International Journal of Scientific and Technological Research
ISSN 2422-8702 (Online)
Vol 4, No.6, 2018

Immobilization of Pectinases by Sequential Layering on Chitosan Beads

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This study was supported by Anadolu University Research Fund with project number BAP1605F452.

Abstract

Chitosan beads were prepared as a support material for immobilization of pectinases using a sequential layering approach. Glutaraldehyde (GA) was used as an activating agent for covalent binding. Different concentrations of cross-linking agent were examined and the highest specific activity of 7.8 U/mg was observed at 3% GA concentration, which was used in the multilayered immobilization studies. Three layers of pectinases were covalently immobilized on the support. As the layer number increased the total bound protein content and the activity of the chitosan beads increased; however, their specific activity significantly decreased from 9.1 U/mg in the first layer to 2.9 U/mg in the 3-layered structure. The results suggested that increasing the density of pectinases on chitosan support might have decreased the catalytic ability of enzymes due to either restriction to the protein backbone or due to substrate accessibility limitations. Therefore, optimal protein loading should be pursued for utilization of immobilized pectinases with highest specific activity.

Keywords: Enzyme Immobilization, Pectinase, Chitosan Support, Glutaraldehyde

1. Introduction

It is difficult to regenerate enzymes produced by living organisms for industrial and analytical purposes without losing their activity and operational stability and due to the obstructions for their repeated or continuous usage (Wu, He, Zhao, Qian, & Li, 2013). Since their isolation and purification is economically constraining, various immobilization processes have been developed in order to benefit from their extended use. Immobilization is defined as the process of confining enzyme molecules in a distinct support or matrix. It may involve physical or chemical attachment of an enzyme to a carrier; entrapment (encapsulation) or cross-linking of the biocatalyst aiming to maintain the catalytic activity of the enzyme molecules and to provide their repeated and continuous use (Sheldon & van Pelt, 2013; Zhang, Yuwen, & Peng, 2013).

Binding the enzymes to a support via covalent bonding has a superior holding characteristics compared to physical or ionic immobilization due to minimal shedding and leakage of enzyme (Sheldon & van Pelt, 2013). Yet, this method might have a significant drawback in cases when the enzyme is inactivated due to unfavorable enzyme conformation during attachment to the support, steric hindrance or strong strength of the covalent binding (Zhang et al., 2013). Immobilization by covalent bonding is an irreversible method; the process is carried out with the assistance of a multifunctional reagent which acts as a connector molecule (Nguyen & Kim, 2017). Glutaraldehyde is one of the most widely used reagents in the fabrication of immobilized biocatalysts (Ramirez, Brizuela, Iranzo, Arevalo-Villena, &

Perez, 2016). Glutaraldehyde is a bi-functional reagent that is able to react with different enzyme components, mainly involving primary amino groups of proteins as well as amine-functionalized supports (Barbosa et al., 2014; Nguyen & Kim, 2017). Its mode of action during immobilization is quite simple and efficient and, in some cases, it may enhance enzyme stability by providing more favorable protein configuration due to multipoint attachment (Betancor et al., 2006).

Pectinases are one of the most commercially exploited group of enzymes in the biotechnology sector and their industrial production and use steadily increase (Kashyap, Vohra, Chopra, & Tewari, 2001; Rebello et al., 2017). Predominantly, these enzymes are responsible for the degradation of pectin, long and complex structural polysaccharides found in the call walls of plant cells (Kashyap et al., 2001). They have a broad industrial application in various fields such as wine industry; food industry; paper industry for bleaching of pulp and waste paper recycling; in the processing of fruit–vegetables, tea–coffee, animal feed; extraction of vegetable oil and scouring of plant fibers (Buyukkileci, Lahore, & Tari, 2015; Garg et al., 2016; Grassin & Fauquembergue, 1996). Due to the tremendous demand for the enzyme, studies have been focusing on the immobilization of pectinase on various supports for facilitation of multiple-use of the biocatalyst in industrial applications. Numerous carriers were employed as a support during immobilization, among which sodium alginate and chitosan (Gur, Idil, & Aksoz, 2018), magnetic cornstarch microspheres (B. W. Wang et al., 2013), oxidized pulp fiber (Wu et al., 2013), sodium alginate and grafted alginate-agar beads (Li et al., 2007; Wahab et al., 2018), celite (Chauhan, Vohra, Lakhanpal, & Gupta, 2015) can be listed.

Since pectinases have significant application in food industry, it is imperative to immobilize them on natural, inert, non-toxic, biodegradable and biocompatible supports. Chitosan is a promising candidate as a carrier in these immobilization studies. It is a high molecular polysaccharide with great abundance in nature, and can be produced by partial deacetylation of chitin found in fungal species and exoskeletons of sea creatures (Kamburov & Lalov, 2012). This polymer, comprised of D-amino glucose monomers, contains two reactive functional groups: amino ($–NH_2$) and hydroxyl (–OH) groups (Nitsae, Madjid, Hakim, & Sabarudin, 2016), amenable to further modification (Kamburov & Lalov, 2012). Since chitosan is soluble in acidic solutions such as acetic acid, nitric acid and hydrochloric acid (Nitsae et al., 2016), the alterations in chitosan structure are crucial when used as carriers of enzymes working under acidic conditions. In covalent immobilization of enzymes to chitosan support, the stabilization of the carrier can be achieved with the cross-linking action of glutaraldehyde.

In this study, *Aspergillus niger* pectinase was covalently bound to chitosan beads, activated with glutaraldehyde. In general, after fabrication of the chitosan support, the carrier is treated with appropriate concentration of activating agent (glutaraldehyde), the excess of the cross-linker is removed and support is exposed to an enzyme solution for the immobilization of the biocatalyst (Liu, Li, Li, He, & Zhao, 2010). Using similar methodology, we investigated the effect of establishing several layers of enzymes on the support. To our knowledge, using layer-by-layer approach in formation of immobilized pectinase on chitosan beads has not been reported. The effectiveness of the process was assessed by measurement of protein loading, activity and specific activity of the immobilized enzymes as the number of layers increased.

2. Materials and Methods

2.1 Materials

All chemicals used in the study were standard analytical grade. The enzyme was *Aspergillus niger* pectinase from Sigma. Chitosan, high molecular weight, was purchased from Aldrich. Glutaraldehyde solution (25%) was obtained from Sigma-Aldrich. Apple pectin was obtained from Sigma.

2.2 Preparation of chitosan beads

Chitosan beads were formed by dripping acidic solution of chitosan in 1 M KOH with a syringe. After some preliminary trials, beads with better mechanical properties and physical appearance were prepared by dissolving 2.5 g of chitosan in 100 mL of 1.5% (v/v) acetic acid solution. The mixture was heated to 60℃ on a magnetic stirrer to obtain a homogeneous blend. In a large beaker, a mixture of 200 mL of 1M KOH and 67 mL of ethanol was prepared. Using a syringe, the viscous chitosan solution was added drop-wisely into the KOH solution and the formation of the beads was observed. The obtained beads were washed with distilled water until the wash solution had a neutral pH value and stored in 0.1 M phosphate buffer having pH=7.

2.3 Activation of chitosan beads

Chitosan support was activated by immersing the beads in 1%, 3% and 5% (v/v) glutaraldehyde (GA) solution for 1 h at room temperature. Upon activation, the beads were extensively washed with distilled water to remove excess GA and later stored in 0.1 M phosphate buffer at refrigeration temperature.

2.4 Sequentially-layered immobilization of pectinase on chitosan beads

One gram of commercial pectinase from *A. niger* was dissolved in 100 mL 0.1 M phosphate buffer at pH=7. The glutaraldehyde-activated chitosan beads (3g) were submerged in 20 mL of this enzyme solution and immobilization was conducted for 1 h at room temperature under mild shaking. Then, the beads were separated by filtration and washed 3 times with 20 mL of the same buffer and the wash solutions were collected for analysis. At the end of this step, the first layer of immobilized enzyme was obtained (Figure 1). Then the same beads were subjected to glutaraldehyde solution (1h at room temperature) for the activation of the 1st layer. After extensive washing with water to remove the activating reagent, the beads were again exposed to 20 mL enzyme solution for the formation of the second enzyme layer. The unbound enzymes were removed by three times washing with 20 mL buffer solution. The final layer was formed by activating the 2-layered beads with glutaraldehyde, washing with distilled water and immersing the beads in enzyme solution for 1 h and washing them 3-times with phosphate buffer. All the wash solutions were analyzed for protein content and the amount of enzyme bound to the support was calculated. After each immobilized layer, the activity of the beads was measured. The suggested mechanism of immobilization is depicted on Figure 1.

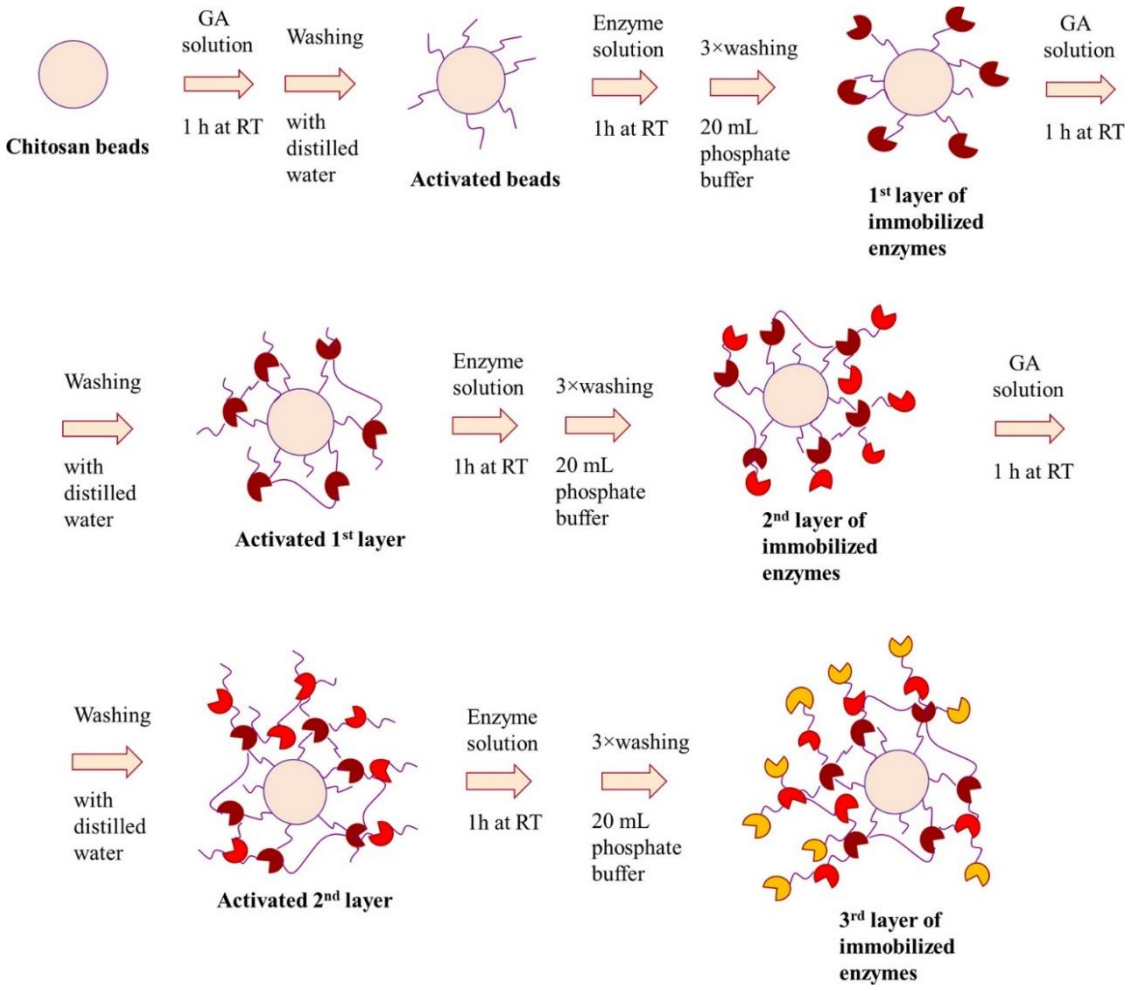


Figure 1. Steps in the immobilization of pectinases using the sequential layering method

2.5 Determination of immobilized protein amount

The protein content of enzyme samples was measured by Bradford method (Bradford, 1976). Shortly, 500 µL appropriately diluted pectinase solution was mixed with 1 mL Bradford solution and after 10 min the absorbance was measured at 595nm in UV-vis spectrophotometer (Shimadzu, Japan). The protein content was calculated from established protein standard curve constructed with bovine serum albumin (BSA) solution (0.005-0.05 mg BSA/mL). Each reported measurement is an average of a triple analysis.

The amount of immobilized pectinase was quantified by measuring the protein content of the pectinase solution before and after immobilization, and the total protein content in the three wash solutions following immobilization. The amount of immobilized enzyme was calculated as:

$$Mass_{immob.} = mass_{initial\ soln} - mass_{final\ soln} - mass_{washing\ solns}$$

2.6 Pectinase activity assay

Pectinase activity was determined using apple pectin as a substrate. 0.5 g of apple pectin was mixed with 2 mL ethanol and dissolved in 100 mL acetate buffer (pH=4.5) and stirred overnight at room temperature. The substrate solution was centrifuged at 5000 rpm to remove any undissolved remains. The pectin hydrolysis was carried out at 40°C and 1 ml-samples were taken from the substrate-enzyme mixture with 5 min intervals for 40 min. The activity assays were conducted by two parallel measurements. The amount of reducing sugars formed during hydrolysis was estimated by the 3,5-dinirtosalicylic acid (DNSA) method (Miller, 1959). Briefly, the hydrolysis samples were mixed with 1.5 mL DNSA reagent, boiled for 15 min, cooled and their absorbance was measured at 540 nm using UV-vis spectrophotometer (Shimadzu, Japan). The standard compound used for the calibration curve was D-(+) galacturonic acid monohydrate. One unit of activity was defined as the amount of enzyme required to liberate 1 µmol galacturonic acid per mL per min under the described conditions.

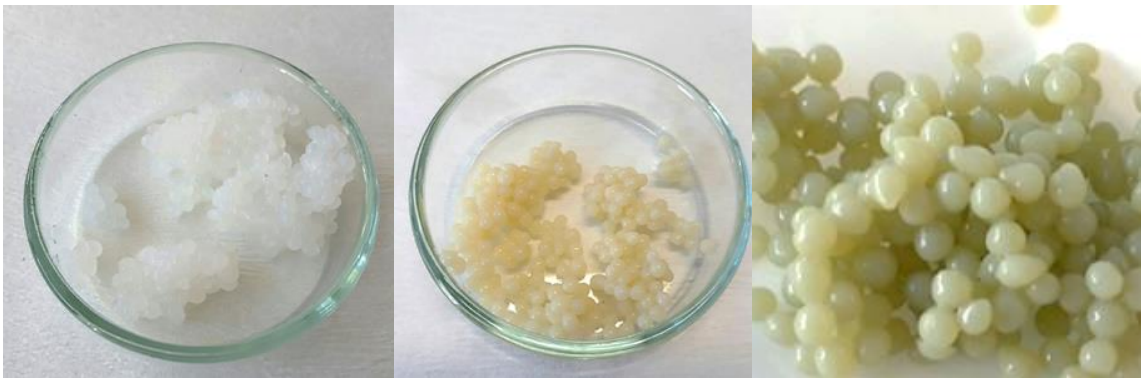
3. Results and Discussion

3.1 Determination of Glutaraldehyde Concentration

The appearance of chitosan beads during the stages of single-layered immobilization of pectinase is shown on Figure 2. Stable chitosan beads (approximately1-3 mm in diameter) were obtained by dropping acetic acid-chitosan mixture into 1 M KOH solution with a syringe (Figure 2-a). These beads were activated by different concentrations of glutaraldehyde (GA) (1%, 3% and 5% (v/v)) to determine the optimum concentration for enzyme immobilization (Figure 2-b). The activated beads were suspended in 1% (w/v) enzyme solution for 1 h at room temperature under mild shaking and the immobilization was achieved (Figure 2-c). After separating the beads from the enzyme solution, they were washed three times with 20 mL phosphate buffer (pH=7) to remove the unbound pectinases. The amount of immobilized pectinase was calculated by subtracting the protein contents of final and wash solutions from the initial enzyme solution. Then, 1 g of immobilized beads was used for the hydrolysis of apple pectin and the activity of the beads was measured. The optimum concentration of the activating agent was assessed in terms of protein loading, beads' activity and specific activity. Specific activity was determined from the ratio of measured bead activity to the amount of protein bound to the chitosan support (U/mg).

In various studies the effect of GA concentration on enzyme immobilization was investigated (Chauhan et al., 2015; Costa, B. Romao, Ribeiro, & Resende, 2013; Ramirez et al., 2016; Sojitra, Nadar, & Rathod, 2017). However, the selection of the optimal concentration was based on different criteria. While some studies focused on the effect of cross-linking agent on loading efficiency (amount of protein/ g support) (Costa et al., 2013), other studies were interested in the activity of immobilized enzymes (U/g support) and continued with the concentration providing highest activity (Chauhan et al., 2015; Ramirez et al., 2016; Sojitra et al., 2017; B. W. Wang et al., 2013). Both approaches in selecting the optimal cross-linking concentration might be misleading. Rather than focusing on protein loading or activity alone, comparing the specific activities (U/g enzyme) of immobilized supports might be more explanatory (Lei, Soares, Shin, Liu, & Ackerman, 2008; M. Wang et al., 2014). Immobilization efficiency should be based on both performance and economical perspective. Having high enzyme loading and/or high enzyme activity but low specific activity might indicate that some portion of the immobilized enzymes are not functioning properly due to overloading/ disorientation/ inactivation/

conformational changes etc. and this might lead to overuse of fairly expensive catalysts. Seeking for immobilization conditions providing the highest specific activity might result in economical utilization of the enzyme and its enhanced catalytic activity. Therefore, our selection of optimal GA concentration was based on the activity per unit enzyme amount (U/mg) on chitosan support.



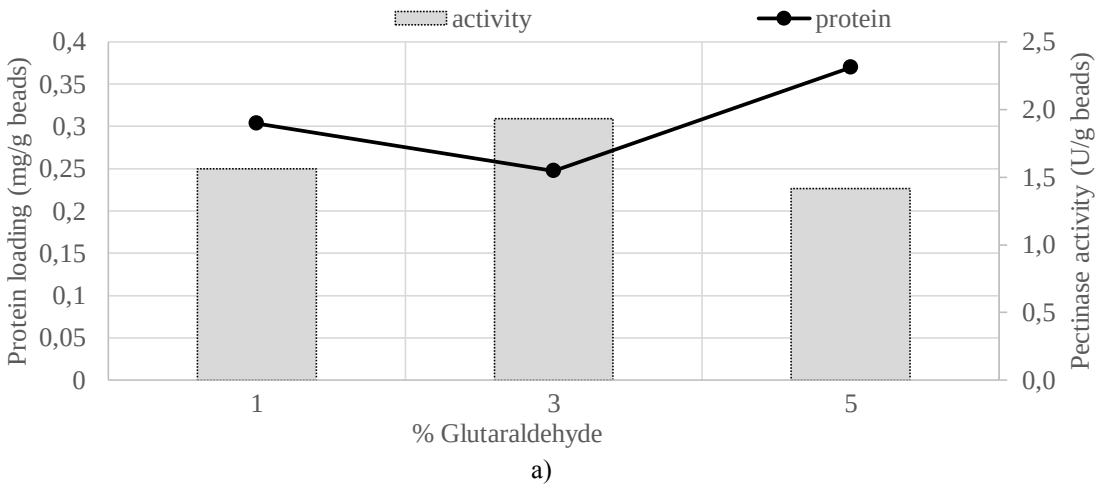
a) b) c)

Figure 2. Typical appearance of chitosan beads after formation (a), after activation (3% GA, 1 h at RT) (b) and after 1-layered immobilization with pectinase (1 h at RT) (c).

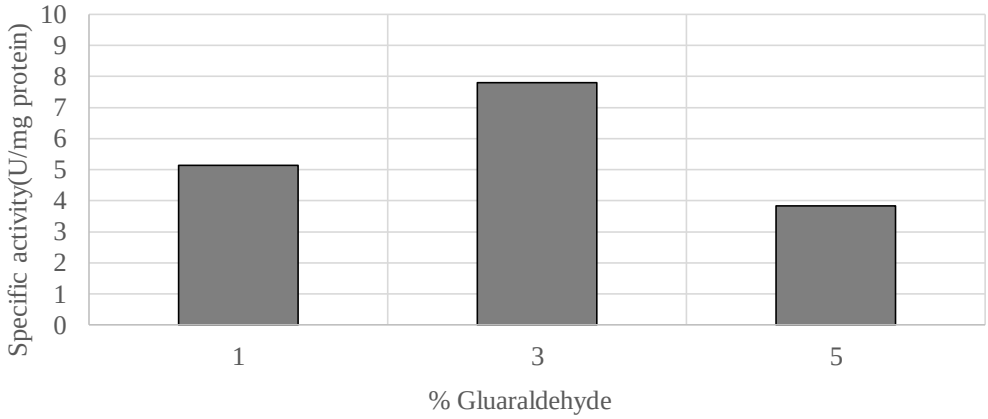
The results on protein loading, pectinase activity and specific activity are illustrated in Figure 3-a. During the immobilization studies, the highest immobilized enzyme amount (0.37 mg/g beads) was observed for 5% GA concentration, followed by 0.30 mg/g beads for 1% GA and 0.25 mg/g beads for 3% GA concentration (Figure 3-a). Usually, increasing GA concentration results in gradual increase of protein loading and after a certain optima a decrease is observed. Chauhan et al. (2015) investigated the effect of GA concentration on binding efficiency of commercial pectinase in the range of 1-3% with 0.5 unit interval. The maximum protein loading was observed at 2.5% GA concentration.

Investigation of the activity of immobilized enzymes revealed that the highest activity of 1.93 U/g beads was achieved at 3% GA concentration. The activities at 1% and 5% GA concentration were found as 1.56 and 1.42 U/g beads, respectively (Figure 3-a). Ramirez et al. (2016) reported the residual activity of pectinase when immobilized on chitosan support with 0.5, 1.0, 2.0 and 3.0% (v/v) GA concentrations. The highest residual activity of 85% was observed in the samples immobilized with 1.0% GA solution. Wang et al. (2013) obtained the highest recovery rate at 3.5% GA when studying the effect of GA concentration between 3.0-3.5-4.0-4.5-5.0% GA.

As mentioned above, the selection of optimal GA concentration was based on the specific activity of immobilized enzyme. The conditions exhibiting the highest specific activity were observed for 3% GA concentration where 7.8 U/mg pectinase specific activity was achieved. Whereas, for 1% GA and 5% GA, specific activities remained as 5.1 and 3.8 U/mg, respectively (Figure 3-b). Therefore, further studies were conducted using 3% (v/v) concentration of the cross-linking agent.



a)



b)

Figure 3. Effect of different concentrations of glutaraldehyde cross-linking agent on a) the protein loading (circles) and activity (columns), and b) specific activity of one-layer-immobilized pectinases on chitosan beads.

3.2 Effect of sequentially layered immobilization

The sequential layered immobilization of pectinases, to our knowledge, was not previously described in the literature. The mechanism of the binding was presented on Figure 1. This layer-by-layer immobilization was investigated by measuring the amount of protein load, the activity of the beads and their specific activity as the number of layers increased.

The results showed that as the number of layers increased, the amount of loaded protein and the activity of the chitosan beads increased (Figure 4-a). The protein content in the first layer was 0.13 mg/g chitosan, when two-layered structure was obtained the total protein load was 0.36 mg/g chitosan, and the three-layered structure contained 0.66 mg enzyme/g chitosan beads. Each step of activation with glutaraldehyde and exposure to enzyme solution resulted in a linear increase in the amount of pectinase bound to the carrier. In terms of activity, however, the increase had an asymptotic behavior. In other words, the increase in activity was not proportional to the amount of protein loading and less activity than expected was observed. This can be better comprehended by examining the specific activities of the samples (Figure 4-b). There was an almost linear decrease in the specific activities of the samples as the number of enzyme layers increased. Starting with 9.1 U/mg specific activity in the single-layered beads, the value decreased to 5.2 U/mg in double-layered and to 2.9 U/mg in the three-layered structures.

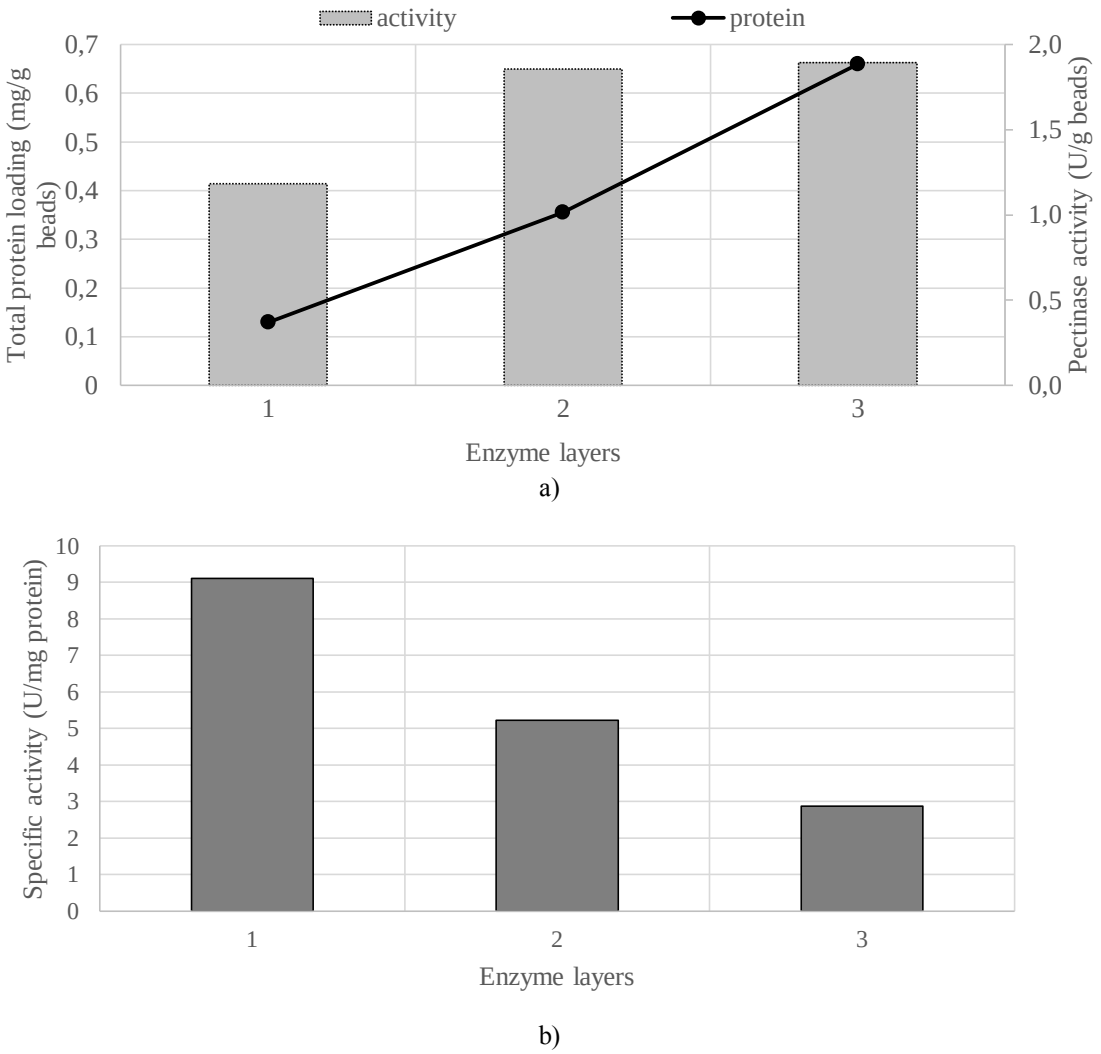


Figure 4. Effect of increasing layers on total a) protein loading and activity and b) specific activity of immobilized pectinases on chitosan beads.

Possible explanation for this decrease might be the fact that as the density of the enzymes on the support's surface increased, the flexibility of enzymes' backbone became restricted. Flexibility of an enzyme is closely related to its catalytic ability and any perturbation that limits protein's flexibility may affect its function (Teilum, Olsen, & Kragelund, 2011). The restricted mobility of the protein backbone, probably resulted in limitation of catalytic activity and/or the access of enzymes' active site to the substrate, decreasing their specific activity. In addition, Nguyen and Kim (2017) reported that utilization of glutaraldehyde might result in severe enzyme modifications in their 3-D arrangement, leading to significant loss of activity. They suggested that addition of an inert protein, like BSA or gelatin, during immobilization may minimize these drastic modifications and preserve the activity of the enzymes (Nguyen & Kim, 2017) . Moreover, the cross-linked matrix of enzymes formed in the multilayered structure might have diffusion limiting-effect on the fairly large pectin substrate. This might be another reason for the decrease in activity of the immobilized enzyme.

Assessing the effectiveness of layered immobilization of pectinases, the higher activity observed in two-layered structure compared to the single layer is an improvement in spite of the low specific activity in the former. Considering the repeated use of the immobilized pectinases, having increased activity with two layers may compensate for the cost of the enzyme and make it useful in industrial applications.

4. Conclusions

Stable chitosan beads were produced by drop-wise addition of acetic acid-chitosan mixture into 1 M KOH solution with a syringe. Different glutaraldehyde concentrations were examined for determination of optimal amount activation agent (glutaraldehyde) in terms of protein loading, beads' activity and specific activity. The optimal conditions exhibiting the highest specific activity were observed for 3% GA concentration and the sequential layering immobilization studies were conducted using this amount of cross-linking agent. Layer-by-layer immobilization results showed that as the number of layers increased, the amount of loaded protein and the activity of the chitosan beads increased. However, the specific activity of the beads decreased, suggesting that significant amount of the enzyme attached to the support were not able to catalyze the hydrolysis reaction of pectin. The possible restriction in the catalytic site of enzyme of substrate diffusion limitations might be the reasons for the decrease in specific activity. Further studies on the evaluation of effectivity of the proposed immobilization method might be conducted using an enzyme involved with smaller substrate so that the accessibility of the enzyme is not restricted.

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