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Use of immobilized Pectinase for Fruit Juice Clarification

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The study was initiated to use for new local support material sources (bentonite) for immobilized pectinase for invest it's in this field, due to huge quantities of this clay in the Western desert of the Republic of Iraq, the aim of the present study to use bentonite for immobilized pectinase, and study of its ability and efficiently for depectinization from fruit juice as application in food technology. The immobilization efficiency of lyophilized pectinase was 88%. PH profile of both free and immobilized enzyme was 4.5 which was stable at 3-6 for 30 min. The maximum temperature was 45 and 50°C, respectively, both enzymes were stable at 40°C for 30 min, while they lost 100 and 64.28% from its initial activity at 70°C respectively. Immobilized pectinase retained initial activity over 18 continue usage; while it retained about 87% of its original activity after 25 continuing usage. Both free and immobilized pectinase retained of 100% of initial activity for 6 and 28 days of storage at 4°C. The required time for pectin hydrolysis by immobilized pectinase was 60 min, with transmittance T_{450} 32.74%, the decrease of viscosity for raw orange juice was 63.9% at the same period.

Keywords: Enzymes application, immobilized enzymes, pectinase, bentonite clay, orange juice clarification.

INTRODUCTION

The global markets are attending an increasing growth in the natural fruit juices industry, this prompted the producers to meet the consumer's desire for providing high quality products through the use of up to date technologies in production, including the use of enzymes which it considered as high biocatalysts ability that use to overcome the manufacturing problems that causes by naturally content of fruits such as the compounds which causing turbidity in fruit juices (Deboni et al. 2014).

Pectin is found the cell walls of the fruits such us oranges, apple, pears and peach, the presence of it in the juices of these fruits was considered the main of the manufacturing problems that must be removed in order to obtain high clear juice with low viscosity, natural flavor, long shelf life and acceptability for consumer. Pectin is a polysaccharide with high molecular weight; the linear chain was composed of α-(14)-linked D-galacturonic acids. The presence of pectin is undesirable in fruit juice due to it is a typical gelling agent, interacts with protein forming a complex which considered as an appropriate source of microbial growth thereby decrease shelf life of the juice and increase viscous of juice that effect of flow ability and filtration during steps of production. Thus, removable of pectin is essential to increase of shelf life of fruits juices (Al-Soufi, 2016b).

Pectinase (endopolygalacturonases, EC 3.2.1.15) is one of the pectinolytic enzymes that widely used commercially in fruits juice clarifying process and increasing juice extraction due its ability to hydrolysis pectin or pectic acid through

its activity on polysaccharide hydrolysisand carbohydrate esterases (Chauhan et al. 2013; Ramirez et al. 2013). However, using of free enzyme in processing implementations will not economically due to decrease of its stability, difficulty of reuse, which make them inappropriate for large scale production system, therefore, to conquer these problems, immobilized enzyme is one of the appropriate techniques due to its ability to provides various features for enzymes, such as, decrease of cost, increase of catalytic properties (activity, pH and thermal stability) and reusability (Alsoufi, 2018; Alsoufi and Aziz, 2019).

Generally, Many studies of immobilized pectinase have been executed with various support materials such as magnetic nanoparticles (Ramankannanet al. 2013; Mosafaet al. 2014; Magroet al. 2019), calcium alginate (Bograet al. 2013; Martín et al. 2019), sodium alginate (Al-Soufi, 2016b), Chitosan (Ramirez et al. 2016), Celite (Chauhan, 2015), Polyvinyl alcohol gel (Cerretiet al. 2017).

Bentonite clay was use as a support material in immobilized enzymes in (Al-Soufi, 2015; Alsoufi, 2018; Alsoufi, 2019; Alsoufi and Aziz, 2019; Alsoufiand Aziz, 2020), due to its functional nature properties that it's providing acidic site for enzyme binding over NH_2 group (Alsoufi, 2018), and its ability for activated and link with glutaraldehyde to making covalent bond between enzyme and clay for increase of stability (Al-Soufi, 2015), thus, this study aimed to use bentonite for immobilized pectinase, and study of its ability and efficiently for depectinization from fruit juice.

MATERIALS AND METHODS

Chemicals

Lyophilized pectinase powdered (EC: 3.2.1.15) (Sigma-Aldrich), bentonite clay, was obtained from Baghdad.

Pectinase assay

The activity of free and immobilized pectinase was estimate by measuring the release of reducing sugars (galacturonic acid) as a result of the pectin hydrolysis by pectinase according to Ramankannan et al. (2013) by mixed 0.5 mL or 2 g/L of enzyme pectinase with 1 mL of substrate solution (2 mg of pectin in 0.01 mM sodium acetate buffer pH 4), then incubate for 60 min at 50°C under shaking condition. The reducing sugars in the supernatant were estimated by method of Miller (1959) using dinitrosalicylic acid (DNS) at 540 nm, the activity of pectinase was calculated by using galacturonic acid calibration curve. The unit of enzyme activity (IU/mg) is defined as an amount of galacturonic acid released (µmol/mg) from pectin by an action of enzyme used.

Protein estimation

Protein (mg/mL) was estimated according to the method of Bradford (1976).

Activation of bentonite clay

The activation took place by stirring bentonite with 10% 3-APTES solution in acetone (v/v) for 1 hour at 25°C, then filtered, washed with acetone and dried in oven at 80°C, the clay was treated with 10% aqueous glutaraldehyde solution (v/v) for 60 min and filtered, washed, dried at 25°C and stored in 0.1 mM phosphate buffer pH 6 at 4°C until use to immobilized pectinase as in the method of Alsoufi (2018).

Immobilization of pectinase

The enzyme was immobilization according to Al-Soufi (2016b) by mixed 1 ml of pectinase solution (10 mg/mL pectinase in 0.1 M sodium acetate buffer pH 4) with 0.5 g of activated bentonite clay and stirred for 1 h at 4°C, then washing it for three times with 20 mL of 0.1 M sodium acetate buffer pH 4 and centrifuged for each time at 10000 rpm for 10 min to ensure removal all free pectinase in supernatant, the immobilized pectinase (precipitate) was stored in the same buffer of washing at 4°C until use.

Immobilization efficiency

Immobilization efficiency (IE) of pectinase was calculated with the following equation by Alsoufi and Aziz (2020).

$$\mathrm{IE}(\%) = \frac{\mathrm{IM}}{\mathrm{FA}} \times 100$$

IM: Specific activity (U/mg) of immobilized pectinase.

FA: Specific activity (U/mg) of free pectinase.

Effect of pH on activity and stability

The effect of pH profile on activity and stability for the free and immobilized pectinase was measuring by using 0.5 M sodium acetate buffer at pH 3, 3.5, 4, 4.5, 5, 5.5 and 6 at 30°C by using 0.5 mL and 10 mg, respectively. The relative activity (%) was estimated through a method of Alsoufi and Aziz (2019).

Effect of temperature on activity and stability

Free and immobilized pectinase profile activity was estimated at a different range of temperature

(30-70°C) by using 0.5 M sodium acetate buffer pH 4.5 for 10 min, while, effect on stability was measuring by incubated both type of enzyme at the same range of temperatures for 30 min, followed by incubation in ice bath, remaining activity (%) was estimated by Alsoufi (2019).

Orange juice preparation

Orange juice was prepare according to the method described by Al-Soufi (2016b) through using manual squeezing and centrifugation at 3000 rpm for 10 min, precipitate was neglect and get supernatant.

Determination of the optimal time immobilized pectinase for clarification

Immobilized pectinase 2.5 gm (10 mg/mL) was adding to 1000 mL of orange juice and incubation under stirring for 0-90 min at 40°C following the method described by Al-Soufi (2016b). The degree of juice clarification was determined by measuring the transmittance by using a spectrophotometer at 450 nm.

Viscosity

Orange juice viscosity was estimated by using an Ostwald capillary tube as method described by Al-Soufi (2016b), the decrease of viscosity (%) was calculated with the following equation.

$$\mathrm{DV}(\%) = 100 - \frac{\mathrm{V}}{\mathrm{V0}} \times 100$$

DV: Decrease of viscosity.

V: Viscosity at time.

V0: Viscosity at zero time.

Effect of storage on activity

Immobilized pectinase was stored in 0.5 M sodium acetate buffer at pH 4.5 for 30 days at 4°C through a method of Alsoufi (2019).

Immobilized pectinase recycling

Immobilized enzyme activity was followed up for 25 cycles according to the method described by Alsoufi and Aziz (2020).

RESULTSAND DISCUSSION

Immobilization efficiency

Pectinase was immobilized by covalent onto activated bentonite by glutaraldehyde; the efficiency of immobilization was 88% from the original enzyme amount.

The determination of immobilized efficiency represented one of the important steps of immobilization process; the aim of selecting the support material is due to its capacity to immobilize the large amount of free enzyme without any decrease in the activity and stability (Nasha et al. 2012). In this regards, Chauhan et al. (2015) refer that the efficiency of immobilized pectinase with celite through adsorption was 43.18%. It was 50.6% for immobilized magnetite nanoparticles (Mosafa et al. 2015). Whilst it was 80% for immobilized by chitosan (Ramirez et al. 2016), and it was 87% for immobilized pectinase on sodium algenate (Al-Soufi, 2016b).

Effect of pH on pectinase activity

The effect of pH on the activity of free and immobilized pectinase was 4.5 (Figure 1) and they are stable at pH range 3-6 for 30 min (Figure 2).

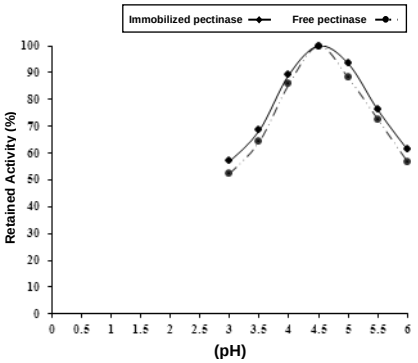


Figure 1: Effect of pH on activity of free and immobilized pectinase on bentonite.

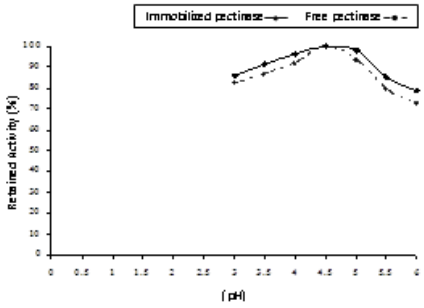


Figure 2: Effect of pH on stability of free and immobilized pectinase on bentonite.

According to the related study, the optimum pH values of free and immobilized pectinase were assayed between 4 and 5. It was 5 and 4.5 for free and immobilized pectinase by adsorption on an alginate-coated chitin support were respectively (Ramirez et al. 2013), and it was 4.5 for both free and immobilized pectinase with polyvinyl alcohol gel (Cerreti et al. 2017). While, it

was 4 for free and immobilized pectinase on Nano, Micro and Macro-chitosan-magnetic particles (Magro et al. 2019). Similarly, Martín et al. (2019) noted that the maximum activity of free and immobilized pectinase by calcium alginate hydrogels was at pH 4. The observed change in the relative activity for immobilized pectinase may be due to the acidic that will provide the favorable environment for the pectinase to actagainst pectin via increased affinity of the enzyme towards the substrate (Ramankannan et al. 2013; Al-Soufi, 2016b).

Effect of temperate on pectinase activity

The maximum temperature for free and immobilized pectinase activity was 45 and 50°C, respectively, (Figure 3), both enzymes were stable at 40°C for 30 min, while they lost 100 and 64.28% respectively, from its initial activity at 70°C at the same time (Figure 4), that mean improved of heat resistance stability for pectinase by immobilization.

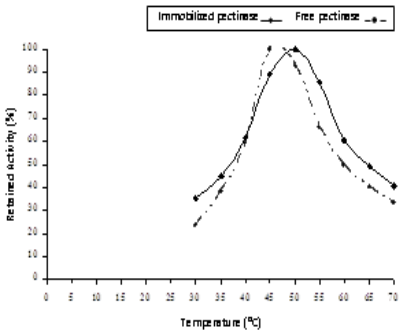


Figure 3: Effect of temperature (°C) on activity of free and immobilized pectinase on bentonite.

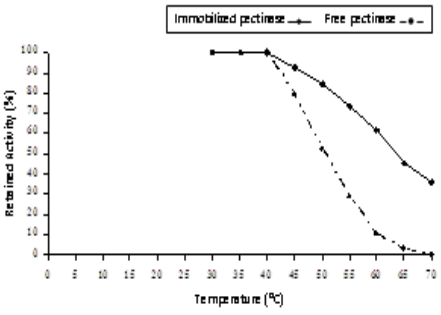


Figure 4: Effect of temperature (°C) on stability of free and immobilized pectinase on bentonite.

Generally, immobilization studies referring to increase thermal stability for immobilized enzyme due to improvement of resistance against increase of temperature at reaction of enzyme (Alsoufi, 2018). Based on that, most researcher were referring to this fact as a similarly, Mosafa et al. (2014) refer that the optimum temperature for free and immobilized pectinase on silica-coated magnetite nanoparticles was 40 and 50°C, respectively. Also, Martín et al. (2019) observed that the maximum activity for immobilized pectinase by calcium alginate was 50°C, and they don't observed any changes in enzymatic activity at this temperature. While it different in the others, such as, Bogra et al. (2013) who referred that the optimum temperature for free and immobilized pectinase on calcium alginate beads were 55 and 60°C, respectively, they kept about 60 and 30% of its original activity at 65°C, respectively. On the other hand, Ramirez et al. (2016) noted that optimum temperature for free and immobilized pectinase on chitosan was 40 and 45°C respectively, and the immobilized pectinase was more stable at high temperature than free pectinase. Likewise, Chauhan et al. (2015) refer that maximum temperature activity for free and immobilized pectinase with celite through adsorption was 40 and 45°C, respectively. Whilst, Magro et al. (2019) found that the free pectinase showed the highest activity at 50°C. While the immobilized enzyme with Nano-CMag, Micro-CMag and Macro-CMag showed highest activity at 60°C. Immobilization exhibit an improvement of thermal stability of pectinase, the responsible for that back to the covalent bond between pectinase and the support which can increase in the stability of the enzyme and increase activation energy of the thermal inactivation (Mosafa et al. 2014).

Effect of immobilized pectinase recycling

Immobilized pectinase retained initial activity over 18 continue usage; while it retained about 87% of its original activity after 25 continue usage (Figure 5).

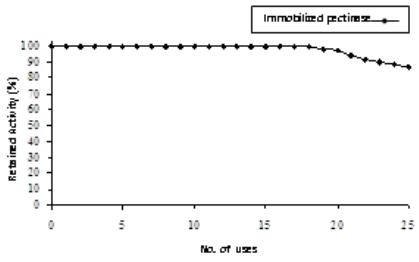


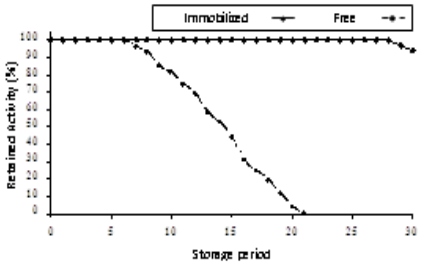
Figure 5: Effect of recycling of immobilized

pectinase on bentonite.

In this regard, as a similarly, Magro et al. (2019) found that immobilized pectinase on Macro-chitosan-magnetic particles was retaining about 85% of its original activity over 25 cycles. While, Ramirez et al. (2016) referred that immobilized pectinase on chitosan remained all activity after 9 consecutive cycles and kept nearly 70% of its original activity over 15 cycles at 30°C. On the other hand, both Deng et al. (2019) and Martín et al. (2019) explained that immobilized pectinase by calcium alginate retained about 20 and 37% of their initial activity after 10 and 6 cycles, respectively. Moreover, Bogra et al. (2013) refer that the immobilized pectinase in calcium alginate beads could be used for at least 10 batch cycles.Whereas, Chauhan et al. (2015) noted that immobilized pectinase to the celite through adsorption retained almost 50% of activity after 3 cycles of reuse. The recycling of immobilized pectinase considered an important factor for any industrial applications in this field to make the process economically feasible, the losing of immobilized pectinase activity during reuse cycle attributed to leakage of enzyme from bentonite due to wash steps (Sojitraet al. 2017; Alsoufi, 2019).

Effect of storage on immobilized pectinase activity

The free and immobilized pectinase retained of 100% of initial activity for 6 and 28 days of storage at 4°C (Figure 6).



*****storage period (day) in figure 6 not clear**

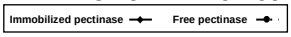


Figure 6: Effect of storage period stability for free and immobilized pectinase on bentonite at 4°C.

In this context, Bogra et al. (2013) found that the storage of immobilized pectinase in calcium alginate beads that at 4°C retain of all activity up to 90 days. Whereas, Mosafa et al. (2014) observed that the immobilized pectinase on silica-coated magnetite nanoparticles retained more than 70% of its original activity meanwhile 30 days at 4°C. While, Sojitra et al. (2017) noticed that immobilized pectinase onto chitosan magnetic nanoparticles retained 89% residual activity over 15 days at 28±2°C. Furthermore, Martín et al. (2019) referred that the free pectinase retain nearly 72% of its initial activity through 5 weeks of storage at 4°C, and the immobilized pectinase by calcium alginate kept all its activity over 11 weeks of storage at the same temperature. The stability of the enzyme through storage period is considered one of the important parameters that use for determination the range of appropriate for use it in industrials application, these type of enzymes will have improved stability as a result of neutralization of charged which it is responsible for interaction between the enzyme and immobilization support materials that will provide stronger contacts of conjugated structural and higher stabilization of the enzyme, while, after long storage periods, immobilized enzymes will lost activity as a result for microorganisms contamination in the stocked solution (Nawaz et al. 2015; Martín et al. 2019; Alsoufi and Aziz, 2020).

Application

The determination of the optimal time for clarification of orange juice by immobilized pectinase showed that the required time for pectin hydrolysis was 60 min, while, transmittance T_{450} (%) increased from 12.57% for the raw orange juice to be 14.68, 21.15, 26.94, 32.74, 32.74 and 32.74% at incubation times 0, 15, 30, 45, 60, 75 and 90 min, respectively (Figure 7).

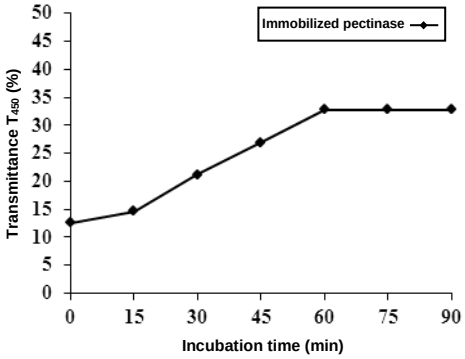
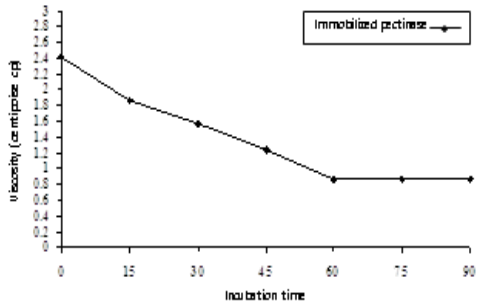


Figure 7: Determination of the optimum time (min) for free and immobilized pectinase on bentonite to hydrolysis pectin in raw orange juice

The use of immobilized pectinase to a

decrease in the viscosity of raw orange juice from 2.41 to 1.86, 1.57, 1.24, 0.87, 0.87, and 0.87 centipoise (cp) at an incubation period of 0, 15, 30, 45, 60, 75 and 90 min, respectively. The decrease in viscosity was recorded at its highest rate at the incubation period of 60 min, and there was no change in the value of viscosity when increasing the incubation period to 90 min (Figure 8), the decrease of viscosity for raw orange juice was 63.9%.



*** incubation **time (min) in figure 8 not clear**

Figure 8: The ability of immobilized pectinase on bentonite to decrease viscosity of raw orange juice.

Immobilized pectinase can be reusing in food industries of raw fruit juice clarification for much period, while free pectinase can be use just one time (Martín et al. 2019). In this context, Bogra et al. (2013) referred that the viscosity of orange juice that treated with immobilized pectinase in calcium alginate beads was a decrease of about 56% and the filter ability was increased about by 260%, the juice remain clear after 60 day of storage at 4°C. Whereas, Mosafa et al. (2014) observed that immobilized pectinase on silica-coated magnetite nanoparticles reduced the viscosity of apple juice from 1.12 to 0.92 mm^2s^{-1}. In addition, Magro et al. (2019) used immobilized pectinase on nano, micro and macro-chitosan-magnetic particles for clarification of grape, apple and orange juices, respectively. While, Sojitra et al. (2017) noticed that the use of immobilized pectinase onto chitosan magnetic nanoparticles was breakdown pectin in apple juice and observe reduce of up to 74% after 150 min of treatment at 50°C. In addition, Martín et al. (2019) use immobilized pectinase by calcium alginate for grape juice clarification and observed decreased significantly of turbidity during 150 min at 20°C. Similarly, Deng et al. (2019) use immobilized pectinase on calcium alginate microspheres for apple juice clarification; they noticed that the light transmittance of apple juice was improved to 96.8%.

Pectin in fruit juice is responsible for the cohesion, turbidity and general appearance, while, it's responsible for increase of viscosity, extend and obstruction processing step of juices clarification, thus, raw fruit juice must be treated with pectinase to hydrolysis pectin and make juice more acceptable for consumers (Martín et al. 2019). Therefore, immobilized pectinase was in use for this industrial application due to its many advantages such as activity, thermal stability and reusability for many times (Alsoufi, 2016b).

CONCLUSION

This study showed the ability of immobilized pectinase by bentonite clay for hydrolysis of pectin and clarification of raw orange juice with high efficiency. However, more detailed studies on the immobilization of others enzymes on bentonite for use in food technology are required.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

Mohammed A Alsoufi: designed the study, collection of data, analysis, interpreted the data and drafted the manuscript.

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