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IMMOBILIZATION OF ß-GLUCOSIDASE IN CA-ALGINATE GEL BY

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ABSTRACT

ß-Glucosidase (ß-D-glucoside glucohydrolase) was immobilized by encapsulating in calcium alginate gel beads and the kinetic parameters of immobilized form were determined. The retention activity of bound enzyme was 80.4% of the soluble form at 4% (W/V) sodium alginate. The optimum pH was 4.8 for immobilized enzyme compared with free enzyme (pH 4.5), the optimum temperature for immobilized form reached 50°C. Glucose inhibited free and immobilized forms of â-glucosidase and this inhibition increase with increasing glucose concentration and the enzyme activities decreased to 59.3% and 67.8% of its original values for immobilized and free enzyme, respectively.

The effect of substrate concentration on reaction velocity of enzyme pointed out that V_{max} and K_m for immobilized form were 138.1 mM and 1.80 mM in absence of glucose. While these values for soluble enzyme were 201.0 mM, 0.45 mM in absence of glucose. On the other hand, K_i of free and immobilized enzyme were 0.30 mM, and 1.30 mM, respectively. Reusability of immobilized form lost 29.8% of its original immobilized activity after 7 times.

INTRODUCTION

ß-Glucosidase (ß-D-glucoside glucohydrolase, E.C.3.2.1.21) is an important enzyme for its role in the hydrolysis of cellobiose to glucose. Immobilization of ß-glucosidase would allow its recovery and capability of reuse of this enzyme. In addition, the immobilization of ß-glucosidase enzyme may solve to a great extent the abovementional problem of the produced glucose during the enzymatic attack. Consequently, immobilization is considered as a practical economic improvement (Sundstrom, *et al.*, 1981).

Woodward and Arnold (1981) studied the inhibition of free ß-glucosidase activity by isomers of glucose using cellobiose (10 mM) as substrate. They found that at pH 4.8 the enzymatic hydrolysis was inhibited competitively by glucose (α- and ß-anomers) with K_i value equalled 0.5 mM. The inhibitition percent reached to 49% and 27% for α- and ß-anomers of glucose, respectively.

Rao, *et al.*, (1983) immobilized ß-glucosidase enzyme on polyamide as an inexpensive support. They found that K_m and V_{max} of soluble and immobilized form were (2.0 mM, 0.4 µmoles/min/mg) and (0.40 mM, 0.16 µ moles/min/mg)with cellobiose as substrate.

Jain and Ghose (1984) studied the kinetic constant of free and immobilized ß-glucosidase by entrapment in calcium alginate gel beads. They found that Michaelis constant (K_m), maximum reaction velocity (V_{max}), and the inhibition constant (K_i) were (12.18 mM, 1.33 µmoles/min/mg, and 1.72 mM) and (31.0mM, 1.05 µmoles/min/gm, and 2.20 mM) for free and immobilized forms, respectively.

Kantham and Jaannathan (1985) evaluated the kinetic constant ofß-D-glucosidase from *Penicillium funiculosum*. They found that (K_m), (V_{max}),and (K_i) values were 2.0×10^{-4}M, 171µmoles/min/gm, and 3.2×10^{-5} M, respectively.

Dekker (1986) studied the kinetic, inhibition, and stability properties of a commercial ß-D-glucosidase preparations from *Aspergillus niger*. He found that the K_m, and (V_{max}) values of enzyme preparation were 5.63 mM and 33.74 µmoles/min/mg enzyme with cellobiose (50mM) as substrate. Also, they found that the inhibition constant (K_i) was 3.0 mM and inhibition percentage reached to 63% in the presence of 15% glucose.

Mitchell, *et al.*, (1991) immobilized ß-D-glucosidase enzyme from *Aspergillus niger* on ion exchange resin using glutaraldehyde as crosslinking. They found that K_m and V_{max} equalled to (5.6 mM, 33.7µmoles/min/mg) for free enzyme and 10.1mM, 117 µmoles/min/mg for immobilized forms.

The aim of the present work is to study the immobilization of ß-D-glucosidase from *Aspergillus niger* by entrapment on Ca-alginate gel beads as an inexpensive support. Also, kinetic behavior of free and immobilized form of ß-D-glucosidase were evaluated, since these parameters are very important from the industrial and economical point of view.

MATERIAL AND METHODS

1. ß-D-glucosidase enzyme (cellobiase, Novozym 188; E.C.3.2.1.21 ß-D-glucosid glucohydrolase) was supplied from NOVO Laboratories, INC., Danbury, USA. Immobilized ß-D-glucosidase enzyme was prepared on calcium alginate gel beads (CAGB) according to the methods described by Hahn-Hägerdal (1984). D (+) cellobiose and D (+) glucose sugars as standards were purchased from Sigma chemical Co. (st. Louis, MO).

2. 1. Effect of Na- alginate concentration on preparations of immobilized ß-D-glucosidase enzyme:

Different concentrations of sodium algenate, i.e. 1,2,3, and 4% were used to immobilize ß-D-glucosidase (55 units, 3.85 mg protein/ml). The resulting thick sodium alginate-ß -glucosidase suspension was pumped drop by drop into a stirred 2% (w/v) calcium chloride solution. The formed beads were then filtered, washed, suspended in a 0.05 M acetate buffer (pH 4.8) and stored at 4°C. The effectiveness factor and retention activity of immobilized enzyme were calculated according to Woodward, (1985).

2. 2. Effect of pH and temperature on the activity of immobilized enzyme in Ca-alginate gel:-

The effect of pH on the activity of immobilized form were tested on different pH values, i.e. 3.3, 3.6, 3.9, 4.2, 4.5, 4.8, 5.1, 5.4, 5.7 and 6.0 acetate buffer. While, the effect of temperature on the activity of immobilized form were incubated at different temperatures, i.e. 30, 35, 40, 45, 50, 55, 60, 65 and 70°C for 30 min. The activity of the enzyme was determined by measuring the resultant glucose using the glucose oxidase method as described by Keston (1956).

2. 3. Kinetics of free and immobilized ß-D-glucosidase with different concentrations of glucose as an inhibitor:

Four concentrations of glucose i.e. 1, 2, 5 and 10 mM in acetate buffer (pH 4.8) were used in this experiment with different concentrations of cellobiose as substrate, i.e. 0.68, 1.35, 2.70, 5.40 and 10 mM. The enzymatic reactions were achieved with free and immobilized ß-D-glucosidase. The activity was determined and expressed as reaction velocity. K_m and K_i were obtained

1978).

Lineweaver-Burk technique (1954) were applied by plotting $\frac{1}{(S)}$ against $\frac{1}{V}$ **where**:

$$\frac{1}{V} = \frac{Km}{V_{max}} \cdot \frac{1}{(S)} + \frac{1}{Vmax} \text{ (in absence of inhibitor) and}$$

$$\frac{1}{V} = \frac{Km\left(1 + \frac{I}{Ki}\right)}{Vmax} \cdot \frac{1}{(S)} + \frac{1}{V_{max}} \text{ (in the presence of inhibitor), as}$$

mentioned by Plummer (1978).

2. 4. Effect of glucose concentration on the activity of free and immobilized ß-D-glucosidase enzyme:

Different concentrations of glucose solutions, i.e., 1, 2, 5, and 10 mM in acetate buffer (50 mM, pH 4.8) were used with cellobiose as a substrate concentration (10 mM) in the same acetate buffer solution. Enzyme dilution of

ß- glucosidase enzyme (1:200; 0.77 mg protein/ml) for free enzyme and a quantity of enzyme support-complex containing the same amount of protein enzyme in the case of immobilized form was used in this experiment. The mixture of reaction was incubated for 30 min. at 50°C and pH 4.8. The resulting glucose was colorimetrically determined by the glucose oxidase method as described before.

2. 5. Reuse of immobilized enzyme:-

The reaction mixture of cellobiose (5 ml, 10 mM) and immobilized ß-D-glucosidase form (100 mg wet beads gel, 44.2 U) were incubated at 50°C for 20 min. The suspension was filtered and the residue washed with 10 ml of 0.05 m acetate buffer pH 4.8. The filtrate syrup was estimated for glucose. The immobilized enzyme was resuspended in fresh substrate and the procedure repeated.

RESULTS AND DISCUSSION

3.1. The effect of Na-alginate concentration on preparations of immobilized ß-glucosidase enzyme was studied and the results are shown in Table (1).

From these results the retention activity of immobilized enzyme increased progressively with the increase of sodium alginate. Effectiveness factor and retention activity of immobilize ß- glucosidase were 119.4% and 68.4%, respectively at a concentration 2 %, (w/v) sodium alginate. On the other hand, at 4% (w/v) sodium alginate, the effectiveness factor and retention activity were 140.3% and 80.4% of the original enzyme activity.

Table (1): Effect of Na-alginate concentrations on preparations of immobilized ß-glucosidase enzyme.

Na-alginate ration (W/V) %	Activity (μ mol glucose/min/ml						Effectiveness factor 1 00 (%)	Retention activity 1 00 (%)
	Enzyme added		In washing		Entrapped within Ca-Alginate			
	Units (A)°	Protein mg/ml	Units (B)	Protein mg/ml	Theoretical (A-B)=C	Actual x (D)*		
1	55	3.85	9.0	1.7	46.0	26.6	57.83	48.36
2	55	3.85	23.5	2.8	31.5	37.6	119.37	68.40
3	55	3.85	23.5	2.76	31.5	41.9	133.02	76.20
4	55	3.85	23.5	2.68	31.5	44.2	140.32	80.40

o Units refer to the amount of total activity contained in 1.0 ml enzyme

* Units refer to the activity found in the total number of gel spheres produced in the experiments.

$$\text{Effectiveness factor} = \frac{\text{Activity of entrapped enzyme (actual complex)}}{\text{Activity of free enzyme - Activity loss in washing}} \times 100$$

$$\text{Retention activity} = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of free enzyme}} \times 100$$

In general, the obtained results indicate that ß- glucosidase enzyme was immobilized on Ca-alginate gel. This concentration of Na-alginate ,4% (w/v) is in good agreement with that stated by Jain and Ghose (1984). They mentioned that ca-alginate revealed as a very effective support for ß- glucosidase immobilization through encapsulated or covalent bonds between ß- glucosidase molecules prior to their entrapment.

3. 2. Thermal stability of native and immobilized form:-

The effect of incubation time on the relative activities of both native and immobilized ß- glucosidase enzyme is shown in and Fig. (1) which indicated that the immobilized enzyme retained most of its activity (98.5%) after 4 hr at 50°C. On the other hand, the relative activity of native enzyme decreased to 95.6% under the same conditions.

3. 3. Effect of pH on the activity of immobilized ß- glucosidase in Ca-alginate gel beads:-

The plot of pH values versus activity of immobilized enzyme may reveal the change in optimum pH of immobilized enzyme in comparison with free enzyme which had a pH optimum of 4.5. The immobilized enzyme had a pH optimum of 4.8 with maximum activity of 45.7 µmoles glucose/ min/100 mg wet beads (Table 2 and Fig. 2). Our findings support the data reported by other workers (Mattean and Saddler, 1982 and Dekker, 1986).

Table (2): Effect of pH on the activity of immobilized in Ca-alginate gel.

pH's	Activity (Units) µmol glucose/min	
	Native enzyme	Immobilized enzyme
3.3	29.5	23.1
3.6	44.5	25.2
3.9	45.7	26.1
4.2	47.2	37.7
4.5	55.8	42.3
4.8	51.1	45.7
5.1	50.2	39.2
5.4	43.9	31.16
5.7	43.0	28.00
6.0	41.7	21.40

The differentiation in optimum pH values of soluble and immobilized enzyme may be due to the conformation changes in the immobilized enzyme which occurred during immobilization process. These results indicate the importance of such acid media (pH 4.6-4.8) to fit the nature of the active site in ß- glucosidase enzyme. Underkofter, *et al.*, (1965) stated that the formation of

glucose from liquefied maltose and cellobiose at lower pH reduces colour formation in the resulted glucose syrup and preventing microbial growth during the long period.

3. 4. Effect of temperature:

The effect of temperature on the activity of immobilized ß- glucosidase in Ca-alginate gel beads are presented in Table (3) and Fig. (3). The enzyme activity increased with increasing temperature and reach its maximum value at 50°C and tend to decrease at higher temperature. Such values for optimum temperature is in good agreement with that obtained by Jain and Ghose (1984).

Table (3): Effect of temperature on the activity of immobilized ß-glucosidase in Ca- alginate gel.

Temperature's C°	Activity (Units) µmol glucose/min	
	Native enzyme	Immobilized enzyme
30	41.9	27.3
35	42.6	29.6
40	48.8	30.4
45	50.0	36.1
50	55.8	39.6
55	46.8	28.0
60	39.4	20.3
65	20.1	18.6
70	16.2	13.2

3. 5. Effect of glucose concentration on the reaction activity of free and immobilized ß- glucosidase enzyme:-

The effect of glucose concentration on the reaction activity of free and immobilized ß- glucosidase enzyme was studied and the results are illustrated in Table (4) and Fig. (4). The results indicate that glucose acts as an inhibitor for free and immobilized ß-D-glucosidase on calcium alginate gel beads and the relative activity decreased with increasing glucose concentration to reach its lowest value i.e., 67.8% and 59.3% of its original values of free and immobilized enzyme, respectively at 10 mM glucose concentration.

The higher decrement in the activity of immobilized enzyme on calcium alginate may be due to partially denaturation of protein enzyme during coupling or leakage out of the gel matrix.

The obtained results illustrate that the presence of glucose in the media of reaction inhibited the enzyme entrapped within calcium alginate to a great extent than the soluble enzyme. This observation could be supported from the fact that the presence of high concentration of glucose around the support can bind glucose sugar on the support as reported by Woodward (1985).

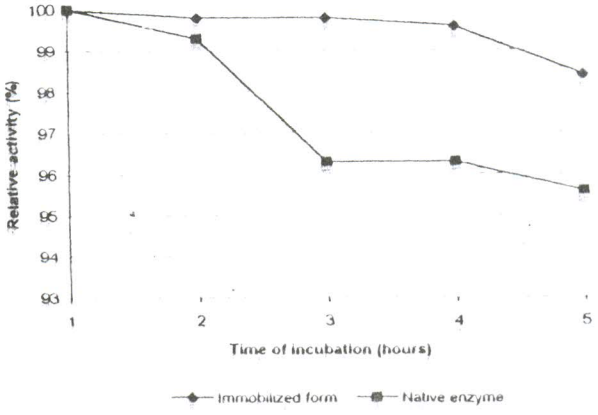

Fig (1): Thermal stability of native and preparations of immobilized B-glucosidase in (4%) Ca-alginate gel

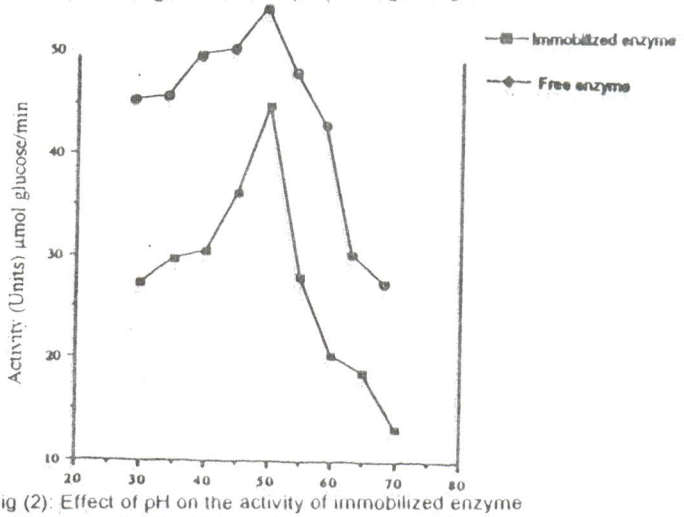

Fig (2): Effect of pH on the activity of immobilized enzyme in Ca-alginate gel.

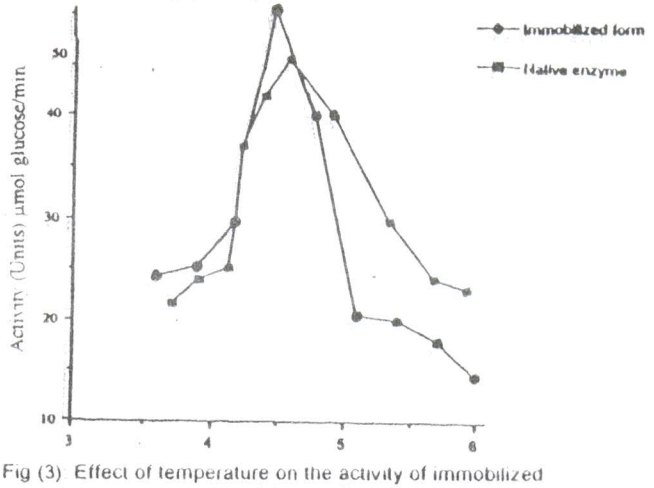

Fig (3): Effect of temperature on the activity of immobilized enzyme ß-glucosidase in Ca-alginate gel.

Table (4): Effect of glucose concentration on free and immobilized ß-glucosidase activity.

Glucose concentration mM	Free enzyme			Immobilized enzyme		
	Glucose content mM/L	Reaction activity µmol G/min	Relative activity %	Glucose content mM/L	Reaction activity µmol G/min	Relative activity %
0	36.18	10.05	100.00	24.86	6.91	100.00
1	29.84	8.29	82.49	23.09	6.42	92.91
2	28.49	7.92	78.81	21.58	5.60	81.04
5	26.87	7.47	74.33	18.00	5.00	72.36
10	24.53	6.81	67.76	14.77	4.10	59.33

Generally, our results indicate that the soluble and immobilized ß-D-glucosidase forms were inhibited by the addition of different concentration of glucose to the media of reaction mixtures our data are in agreement with that obtained by Jain and Ghose (1984) and Dekker (1986)

3. 6. Kinetics of free and immobilized ß-D-glucosidase with different concentrations of glucose:-

The effect of substrate concentration on reaction activity of free and immobilized ß-D-glucosidase enzyme in the presence different concentrations of glucose i.e. 1, 2, 5 and 10 mM are shown in Tables (5, 6) and Fig. (5 , 6)

From these results the rate of reaction velocities increased progressively with the increase of substrate concentration in absence of glucose in case of free and immobilized forms. The maximum velocities (V_{max}) were 201.0 mM/L for free enzyme and 138.1 mM/L for immobilized enzyme in calcium alginate gel beads. While, in the presence of glucose the velocity decreased with the increase of glucose concentration in the reaction mixture. The maximum velocities at different concentrations of glucose (1, 2, 5 and 10 mM) were (168.3 mM, 158.3 mM/L, 149.3 mM/L and 147.4 mM/L) for free enzyme. While, the maximum velocities in case of immobilized form on calcium alginate were 128.3 mM/L, 119.9 mM/L, 100.0 mM/L and 65.4 mM/L, respectively at 10 mM cellobiose as substrate.

The differentiation in Vmax values for free and immobilized ß-D-glucosidase in Ca-alginate under the above conditions may be due to the conformation changes in the immobilized forms which occured during immobilization and limitation on substrate diffusion into and through the supports (Woodward, 1985).

K_m and K_i were found to be 0.45 mM cellobiose and 0.30 mM glucose for free enzyme and 1.8 mM cellobiose and 1.30 mM glucose for immobilized form in calcium alginate gel beads. The proportionally lower K_m value for

Table (5): Kinetics inhibition of free Aspergillus niger ß-glucosidase enzyme.

Substr oncentration M [S]	1/[S]	Free enzyme			I=1mM Glucose			I=2mM Glucose			I=5mM Glucose			I=10mM Glucose		
		Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$
0.68	1.48	162.8	120.6	0.83	153.8	52.0	1.92	147.8	34.7	2.88	149.5	18.5	5.41	129.1	11.5	8.70
1.35	0.74	166.0	150.8	0.66	160.5	82.2	1.22	150.0	58.9	1.70	161.4	33.7	2.97	132.3	21.7	4.61
2.70	0.37	174.0	172.3	0.58	164.1	116.7	0.86	153.0	91.1	1.10	164.0	57.7	1.73	139.5	39.2	2.55
5.40	0.19	195.0	185.8	0.54	168.3	147.7	0.68	155.6	125.3	0.80	160.5	89.7	1.11	145.7	65.5	1.53
10.00	0.10	201.0	192.3	0.52	165.8	168.2	0.59	158.3	151.6	0.66	149.3	120.4	0.83	147.4	95.0	1.05

Table (6): Kinetics inhibition of immobilized Aspergillus niger in Ca-alginate gel

Substr oncentration M [S]	1/[S]	Immobilized form			I=1mM Glucose			I=2mM Glucose			I=5mM Glucose			I=10mM Glucose		
		Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$	Glucose content mm/1L	Reaction velocityt (V)	$1/V\times10^{-2}$
0.68	1.48	41.8	37.9	2.64	32.6	24.3	4.12	25.7	22.4	4.46	15.8	16.2	6.17	9.6	10.5	9.52
1.35	0.74	69.1	59.2	1.69	54.3	41.1	2.43	44.5	38.4	2.60	28.8	28.8	3.47	18.1	19.5	5.13
2.70	0.37	112.1	82.9	1.21	97.8	63.4	1.58	69.9	60.0	1.67	48.9	47.6	2.10	32.6	34.1	2.93
5.40	0.19	123.0	103.6	0.97	108.7	86.9	1.15	97.8	83.7	1.19	75.2	70.8	1.41	44.3	54.7	1.83
10.00	0.10	138.1	117.0	0.85	128.3	104.7	0.96	119.9	102.2	0.98	100.0	91.3	1.10	65.4	75.8	1.32

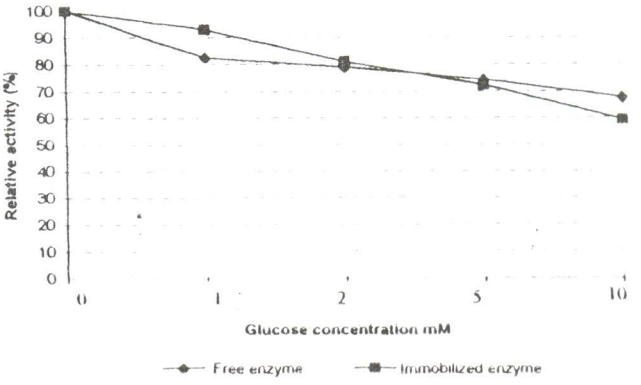

Fig (4): Effect of glucose concentration on the reaction activity of free and immobilized B-glucosidase.

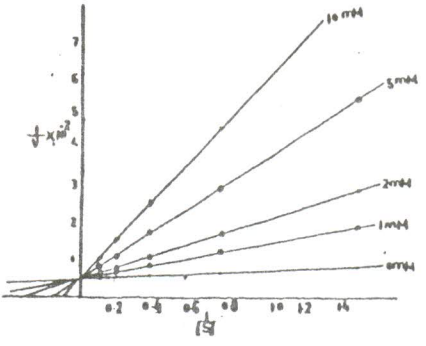

Fig (5): Kinetics inhibition of free Aspergillus niger ß-glucosidase enzyme.

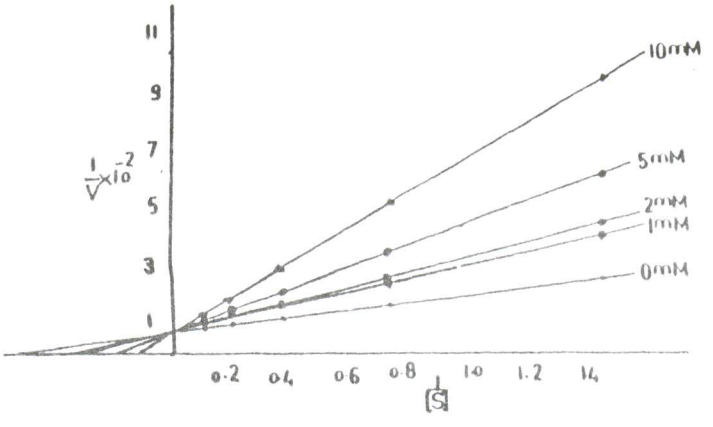

Fig (6): Kinetics inhibition of immobilized Aspergillus niger in Ca-alginate gel.

immobilized enzyme may be attributed to the binding of support to soluble enzyme which increase the affinity between the substrate and Ca-alginate-enzyme complex, and hence lower K_m can be observed. Lineweaver and Burk plotts of free and immobilized ß-D-glucosidase on Ca-alginate in absence and presence of glucose at the same abovemetioned concentrations are shown in Figs. (5, 6). From the obtained results, it may be observed that K_m was affected while V_{max} was constant which mean that the inhibition is competitive type and these are in agreement with that reported by Woodward and Arnold (1981), Dekker (1986); Mitchell, *et al.*, (1991).

A repolt of the slopes from the lineweaver-Burk were plotted versus glucose concentration to distinguish between the different types of competitive inhibition and as shown in and Fig. (7) the slopes were not a linear line in case of free and immobilized ß-D-glucosidase enzyme in calcium alginate beads. This means that the inhibition of these enzymes by glucose in partially competitive as reported by others (Woodward and Wohlpart, 1982 and Dekker, 1986).

3.7. Reusability of ß-glucosidase-alginate gel beads:-

The results shown in Table (7) indicated that, activity of immobilized enzyme after 7 times decreased by about 29.8% of its original levels.

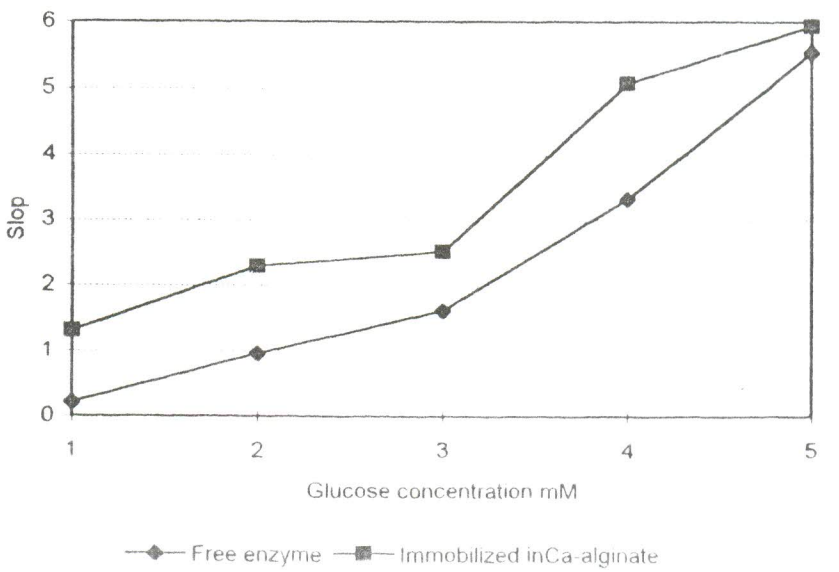

Fig (7): Replot of slops of lineweaver-Burk plot versus glucose.

Table (7): Reusability of immobilized ß-glucosidase alginate gel beads.

No. of reuse	Activity (µmol glucose/min/100 mg beads
Initial	100
1	100
2	99.2
3	99.6
4	100.0
5	96.8
6	87.6
7	70.2

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تحميل إنزيم بيتا-جلوكوسيديز فى الكالسيوم-الجينيت جل

إبراهيم محمد عبدالعليم ، فرحات فوده على فوده

قسم الأراضى والكيمياء الزراعية -كلية الزراعة بمشتهر -جامعة الزقازيق-فرع بنها

يهدف هذا البحث إلى دراسة إمكانية تحميل إنزيم بيتا-جلوكوسيديز الذائب فى الكالسيوم الجينيت جل كدعامة رخيصة وتكوين كبسولات وكذلك دراسة السلوك الحركى للإنزيم الحر والمحمل وذلك لإمكانية إستخدام الإنزيم المحمل عديد من المرات وهذه النقطه مهمة من الناحية الصناعية والإقتصادية حيث أن هذا الإنزيم مهم فى عمليات تحليل السليلوز وإنتاج شراب الجلوكوز.

أوضحت الدراسة أن أعلى قوة ربط الكالسيوم-الجينيت جل لإنزيم بيتا-جلوكوسيديز تساوى ٤و٨٠٪ من كمية الإنزيم المضافة إلي الكالسيوم-الجينيت وذلك على تركيز من الصوديوم-الجينيت يساوى ٤٪.

وقد أوضحت النتائج أن درجة الحموضة المثلى pH للإنزيم المحمل تساوى ٨و٤ بينما كانت للإنزيم الحر٥و٤ وأن درجة الحرارة المثلى للإنزيم الحر ومعقد الإنزيم -كالسيوم-الجينيت جل تساوى ٥٠ درجة مئوية. أما بالنسبة لتأثير الجلوكوز كمثبط لإنزيم بيتا-جلوكوسيديز الحر والمحمل فإن معدل التفاعل وصل إلى أقصاه ٨و٦٧٪ للإنزيم الحر و ٣و٥٩٪ للإنزيم المحمل وذلك عند تركيز ١٠ ملليمول جلوكوز.

أظهرت الدراسة الحركية لهذا الإنزيم فى وجود أو غياب المثبط أن السرعة القصوى وثابت ميكاليس كانت لمعقد الإنزيم ١و١٣٨ ملليمول /لتر ،٨و١ملليمول أما هذه الثوابت للإنزيم الحر فكانت ٢٠١ ملليمول /لتر، ٤٥و٠ ملليمول وذلك فى غياب المثبط وقد يعزى إنخفاض ثابت ميكاليس للإنزيم المحمل عن الإنزيم الحر أن إرتباط الكالسيوم-الجينيت مع الإنزيم أدى إلى زيادة جاذبية مادة التفاعل للإنزيم. بينما كان ثابت ميكاليس فى وجود المثبط يساوى ٣و٠ ملليمول للإنزيم الحر، ٣و١ ملليمول للإنزيم المحمل. أما بالنسبة لإستخدام الإنزيم المحمل عديد من المرات فإنه أمكن إستخدامه ٧ مرات مع فقد ٨و٢٩٪ من درجة نشاطه وهذه النقطة مهمة من الناحية التكنولوجية للإنزيمات الصناعية.