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Immobilization, Optimization and Properties of Pea Invertase within Sodium Alginate Gel.

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ABSTRACT

The aim of this study was to prepare immobilized invertase with high hydrolytic activity for biotechnological applications. Pea invertase was immobilized in alginate beads using 2% sodium alginate. Optimization condition of entrapped free invertase in sodium alginate was investigated. Immobilized process depends on the amount of free enzyme added and on the concentration of sodium alginate used. At optimum condition for immobilization (1499.9 µg fructose invertase activity and 2% sodium alginate), complete immobilization of all enzyme added with high specific activity (14600 U/mg) and immobilization efficiency (739.2). Degree of hydrolysis sucrose was 33.52% under the standard assay conditions. Optimum conditions for invertase activity were affected by immobilization. The hydrolytic activity of the immobilized invertase was found to be influenced by temperature and pH. The immobilized invertase showed high invertase activity at wide pH range (5.0 to 6.5) with optimum at pH 5.0 and wide range of temperature from 45°C to 55°C. The immobilized process is simple and invertase does not leak out of beads. This method can be used for the industrial production of invert sugar.

Keywords: Invertase (EC 3.2.1.26), immobilization, sodium alginate, optimization, characterization.

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INTRODUCTION

Invertase (E 3.2.1.26) is a sucrose hydrolyzing enzyme producing an equimolar mixture of glucose and fructose named invert sugar [1]. The enzymatic process is more expensive than acid hydrolysis, due to the relatively high cost of invertase. To reduce the cost of final product, the application of immobilized invertase has been considered an appropriate solution. The immobilized enzyme has many advantage as 1) it can reused many times and still remain active, 2) immobilization protects the activity of the enzyme from un-favorable conditions, 3) the separation and recovery of enzyme is easy and convenient, 4) it can be used in continuous system for the production of invert syrup from sucrose solution, 5) the application of immobilized enzyme provides considerable reduction in the operating costs [2-3].

Several attempts have been made to obtain a highly active and stable immobilized preparation suitable for commercial application. Many studies were focused on the support for immobilization of invertase in different aspects namely polyvinylalcohol [4], polyacrylamide [5], chitosan [6], hen-egg white and diethylamino ethylcellulose [7]. There are varieties of methods used to immobilize enzymes. Three of the most common being adsorption, entrapment, and cross linking or covalently binding to the support [8]. The gel entrapment method has the advantage that it preservers a high level of enzyme activity since enzyme molecules are physically retained and shield by the matrix and not chemically bound to it. Diffusion effects are drawback in gel entrapment protocols as the substrate must diffuse through the gel to reach enzyme molecules buried inside the matrix. Immobilized enzymes have an enormous potential as catalysts in chemical processes in wide range of industries and medicine. They offer a distinct advantage over classical catalyst due to their specificity, high catalytic efficiency at low temperatures.

A previous study investigated a novel invertase isolated and partial purified from pea pods with high specific activity (168.08 U/mg) with high thermo stability and degree of hydrolysis sucrose 3.9% [9]. It retained 88.6 % of its initial activity when storage at 4°C for 270 days. These results indicated that the enzyme could be potentially served in industry for invert syrup production. In this work, the partial purified invertase was immobilized. The condition of immobilization was optimized for preparation high active immobilized invertase. Its properties were investigated including optimum pH, temperature .....etc.

MATRIALS AND METHODS

Material

Free invertase (EC 3.2.1.26) was prepared from pea pods as described previously [9]. Sucrose was purchased from Merck Chemical Co. All other chemicals were of analytical grade.

Immobilization of free invertase by

Covalent binding

Chitosan (0.5 g) was shaken in 6.25 ml acetate buffer (0.05M, pH 5.0) containing 2.25 % glutaraldehyde for 2 hrs at 30°C. The solubilized chitosan was precipitated by the addition of 1ml 0.1 M NaoH. The precipitated was collected by filtration and washed with distilled water to remove the excess glutraldehyde. The wet chitosan was mixed with 1 ml of enzyme solution and shaken for 3 hrs at 30°C with gentle agitation. The unbound enzyme was removed by washing with the same buffer [10]. Invertase activity and protein concentration were determined in wash solution (unbound enzyme). The actual immobilized invertase activity was determined in the precipitated enzyme.

Entrapment

Entrapment of the free invertase in sodium-alginate beads was carried out according to Vu and Le [3]. In experiment, 10 ml of 2% sodium alginate solution were mixed with the 1 ml invertase. The whole mixture was made into beads by drop wise the alginate solution into 0.1 M $CaCl_2$ by dropper (Hamiliton). The formed beads were retained in the stirred $CaCl_2$ solution (using a magnetic stirrer) at least 2 hrs for gel hardening. The resulting beads left in refrigerator at 11°C overnight for 24 hrs, then collected and washed with distilled water for 3 times to remove the unbound enzyme. Before using, the beads were immersed and kept in 0.1 M sodium

acetate buffer of pH 5.0. Invertase activity and protein concentration were determined in calcium chloride and in the wash solutions (unbound enzyme). The actual immobilized invertase activity was determined in the prepared beads.

Free and Immobilized invertase activity

The standard reaction mixture contained 1% (w/v) of the substrate (Sucrose) dissolved in 0.1M sodium acetate buffer, pH 5.0 and appreciable amount of free and immobilized invertase in a total final volume of 1.0 ml. The reaction mixture was incubated at 40°C in water bath for 1.0 hr. The enzyme activity was accomplished by measurement of the liberated reducing sugar (fructose and glucose) by the procedure described by Somogyi [11] and Nelson [12] method, using fructose as standard. Numbers of beads and weight were determined for each preparation. One unit of enzyme is defined as the amount of enzyme which will catalyze the formation of one µmole of reducing sugar (as fructose) per hr under the standard assay conditions. The specific activity is expressed as units per mg of proteins. The actual immobilized invertase activity is defined as µg fructose per hr under the standard assay conditions.

Protein determination

The protein concentration was determined by method of Lowry *et al*. [13] using bovine serum albumin as a standard.

Relative activity

Relative activity was expressed as a percentage of the maximum activity under the standard assay condition.

Determination of invertase immobilization yield

Immobilization yield IY was calculated as the percent ratio of the bound invertase activity to the activity of the free invertase added.

$$IY=A-B/A \times 100$$

where:

A is the total amount of free enzyme activity added to the immobilization solution.
B is the amount of residual enzyme activity in calcium solution and in the washing solution of gel beads in the immobilization procedure.
Both A and B were evaluated from the amount of reducing sugars produced enzymatically in the corresponding solutions.

Determination of immobilized efficiency

Immobilization efficiency is defined as the ratio of the actual immobilized invertase activity in µg fructose to the activity of the bound free invertase in µg fructose.

Determination degree of hydrolysis

Degree of hydrolysis percentage was calculated by using the following formula:

$$\text{Hydrolysis (\%)} = TRS/S^{\circ} \times 100,$$

where

TRS is the total reducing sugar (glucose and fructose) in mM, S° is the initial sucrose concentration in mM.

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Optimization conditions of immobilization method

Effect of free invertase concentrations added on the immobilization yield and efficiency

Free invertase was immobilized at different concentrations of free enzyme added from 1499.9 to 3749.6 µg fructose in four groups (B_1 to B_4) and 2% sodium alginate. Immobilization yield and efficiency were determined in each group. Relative activity for immobilized invertase in each group (B_1 to B_4) at different immobilized invertase concentrations from 0.01 to 0.2 mg wt. /reaction mixture also was estimated.

Effect of sodium alginate concentrations on immobilization yield and efficiency

Free invertase (1499.9 µg fructose) was immobilized with different concentration of sodium alginate (2 %, 4 %, 6 % and 8 %). Immobilization yield and efficiency were determined in each group.

Statistical analysis:

Data are expressed as the mean ± standard error from at least three experiments.

RESULTS AND DISCUSSION

Effect of immobilization on the activity of the free invertase

Free invertase solution (1499.9 µg fructose) was immobilized within 2% sodium alginate. The resultant of entrapping beads showed complete immobilization of all added enzyme (100%) with high activity (1108×10^3 µg fructose) and immobilization efficiency 739.2 (Table 1). Degree of hydrolysis sucrose by the immobilized invertase was calculated to be 33.52%, while for the free invertase was 3.9% as calculated before [9]. Enzyme leakage test has shown that there was no invertase activity in the washing solution, which indicated that the immobilization of the free invertase within sodium alginate was complete and without loss of enzyme. Specific activity of the immobilized invertase (14600 U/mg) was higher by 86.9% fold than that of the free one calculated before, [ ] (Table 2).

Table 1: Immobilization yield, efficiency and degree of hydrolysis of immobilized invertase

Free invertase activity				Immobilized invertase activity			
Added (µg fructose)	Unbound (µg fructose)	Bound		No. of beads	Actual activity (µg fructose) X 10^3	Immobilization efficiency	Degree of hydrolysis (%)
		Activity (µg fructose)	Immobilization yield (%)				
1499.0	0.0	1499.9	100	2738	1108 X 10^3	739.2	33.52

Table 2: Specific activity and immobilization efficiency of immobilized invertase

Steps	Specific activity (U/mg)	Purification Fold	Invertase activity (µg fructose)	Immobilization efficiency
Free invertase	168.08 ± 0.02	1.0	1499.0	----------
Immobilized invertase	14600 ± 0.00014	86.9	1108 X 10^3	739.2

Immobilization of invertase by covalent binding indicated the unsuitability of this method with the used carriers chitosan. This could be due to the weak binding between the carriers and the enzyme.

From the above results, we choice entrapment of the free invertase within sodium alginate beads method for preparation immobilized invertase. Immobilization of invertase using alginate was tried by various researchers. Yeast invertase was immobilized in alginate capsule with 87% [14] and 79.34% yield [3]. Previously, it was found that invertase quickly leaked out of alginate beads [15-16] .

Optimization conditions of immobilization method

Effect of free invertase concentrations added on the immobilization yield and efficiency

Results illustrate that the immobilization depends on the amount of free enzyme added (Table 3). This relationship was found to be reverse proportional. This behaviour could be caused by diffusional restrictions of sucrose in beads membrane so that note all the activity of the immobilized invertase was fully utilized. The immobilized process in group B_1 was optimum with 100% yield and immobilization efficiency 765.9. Immobilized invertase activity was maximum in group B_1 at 0.025 mg wt /reaction mixture (Table 4). Figure (1) shows that as increase the concentration of enzyme added lead to increase the beads weight. The most active one was used for further study.

Effect of sodium alginate concentrations on immobilization yield and efficiency

Table (5) illustrate that the immobilization yield and efficiency was maximum when using 2% sodium alginate. Figure (2) was illustrates shape, weight and number of beads at different concentrations of sodium alginate. This shows that as the concentrations of sodium alginate increase, the beads weight increase.

Properties of the immobilized invertase

Effect of different pH on the immobilized invertase activity

Optimum pH was determined by individually changing the pH from 3.0 to 9.2 in the reaction mixture. High invertase activity at pH range from 5.0 to 6.0 with optimum a pH 5.0 (Figure 3). This result is **agree with Tan *et al*., [17], Raja and Meena [18],** Sungunn and Sanjay [19]. It was greater than those isolated from *Saccharomyces cerevisiae with pH 3.6, 4.3 and 4.5* [7,14,20]. The free pea invertase activity was maximum at wide range pH from 5.0 to 7.4 with optimum at 6.5 as reported before [9]. The optimum pH of the immobilized invertase was shifted towards acidic side when compared with the free enzyme. This might be explained by partitioning of protons. Negatively charged groups of the matrix will tend to concentrate protons (thus lowering the pH) around the enzyme. Therefore, the pH around the enzyme will be lower than that of the bulk phase from which the measurement of pH is carried out. As a result, the immobilized enzyme will be appearing to shift its pH activity profile down words [21].

Effect of different temperatures on the immobilized invertase activity

Optimum temperature was determined by individually changing the incubation temperature from 30°C to 60°C. As determined before [ ], the optimum temperature for the free invertase activity was 45°C, high activity of the immobilized invertase at wide range of temperature from 45 to 55°C with optimum at 50°C (Figure 4). The increase in optimum temperature can be explained by alterations of the physical and chemical properties of the enzyme upon immobilization. This result was consistent with [7,17, 19, 22-24].

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Table (3): Effect of invertase concentrations on immobilization yield and efficiency

Group number	Free invertase					Immobilized invertase		Immobilization efficiency	
	Volume (ml)	Added (µg fructose)	Unbound activity (µg fructose)	Bound		No. of beads	Actual activity (µg fructose X 10^3)	Times	Relative activity (%)
				Activity (µg fructose)	Immobilization yield (%)				
B_1	1.0	1499.9	0.0	1499.9	100	2738	1148	765.9	100
B_2	1.5	2249.8	0.0	2249.8	100	2138	427.6	190.1	24.8
B_3	2.0	2999.6	26.1	2973.5	99.13	2170	609.8	203	26.5
B_4	2.5	3749.6	29.7	3719.8	99.2	2219	329.3	87.8	11.5

Table (4): Effect of immobilized enzyme concentrations on invertase activity

Immobilized beads (mg wt / R.M)	Relative invertase activity (%)			
	B_1	B_2	B_3	B_4
0.01	100	76.27	71.2	75.82
0.025	107.02	54.4	74.31	77.33
0.05	96.53	57.07	55.11	78.31
0.1	25.96	20.09	18.66	0.0
0.15	21.24	19.11	0.8	0.0
0.2	12.88	1.42	0.0	0.0

Relative activity was expressed as a percentage of invertase activity at 0.01 mg wt. /R.M. in group B_1

Table (5): Effect of sodium alginate concentrations on the immobilization yield and efficiency

Concentrations of Sodium alginate (%)	Free invertase				Immobilized invetase		Immobilization efficiency	
	Added (µg fructose)	Unbound activity (µg fructose)	Bound		No. of beads	Actual activity (µg fructose X 10^3)	Times	Relative activity (%)
			Activity (µg fructose)	Immobilization yield (%)				
2	1499.9	0.0	1499.9	100	2547.8	1068	712.5	100
4		85.2	1414.7	94.3	1126.9	280	186.8	26.2
6		93.1	1406.8	93.8	954.4	151	100.7	14.1
8		97.7	1402.1	93.5	723.5	57.9	86.79	12.2

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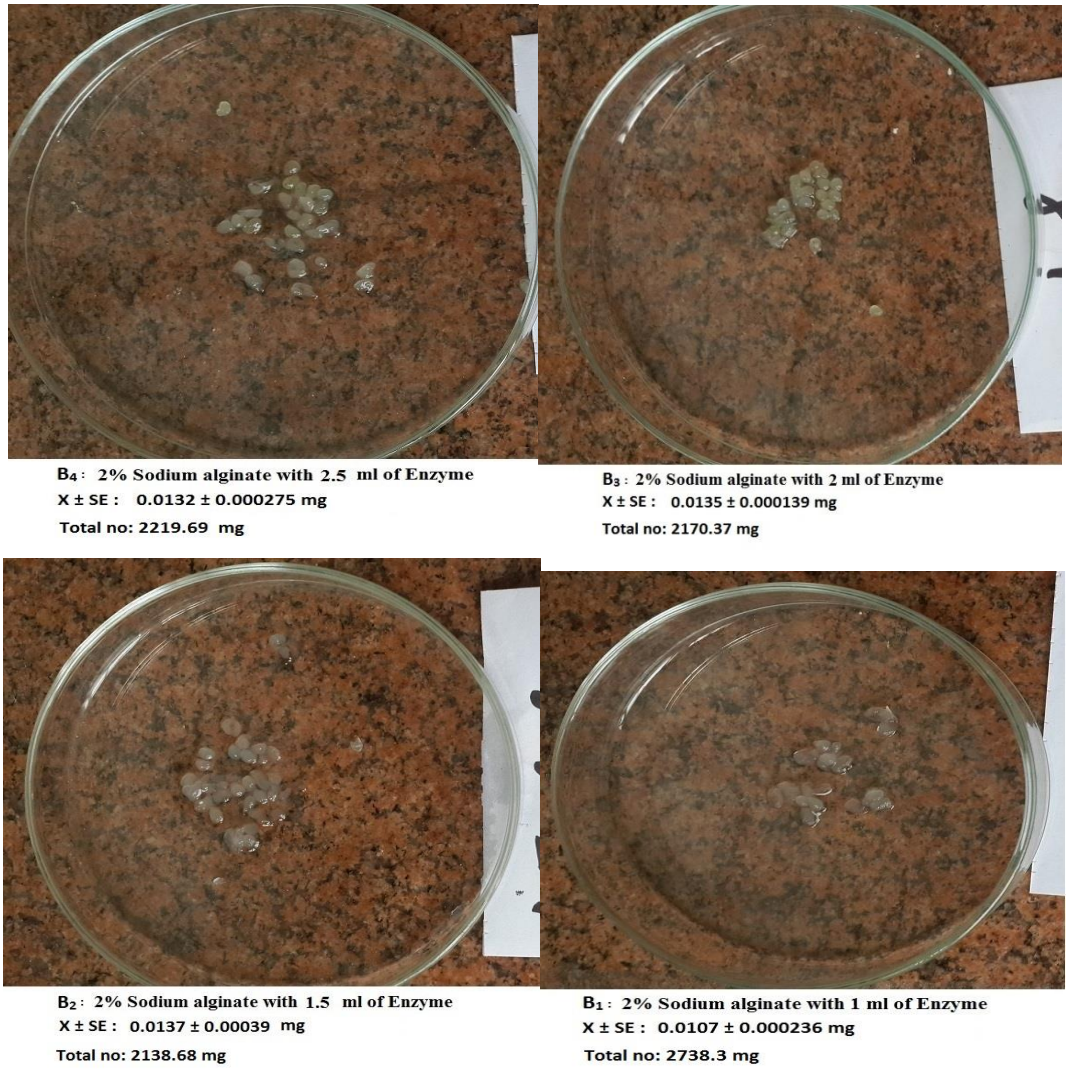


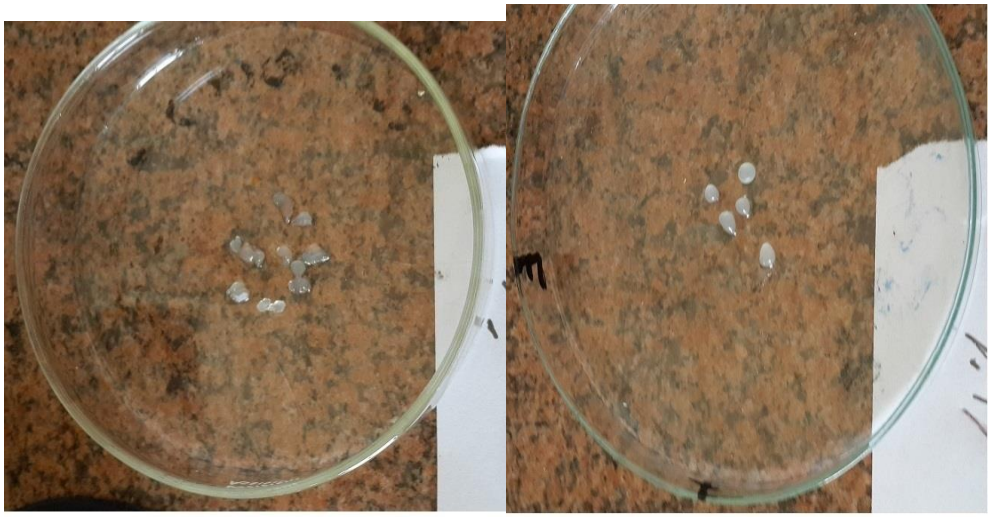
B_4 : 2% Sodium alginate with 2.5 ml of Enzyme
X ± SE : 0.0132 ± 0.000275 mg
Total no: 2219.69 mg

B_3 : 2% Sodium alginate with 2 ml of Enzyme
X ± SE : 0.0135 ± 0.000139 mg
Total no: 2170.37 mg

B_2 : 2% Sodium alginate with 1.5 ml of Enzyme
X ± SE : 0.0137 ± 0.00039 mg
Total no: 2138.68 mg

B_1 : 2% Sodium alginate with 1 ml of Enzyme
X ± SE : 0.0107 ± 0.000236 mg
Total no: 2738.3 mg

Figure (1): Effect of invertase concentrations added on weight and number of beads in each group.



2% Sodium alginate with 1 ml of Enzyme
X ± SE : 0. 0 115 ± 0.0015 mg
Total no: 2547.8 mg

4% Sodium alginate with 1 ml of Enzyme
X ± SE : 0.021 ± 0.0016 mg
Total no: 1126.92 mg

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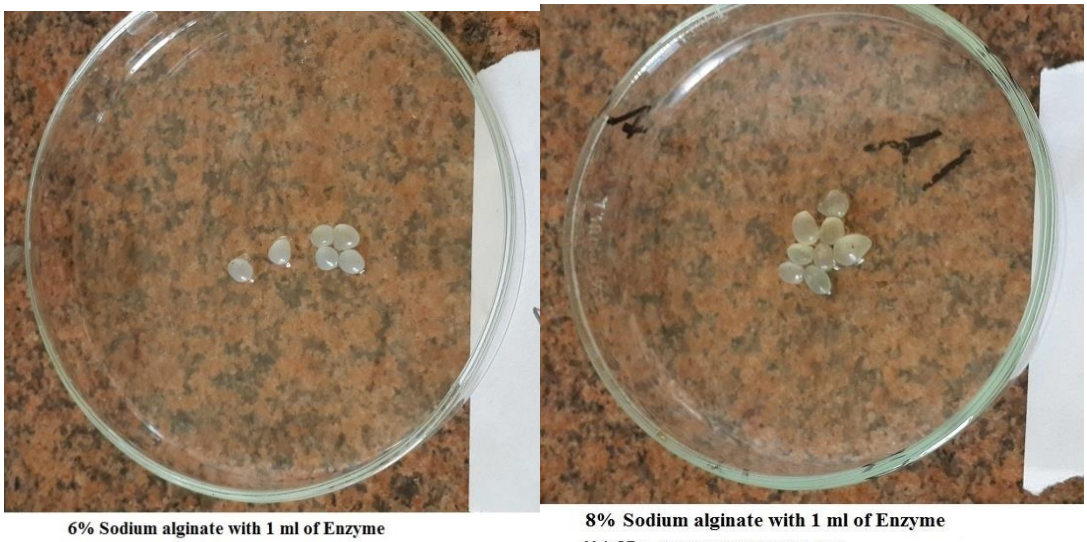

Figure (2): Effect of sodium alginate concentrations on weight and number of beads in each group.

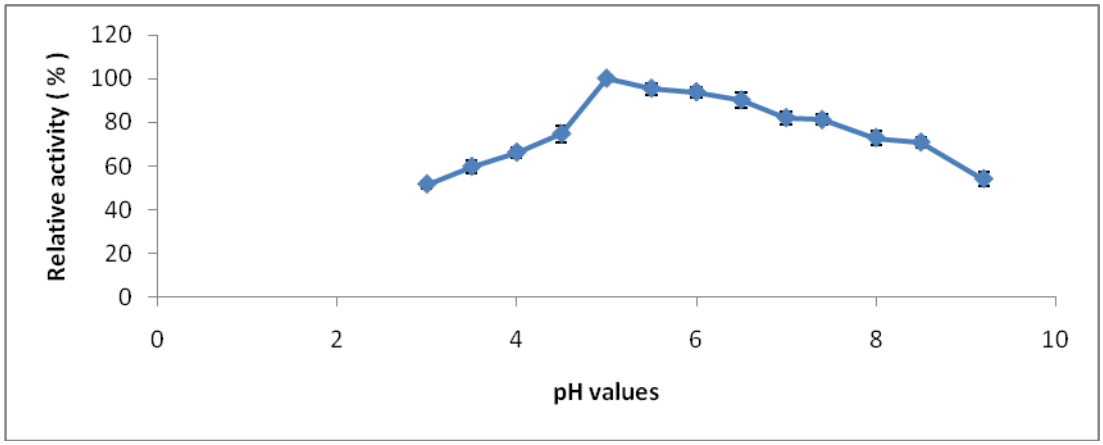

Figure (3): Effect of different pH on immobilized invertase activity.
***Relative activity was expressed as a percentage of maximum invertase activity.**

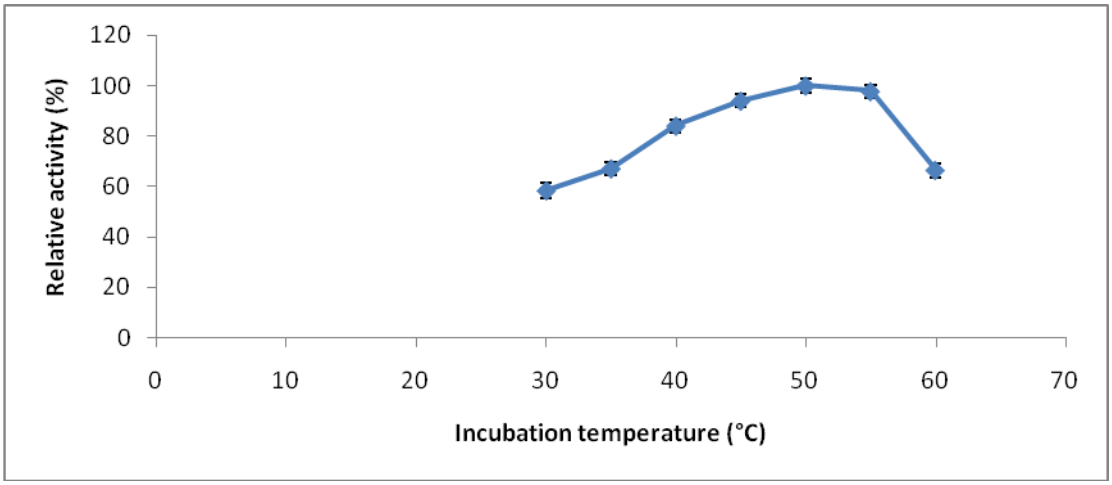

Figure (4): Effect of different temperatures on immobilized invertase activity.
***Relative activity was expressed as a percentage of maximum invertase activity.**

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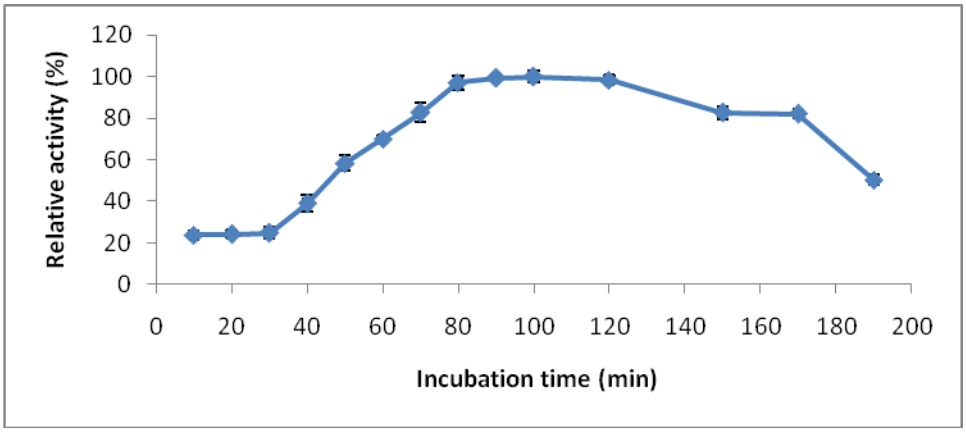

Figure (5): Effect of different incubation times on immobilized invertase activity.
***Relative activity was expressed as a percentage of maximum invertase activity.**

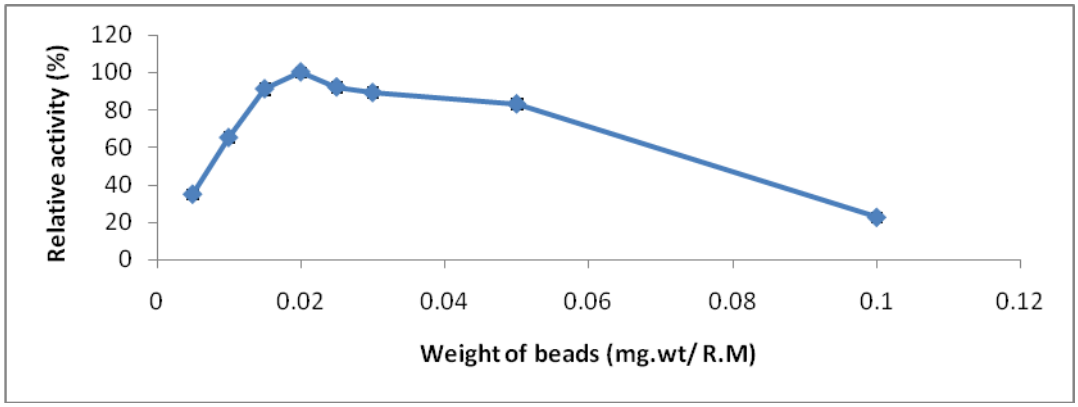

Figure (6): Effect of different immobilized enzyme concentrations on invertase activity.
***Relative activity was expressed as a percentage of maximum invertase activity.**

Activity of the immobilized invertase at different incubation times

The activity of the immobilized invertase was determined at different incubation times up to 180 mins. It was increased up to 80 mins fig (5). It could be concluded that increasing time leads to excess products which may inhibit the enzyme activity.

Activity of the immobilized invertase at different enzyme concentration

The invertase activities were estimated at different concentrations of immobilized invertase ranging from 0.005 to 0.35 mg wt /reaction mixture. Figure (6) illustrates that the invertase was increased proportionally in a linear relationship with the increase of enzyme concentration up to 0.02 mg/reaction mixture.

CONCLUSIONS

Entrapment free invertase within sodium alginate was found to be the best one for prepare immobilized invertase. The method is simple and inexpensive. The prepared immobilized invertase shows high specific activity (14600 U/mg) with high degree of hydrolysis sucrose (33.52%) at pH 5.0 and 50°C. The prepared enzyme may be suitable for industrial application in sucrose hydrolysis for fructose syrup production. More research is needed to fully explore this new immobilized enzyme and its potential application in invert syrup production.

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