**Annals of Agric. Sci., Moshtohor**
**Vol. 50(2) (2012), 173 – 184**

**ISSN 1110-0419**
**http://annagricmoshj.com**

# Kinetic parameters of immobilized pectinase enzyme

**Saad, S.M.M.; Foda, F.F.A.; Abdel-Aleem, I.M. and Gamal El-Deen, M.S.**

Biochem. Dept., Fac. of Agric. Moshtohor, Benha Branch, Egypt

**Abstract**

Pectinase (E.C.3.2.1.15), pectinex ultra SP-L enzyme from *Aspergillus aculeatus* has been immobilized on different supports materials i.e. porous glass beads, chitin, chicken bone, sand and calcium alginate also the kinetic parameters of immobilized forms were determined. The retention activities of bounded enzyme preparations were found to be 81.86, 74.07, 78.39, 75.85 and 77.09% for the above mentioned supports, respectively. The optimum pH was 4.8 for immobilized forms on sand and Ca-alginate gel beads, but it was 4.8 for chitin enzyme complex. The other preparations of pectinex ultra SP-L on glass beads and chicken bone had the maximum activity at pH's 5.2 and 5.0, respectively. On the other hand, optimum temperature reached 55° for immobilized enzyme on chicken bone, 50°C for immobilized enzyme on activated sand and Ca-alginate but immobilized forms on glass beads and activated chitin were found to be at 60°C.

Kinetic behaviors of the immobilized enzyme with different supports were determined. The free enzyme showed $K_m$ value of 0.74 g/100 g pectin which was increased after immobilization to 0.81, 0.79 and 1.01 g/100 g pectin for immobilized pectinex ultra SP-L on glass beads, activated chitin and Ca-alginate, respectively. A decrease in $V_{max}$ values occurred upon enzyme immobilized for all supports. Also, the immobilized enzyme on the above mentioned supports can be used 4 times with only lost about 5.52, 9.23, 5.81, 6.36 and 11.11% of its original activity. Enzymatic saccharification processes were 90.75, 86.78, 88.10, 82.84 and 74.95%, respectively for immobilized pectinase enzyme on glass beads, chitin, chicken bone, sand and Ca-alginate, respectively.

**Key word:** Pectinase enzyme – Immobilization - *Aspergillus aculeatus* - Pectinex ultra SP-L Enzyme - Glass beads – Chitin - Chicken bone - Sand - Calcium alginate.

## Introduction

Pectinases are a complex group of enzymes that degrade various pectic substances present in plant tissues. Pectinases have potential applications in fruit, paper and textile industries. Apart from these industrial applications, these enzymes possess biological importance in protoplast fusion technology and plant pathology. Since many applications of pectinases in various fields, it is important to understand the nature and properties of these enzymes for efficient and effective usage **(Gummadi and Panda, 2003)**.

Enzyme immobilization in the food processing industry helps in development of continuous process, economic organization of operations, automation and decrease of labor, greater control over reactions, high volumetric productivity and low residence time. Utilization of immobilized enzymes give products that are relatively pure, which is an important factor especially in food processing and pharmaceutical industries where contamination could be of serious toxicological, sensory or immunological consequences. Enzymes can be immobilized on a variety of natural and synthetic supports. The choice of support and/or technique depends on the nature of the enzyme, its substrate and its application. Attempts have been carried out to immobilize commercial pectinase preparations on various supports for fruit processing and endopoly-galacturonase **(Demiral *et al.*, 2004)**.

**Csanadi and Sisak (2006)** studied immobilization of pectinex ultra SP-L pectinase on an anion exchange resin. The temperature and pH optima of the solid-phase biocatalyst were 53°C and 5.6, respectively. Also, the immobilized form showed almost no decrease in its activity during 12 reaction cycles.

**Lei *et al.* (2007a)** used chitosan-tethered silica particle from a layer-by-layer approach for pectinase immobilization. Kinetic parameters of free and immobilized pectinase were calculated as 8.28 and 9.98 mg pectin $ml^{-1}$, respectively for $K_m$ and 1.165 $g^{-1}$ pectin/g enzyme, 1.124 $g^{-1}$ pectin/g particle for $V_{max}$ of free and immobilized enzymes, respectively.

**Lei *et al.* (2007b)** studied the combined magnetic and chemical covalent immobilization of pectinase on composites membranes. $V_{max}$ values for free and immobilized pectinase enzymes were 1.165, 1.112 $g^{-1}$ pectin/g enzyme, respectively. However, $K_m$ value of immobilized enzyme (9.86 g pectin $ml^{-1}$) was higher than that of free enzyme (8.28 g pectin $ml^{-1}$). They added that the pH optimum and temperature optimum of free and immobilized pectinase were found to be pH 6.0 and 65°C.

**Li *et al.* (2007)** reported that pectinase was immobilized onto alginate support using glutaraldehyde as a coupling agent, which yielded relatively high level of residual activity (66%).

**Li *et al.* (2008)** evaluated the immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. They found that that optimal temperature for pectinase activity changed from 40 to 50°C after immobilization; however, the optimal pH remained unchanged. The immobilized enzyme also exhibited great operational stability, and an 81% residual activity was observed in the immobilized enzyme after 10 batch reactions.

**Li *et al.* (2009)** immobilized pectinase enzyme on $Fe_3O_4/SiO_2$ as solid support by covalent attachment. They found that the immobilized enzyme retained >50% of its initial activity over 30 days, and the optimum temperature and pH also increased to the ranges of 50-60°C and 3.0-4.7, respectively.

**Buga *et al.* (2010)** studied the physico-chemical characteristics of immobilized polygalacturonase from *Aspergillus niger* (SA6) by entrapment using calcium alginate. They found that the $K_m$ and $V_{max}$ values of the immobilized form were 11.1 mg $ml^{-1}$ and 1.65 µmole/min/mg, respectively. The optimum pH and temperature of the immobilized polygalacturonase were 4.5 and 40°C, respectively. The activity of the immobilized polygalacturonase reduced to 34.56 and 14.81% of the initial activity in the second and third catalytic cycles, respectively.

**Jun *et al.* (2011)** evaluated the acidic endo-polygalacturonase (PGAI) from *Bispora* sp. MEY-1. The $K_m$ and $V_{max}$ values for polygalacturonic acid were found to be 1.25 mg $ml^{-1}$ and 2526 µM/min/mg, respectively.

The aim of the present work is to study the kinetic parameters of pectinase enzyme complex after immobilization such as pH, temperature and substrate concentration. Also, attempts were carried out for immobilization of these enzymes which are considered as the most popular enzymes used for industrial purposes by using supports i.e. glass beads, chitin, chicken bone, sand and sodium alginate. Besides that, the optimum conditions of immobilized application for continuous conversion of pectin extracted from orange peels were investigated and compared with those free enzymes. This point is very from the economical point of view.

## Materials and methods

### 1. Enzyme source:

Commercially available pectinase (E.C. 3.2.1.15), pectinex ultra SP-L enzyme from *Aspergillus aculeatus* was used without further purification and obtained from NOVO Nordisk ferment (NOVO INDUSTRIA, A/S Comp. DENMARK) and was stored at 4°C.

### 2. Supports for the immobilization techniques:

Glass beads, chicken bone and sodium alginate were obtained from Sigma Chemical Co. (ST Louis, MO 63178 USA). Chitin was purchased from Alderich Chem. Co. Sand was obtained from Sina desert, Egypt.

### 3. Chemical reagents:

The commercial substrates used in this study were polygalacturonic acids (PGA) purchased from British Drug House LTD [B.D.H.] to be used as standard references.

Glutaraldehyde, 3-Aminopropylethoxy Silane (APTS), Na-alginate, sodium metaperiodate, cyanoborohydride and ethylenediamine were obtained from Sigma Chemical Co. All other reagents were purchased from El-Gomharya Chemical Company, Cairo, Egypt.

### 4. Experimental methods:

#### 4.1. Assay of soluble and immobilized enzyme activites:

Both soluble and immobilized preparations pectinex ultra SP-L activities were determined spectrophotometrically as described by **Demirel *et al.* (2003)** using polygalacturonic acid as a substrate.

#### 4.2. Determination of reducing sugars:

Reducing sugars (D-galacturonic acid) were determined by **Nelson's colorimetric method (1944)**, modified by **Somogyi (1952)**.

### 5. Immobilization methods for pectinex ultra SP-L enzyme:

In general enzymes are immobilized with different techniques. The enzymes can be adsorbed to insoluble materials, entrapment in matrix gels, cross-linked with bifunctional reagents, or covalently bound to an insoluble carrier via reaction between functional groups of the protein and reactive groups on the carrier surface **(Walt, 1987)**.

#### 5.1. Immobilization of pectinase enzyme on porous glass beads:

Porous glass beads were firstly activated by 3-aminopropyl triethoxy silane (APTS) to immobilize pectinase (pextinex ultra SP-L) enzyme. The silanization procedure was performed as described by **Weetall (1993)**. The α-amino groups of lysine of enzyme molecules were attached to the aminopropyl glass beads via glutaraldehyde (2.5%) which produce activated modified alkylamine glass derivative.

#### 5.2. Immobilization of pectinase enzyme on chitin:

Immobilization of pectinex (pectinex ultra SP-L) enzymes on chitin (chitosan polysaccharide) were carried out according to the method described by **Lei *et al.* (2007a)**.

#### 5.3. Immobilized of pectinase enzyme on chicken bone (Biobone):

Chicken bone was used as support for the immobilization of enzymes (pectinase) by the method described by **Schafhauser and Storey (1992)**.

#### 5.4. Immobilization of enzymes on sand:

This enzyme was immobilized on sand according to the method described by **Weetall (1993)**.

**5.5. Entrapment with calcium alginate gels:**

Pectinase enzyme was entrapped with calcium alginate according to the method described by **Buga *et al.* (2010)**.

**6. Determination of protein content:**

Enzyme protein content was determined by the method described by **Bradford (1976)**.

**7. Factors affecting on the reaction activity and reaction velocity of immobilized pectinex ultra SP-L enzyme:**

**7.1. Effect of pH on the reaction activity:**

The activity of pectinase (pectinex ultra SP-L) enzyme preparations were tested on different pH values, of 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.6, 5.8 and 6.0 in acetate buffer (0.2 M), using 2.5 ml of pectin (1%) as substrate and 0.5 ml diluted enzyme. The incubation period was 15 min at 40°C and the resulted reducing sugar was carried out according to the method mentioned by **Somogyi (1952)**.

**7.2. Effect of temperature on the reaction activity:**

The activity of pectinase enzyme preparations were tested at different temperatures i.e., 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75°C, using 2.5 ml of pectin solution (1%) in acetate buffer (0.2 M, pH 4.8) as substrate. Determination of the resulted reducing sugar was carried out according to the method mentioned before.

**7.3. Effect of substrate concentration on reaction velocity of immobilized pectinase enzyme:**

The effect of substrate concentration on reaction velocity of immobilized pectinase (pectinex ultra SP-L) enzyme forms were tested by using different concentrations of pectin solutions, of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0% in acetate buffer (0.2 M), pH 4.8). The process was carried out at 55°C for 15 min in water bath and the produced reducing sugar was carried out as mentioned before by **Nelson's (1944)** and which was modified by **Somogyi (1952)**.

$V_{max}$ and $K_m$ values of immobilized pectinase enzyme were determined by measuring the activities reaction rates (under the optimum conditions) in the presence of various substrate concentrations. Also, $K_m$ and $V_{max}$ were determined using the **Lineweaver-Burk (1954)**.

**8. Stability of free and immobilized preparations on different supports:**

The stability assays of different preparations of pectinase (pectinex ultra SP-L) enzyme were performed at optimum temperature and optimum pH for immobilized forms in an aqueous medium (0.2 mM acetate buffer) using the same amount of enzyme.

**9. Reuse of immobilized enzymes:**

The activity and relative activity (%) of immobilized pectinase preparations on different supports were evaluated according the method described by **Woodward (1985)** after 6 times with reported washing.

**10. Effect of incubation time on the enzymatic hydrolysis of pectin:**

Different incubation periods i.e. 60, 90, 120, 150, 180, 210 and 240 nin were tested for continuous hydrolysis of pectin by using free and immobilized forms on different supports. The reaction mixtures were carried out at determined optimum temperature and pH for each enzyme forms. The resulting reducing sugars (as galacturonic acid) were measured according to the method described by **Somogy (1952)**.

## Results and discussion

**1. Effect of different methods supports and immobilization parameters on the retention and reaction activity of immobilized pectinex ultra SP-L enzyme:**

The present part of this study deals with attempts for immobilization of pectinase (pectinex ultra SP-L) enzyme by using various immobilization techniques and different support materials in order to fined the optimal immobilization method and support for this enzyme. This enzyme has been immobilized on different supports i.e. porous glass beads, chitin, chicken bone, sand and calcium alginate.

**1.1. Retention activity of preparations pectinex ultra SP-L enzyme on different supports:**

The pectinase enzyme was immobilized on different support materials i.e. glass beads, chitin, chicken bone, sand and Ca-alginate by using different methods of the immobilization process. The retention activity and stability of each immobilized forms are of major importance. The quantity of the enzyme bounded to each support besides the retention activity after immobilization has to be considered in assessing such support materials. The effects of different supports and methods on the retention activity of pectinex ultra SP-L enzyme preparations were evaluated and the obtained results are presented in Table (1). From the obtained data, this enzyme has been bounded with or within all the evaluated different supports but with different retention activities. The highest efficiency loading capacity of immobilized enzyme form was found with a support of glass beads, which simply means that the support was bounded with the high amount of protein enzyme added. The retention activity was 81.86%. In case of pectinase-alginate system, activity retention of immobilized pectinase was 77.09%. On the other hand, chitin and sand as solid supports showed the lowest bounded material which was accompanied with the lowest retention activities which amounted to 75.85 and 74.07% with sand and chitin, respectively.

The decrement in the retention activity of the pectinex ultra SP-L enzyme preparations with chitin and sand may be due to the weaker boding between the enzyme and the above supports. Also, it may be due to the desorption of the enzyme from the support as they effect on such linkages. Besides, that the enzyme may be conformationally different when fixed on support or cause diffusion limitations which would markedly decrease the observed enzyme activity.

However, the retention activity of bound pectinex ultra pectinex ultra SP-L enzyme on to granular chicken bone (Biobone) by noncovalent interaction equaled to 78.39%. The noticed decrement in the relation activity of the pectinase preparations with above different supports may be due to perturbation of catalytic pathway of the enzymic reaction would reflect events arising from the fact that enzyme-substrate interactions occur in a different micro environmental effects when an enzyme is immobilized on a solid support **(Vaillant *et al.*, 2000 and Karakus and Pekyardimci, 2009)**.

## 2. Properties and characterization of immobilized pectinex ultra SP-L enzyme on different supports:

The optimum factors influence the immobilized enzyme reaction e.g. pH, temperature and substrate concentration were determined for the immobilized pectinex ultra SP-L enzyme within or on different supports under investigation. Also, stability and reuse of immobilized preparations with different supports were evaluated.

### 2.1. Effect of pH on the activity of immobilized forms:

The obtained enzyme activities for immobilized pectinex ultra SP-L enzyme forms are presented in Table (2) and illustrated in Fig. (1).

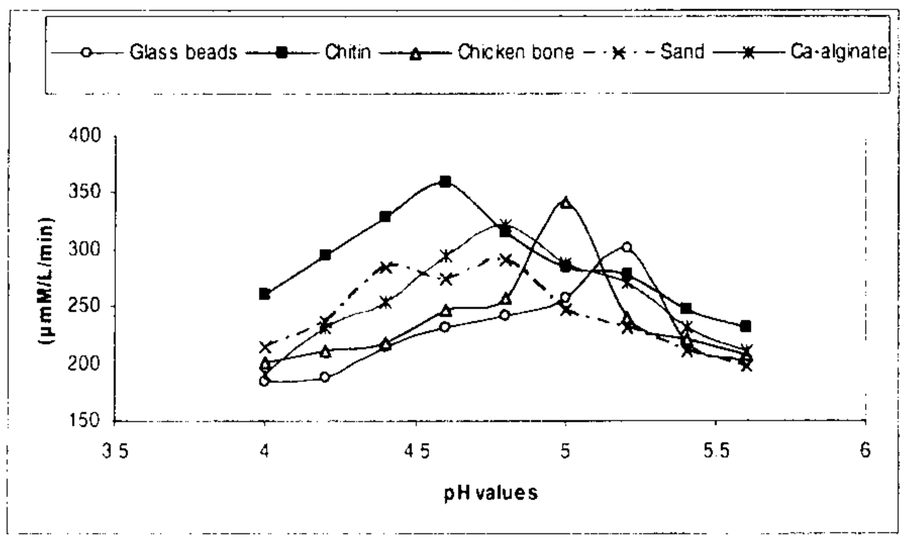



**Figure 1.** Effect of pH on the reaction activity of immobilized pectinase (pectinex ultra SP-L) on different supports.

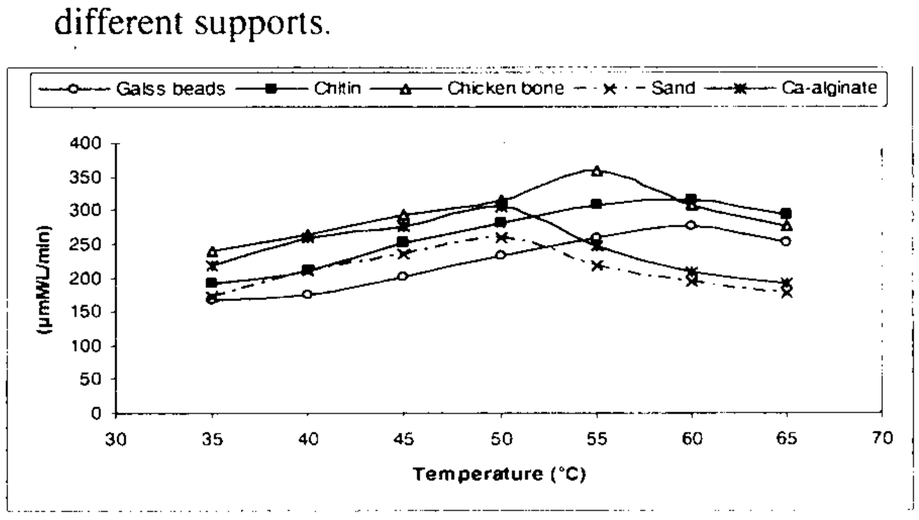
The maximum activity was observed at pH 4.8 for free pectinase as mentioned before. While, the immobilized enzyme on activated sand and entrapped within Ca-alginate gel beads had the same pH value. The maximum activities were found to be 290.70 and 321.35 µmM/L/min for immobilized forms on sand and Ca-alginate gel beads, respectively. However, chitin bounded enzyme complex exhibits the maximum activity at pH 4.6 and which amounted 358.96 µmM/L/min. On the other hand, immobilized pectinex ultra SP-L enzyme preparations on glass beads and chicken bone (Biobone) gave the highest reaction activity at pH's 5.2 and 5.0, respectively.

These results are different from the obtained results of such of observation that pectinase immobilized enzyme on alginate.

### 2.2. Effect of temperature on the reaction activity of immobilized pectinase on different supports:

The obtained results are presented in Table (3) and Fig. (2). The optimum temperatures for immobilized pectinase forms on activated sand and Ca-alginate gel beads were obtained at 50°C. However, this enzyme was immobilized on chicken bone (Biobone) showed the maxium activity at 55°C which amounted to 358.96 µM/L/min. On the other hand, the maximum reaction activities of immobilized pectinex ultra SP-L enzyme on glass beads and activated chitin were found to be at 60°C and equaled 276.91 and 314.52 µmM/L/min, respectively.

**Figure 2.** Effect of temperature on the reaction activity of immobilized pectinase (pectinex ultra SP-L) on different supports.

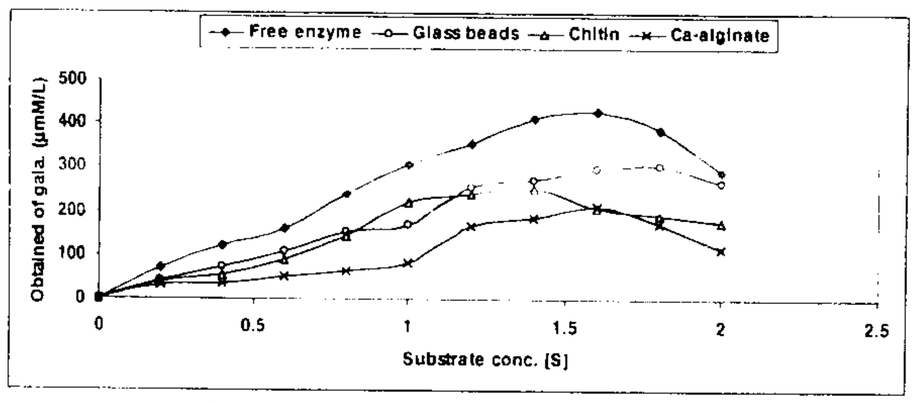



The temperature-activity curves coincided with the results of pectinex ultra SP-L immobilized on magnetic duolite-polystyrene particles **(Demirel *et al.*, 2004)**, silica-coated chitosan particle from a layer by layer approach for pectinase immobilization **(Lei and Bi, 2007a)**, pectinase adsorbed on a noval type of colloidal particles **(Lei *et al.*, 2007b)** and pectinase immobilized on an activated agar-gel support by multipoint attachment **(Li *et al.*, 2008)**. However, these results differed from the previous studies by **Li *et al.* (2007 and 2008)** in which the optimal temperature of 40°C for pectinase immobilized on the alginate support remained unchanged.

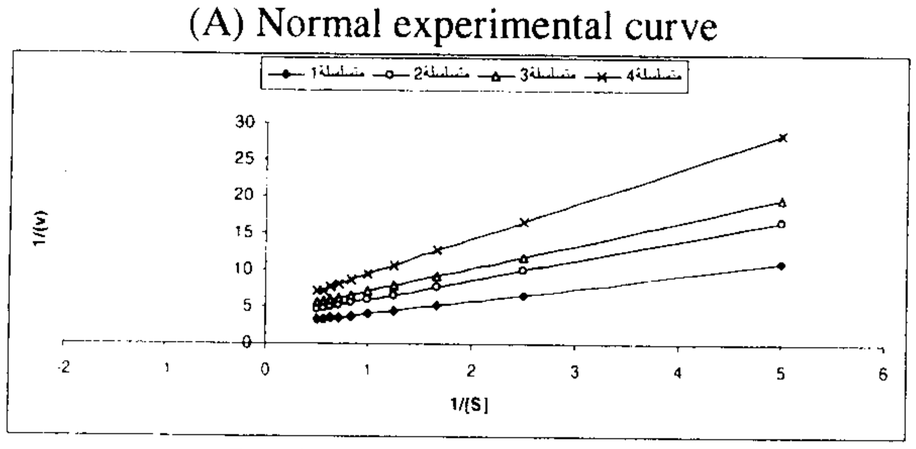
### 2.3. Effect of substrate concentration on the reaction activity of immobilized pectinase on some different supports:

The effect of substrate concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0% on the reaction velocity of immobilized pectinase on different supports such as glass beads, chitin and Ca-

alginate gel beads were estimated. $K_m$ and $V_{max}$ calculated from the equations of these plots and the results were summarized at Table (4) and illustrated in Fig. (3a, b). The maximum reaction velocity ($V_{max}$) were 304.38, 253.08 and 212.04 μmM/L/min for pectinex ultra SP-L enzyme for immobilized enzyme on glass beads, activated chitin and Ca-alginate gel beads, respectively. These values for immobilized forms were lower than that the native form (430.92 μmM/L/min).




(A) Normal experimental curve




(B) Lineweaver and Burk Plots

**Figure 3.** Effect of substrate concentration on reaction velocity of free and immobilized pectinase (pectinex ultra Sp-L) enzyme on different supports

These decrease in $V_{max}$ after immobilization largely reflect the percentage of enzyme initially immobilized on different supports, also this observation can be attributed to steric effect which resulting from the banding between the active centers of the enzyme and the activated support.

$K_m$ of immobilized enzyme on different supports can be obtained by the half point of the experimented curve as showing in Fig. (3a). The values of $K_m$ were 0.81, 0.79 and 1.01 g/100 g pectin for immobilized on glass beads, activated chitin and Ca-alginate, respectively, if compared with native pectinase ($K_m$ = 0.74 g/100 g pectin).

The above mentioned obtained results indicated that the $K_m$ values of immobilized enzyme forms were higher than that of free enzyme. This increase in the $K_m$ values was either due to the conformational change of the enzyme resulting in a lower possibility of forming a substrate-enzyme complex, or to the lower accessibility of the substrate to the active sites of the immobilized enzyme caused by the increased diffusion limitation. Also, $K_m$ values of immobilized forms were determined by **Lineweaver and Burk (1954)** technique and shown in Fig. (3b). These results differ partially with those reported by **Lei and Bi (2007a,b), Lei *et al.* (2007a,b), Li *et al.* (2008) and Buga *et al.* (2010)**.

**3. Stability of native and immobilized pectinex ultra SP-L enzyme on different supports:**

The stability of the native and immobilized pectinex ultra SP-L enzyme on porous glass beads, chitin, chicken bone, sand and Ca-alginate were investigated by measuring the enzyme activities or relative activities at certain time intervals. The obtained results are given in Table (5) and illustrated in Fig. (4).

On the basis of relative activity, it could be observed that the native enzyme lost about 19.13% after 120 hrs. On the other hand, the immobilized pectinex ultra SP-L enzyme on soild supports lost about 7.77, 5.33, 5.87 and 7.57% compared with the original activity for glass beads, chitin, chicken bone and sand, respectively after 120 hrs. Moreover, the entrapped pectin within Ca-alginate lost about 10.35% of all its initial activity after 120 hrs. These results agree with that reported by **Lei *et al.* (2007a, b), Li *et al.* (2008) and Karakus and Pekyardimci (2009)**.

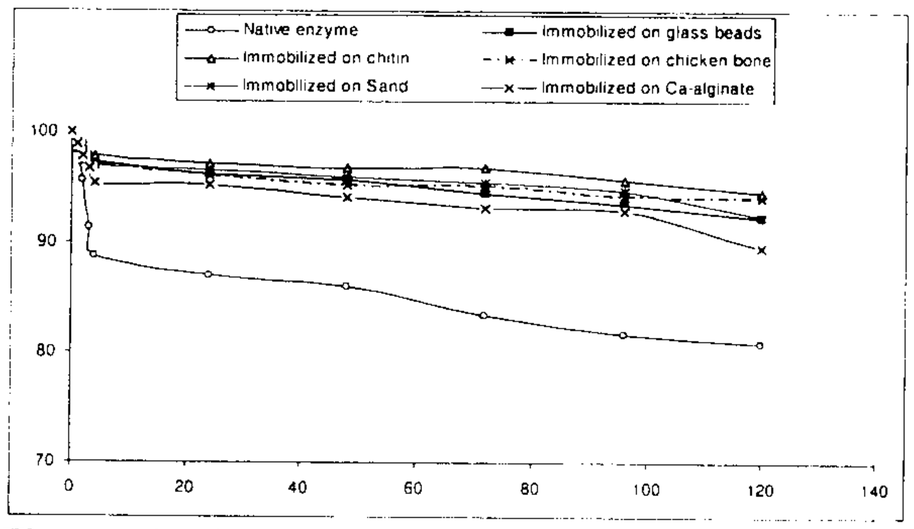



**Figure 4.** Stability of native and preparations of immobilized pectinase (pectinex ultra Sp-L) enzyme on different supports.

**4. Reusability of immobilized pectinex ultra SP-L enzyme on different supports:**

In the batch reaction repetitions 6 times, the activity and relative activity of the immobilized pectinex ultra SP-L enzyme on different supports under investigation were evaluated and the obtained results are shown in Table (6) and illustrated in Fig. (5).

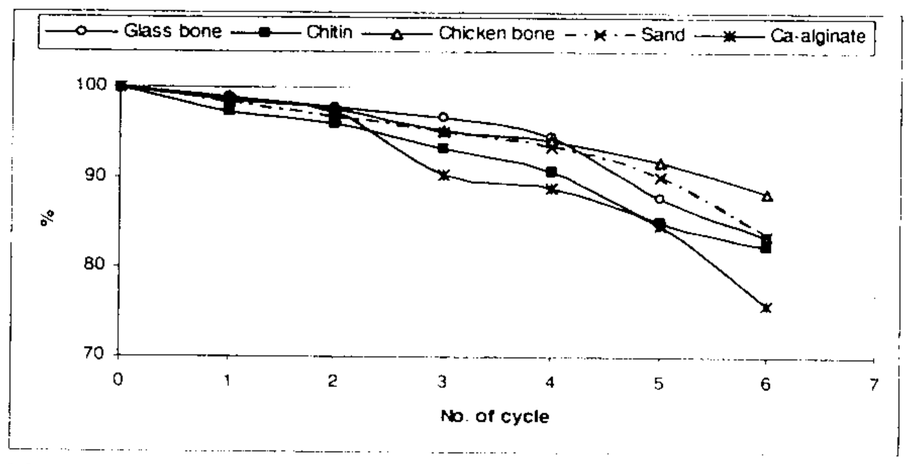



**Figure 5.** Reusability of immobilized pectinase (pectinex ultra SP-L) enzyme on different supports.

**Table 1.** Effect of different supports on the immobilization of pectinase (pectinex ultra SP-L) enzyme.

| Supports | Activity of preparations enzyme | | | | | Effectiveness factor [(D/C) x 100] (%) | Retention activity [(D/A) x 100] (%) |
|---|---|---|---|---|---|---|---|
| | Enzyme added (A) | Enzyme protein mg/ml | In washing (B) | Adsorbation in supports | | | |
| | | | | Theoretical (A-B) = C | Actual complex (D) | | |
| Glass beads | 118.0 | 18.75 | 34.10 | 83.90 | 96.60 | 115.14 | 81.86 |
| Chitin | 118.0 | 18.75 | 41.20 | 76.80 | 89.50 | 116.54 | 75.85 |
| Chicken bone | 118.0 | 18.75 | 32.45 | 85.55 | 92.50 | 108.12 | 78.39 |
| Sand | 118.0 | 18.75 | 42.20 | 75.80 | 87.40 | 115.30 | 74.07 |
| Ca-alginat | 59.0 | 9.70 | 10.50 | 48.50 | 45.60 | 94.02 | 77.29 |

$$\text{Effectiveness factor} = \frac{\text{Activity of immobilized enzyme (Actual complex)}}{\text{[Activity of free enzyme (A) - Activity loss in washing (B)]}} \text{x100} = \text{(D/C) x100}$$

$$\text{Retention activity} = \frac{\text{Activity of immobilized form (D)}}{\text{[Activity of free enzyme (A)]}} \text{x100} = \text{(D/A) x100}$$

**Table 2.** Effect of pH on the reaction activity of immobilized pectinase (pectinex ultra SP-L) on different supports.

| pH's | Glass beads | | Chitin | | Chicken bone | | Sand | | Ca-alginate | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Obtained of Gala.* (µmM/L | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L | Activity (µmM/L/min) |
| 4.0 | 2769.12 | 184.61 | 3897.28 | 259.82 | 3025.52 | 201.70 | 3230.64 | 215.38 | 2871.68 | 191.44 |
| 4.2 | 2820.40 | 188.03 | 4410.08 | 294.00 | 3179.36 | 211.96 | 3538.32 | 235.89 | 3487.04 | 232.47 |
| 4.4 | 3230.64 | 215.38 | 4922.88 | 328.19 | 3281.92 | 218.79 | 4256.24 | 283.75 | 3794.72 | 252.98 |
| 4.6 | 3487.04 | 232.47 | 5384.40 | 358.96 | 3692.16 | 246.14 | 4102.40 | 273.49 | 4410.08 | 294.01 |
| 4.8 | 3640.88 | 242.27 | 4717.76 | 314.52 | 3846.00 | 256.40 | 4360.50 | 290.70 | 4820.32 | 321.35 |
| 5.0 | 3846.00 | 256.40 | 4256.24 | 283.75 | 5128.00 | 341.87 | 3692.16 | 246.14 | 4307.52 | 287.17 |
| 5.2 | 4512.64 | 300.84 | 4153.68 | 276.91 | 3589.60 | 239.31 | 3487.04 | 232.47 | 4051.12 | 270.07 |
| 5.4 | 3281.19 | 218.79 | 3692.16 | 246.14 | 333.20 | 222.21 | 3179.36 | 211.96 | 3487.04 | 232.47 |
| 5.6 | 3025.52 | 201.70 | 3487.04 | 232.46 | 3128.08 | 208.53 | 2974.24 | 198.28 | 3179.36 | 211.96 |

* Galacturonic acid

**Table 3.** Effect of temperature on the reaction activity of immobilized pectinase (pectinex ultra SP-L) on different supports.

| Temp. (°C) | Glass beads | | Chitin | | Chicken bone | | Sand | | Ca-alginate | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Obtained of Gala.* (µmM/L) | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L) | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L) | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L) | Activity (µmM/L/min) | Obtained of Gala.* (µmM/L) | Activity (µmM/L/min) |
| 35 | 2512.72 | 167.51 | 2871.68 | 191.44 | 3589.60 | 239.31 | 2564.00 | 170.93 | 3281.92 | 218.79 |
| 40 | 2615.28 | 174.35 | 3179.36 | 211.96 | 3948.56 | 263.24 | 3179.36 | 211.96 | 3897.28 | 259.82 |
| 45 | 3025.52 | 201.70 | 3794.72 | 252.98 | 4410.08 | 294.01 | 3538.32 | 235.89 | 4153.68 | 276.91 |
| 50 | 3487.04 | 232.47 | 4204.96 | 280.33 | 4717.76 | 314.52 | 3897.28 | 259.82 | 4563.92 | 304.26 |
| 55 | 3897.28 | 259.82 | 4615.20 | 307.68 | 5384.40 | 358.96 | 3281.92 | 213.79 | 3692.16 | 246.14 |
| 60 | 4153.68 | 276.91 | 4717.76 | 314.52 | 4615.20 | 307.68 | 2922.96 | 194.86 | 3128.08 | 208.54 |
| 65 | 3794.72 | 252.98 | 4410.08 | 294.00 | 4153.68 | 276.91 | 2666.56 | 177.77 | 2871.68 | 191.45 |

*** Galacturonic acid**

**Table 4.** Effect of substrate concentration on reaction velocity of free and immobilized pectinase (pectinex ultra Sp-L) enzyme on different supports.

| Substrate conc. (%) | 1/[S] | Free enzyme | | | Glass beads | | | Chitin | | | Ca-alginate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Obtained of Gala* (µmM/L/min) | Reation velocity (V) | $(1/V)x10^{-3}$ | Obtained of Gala* (µmM/L/min) | Reation velocity (V) | $(1/V)x10^{-3}$ | Obtained of Gala* (µmM/L/min) | Reation velocity (V) | $(1/V)x10^{-3}$ | Obtained of Gala* (µmM/L/min) | Reation velocity (V) | $(1/V)x10^{-3}$ |
| 0.2 | 5.00 | 71.82 | 91.69 | 10.90 | 41.04 | 60.27 | 16.59 | 37.62 | 51.13 | 19.56 | 30.78 | 35.05 | 28.53 |
| 0.4 | 2.50 | 123.12 | 151.20 | 6.61 | 75.24 | 100.62 | 9.94 | 54.72 | 85.07 | 11.76 | 34.20 | 60.15 | 16.63 |
| 0.6 | 1.67 | 160.74 | 192.95 | 5.18 | 109.44 | 129.52 | 7.72 | 88.92 | 109.24 | 9.15 | 51.30 | 79.02 | 12.66 |
| 0.8 | 1.25 | 239.40 | 223.85 | 4.47 | 153.90 | 151.24 | 6.61 | 143.64 | 127.34 | 7.85 | 64.98 | 93.72 | 10.67 |
| 1.0 | 1.00 | 307.80 | 247.66 | 4.04 | 171.00 | 168.17 | 5.95 | 222.30 | 141.39 | 7.07 | 82.08 | 105.49 | 9.48 |
| 1.2 | 0.83 | 355.68 | 266.55 | 3.75 | 256.50 | 181.72 | 5.50 | 239.40 | 152.61 | 6.55 | 167.58 | 115.13 | 8.69 |
| 1.4 | 0.71 | 400.14 | 281.91 | 3.54 | 273.60 | 192.82 | 5.19 | 253.08 | 161.79 | 6.18 | 184.68 | 123.18 | 8.12 |
| 1.6 | 0.63 | 430.92 | 294.65 | 3.39 | 297.54 | 202.08 | 4.95 | 208.62 | 169.42 | 5.90 | 212.04 | 129.94 | 7.69 |
| 1.8 | 0.56 | 386.46 | 305.38 | 3.27 | 304.38 | 209.92 | 4.76 | 191.52 | 175.89 | 5.69 | 174.42 | 138.83 | 7.20 |
| 2.0 | 0.50 | 290.70 | 314.54 | 3.18 | 265.44 | 216.64 | 4.62 | 177.84 | 181.42 | 5.51 | 116.28 | 140.89 | 7.10 |

*** Galacturonic acid.**

**Table 5.** Stability of native and preparations of immobilized pectinase (pectinex ultra Sp-L) enzyme on different supports.

| Type of support | | Activity and relative activity (%) Time of incubation (h) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 24 | 48 | 72 | 96 | 120 |
| Native enzyme | | 399.30 | 386.46 | 376.20 | 359.10 | 348.84 | 342.00 | 338.56 | 328.32 | 321.48 | 318.06 |
| | % | 100 | 98.26 | 95.65 | 91.30 | 88.69 | 86.96 | 86.09 | 83.48 | 81.74 | 80.87 |
| Immobilized on glass beads | | 307.80 | 304.38 | 304.04 | 300.29 | 299.58 | 296.17 | 294.47 | 290.69 | 287.98 | 283.88 |
| | % | 100 | 98.98 | 98.78 | 97.56 | 97.33 | 96.22 | 95.67 | 94.44 | 93.56 | 92.23 |
| Immobilized on chitin | | 315.16 | 310.91 | 310.75 | 308.60 | 308.35 | 306.34 | 305.14 | 305.07 | 301.18 | 298.36 |
| | % | 100 | 98.65 | 98.60 | 97.92 | 97.84 | 97.20 | 96.82 | 96.80 | 957.76 | 94.67 |
| Immobilized on chicken bone | | 345.40 | 341.98 | 338.56 | 335.14 | 335.28 | 331.99 | 328.82 | 328.65 | 325.54 | 325.13 |
| | % | 100 | 99.01 | 98.02 | 97.05 | 97.07 | 96.12 | 95.20 | 95.15 | 94.25 | 94.13 |
| Immobilized on Sand | | 253.08 | 249.66 | 246.25 | 245.59 | 245.21 | 244.32 | 242.93 | 241.67 | 239.87 | 233.92 |
| | % | 100 | 98.65 | 97.30 | 97.09 | 96.89 | 96.54 | 95.99 | 95.49 | 94.78 | 92.43 |
| Immobilized on Ca-alginate | | 215.15 | 212.83 | 210.52 | 208.05 | 205.25 | 204.93 | 202.61 | 200.30 | 199.81 | 192.88 |
| | % | 100 | 98.92 | 97.85 | 96.70 | 95.40 | 95.25 | 94.17 | 93.10 | 92.87 | 89.65 |

**Table 6.** Reusability of immobilized pectinase (pectinex ultra SP-L) enzyme on different supports.

| No. of cycles | Glass beads | | Chitin | | Chicken bone | | Sand | | Ca-alginate | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Activity* (µmM/L/min) | Relative activity (%) | Activity* (µmM/L/min) | Relative activity (%) | Activity* (µmM/L/min) | Relative activity (%) | Activity* (µmM/L/min) | Relative activity (%) | Activity* (µmM/L/min) | Relative activity (%) |
| Before fresh | 307.68 | 100 | 253.08 | 100 | 294.12 | 100 | 208.62 | 100 | 246.24 | 100.00 |
| 1 | 304.38 | 98.93 | 246.24 | 97.30 | 290.70 | 98.84 | 205.20 | 98.36 | 242.82 | 98.61 |
| 2 | 300.96 | 97.82 | 242.82 | 95.95 | 287.28 | 97.67 | 201.78 | 96.72 | 239.40 | 97.22 |
| 3 | 297.54 | 96.70 | 235.98 | 93.24 | 280.44 | 95.35 | 198.36 | 95.08 | 222.30 | 90.28 |
| 4 | 290.70 | 94.48 | 229.72 | 90.77 | 277.02 | 94.19 | 194.94 | 93.44 | 218.88 | 88.89 |
| 5 | 270.18 | 87.81 | 215.46 | 85.14 | 270.18 | 91.86 | 188.10 | 90.16 | 208.62 | 84.72 |
| 6 | 256.50 | 83.37 | 208.62 | 82.43 | 259.92 | 88.37 | 174.42 | 83.61 | 186.68 | 75.81 |

* µmM of galacturonic acid.

**Table 7.** Enzymatic saccharification of pectin by free and immobilized pectinase (pectinex ultra SP-L) enzyme with different supports.

| Time of incubation (min) | Free enzyme | | Immobilized forms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Glass beads | | Chitin | | Chick bone | | Sand | | Ca-alginate | |
| | Obtained of *gala. (µmm/L) | Conversion (%) | Obtained of *gala. (µmm/L) | Conversion (%) | Obtained of *gala. (µmm/L) | Conversion (%) | Obtained of *gala. (µmm/L) | Conversion (%) | Obtained of *gala. (µmm/L) | Conversion (%) | Obtained of *gala. (µmm/L) | Conversion (%) |
| 60 | 2358.88 | 60.48 | 2974.24 | 76.26 | 2871.68 | 73.63 | 2999.88 | 76.92 | 2615.28 | 67.06 | 2681.94 | 68.77 |
| 90 | 2512.77 | 64.43 | 3179.36 | 81.52 | 3256.28 | 83.49 | 3051.16 | 78.23 | 2900.82 | 74.38 | 2922.96 | 74.95 |
| 120 | 2820.40 | 72.32 | 3487.04 | 84.41 | 3487.04 | 86.78 | 3358.84 | 86.12 | 2943.47 | 75.47 | 2820.40 | 72.32 |
| 150 | 3025.52 | 77.58 | 3538.32 | 90.75 | 3307.56 | 84.81 | 3435.76 | 88.10 | 3020.39 | 77.45 | 2630.66 | 67.45 |
| 180 | 3281.92 | 84.15 | 3333.20 | 85.47 | 3205.00 | 82.18 | 3292.18 | 84.41 | 3230.64 | 82.84 | 2461.44 | 63.11 |
| 210 | 3180.84 | 81.56 | 3076.80 | 78.89 | 3153.72 | 80.86 | 3194.74 | 81.92 | 2834.52 | 72.68 | 2384.52 | 61.14 |
| 240 | 3128.08 | 80.21 | 2974.24 | 76.26 | 3102.44 | 79.55 | 2897.32 | 74.29 | 2666.56 | 68.37 | 2307.60 | 59.17 |

*gala: Galacturonic acid

The immobilized enzyme on each glass beads, chitin and sand lost 16.63, 17.57 and 16.49% of the original activity after 6 cycles. This may be due to readsorbed of pectinase enzyme on the above mentioned supports from surface area of these supports and physical loss of weakly bound enzyme from the support. On the other hand, the immobilized form on Ca-alginate gel beads last about 24.19% and 75.81% relative activity after consecutively repeating the reactions 6 times. This value of 75.81 was lower than that obtained by using the alginate support under the same condition (80%) **by Li *et al.* (2007**a). Entrapped enzymes usually suffer gradual loss of activity after several catalytic cycles due to leakage of the enzyme into the surrounding medium.

Moreover, Biobone enzyme complex exhibited the highest relactive activity (88.37%), of the initial activity after 6 cycles. Consequently, it can be used for industrial applications and Ca-alginate for food industry (fruit processing).

**5. Saccharification of pectin by immobilized pectinase:**

Saccharification processes include the stages of conversion of extracted pectin from citrus peel as substrate into mixture of mono- and oligomers containing of D-galacturonic acid as reducing sugars. These processes were carried out in stirred reactor at optimum temperature and pH for each immobilized pectinase preparations under investigation at 200 rpm. Also, enzymatic saccharification were accomplished for different periods of time, 60, 90, 120, 150, 180, 210, and 240 min.

The obtained results of these processes are presented in Table (7) and Fig. (6). From these results, the highest conversion values of saccharification process was 84.15% for native pectinase enzyme with 20 g/L extracted citrus pectin at period of reaction of 180 min (3 hrs).

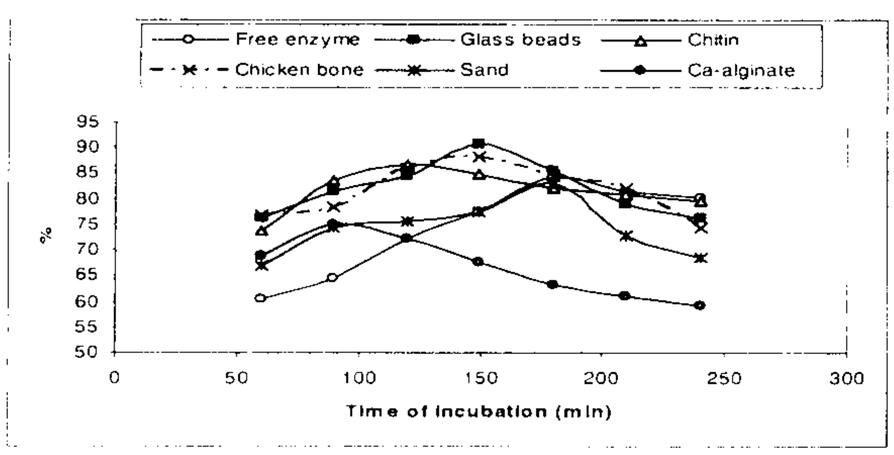



**Figure 6.** Enzymatic sacchrification of pectin by free and immobilized pectinase (pectinex ultra SP-L) enzyme with different supports.

On the other hand, conversion percent of extracted citrus pectin with immobilized pectinase on activated glass beads reached its maximum value of 90.75% at period time of 150 min (2½ h) when compared with other immobilized forms. This may be due to the activity of the immobilized pectinase, especially in a covalently bound system on activated glass beads, being resistant to heat and denaturing agents than that of the soluble form. Also, the stability of the immobilized form was increased by immobilization attachment **(Karakus and Pekyardimici, 2009)**. However, immobilized pectinase within Ca-alginate was the lowest value (74.95%) of saccharification process at period time of 90 min (1½ h). The reduction of conversion with Ca-alginate may be due to heat inactivation and gradual loss of activity due to enzyme leakage into the surrounding medium **(Buga *et al.*, 2010)**.

In general, from the above results, it might be concluded that the maximum values of saccharification process were 86.78, 88.10 and 82.84%, respectively for immobilized pectinase on activated chitin, chicken bone (Biobone) and silanized sand.

Such result might be attributed to selected the carrier material and nature of the attachment for using enzyme immobilization. The decrement in the saccharification process for sand and chitin may be due to a desorption of the enzyme from the support as they effect on such linkages. Changes in experimental conditions such as pH, ionic strength, temperature and type of solvent can cause desorption **(Vaillant *et al.*, 2000, Lei and Bi, 2007a, b).**

Such values of saccharification processes of extracted citrus pectin by using free and immobilized pectinase (pectinex ultra SP-L) on different above supports are partially different from those to obtained by **Demirel *et al.* (2004) and Li *et al.* (2008)**.

## References

**Bradford, M.M. (1976).** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Biochemistry, 72: 248-254.

**Buga, M.L.; Ibrahim, S. and Nok, A.J. (2010).** Physico-chemical characteristics of immobilized polygalacturonase from *Aspergillus niger* (SA6). African J. Biotechnology 9(52): 8934-8943.

**Csanadi, Z. and Sisak, C. (2006).** Immobilization of Pectinex Ultra SP-L and its application to production of fructooligo-saccharides. Acta Alimentaria (Budapest), 35(2): 205-212.

**Demirel, D.; Boyaci, I.H. and Mutlu, M. (2003).** Determination of kinetic parameters of pectolytic enzymes at low pectin concentrations by a simple method. Eur Food Res. Technol., 217: 39-42.

**Demirel, D.; Özdural, A.R. and Multu, M. (2004).** Performance of immobilized pectinex ultra SP-L on magnetic duolite-polystyrene composite particles. Part I: a batch reactor study. J. Food Engineering, 64: 417-421.

**Gummadi, S.N. and Panda, T. (2003).** Purification and biochemical properties of microbial pectinases—a review. Process Biochemistry, 38(7): 987-996.

**Jun, Y.; Ying, L.H.; Jiang, L.; Kun, W.; Ping, C.H.; Guo, B.Y.; Zheng, Y.T.; Liu, F.Y. and**

**Bin, Y. (2011)**. Cloning, expression and characterization of an acidic endo-polygalacturonase from *Bispora* sp. MEY-1 and its potential application in juice clarification. Process Biochem., 46(1): 272-277.

**Karakus, E. and Pekyardimci, S. (2009)**. Immobilization of apricot pectinesterase (*Prunus armeniaca* L.) on porous glass beads and its characterization. J. Molecular Catalysis B: Enzymatic 56: 13-19.

**Lei, Z. and Bi, S. (2007a)**. The silica-coated chitosan particle from a layer-by-layer approach for pectinase immobilization. Enzyme and Microbial Technolo., 40(5): 1442-1447.

Lei, Z. **and Bi,** S. (2007b). Preparation and properties of immobilized pectinase onto the amphiphilic PS-*b*-PAA diblock copolymers. J. Biotechnol., 128(1): 112-119.

**Lei, Z.; Bi, S. and Yang, H. (2007a)**. Chitosan-tethered the silica particle from a layer-by-layer approach for pectinase immobilization. Food Chem., 104: 577-584.

**Lei, Z.; Bi, S.; Hu, B. and Yang, H. (2007b)**. Combined magnetic and chemical covalent immobilization of pectinase on composites membranes improves stability and activity. Food Chem., 105: 889-896.

**Li, L.Z.; Na, R.; Li, L.Y.; Na, L. and Bo, M. (2009).** $Fe_3O_4/SiO_2$ PSStNa polymer nanocomposite microspheres (PNCMs) from a surface-initiated atom transfer radical polymerization (SI-ATRP) approach for pectinase immobilization. J. of Agricultural and Food Chem., 57(4): 1544-1549.

**Li, T.; L., S.; Wang, N. and Tain, L. (2008).** Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. Food Chem., 109: 703-708.

**Li, T.P.; Wang, N.; Zhao, Q.C.; Guo, M. and Zhang, C.Y. (2007).** Optimization of covalent immobilization of pectinase on sodium alginate support. Biotechnology Letters. Doi: 10.1007/s10529-007-9409-3.

**Lineweaver, H. and Burk, D. (1954).** Methods of plotting enzyme kinetic data. J. Am. Chem. Soc., 56: 658-663.

**Nelson, N. (1944).** A photometric adaptation of the Somogyi determination of glucose. J. Biol. Chem., 153: 375-380

**Schafhauser, D.Y. and Storey, K.B. (1992).** Immobilization of amyloglucosidase onto granular chicken bone. Applied Biochem. and Biotenhol., 32: 89-109.

**Somogyi, M. (1952).** Notes on sugar determination. J. Biol. Chem. 95: 18-23.

**Vaillant, F.; Millan, A.; Millan, P.; Dornier, M.; Decloux, M. and Reynes, M. (2000).** Co-immobilized pectinlyase and endocellulase on chitin and nylon supports. Process Biochemistry, 35(9): 989-996.

**Walt, D.R. (1987).** Immobilized cells and enzymes. American Chemical Society Washington, D.C. 20036.

**Weetall, H.H. (1993).** Preparation of immobilized proteins covalenty coupled through silane coupling agents to inorganic supports. Appl. Biochem. and Biotechnol., 41: 157-188.

**Woodward, J. (1985).** Immobilized enzymes: Adsorption and covalent coupling immobilization of enzyme on Co A-sepharose. IRL press, Oxford OX8IJJ, England.

# حركيات إنزيم البكتينيز المحمل على دعامات مختلفة

صلاح مصطفى محمود سعد فرحات فودة على فودة
إبراهيم محمد عبدالعليم محمد سامى جمال الدين

قسم الكيمياء الحيوية – كلية الزراعة– جامعة بنها – مصر

يهدف البحث إلى دراسة تحميل إنزيم البكتينيز باستخدام دعامات مختلفة وهى حبيبات الزجاج، الكيتيت المنشط، مسحوق عظم الدجاج والرمل المنشط وجل ألجينات الكالسيوم لتحويله من الصورة الذائبة إلى الصورة غير الذائبة بغرض إمكانية استخدامه فى النظام المستمر وأيضا تم دراسة الظروف المثلى والسلوك الحركى للأنزيم المحمل مقارنة مع حالته الذائبة وكذلك استخدامه عديد من البوليمرات حيث أن هذه النقطة ذات أهمية صناعية وإقتصادية نظرا لأهمية هذا الأنزيم فى المجالات الصناعية والغذائية .

أوضحت الدراسة أن قوة ربط الدعامات المختلفة لأنزيم البكتينيز هى ٨١,٨٦، ٧٤,٠٧، ٧٨,٣٩، ٧٥,٨٥، ٧٧,٠٩% من كمية الأنزيم المضافة للدعامات حبيبات الزجاج، الكيتين، مسحوق عظم الدجاج والرمل المنشط وجل ألجينات الكالسيوم على التوالى.

كذلك أوضحت الدراسة أن الظروف المثلى لتحميل هذا الأنزيم من درجة الـ pH المثلى هى ٥,٢، ٥,٠ وذلك عند استخدام كل من حبيبات الزجاج ومسحوق عظم الدجاج بينما كانت ٤,٨ للصورة الحرة والأنزيم المحمل على الرمل المنشط وجل ألجينات الكالسيوم بينما الأنزيم المحمل على الكيتين النشط أعطى أعلى درجة نشاط على درجة الـ pH ٤,٦. بينما أوضحت الدراسة أن درجة الحرارة المثلى لصورة الأنزيم المحمل على دعامات مختلفة هى ٥٠°م لكل من الرمل المنشط وجل ألجينات الكالسيوم ، ٥٠°م للصورة الحرة بينما كانت ٥٥°م عند استخدام مسحوق عظم الدجاج. كما أن الأنزيم المرتبط بالدعامات المختلفة أظهر درجة ثبات عالية مقارنة للأنزيم الحر.

أظهرت الدراسة الحركية لهذا الأنزيم أن ثابت ميكاليس منتن للصورة الحرة ٠,٧٤ جم/١٠٠ جم بكتين بينما لمعقد الأنزيم مع الدعامات المختلفة فكانت ٠,٨١، ٠,٧٩، ١,٠١ جم/١٠٠ جم بكتين مستخلص لكل من حبيبات الزجاج والكيتين النشط وجل ألجينات الكالسيوم على التوالى. وقد يعزى زيادة ثابت ميكاليس منتن للأنزيم المحمل على الدعامات المختلفة عن الأنزيم الحر إلى انخفاض جاذبية المادة المتفاعلة نتيجة لوجود طبقة بين الأنزيم المحمل ومحلول التفاعل.

كما أوضحت الدراسة أن السرعة القصوى للأنزيم الحر تساوى ٤٣٠,٩٢ ميكروملليمول/لتر/دقيقة بينما للأنزيم المحل فكانت ٣٠٤,٣٨، ٢٥٣,٠٨، ٢١٢,٠٤ ميكروملليمول/لتر/دقيقة على كل من حبيبات الزجاج والبكتين المنشط وجل ألجينات الكالسيوم.

أما بالنسبة لإعادة إستخدام الأنزيم المحمل عديد من المرات فقد أظهرت الدراسة أن الأنزيم المحمل على الدعامات المختلفة أمكن استخدامها ٤ مرات مع فقد ٥,٥٢، ٩,٢٣، ٥,٨١، ٦,٣٦، ١١,١١% من درجة نشاطه الأصلى.

كما أوضحت الدراسة أن درجة التسكر للبكتين المستخلص كانت ٨٤,١٥% فى حالة الأنزيم الحر عند تركيز ٢٠ جم/لتر بكتين مستخلص بعد فترة تحضين ١٨٠ دقيقة بينما كانت نسبة التحويل للأنزيم المحمل على حبيبات الزجاج ٩٠,٧٥% بعد فترة ١٥٠ دقيق بينما كانت أقل نسبة نسبة تسكر مع الأنزيم المحمل على وجل ألجينات الكالسيوم تساوى ٧٤,٩٥% بعد فترة تحضين ٩٠ دقيقة كما أوضح الأنزيم المحمل على الدعامات المختلفة درجة تسكر تساوى ٨٦,٧٨، ٨٨,١٠، ٨٢,٨٤% للأنزيم المحمل على الكيتين المنشط ومسحوق عظم الدجاج والرمل النشط.