khodaiyan, & Hosseini, 2019b). The 2892.42 cm^{-1} signal is attributed to C–H stretching in CH, CH₂ and CH₃ groups (Pasandide, Khodaiyan, Mousavi, & Hosseini, 2018). Also, the peak of 1648.61 cm^{-1} was related to O–H bonding in water molecules (Shahabi-Ghahfarrokhi et al., 2015). Finally, the broad area at $800\text{--}1200\text{ cm}^{-1}$, as finger print area for carbohydrates, is attributed to the carbohydrate rings and side groups including C–O–C, C–OH and C–H groups (Ghasemlou, Khodaiyan, Oromiehie, & Yarmand, 2011).

3.2. Influence of pH and temperature on the activity and stability of free and immobilized pectinase

The optimization of pH and temperature are the most important steps for the evaluation of the free and immobilized pectinase activity. The pectinase activity in the different pH and temperature was reported based on relative activity. In this method, the maximum activity in optimal conditions is considered as 100% and other conditions are compared with it (de Oliveira et al., 2018). Also, in this stage, the pH and thermal stability of both free and immobilized pectinase was evaluated based on residual activity in the sense that the initial activity of pectinase in various pH and temperature is taken as 100% and the residual activity of the enzyme after incubation time (see to Section 2.7) is reported as a fraction of 100 (Rehman, Nawaz, et al., 2014).

Fig. 3a showed the free and immobilized pectinase activity in various pH and a constant temperature (50°C) and reaction time (15 min). As can be seen, the maximum activity of the free pectinase was obtained in pH of 5.0, while after immobilization of pectinase on glass bead, the optimum pH was shifted from 5.0 to 5.5. The change in the optimum pH of the enzyme depends to the type of enzyme and the structure of the applied cross-linker. The alteration of optimum pH in the immobilized pectinase can be due to increase in structural and conformational stability of pectinase by immobilization process

(Rehman, Aman, et al., 2014). Also, this result was in line with the data reported by de Oliveira et al. (2018). These researchers reported that the optimum pH of the free pectinase was changed by its immobilization into alginate beads.

In addition, based on Fig. 3b, the obtained results showed that the immobilization of pectinase led to an increase in pH stability of the enzyme, so that the curve related to the immobilized pectinase was higher than free enzyme at all the applied pH values. These observations also were in accordance with previous studies (Li et al., 2007; Rehman, Nawaz, et al., 2014).

The effect of temperature on the free and immobilized pectinase was presented in Fig. 3c. For this purpose, the reaction temperature was varied from 30 to 90°C , while the pH (5.0 for free pectinase and 5.5 for immobilized enzyme) and reaction time (15 min) were constant. The maximum activity of both the free and immobilized pectinase was obtained at 50°C . Also, the immobilized pectinase retained more than 75% activity in temperature range of $60\text{--}90^\circ\text{C}$, whereas the free pectinase lost activity rapidly beyond 50°C . As can be seen in Fig. 3c, the optimum temperature of both the free and immobilized enzyme was similar and no changes were observed in this factor by immobilization process. It seems that the change or no-change optimum temperature of the enzyme by immobilization process depends on the nature of the enzyme, the type of immobilization method and applied cross-linker. For example, Rehman, Nawaz, et al. (2014) reported that the optimum temperature of pectinase was not changed as a result of its immobilization into chitosan beads, while in another study, the same researchers found that this parameter for the free pectinase was shifted from 45 to 50°C by its immobilization on the agar-agar gel (Rehman, Aman, et al., 2014).

The thermal stability of the free and immobilized pectinase was showed in Fig. 3d,e. Although the thermal stability of both enzymes was remarkable in low temperature, this parameter was higher for the immobilized pectinase so that the polyaldehyde kefiran cross-linked enzyme retained 100% of the initial activity after 90 min at 30°C . Also, in high temperatures, the thermal stability of the immobilized pectinase was higher than the free enzyme. As can be seen, the residual activity of the free enzyme reached to zero after incubation time of 90 min at 60°C , while the immobilized pectinase retained 56.58% of the original activity after same incubation time and temperature. These observations were in line with reported data by previous studies (Li, Li, Wang, & Tain, 2008; Rehman et al., 2016).

3.3. Specific activity of the free and immobilized pectinase and kinetic parameters

In this stage, the specific activity of the free and immobilized pectinase was evaluated in optimal reaction conditions (pH of 5.0 and temperature of 50°C for the free enzyme and pH of 5.5 and temperature of 50°C for the immobilized pectinase). This parameter for the free and immobilized pectinase was 2.93 ± 0.13 and 1.87 ± 0.24 unit/mg, respectively. As can be seen, the specific activity of the immobilized pectinase was lower than the free type, which can be attributed to diffusion limitation effect caused by the polymeric network of the polyaldehyde kefiran (Lei & Jiang, 2011). In addition, the specific activity of the immobilized pectinase in the current study was higher than the reported data (1.349 unit/mg) for the immobilized pectinase onto the macroporous polyacrylamide microspheres by Lei and Jiang (2011). Also, in another study, Lei and Bi (2007) reported that the specific activity of the silica-coated chitosan particle immobilized pectinase was 1.028 unit/mg, which was lower than the obtained data in this study.

In this stage, the kinetic parameters were calculated at temperature of 50°C and pH of 5.5 and their results were given in Table 1. The Michaelis–Menten constant (K_m) and the maximum reaction rate ($V_{\max}$) was obtained according to $1/[V]$ against $1/[S]$ plot. K_m showed the pectin concentration when the reaction velocity is $0.5 V_{\max}$ and $V_{\max}$ indicated the maximum enzymatic reaction velocity when all the

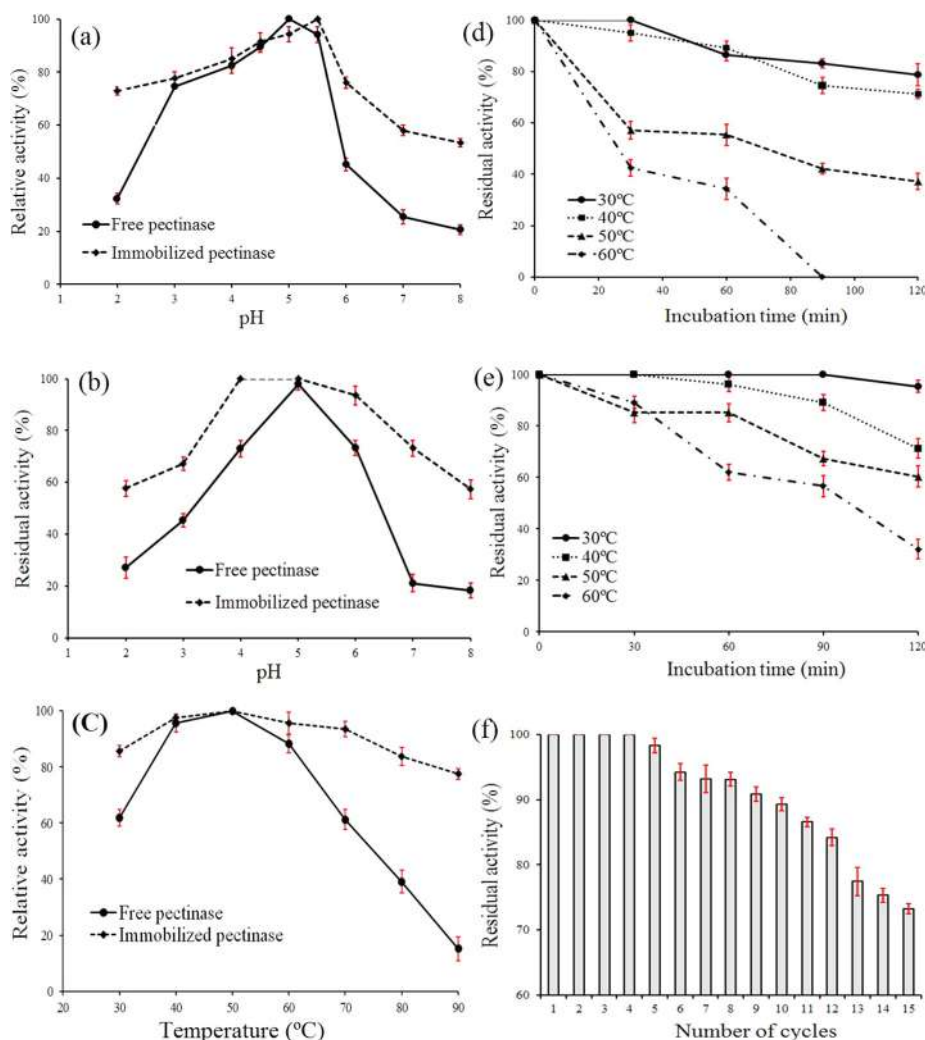


Fig. 3. Effect of pH on the activity (a) and stability (b) of the free and immobilized pectinase. (c) Effect of temperature on the activity of the free and immobilized pectinase. Thermal stability of the free (d) and immobilized (e) pectinase. (f) Reusability of the immobilized pectinase on the glass beads.

Table 1

Kinetic parameters for free (FE) and immobilized (IE) pectinases at reaction temperature 50 °C and pH of 5.5.

Kinetic parameter	Unit	FE	IE
K_m	mg pectin/mL	9.71	11.58
V_{max}	$\mu\text{mol}\cdot\text{min}^{-1}/(\text{mg of protein})$	3.07	2.17

pectinases are saturated with pectin as substrate. As can be seen, K_m and V_{max} of the immobilized pectinase were higher and lower than the free pectinase, which can be due to the diffusion limitation effects created by the polymeric network and three-dimensional structure of the polyaldehyde kefiran (Rehman, Aman, et al., 2014). Similar results were obtained about the kinetic parameters of pectinases after immobilization by the different methods (Lei & Bi, 2007; Rehman et al., 2013, 2016).

3.4. Reusability of the glass bead immobilized pectinase

The reusability is a very important factor in the industrial application of enzymes, because this factor has a reverse relationship with process cost. In this study, the reusability of the glass bead immobilized pectinase was evaluated at reaction temperature of 50 °C and pH of 5.5 as optimum conditions. As can be seen in Fig. 3f, the immobilized

pectinase showed no activity reduction after 4 cycles and also, retained ~90 and ~73% of its initial activity after 10 and 15 cycles, respectively. These results showed that the method used to immobilize the enzyme in this study was very effective, because in a similar study the immobilized pectinase onto the

macroporous polyacrylamide microspheres retained ~75 and ~65% of the original activity after the same number of cycles, respectively (Lei & Jiang, 2011). In addition, the pectinases immobilized on the chitosan beads lost ~20% of its original activity after 7 cycles (Rehman, Nawaz, et al., 2014), and the alginate beads immobilized pectinases lost ~70% of its initial activity after 8 cycles (de Oliveira et al., 2018). Also, Andriani, Sunwoo, Ryu, Prasetya, and Park (2012) reported the calcium alginate immobilized cellulase lost 90% of its activity after 4 cycles and Anwar, Qader, Raiz, Iqbal, and Azhar (2009) stated that the calcium alginate immobilized protease lost 100% of its activity after 3 cycles. By comparing the results obtained in this study with other papers, it can be stated that the type of enzyme, cross-linker and support and also, the process conditions can be effective on this property.

3.5. Surface characterization by SEM and AFM

Fig. 4a,b showed the surface morphology of glass bead before and after the immobilization of pectinase by polyaldehyde kefiran. It is quite obvious that the surface of glass bead was changed after pectinase immobilization. The original glass bead had a very smooth surface,

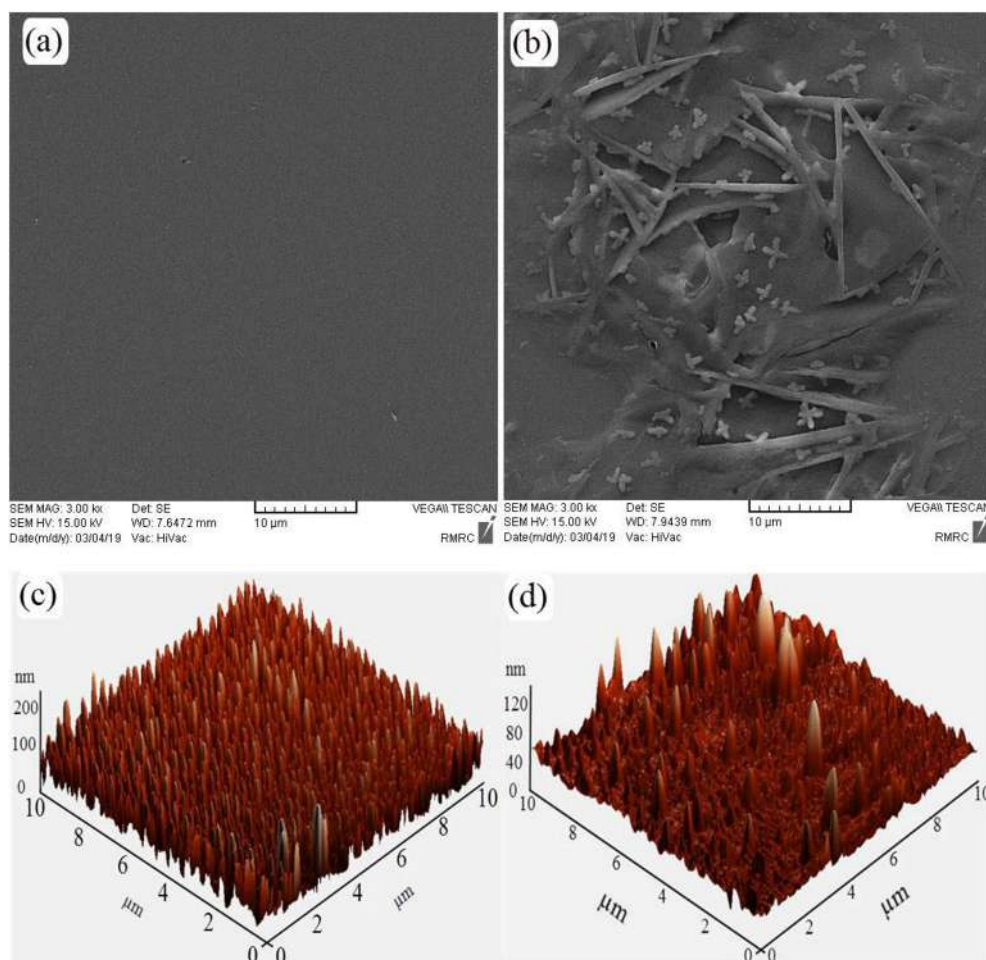


Fig. 4. SEM (up) and AFM (down) images of glass bead before (left) and after (right) pectinase immobilization by polyaldehyde kefiran.

while after immobilization, a polymeric network was seen on the surface which was probably attributed to polyaldehyde kefiran attached on the bead. These observations were in accordance with the obtained images from aminopropyl glass bead immobilized peroxidase by Marchis, Cerrato, Magnacca, Crocellà, and Laurenti (2012). AFM images of glass bead before and after immobilization were also presented in Fig. 4c,d. It was observed that by immobilization, the surface of glass bead was changed from uniform to non-uniform. These observations, in addition to confirming the obtained results by SEM, also were in line with the results reported by Hirsh et al. (2010) for polystyrene film immobilized cellulase and by Libertino et al. (2008) for the glucose oxidase immobilized on SiO₂ Surfaces.

4. Conclusion

In present study, the oxidized kefiran, as a new cross-linker, was applied to immobilize the pectinase enzyme on the aminopropyl glass bead. The obtained results showed that the maximum activity of the immobilized pectinase (1.87 ± 0.24 unit/mg) was in temperature of 50 °C and pH of 5.5, while this factor for free enzyme (2.93 ± 0.13 unit/mg) was obtained at the same temperature and pH of 5.0. Also, the thermal and pH stability of the immobilized pectinase was higher than the free enzyme at all temperatures and pH investigated. Kinetic parameter such as K_m and V_{max} were 11.58 mg/mL and $2.17 \mu\text{mol} \cdot \text{min}^{-1} / (\text{mg of protein})$ for the immobilized pectinase and 9.71 mg/mL and $3.07 \mu\text{mol} \cdot \text{min}^{-1} / (\text{mg of protein})$ for the free enzyme, respectively. In addition, the immobilized pectinases indicated a great reusability so that they retained 100% and ~73% of their initial

activity after 4 and 15 cycles, respectively. Therefore, according to the obtained results, it is suggested that polyaldehyde kefiran can be used as a suitable cross-linker to immobilize the various enzymes on the different supports.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- 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.
- Anwar, A., Qader, S. A. U., Raiz, A., Iqbal, S., & Azhar, A. (2009). Calcium alginate: A support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. *World Applied Sciences Journal*, 7(10), 1281–1286.
- Bódalo, A., Gomez, E., Gomez, J. L., Bastida, J., Maximo, M. F., & Diaz, F. (1991). A comparison of different methods of β -galactosidase immobilization. *Process Biochemistry*, 26(6), 349–353.
- Börjesson, M., Larsson, A., Westman, G., & Ström, A. (2018). Periodate oxidation of xylan-based hemicelluloses and its effect on their thermal properties. *Carbohydrate Polymers*, 202, 280–287.
- 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 Technology*, 109, 9–18.
- Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β -galactosidase immobilized on functionalized glass beads. *Food Chemistry*.
- Ghasemlou, M., Khodaiyan, F., Oromiehie, A., & Yarmand, M. S. (2011). Development and characterisation of a new biodegradable edible film made from kefir, an exopolysaccharide obtained from kefir grains. *Food Chemistry*, 127(4), 1496–1502.
- Gomez, J. L., Bodalo, A., Gomez, E., Bastida, J., Hidalgo, A. M., & Gomez, M. (2006). Immobilization of peroxidases on glass beads: An improved alternative for phenol removal. *Enzyme and Microbial Technology*, 39(5), 1016–1022.
- Guo, J., Ge, L., Li, X., Mu, C., & Li, D. (2014). Periodate oxidation of xanthan gum and its crosslinking effects on gelatin-based edible films. *Food Hydrocolloids*, 39, 243–250.
- Gupta, B., Tummalapalli, M., Deopura, B. L., & Alam, M. S. (2013). Functionalization of pectin by periodate oxidation. *Carbohydrate Polymers*, 98(1), 1160–1165.
- Hamidi, M., Kennedy, J. F., Khodaiyan, F., Mousavi, Z., & Hosseini, S. S. (2019). Production optimization, characterization and gene expression of pullulan from a new strain of *Aureobasidium pullulans*. *International Journal of Biological Macromolecules*, 138, 725–735.
- Hirsh, S. L., Bilek, M. M. M., Nosworthy, N. J., Kondyurin, A., Dos Remedios, C. G., & McKenzie, D. R. (2010). A comparison of covalent immobilization and physical adsorption of a cellulase enzyme mixture. *Langmuir*, 26(17), 14380–14388.
- Hosseini, S. S., Khodaiyan, F., Kazemi, M., & Najari, Z. (2019). Optimization and characterization of pectin extracted from sour orange peel by ultrasound assisted method. *International Journal of Biological Macromolecules*, 125, 621–629.
- Kahraman, M. V., Bayramoglu, G., Kayaman-Apohan, N., & Gungor, A. (2007). α -Amylase immobilization on functionalized glass beads by covalent attachment. *Food Chemistry*, 104(4), 1385–1392.
- Kazemi, M., Khodaiyan, F., & Hosseini, S. S. (2019a). Utilization of food processing wastes of eggplant as a high potential pectin source and characterization of extracted pectin. *Food Chemistry*, 294, 339–346.
- Kazemi, M., Khodaiyan, F., & Hosseini, S. S. (2019b). Eggplant peel as a high potential source of high methylated pectin: Ultrasonic extraction optimization and characterization. *LWT*, 105, 182–189.
- Koliya, F., Chaudhary, J. P., Vadodariya, N., & Meena, R. (2016). Synthesis of bio-based aldehyde from seaweed polysaccharide and its interaction with bovine serum albumin. *Carbohydrate Polymers*, 150, 278–285.
- Kim, U.-J., Wada, M., & Kuga, S. (2004). Solubilization of dialdehyde cellulose by hot water. *Carbohydrate Polymers*, 56(1), 7–10.
- 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.
- Li, H., Wu, B., Mu, C., & Lin, W. (2011). Concomitant degradation in periodate oxidation of carboxymethyl cellulose. *Carbohydrate Polymers*, 84(3), 881–886.
- Li, T., Li, S., Wang, N., & Tain, L. (2008). Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. *Food Chemistry*, 109(4), 703–708.
- 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.
- Libertino, S., Giannazzo, F., Aiello, V., Scandurra, A., Sinatra, F., Renis, M., & Fichera, M. (2008). XPS and AFM characterization of the enzyme glucose oxidase immobilized on SiO₂ surfaces. *Langmuir*, 24(5), 1965–1972.
- 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.
- Marchis, T., Cerrato, G., Magnacca, G., Crocellà, V., & Laurenti, E. (2012). Immobilization of soybean peroxidase on aminopropyl glass beads: Structural and kinetic studies. *Biochemical Engineering Journal*, 67, 28–34.
- Mu, C., Guo, J., Li, X., Lin, W., & Li, D. (2012). Preparation and properties of dialdehyde carboxymethyl cellulose crosslinked gelatin edible films. *Food Hydrocolloids*, 27(1), 22–29.
- Pasandide, B., Khodaiyan, F., Mousavi, Z., & Hosseini, S. S. (2018). Pectin extraction from citron peel: Optimization by Box-Behnken response surface design. *Food Science and Biotechnology*, 27(4), 997–1005.
- Rehman, H. U., Aman, A., Nawaz, M. A., Karim, A., Ghani, M., Baloch, A. H., & Qader, S. A. U. (2016). Immobilization of pectin depolymerising polygalacturonase using different polymers. *International Journal of Biological Macromolecules*, 82, 127–133.
- 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. (2014a). 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. (2014b). 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.
- Shahabi-Ghahfarrokhi, I., Khodaiyan, F., Mousavi, M., & Yousefi, H. (2015). Green biocomposite based on kefir and cellulose nanocrystals produced from beer industrial residues. *International Journal of Biological Macromolecules*, 77, 85–91.
- Sheldon, R. A. (2007). Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis & Catalysis*, 349(8–9), 1289–1307.
- Zolfi, M., Khodaiyan, F., Mousavi, M., & Hashemi, M. (2014). Development and characterization of the kefir-whey protein isolate-TiO₂ nanocomposite films. *International Journal of Biological Macromolecules*, 65, 340–345.