



A Health-Friendly Strategy for Covalent-Bonded Immobilization of Pectinase on the Functionalized Glass Beads

Seyed Saeid Hosseini¹ · Faramarz Khodaiyan¹ · Seyed Mohammad E. Mousavi¹ · John F. Kennedy² · Seyedeh Zahra Azimi¹

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Abstract

In recent years, polyaldehyde polysaccharides have received an increasing attention as an ideal cross-linker which their aldehyde groups can be crosslinked with amino groups of enzyme. In this study, pectinase was covalently immobilized on the glass beads by polyaldehyde pullulan as the resulting product from periodate oxidation of this polysaccharide and by glutaraldehyde as a common cross-linker. Polyaldehyde pullulan had an aldehyde content of $19.9 \pm 1.3\%$, which was confirmed by FTIR. The protein content of glass beads after enzyme immobilization by polyaldehyde pullulan and glutaraldehyde was 1.83 and 0.41 mg/10 glass beads, respectively. Optimum pH and temperature for pectinase immobilized by both cross-linkers were 5.5 and 50 °C, and the specific activity under these conditions was 2.21 ± 0.18 and 2.56 ± 0.12 unit/mg for enzyme immobilized by polyaldehyde pullulan and glutaraldehyde, respectively. pH stability of the pectinase immobilized by polyaldehyde pullulan was higher than by glutaraldehyde, while both immobilized system has an almost similar temperature stability. The kinetic parameters were determined for both immobilization systems. Also, SEM and atomic force microscopy confirmed the presence of polyaldehyde pullulan on glass bead after immobilization. Eventually, the obtained results suggested that polyaldehyde pullulan can be applied as a high potential alternative to glutaraldehyde for the enzyme immobilization.

Keywords Pectinase · Polyaldehyde pullulan · Immobilization · Specific activity · Kinetic parameters

Introduction

Pectin is a complex polysaccharide with a backbone of (1 → 4) galacturonic acids esterified at some carboxyl groups. This heteropolysaccharide is widely applied in the food and drug industry as emulsifier, gelling agent, and stabilizer (Pasandide et al. 2018). However, this valuable macromolecule causes disadvantages in some products, such as some fruit juices, because it contributes to the creation of the undesirable haze

and sediment in the bottle during their shelf-life (Cerreti et al. 2017; Benucci et al. 2019). Therefore, it seems that its hydrolysis is necessary in production of these products. Pectinase is the used enzyme for this process.

Generally, the pectinases consist of a series of pectin degrading enzymes, which the most important of them are pectinlyase (EC 4.2.2.10), polygalacturonase (EC 3.2.1.15), and pectinesterase (EC 3.1.1.11). The first and second enzyme groups hydrolyze glycosidic bonds in pectin and polygalacturonic acid, respectively, and the third group leads to the de-esterification of pectin and production of substrate for polygalacturonase (Diano et al. 2008). However, the use of free enzyme has disadvantages such as high production cost, instability of process, difficulty in product recovery, and inability of reusability, which can be overcome by immobilization of enzyme on the suitable support (de Oliveira et al. 2018; Rehman et al. 2014; Romo-Sanchez et al. 2014). So far, the different immobilization methods including adsorption, entrapment, and covalent binding were applied to immobilize the various enzymes on the selected support. Gurdas et al. (2012) immobilized β -Galactosidase enzyme

✉ Seyed Saeid Hosseini
Saeid_Hosseini@ut.ac.ir

✉ Faramarz Khodaiyan
Khodaiyan@ut.ac.ir

¹ Bioprocessing and Biodetection Laboratory, Department of Food Science and Engineering, University of Tehran, Karaj 31587-77871, Iran

² Chembiotech Laboratories, Advanced Science and Technology Institute, 5 The Croft, Buntsford Drive, Stoke Heath, Bromsgrove, Worcs B60 4JE, UK

onto duolite A568 resin via adsorption method and applied the obtained enzymatic system for lactose hydrolysis. In another study, Carpio et al. (2011) applied adsorption method for immobilization of glucoamylase onto calcined chicken bone particles in order to the saccharification of liquefied cassava starch. Jana et al. (2015) clarified the apple juice using tannase immobilized onto chitin-alginate by the adsorption-entrapment technique. Although the mentioned researchers reported that the used adsorption and entrapment strategies are good methods to reduce costs, they have a higher probability of the leaching out of enzyme from support and thereby, a lower process stability than the covalent immobilization method (Rehman et al. 2014; Lei and Jiang 2011; Hosseini et al. 2020). A key point in covalent immobilization is the selection of a suitable support. The support should be cheap, stable, and insoluble in reaction medium with high capacity to bind enzyme and also should not have negative effect on its action (Kahraman et al. 2007). All of the above are seen in glass beads.

In many studies, glass beads were used as support for immobilization of different enzymes (Kahraman et al. 2007; Champagne and Ramsay 2010; Gomez et al. 2006). Glutaraldehyde, as the most commonly used cross-linker to connect the enzyme to the glass bead, is a toxic compound due to sensory irritant effects, sensitization of skin and respiratory organs, genotoxicity and carcinogenicity, developmental toxicity, and environmental effects (Takigawa and Endo 2006), and naturally, its use is associated with limitations. Therefore, replacing it with a new safe, yet efficient cross-linker seems to be essential.

In recent years, polyaldehyde polysaccharides have received an increasing attention as an ideal cross-linker which their aldehyde groups can be crosslinked with amino groups of enzyme by Schiff's base formation (Guo et al. 2014). Pullulan is an exo-polysaccharide produced by *Aureobasidium pullulans*, which consists of maltotriose units attached by (1 → 4) linkages and repeated maltotriose units attached to each other by (1 → 6) linkages (Chen et al. 2017; Singh et al. 2017; Hamidi et al. 2019). It seems that this exopolysaccharide can be used as a cross-linker by partial oxidation and the formation of free aldehyde groups on it. Sodium periodate has long been utilized in the production of polyaldehyde polysaccharides by oxidation reaction which in it, the periodate ion cleaves the C₂-C₃ bond in the constituent units of the polysaccharide and leads to the formation of free aldehyde groups (Amer et al. 2016; Borjesson et al. 2018; Li et al. 2011).

In previous study, we immobilized pectinase on the glass bead by polyaldehyde kefiran as a new safe cross-linker and the obtained results were compared with native enzyme (Hosseini et al. 2020). However, according to the different potential of the various polysaccharides to become cross-linkers and also the importance of achieving a suitable cross-

linker comparable to the common unhealthy one (glutaraldehyde), the main objective of the current study was to immobilize pectinase on the glass beads by polyaldehyde pullulan (pectinase immobilized on the glass beads by polyaldehyde pullulan: PIGP) or glutaraldehyde (pectinase immobilized on the glass beads by glutaraldehyde: PIGG) and to investigate the effect of these cross-linkers on the behavior of the immobilized pectinases in different temperature and pH conditions. In addition, the thermal and pH stability and also the kinetic parameters of immobilized enzymes were evaluated.

Materials and Methods

Materials

Glass beads, with diameter of 6 mm, were purchased from Iran Beads Co. (Esfahan, Iran). Pectinex® Ultra Color with a protein concentration of ~80 mg/mL was obtained from Novozymes (Bagsvaerd, Denmark). Pullulan exopolysaccharide was supplied by Hayashibara Co., Ltd. (Okayama, Japan). Apple pectin (USP grade and DE of 72 to 76%) was obtained from Herbstreith & Fox KG (Germany). Sodium periodate, hydroxylamine hydrochloride, 3-aminopropyl triethoxysilane (3-APTES), glutaraldehyde, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide, hydrochloric acid, nitric acid, sulfuric acid, sodium acetate, acetic acid, D-(+)-galacturonic acid monohydrate, potassium chloride, and potassium hydrogen phosphate were bought from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals applied in this study were of analytical grade.

Polyaldehyde Pullulan Production

Pullulan was oxidized by sodium periodate according to Guo et al. (2014). About 5 g pullulan powder was dissolved in 125 mL of the distilled water, and 50 mL of 0.05 g/mL sodium periodate aqueous solution was added to it under stirring. After adjusting pH to 5 with 1 M sulfuric acid solution, the obtained solution was stirred in the dark for 3 h at 40 °C. In the next stage, the oxidized pullulan (polyaldehyde pullulan) was precipitated by adding two volumes of ethanol (96%) and storing in the refrigerator for 3 days. After centrifuging and separating phases, the supernatant was washed with distilled water and ethanol (96%) for three times and freeze-dried for 1 day.

Evaluation of Aldehyde Content of Polyaldehyde Pullulan

The aldehyde content of the oxidized pullulan was determined by forming oxime with hydroxylamine hydrochloride (Li et al. 2011). Briefly, 0.5 g of polyaldehyde pullulan was

dissolved in 25 mL distilled water. After adjusting pH to 5 with NaOH solution, 20 mL of hydroxylamine hydrochloride aqueous solution (0.05 g/mL) was added in same pH and stirred (4 h, 40 °C). In the next step, the obtained solution was titrated with 1 mol/L NaOH. It should also be noted that pullulan aqueous solution in the same concentration and pH was used as blank. The aldehyde content of the oxidized pullulan was calculated as follows:

$$\text{Aldehyde content (\%)} = \frac{(V_1 - V_2) \times C_N}{1000 \times m/M} \times 100 \quad (1)$$

where V_1 and V_2 were the volume (mL) of the NaOH consumed by the polyaldehyde and the native pullulan solutions, respectively. C_N was the molar concentration of the consumed NaOH (1 mol/L). m was the polyaldehyde pullulan dry weight (g), and M was approximately the molecular weight of repeating unit in polyaldehyde pullulan.

FTIR Spectroscopy

FTIR spectra of the pullulan was characterized before and after the oxidation process by a Perkin Elmer FT-IR spectrometer (Perkin Elmer Co., MA, USA) with the KBr disk method. The resolution and the wavenumber range were 4 and 4000–400 cm^{-1} , respectively.

Immobilization of Pectinase on the Glass Bead

Immobilization process was based on Diano et al. (2008) with slight modifications. The details of the immobilization process are shown in Fig. 1.

In the first step, glass beads were immersed in 5% nitric acid aqueous solution at 85 °C for 120 min and then washed by distilled water and dried in 110 °C for 24 h.

About 140 cm^3 (~210 g) of the dried glass beads was immersed in 140 mL of 10 mL/100 mL 3-APTES aqueous solution (this compound was applied to activate the glass beads surfaces by free amino groups (Fig. 1)), and pH of the mentioned mixture was adjusted to 4 by 6 mol/L hydrochloric acid. After immersing at 60 °C for 3 h, the glass beads were washed with distilled water and dried at 80 °C for 24 h.

The glass beads were immersed in 140 mL of glutaraldehyde (2.5 mL/100 mL) or polyaldehyde pullulan (2.5 g/100 mL) aqueous solutions for 2 h at room temperature. After this time, the glass beads were washed with the distilled water and immersed in pectinase enzyme solution (140 mL) for 5 h at 25 °C. Pectinase enzyme solution was prepared by diluting the Pectinex® Ultra Color with sodium acetate buffer solution (pH=4) in a ratio of 1 to 4. Afterward, the glass beads were washed with the sodium acetate buffer solution in same pH to remove the unbound enzyme.

Determination of the Glass Bead Protein Content

The protein content immobilized on the glass beads was evaluated with the Lowry method (Lowry et al. 1951) as follows:

$$\text{IP (mg/10 glass beads)} = C_I - (C_R + C_W) \quad (2)$$

where C_I was the initial amount of protein in the solution used for immobilization, C_R was the amount of the remained protein in solution after immobilization process, and C_W was the amount of protein in the washing solution. In this section, bovine serum albumin (BSA) was applied as standard to draw a calibration curve (0.01–0.5 mg/mL).

Enzyme Activity Assay

The immobilized pectinase activity was determined by the estimation of reducing sugars released by the enzyme, using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). For this purpose, the apple pectin and the galacturonic acid were applied as substrate and standard compound, respectively. Ten glass beads containing the enzymes immobilized by glutaraldehyde or polyaldehyde pullulan cross-linkers were stirred in 2 mL of apple pectin solution (1 g/100 mL) prepared in sodium acetate buffer (pH = 5.5) on the magnetic stirrer. After 15 min at 50 °C, 1 mL of the hydrolyzed pectin solution was poured into a test tube and then 1 mL of DNS was added to it. The obtained mixture was heated in a boiling water bath for 5 min and cooled in an ice-water bath. In the next step, 3 mL of distilled water was added to the mentioned test tube, and finally, its absorbance was read at 540 nm. The pectinase activity was reported based on specific activity.

Specific activity was defined as enzyme units per milligram of protein. It should also be noted that one unit was the amount of enzyme required to produce 1 $\mu\text{mole/min}$ of galacturonic acid under the test conditions.

Effect of pH on the Activity and Stability of the Immobilized Pectinase

The effect of pH on the pectinase activity was evaluated by performing the enzymatic reaction in 0.1 M different buffers (pH 2–8) at constant temperature (50 °C) and process time (15 min). The buffers include KCl-HCl (pH 2), acetate (pH 3–5.5), and KH_2PO_4 -NaOH (pH 6–8). pH stability of the immobilized pectinase was investigated by pre-incubating the glass beads in the mentioned different pH for 30 min and then carrying out the enzymatic reaction for determination of residual activity at pH of 5.5.

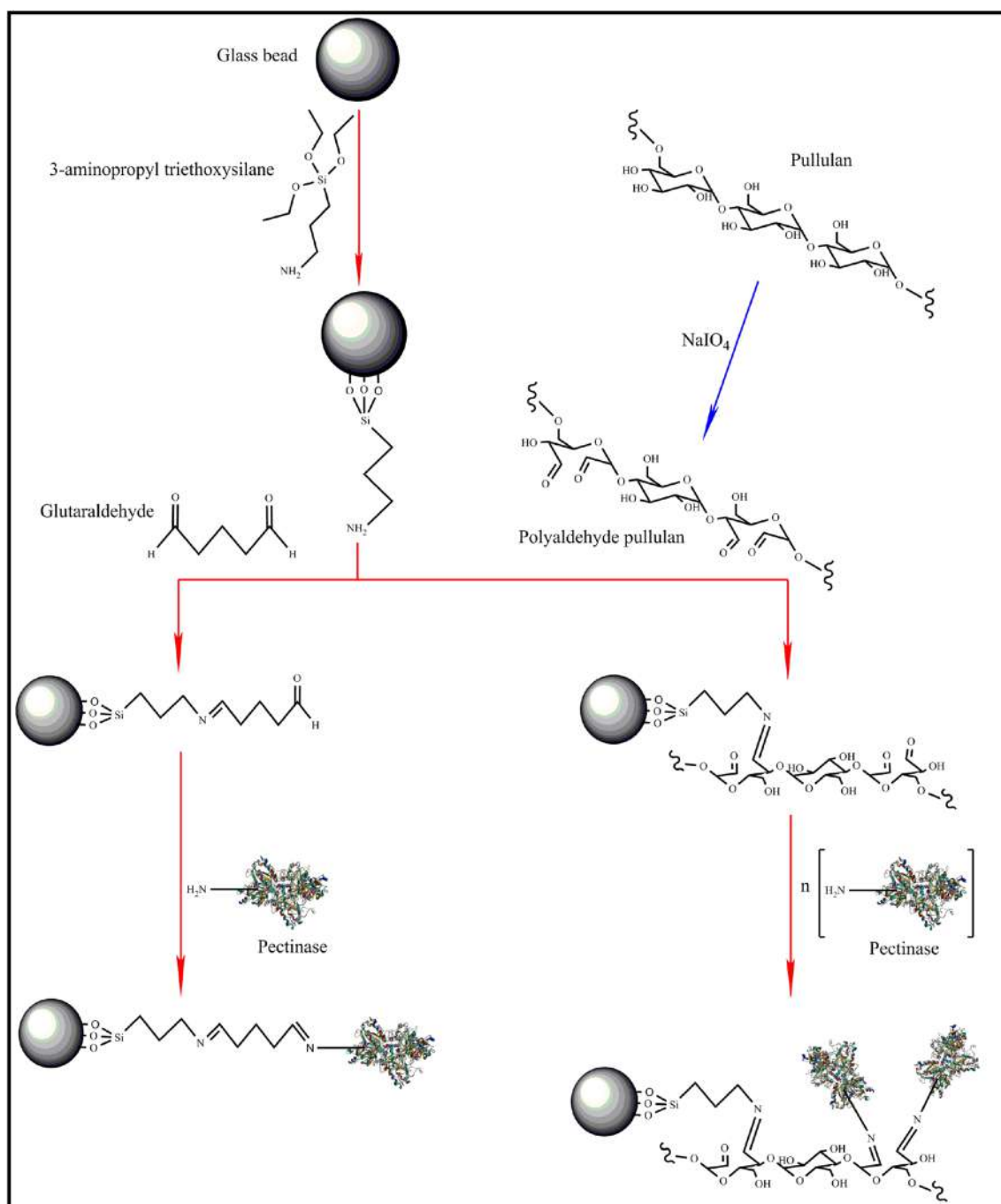


Fig. 1 The schematic representation of polyaldehyde pullulan production and enzyme immobilization on the glass bead

Effect of Temperature on the Activity and Stability of the Immobilized Pectinase

The effect of temperature on the pectinase activity was examined by carrying out the enzymatic reaction at different temperature (30–90 °C) in constant pH of 5.5 and process time of 15 min. Also, thermal stability of the immobilized pectinase was evaluated by pre-incubating the glass beads at different temperature (30–60 °C) and then performing the enzymatic

reaction for the evaluation of residual activity at temperature of 50 °C.

Determination of Kinetic Parameters

The Michaelis–Menten constant (K_m) and the maximum reaction rate (V_{max}) were determined by a Lineweaver–Burk plot according to the equation below:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (3)$$

where $[S]$ was pectin concentration (2–20 mg/mL).

It should also be noted that the enzymatic reaction conditions were based on what was said in the enzyme activity assay section.

Reusability of Immobilized Pectinase

The reusability of the glass bead immobilized pectinase was examined by repeatedly reusing the glass beads. After each step, the glass beads were separated from reaction medium and were washed with the sodium acetate buffer (pH 4) to remove the released enzymes and the remained substrates. Afterward, the glass beads were reintroduced in fresh pectin solution. The reaction conditions were in accordance with the enzyme activity assay section.

Scanning Electron Microscopy Analysis

The surface morphology properties of glass beads were evaluated before and after immobilization process by a scanning electron microscope (Vega3, Tescan Co. Ltd., Brno, Czech Republic) at the accelerating voltage of 15 KV after being coated with gold.

Atomic Force Microscopy Analysis

An atomic force microscope (AFM, NT-MDT, Russia) was applied to study the surface topography before and after immobilization process at room temperature.

Results and Discussion

Preparation of the Polyaldehyde Pullulan

Periodate oxidation is known as a highly specialized reaction in which 1,2-dihydroxyl groups are converted into two free aldehyde groups (Guo et al. 2014). Theoretically, in this reaction, 1 mol of sodium periodate can oxidize 1 mol of pullulan and produce the aldehyde groups on it (Li et al. 2011). However, it is never possible to complete oxidation due to the reaction between the produced aldehyde groups and neighbor hydroxyl groups and formation of hemiacetals (Gupta et al. 2013). After the end of the reaction, the aldehyde groups created on the polysaccharide is usually measured by titration with an alkali, especially sodium hydroxide in presence of hydroxylamine hydrochloride. In the current study, pullulan exopolysaccharide was oxidized by the mentioned method and then the produced supernatant was applied to connect

pectinase to the activated glass beads (glass bead+3-APTES). The results showed that the aldehyde content of the obtained product was $19.9 \pm 1.3\%$, which was lower than the reported data for xanthan ($22.52 \pm 1.91\%$ (Guo et al. 2014)) and kefiran ($23.6 \pm 0.9\%$ (Hosseini et al. 2020)) gums in same conditions. However, it is certain that the aldehyde content is dependent on the type and molecular weight (please see to Eq. 1) of gum used, sodium periodate concentration, and the reaction conditions, and changing any of these factors may change the obtained results.

FTIR Spectra Analysis

In order to confirm the formation of aldehyde groups on the oxidized pullulan, the FTIR spectrum obtained before and after oxidation reaction was investigated (Fig. 2). As can be seen, the native pullulan has several characteristic peaks including signals at 3437.06, 2925.52, 1641.50, 1402.21, 1031.14, and 663.55 cm^{-1} , which are due to O–H stretching, C–H stretching, O–C–O stretching, C–O–H banding, C–O stretching, and α -configuration of D-glucopyranoses, respectively (Choudhury et al. 2011; Haghghatpanah et al. 2020; Kazemi et al. 2019; Sugumaran et al. 2014). In addition to the signals mentioned, two new peaks were observed in FTIR spectrum of the oxidized pullulan (Fig. 2b), which confirmed the presence of the aldehyde groups on the treated exopolysaccharide: the signals at 1742.32 and 881.76 cm^{-1} . These peaks are due to aldehydic carbonyl groups and hemiacetal bonds created by the reaction between aldehyde groups and neighboring hydroxyl groups, respectively (Fan et al. 2001; Kholiya et al. 2016).

Protein Content of the Enzyme Cross-Linked Glass Beads

To evaluate the adequacy of the immobilization method, the protein content of the glass beads after the immobilization of pectinase was determined. The obtained results showed that the amount of protein immobilized by glutaraldehyde and polyaldehyde pullulan on the glass beads was 0.41 and 1.83 mg/10 glass beads, respectively. The higher protein content of the glass beads that were attached to pectinase by polyaldehyde pullulan was predictable, because the more presence of the free aldehyde groups in the mentioned cross-linker structure can provide more sites for binding to the protein.

pH Profile for Activity and Stability of the Immobilized Pectinase

In this section, the pH effect on the activity and stability of the PIGP and PIGG was evaluated in the pH range of 2–8 (Fig. 3a, b). In these figures, the maximum activity of the

Fig. 2 FTIR spectra of **a** native pullulan and **b** oxidized pullulan

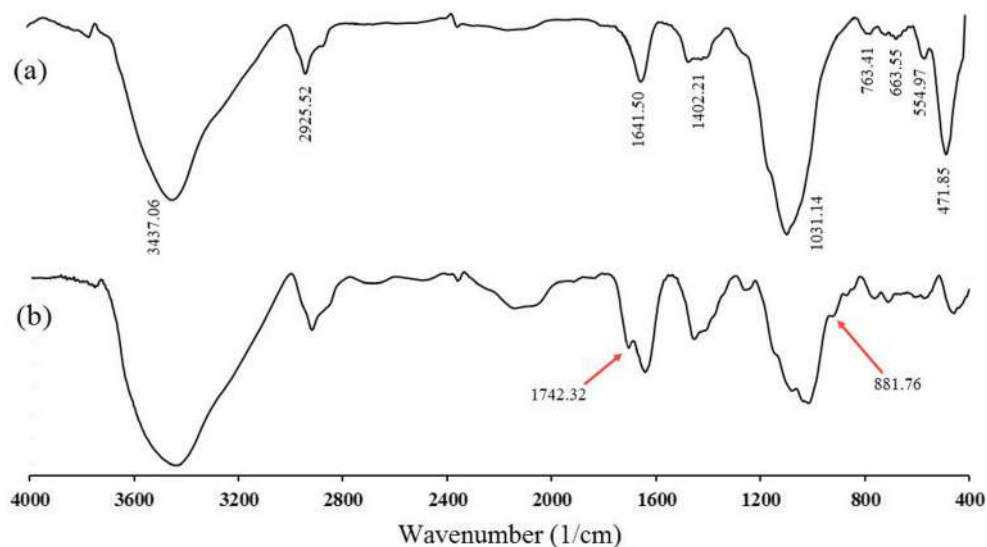
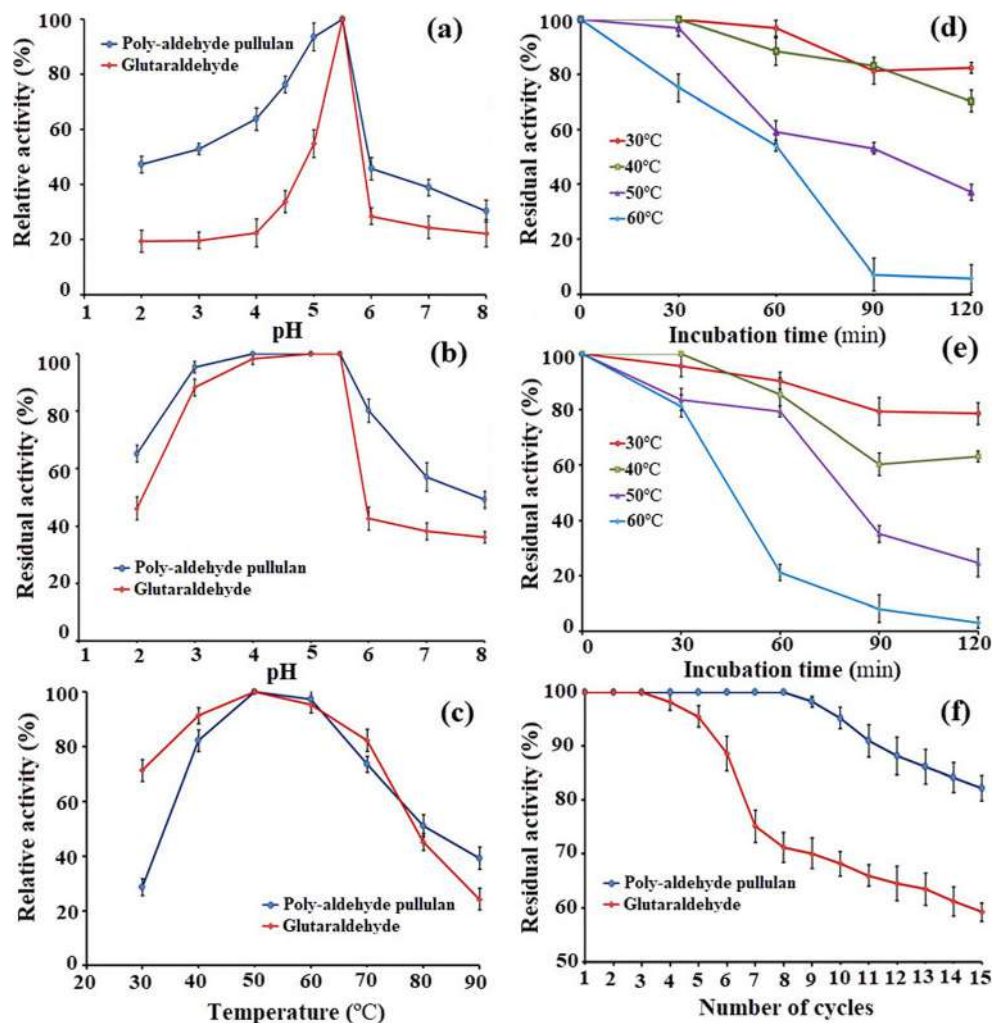


Fig. 3 Effect of pH on **a** the activity and **b** stability of the PIGP and PIGG (reaction condition: pH of 2–8, temperature of 50 °C, and process time of 15 min). **c** Effect of temperature on the activity of the PIGP and PIGG (reaction condition: temperature of 30–90 °C, pH of 5.5, and process time of 15 min). Thermal stability of **d** the PIGP and **e** PIGG (reaction condition: temperature of 30–60 °C, pH of 5.5, and process time of 15 min). **f** Reusability of the immobilized pectinase on the glass beads (reaction condition: temperature of 50 °C, pH of 5.5, and process time of 15 min). The values are mean $\pm$ SD of triplicate measurements



immobilized pectinases was considered as 100% and the rest of the data was compared with it. As can be seen in Fig. 3a, the optimum pH for both systems was 5.5, which was similar to the obtained data for the pectinase immobilized on the glass beads by polyaldehyde kefir and was higher than free enzyme (pH 5.0) (Hosseini et al. 2020). Also, at all examined pHs, the relative activity of the PIGP was higher than PIGG. However, the different studies illustrate that the optimum pH of pectinase in different immobilization methods can be different. Lei and Jiang (2011) reported that the optimum pH for activity of the pectinase immobilized on the macroporous polyacrylamide microspheres by glutaraldehyde was 3.5. In another study, this factor for the pectinase immobilized on the Dilbead VWR support by dextran aldehyde was 5.0 (Rajdeo et al. 2016). This data shows that the type of the used pectinase, cross-linker, and support can be very effective on the optimum pH of the process.

Figure 3 b indicates that although pH stability of pectinase in the both immobilization systems was appropriate, this factor for PIGP was higher. In mentioned system, the residual activity of the immobilized pectinase was ~95% at pH of 3.0 and 100% at pHs of 4.0, 5.0, and 5.5, whereas this factor for PIGG was 88.16, 98.12, 100, and 100% at pHs of 3.0, 4.0, 5.0, and 5.5, respectively. However, it was observed that this factor was gradually reduced by distance from the optimum pH.

Temperature Profile for Activity and Stability of the Immobilized Pectinase

The effect of reaction temperature (30–90 °C) on the activity of the immobilized pectinases was determined, and the obtained data was presented in Fig. 3c. The pectinase activity for both immobilization methods was increased by increasing the temperature from 30 to 50 °C and then gradually decreased to 39.18 and 24.16% at 90 °C for the PIGP and PIGG, respectively. The mentioned figure showed the optimum temperature for both systems was 50 °C which was similar to the observed results for free pectinase and the immobilized enzyme by polyaldehyde kefir (Hosseini et al. 2020). Here, too, the immobilization method seems to be very effective on the optimum temperature. de Oliveira and co-workers reported that the optimum temperature for alginate bead cross-linked pectinase was 40 °C (de Oliveira et al. 2018), while this factor for agar-gel support cross-linked pectinase was 50 °C (Li et al. 2008).

The thermal stability of the PIGP and PIGG was also determined at 30–60 °C (Fig. 3d, e). In low temperature (30 and 40 °C), the thermal stability of both systems was high, while this factor was sharply decreased with a rise in temperature to 60 °C. As can be seen, the residual activities of the PIGP and PIGG were 5.7 and 3.11% at 60 °C after incubation time of 120 min, respectively. These obtained results were in

accordance with previous reports (Diano et al. 2008; Rajdeo et al. 2016).

Optimum Activity of the Immobilized Pectinase and Kinetic Parameters

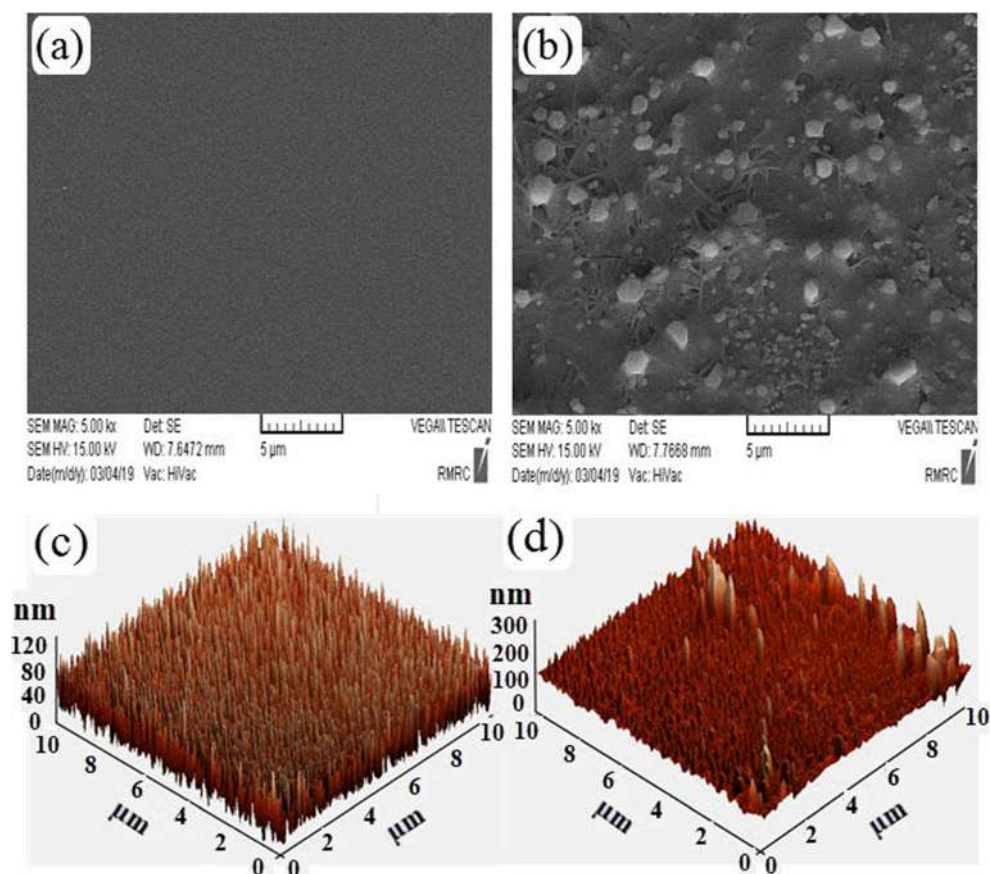
In this section, the specific activity of the PIGP and PIGG was determined under optimal conditions (temperature of 50 °C and pH of 5.5). This parameter for the PIGP and PIGG was 2.21 ± 0.18 and 2.56 ± 0.12 unit/mg, respectively. It is clear that the structure of polyaldehyde pullulan is much more complex than glutaraldehyde, and therefore, it can lead to more resistance to the substrate diffusion and decrease in the substrates access to the immobilized enzyme active site and thereby decrease in specific activity compared with glutaraldehyde (Lei and Jiang 2011). It should also be noted that the specific activity for both immobilization systems was lower and higher than free enzyme and the immobilized pectinase by polyaldehyde kefir (Hosseini et al. 2020).

The kinetic parameters of the immobilized pectinases including Michaelis–Menten constant (K_m) and the maximum reaction rate (V_{max}) were calculated by Lineweaver–Burk plot at temperature of 50 °C and pH of 5.5. The obtained results showed K_m and V_{max} values for PIGP were 11.21 ± 0.24 mg/mL and 2.24 ± 0.18 $\mu\text{mol}\cdot\text{min}^{-1}/(\text{mg of protein})$ and for PIGG were 10.07 ± 0.37 mg/mL and 2.93 ± 0.29 $\mu\text{mol}\cdot\text{min}^{-1}/(\text{mg of protein})$, respectively. Polyaldehyde pullulan can lead to more diffusion limitation for substrate to move in and out of its polymeric network, and for this reason, pectinase immobilized by this cross-linker showed a higher K_m and a lower V_{max} as compared with PIGG (Rehman et al. 2013; Rehman et al. 2016).

Reusability of the Immobilized Pectinases

The reusability of the different enzymes is a key factor for their application in the industry. For this purpose, the reusability of the pectinases immobilized on the glass beads by polyaldehyde pullulan and glutaraldehyde was determined at optimal conditions (temperature of 50 °C and pH of 5.5) and the obtained results were showed in Fig. 3f. As can be seen, the PIGP showed a higher reusability and retained more than 80% of the initial activity after 15 cycles, while this factor for the PIGG was about 59% after the same number of cycles. Decrease in activity of the immobilized pectinases can be related to the leakage of the enzymes from the glass beads during each run and washing of glass beads after each run (de Oliveira et al. 2018). Compared with the previous study, it can be stated that the reusability of PIGP was higher than the pectinase immobilized by polyaldehyde kefir (Hosseini et al. 2020), which can be related to difference in structure of these two polyaldehyde polysaccharide. In another study, Rehman et al. (2014) reported that the chitosan bead

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



immobilized pectinase retained about 80% of original activity after 7 cycles, which was lower than the obtained data in the current study for the pectinase immobilized by polyaldehyde pullulan (100% after 7 cycles). Also, de Oliveira et al. (2018) stated that the residual activity of alginate bead immobilized pectinase was about 30% after 8 cycles, which this result also was much lower than the results obtained in present research. However, it seems that the reusability of the immobilized enzymes depends on the native of enzyme, the type of cross-linker, and support and also the reaction conditions.

Surface Morphology

In this step, SEM images were used to investigate the surface morphology of the glass bead before and after the immobilization of pectinase by polyaldehyde pullulan (Fig. 4a, b). It was observed that the surface of glass bead before the immobilization was completely smooth but after that it was covered by polyaldehyde pullulan strands, and therefore, a considerable extent of irregularity was observed on it. These findings were agreed with previous studies (Rehman et al. 2016; Bilal et al. 2017). In a similar study, Marchis et al. (2012) reported that an irregular shape was created on the glass bead after immobilization of soybean peroxidase by glutaraldehyde. Also, AFM

images of glass bead before and after immobilization in Fig. 4c, d shows that the surface of glass bead was perfectly uniform before immobilization, while it became non-uniform after the immobilization which was in line with the obtained results by SEM. Also, the recent finding was in accordance with the reported data for SiO₂ surface immobilized glucose oxidase by Libertino et al. (2008).

Conclusions

In this study, for the first time, the polyaldehyde pullulan was used for the immobilization of pectinase on the glass beads and the obtained data was compared with glutaraldehyde cross-linked enzyme. The results showed that the immobilization by polyaldehyde pullulan had not effect on the optimum pH and temperature, while led to a decrease in specific activity and an increase in reusability. According to the results obtained in the present study, it can be stated that polyaldehyde pullulan can be used as a high potential alternative to glutaraldehyde for the immobilization of enzyme on the glass beads.

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References

- Amer, H., Nypelo, T., Sulaeva, I., Bacher, M., Henniges, U., Potthast, A., et al. (2016). Synthesis and characterization of periodate-oxidized polysaccharides: dialdehyde xylan (DAX). *Biomacromolecules*, 17(9), 2972–2980.
- Benucci, I., Mazzocchi, C., Lombardelli, C., Cacciotti, I., & Esti, M. (2019). Multi-enzymatic systems immobilized on chitosan beads for pomegranate juice treatment in fluidized bed reactor: effect on haze-active molecules and chromatic properties. *Food and Bioprocess Technology*, 12(9), 1559–1572.
- Bilal, M., Asgher, M., Iqbal, H. M. N., Hu, H., & Zhang, X. (2017). Delignification and fruit juice clarification properties of alginate-chitosan-immobilized ligninolytic cocktail. *LWT*, 80, 348–354.
- Borjesson, M., Larsson, A., Westman, G., & Strom, A. (2018). Periodate oxidation of xylan-based hemicelluloses and its effect on their thermal properties. *Carbohydrate Polymers*, 202, 280–287.
- Carpio, C., Batista-Viera, F., & Ruales, J. (2011). Improved glucoamylase immobilization onto calcined chicken bone particles. *Food and Bioprocess Technology*, 4(7), 1186–1196.
- Cerreti, M., Liburdi, K., Benucci, I., Spinelli, S. E., Lombardelli, C., & Esti, M. (2017). Optimization of pectinase and protease clarification treatment of pomegranate juice. *LWT- Food Science and Technology*, 82, 58–65.
- Champagne, P.-P., & Ramsay, J. A. (2010). Dye decolorization and detoxification by laccase immobilized on porous glass beads. *Bioresource Technology*, 101(7), 2230–2235.
- Chen, X., Wang, Q.-Q., Liu, N.-N., Liu, G.-L., Chi, Z., & Chi, Z.-M. (2017). A glycosyltransferase gene responsible for pullulan biosynthesis in *Aureobasidium melanogenum* P16. *International Journal of Biological Macromolecules*, 95, 539–549.
- Choudhury, A. R., Saluja, P., & Prasad, G. S. (2011). Pullulan production by an osmotolerant *Aureobasidium pullulans* RBF-4A3 isolated from flowers of *Caesulia axillaris*. *Carbohydrate Polymers*, 83(4), 1547–1552.
- 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.
- Diano, N., Grimaldi, T., Bianco, M., Rossi, S., Gabrovská, K., Yordanova, G., Godjevargova, T., Grano, V., Nicolucci, C., Mita, L., Bencivenga, U., Canciglia, P., & Mita, D. G. (2008). Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors. *Journal of Agricultural and Food Chemistry*, 56(23), 11471–11477.
- Fan, Q. G., Lewis, D. M., & Tapley, K. N. (2001). Characterization of cellulose aldehyde using Fourier transform infrared spectroscopy. *Journal of Applied Polymer Science*, 82(5), 1195–1202.
- 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., Tummalaipalli, M., Deopura, B. L., & Alam, M. S. (2013). Functionalization of pectin by periodate oxidation. *Carbohydrate Polymers*, 98(1), 1160–1165.
- Gurdas, S., Gulec, H. A., & Mutlu, M. (2012). Immobilization of *Aspergillus oryzae* β -galactosidase onto Duolite A568 resin via simple adsorption mechanism. *Food and Bioprocess Technology*, 5(3), 904–911.
- Haghighatpanah, N., Mirzaee, H., Khodaiyan, F., Kennedy, J. F., Aghakhani, A., Hosseini, S. S., & Jahanbin, K. (2020). Optimization and characterization of pullulan produced by a newly identified strain of *Aureobasidium pullulans*. *International Journal of Biological Macromolecules*, 152, 305–313.
- 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. <https://doi.org/10.1016/j.ijbiomac.2019.07.123>.
- 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.
- Jana, A., Halder, S. K., Ghosh, K., Paul, T., Vagvolgyi, C., Mondal, K. C., & Mohapatra, P. K. D. (2015). Tannase immobilization by chitin-alginate based adsorption-entrapment technique and its exploitation in fruit juice clarification. *Food and Bioprocess Technology*, 8(11), 2319–2329.
- 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., & Najari, Z. (2019). An integrated valorization of industrial waste of eggplant: Simultaneous recovery of pectin, phenolics and sequential production of pullulan. *Waste Management*, 100, 101–111.
- Kholiya, 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.
- 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, 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, H., Wu, B., Mu, C., & Lin, W. (2011). Concomitant degradation in periodate oxidation of carboxymethyl cellulose. *Carbohydrate Polymers*, 84(3), 881–886.
- 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(1), 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.
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3), 426–428.
- 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.
- Rajdeo, K., Harini, T., Lavanya, K., & Fadnavis, N. W. (2016). Immobilization of pectinase on reusable polymer support for clarification of apple juice. *Food and Bioprocess Technology*, 99, 12–19.
- 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., 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.
- 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.
- Romo-Sanchez, S., Arevalo-Villena, M., Romero, E. G., Ramirez, H. L., & Perez, A. B. (2014). Immobilization of β -glucosidase and its application for enhancement of aroma precursors in Muscat wine. *Food and Bioprocess Technology*, 7(5), 1381–1392.
- Singh, R. S., Kaur, N., Rana, V., & Kennedy, J. F. (2017). Pullulan: a novel molecule for biomedical applications. *Carbohydrate Polymers*, 171, 102–121.
- Sugumaran, K. R., Jothi, P., & Ponnusami, V. (2014). Bioconversion of industrial solid waste-Cassava bagasse for pullulan production in solid state fermentation. *Carbohydrate Polymers*, 99, 22–30.
- Takigawa, T., & Endo, Y. (2006). Effects of glutaraldehyde exposure on human health. *Journal of Occupational Health*, 48(2), 75–87.

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