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Characterization and stabilization of immobilized pea (*Pismus sativum*) invertase and invert sugar production.

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ABSTRACT

Characterization and kinetics studies of immobilized pea (*Pismus* sativum) invertase immobilized by entrapment in alginate beads was the main objective of this work. Immobilized pea invertase catalyzed hydrolysis of sucrose to invert sugar (glucose and fructose). It showed high specific activity (243.3 U/mg) under optimization working conditions including pH 5.0 at 50°C. It was classified as acid invertase. It exhibited a high specificity for sucrose (α 1, 2 - glycosidic linkage) and inulin (β-1, 2 - glycosidic linkage). Inulin was hydrolyzed at the rate of 42% relative to hydrolysis sucrose, where maltose, lactose and cellulose were not hydrolyzed. Maximum enzyme velocity was observed at 2.5 mg sucrose and 5.0 mg inulin per reaction mixture. It followed Michaelis-Menten kinetics. K_m values for the immobilized invertase toward sucrose and inulin were 0.0526 and 0.0658 mg and V_{max} values were 250 and 111.1 U/mg for sucrose and inulin, respectively. These results were indicating that immobilized pea invertase had higher affinity and reaction velocity toward sucrose than inulin. The immobilization of invertase, into alginate with glutaraldehyde crosslinked, exhibited high resistance to inactivation upon exposure to high temperature up to 70°C for 120 min. The immobilized invertase remained fully active after storage in 0.1 M sodium acetate buffer, pH 5.0 for 270 days at 11°C. Degree of hydrolysis sucrose by the immobilized invertase was 3.76 times higher than that of hydrolysis inulin. Immobilized invertase showed high operational stability. It retained 85 and 50 % of its initial activity after 12 and 15 run, respectively. It was successfully employed to produce 18.01 mg invert sugar (glucose and fructose) from sucrose by 15.0 times repeated run with relative production 52.16 % and 100% saturation. Glucose and fructose in invert syrup were identified by TLC. These properties make this enzyme a potential candidate for future use in industrial application especially in invert sugar production, medicine and food industry.

Keywords: Pea (*Pismus* sativum), immobilized invertase (EC 3.2.1.26), sucrose, characterization, stability, invert sugar production.

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INTRODUCTION

Invertase (β- fructofuranoside fructo hydrolase (EC 3.2.1. 26 β- fructofuranosidase) catalyzed the hydrolysis of sucrose yielding glucose and fructose named invert sugar. Studies had been done on invertase extracted from plant tissues such as tomato, ripe grape berries, japanese pear fruit, pea, oat, radish roots and turnip leaves [1-7]. Acid invertase attacked the disaccharides from the fructose residue and also hydrolyzed other fructourosidase such as raffinose and stachyose with low cleavage efficiency, while neutral and alkaline invertase were specific to sucrose only [8].

Enzymatic treatment of sucrose was a major way of producing sweeteners syrup (invert sugar) from sucrose. Invert sugar contains fructose and glucose in approximately equal amounts. It was up to 1.8 times sweeter than sucrose, making it useful in foods and beverages for the health conscious and many industrial application. Fructose was ideal for use in diabetic foods [9-10].

The immobilized enzymes have an enormous potential as catalysts in chemical processes in wide range of medicine and industries. It had advantage over chemical catalytic due to their specificity and high catalytic efficiency at low temperatures. It can be removed from the reaction mixture and pure product was easily obtained. The immobilization of enzymes may increase their potential application for industrial purposes [11].

Firstly, pea invertase was extracted from pea pods (agricultural wastes) as it was a good economic source [6]. It was successfully immobilized into alginate beads by entrapment method [7]. Calcium alginate gel was high mechanical stability, non-toxic and simple procedural requirements for immobilization [12]. The present study focused upon study properties of immobilized pea like (thermal stability, substrate specificity and kinetics). Storage and operational stability also was investigated. The potential use of the immobilized invertase as a biocatalyst for invert sugar production from sucrose was investigation.

MATERIALS AND METHODS

Materials:

Fresh pea pods (*Pismus* sativum) family *Leguminoseae* were bought from local markets. Sucrose, inulin, fructose, glucose, sodium alginate glutraldehyde and all chemicals were of analytical grade.

Methods:

Preparation of immobilized invertase. Immobilized invertase into alginate beads by entrapment method was prepared from fresh pea pods as described previously [7].

Enzyme assay: Invertase activity was determined by measurement the liberated reducing sugar (fructose and glucose) produced from sucrose [7]. The standard reaction mixture contained 1% (w/v) of the substrate (sucrose) dissolved in 0.1M sodium acetate buffer, pH 5.0 and 0.02 g wt. immobilized invertase bead in a total volume of 1.1 ml. It was incubated at 50°C in water bath for 80 min. The total reducing sugars liberated were determined by the procedure described by Somogyi [13] and Nelson [14] methods, using fructose as standard. The activity of invertase was of average values of three repeated measurements.

One unit of immobilized invertase was calculated as amount of enzyme which will catalyzed the formation of 1 µmole of reducing sugar as fructose per min. under the standard assay conditions. The specific activity is expressed as units per mg of proteins. Relative activity was expressed as a percentage of the maximum activity.

Protein determination. The protein concentration was determined according to the method of Lowry *et al*. [15] using bovine serum albumin as a standard.

Determination of substrate specificity. Immobilized invertase were estimated using various disaccharides (sucrose, lactose and maltose) and polysaccharide (cellulose and inulin). They were incorporated in the

reaction mixture separately at the same concentration of sucrose. Relative activity was calculated as a percentage of enzyme activity compared to maximum invertase activity toward sucrose as substrate.

Effect of different substrate concentrations on enzyme activity. The immobilized invertase activity was estimated at different substrate concentrations ranging from 0.25 to 15 mg per reaction mixture with the same amount of enzyme under the standard assay conditions. Relative enzyme activity were calculated as a percentage of maximum enzyme activity. They were plotted against different substrate concentrations.

Determination of kinetic values. Apparent Michaelis constant (K_m) and (V_{max}) values of the immobilized enzymes toward sucrose and inulin were determined according to the method of Lineweaver and Burk [16].

Thermostability. Immobilized invertase beads were pre-heated at different temperatures from 65°C to 80°C for varying time intervals from 15 to 120 min. The remaining enzymes activities were then estimated using the standard assay conditions. Residual activity was expressed as a percentage of the initial activity under the standard assay conditions.

Storage stability of the immobilized invertase. Immobilized invertase beads were stored in 0.1 M sodium acetate buffer pH, 5.0 at 11°C for 270 days. The residual activities at different times intervals were measured by the standard assay procedure as described earlier. The activity of the immobilized invertase was expressed as a percentage of its initial one.

Operation stability of the immobilized invertase. Immobilized invertase bead (0.02 g wt.) was incubated with 0.8 ml of 1% sucrose solution (0.1 M sodium acetate buffer, pH 5.0) at 50°C for 40 min. At the end of each reaction time (40 min), beads was collected and washed with distilled water and re-suspended again in 0.8 ml of freshly prepared substrate to start a new run. The supernatants for each run were collected and assayed for invertase activity. It was repeated for 15 times. Residual enzyme activity was expressed as a percentage of the initial one.

Invert syrup production. The invert sugar was produced enzymatically from sucrose by reuses of beads (repeated batch) for 15 runs under the assay conditions as mention above. The supernatant (invert syrup) for each case were collected and the total reducing sugars were determined as mention before. Relative invert sugar production, invert syrup concentration and invert sugar yield were calculated.

Relative invert sugar production % (hydrolysis measurement) = Invert sugar concentration mg per the initial substrate (sucrose) concentration (mg) × 100.

Invert syrup saturation = Invert sugar concentration (mg) per 100 ml solution.

Analysis of the prepared invert syrup. Invert sugar (syrup) obtained from hydrolysis of sucrose by the immobilized invertase were subjected to one dimensional thin layer chromatography chromatogram on cellulose layer plate [17]. Standard sugars named sucrose, D (-) Fructose, (+) Glucose and the prepared invert syrup were dissolved in deionized water. One micro-litter of each sugar solution was applied to the chromatoplate with the micropipette in the usual manner. The solvent system used was n-butanol- acetone-diethyl amine deionised water (10:10:2:6 v/v/v/v). The plate was sprayed with 5% diphenylamine in ethanol, 4% aniline in ethanol and 85% phosphoric acid (5:5:1 v/v/v).The plate was heated for 10 min at 105°C . Rf of the coloured spots was determine for invert syrup and compare with Rf of standard sugars used.

Statistical analysis. Data were expressed as the mean ± standard error (x ± SE) from at least three experiments.

RESULTS AND DISCUSSION

Pea immobilized invertase showed high invertase activity at standard optimum conditions including 0.02 g enzyme wt. (0.033 µg enzyme protein) at wide pH range from 5.0 to 6.5 with optimum at 5.0 and wide range of temperature from 45 to 55°C after 80 min as determined previously. It was defined (classified) as acid invertase. It had high specific activity (243 U/mg) with degree of hydrolysis 33.52 % toward sucrose.

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Immobilized acid invertase catalyzed hydrolysis of sucrose (α 1, 2 - glycosidic linkage) and inulin (β-1, 2 - glycosidic linkage). It failed to hydrolyze lactose (β- 1,4 - glycosidic linkage), maltose (α – 1,4 – glycosidic linkage) and cellulose (polymer of glucose β – 1,4 – glycosidic linkage). It hydrolyzed inulin with 42% and 49 % of its activity at 2.5 and 5.0 mg/R.M., respectively when compared with sucrose at 2.5 mg per reaction mixture under the same standard assay conditions. Similarly, invertase enzyme isolated previously from turnip leaves and yeast hydrolyzed sucrose and failed to hydrolyzed maltose and lactose [18-19]. Sturm [8] and Fotopoulos [20] was reported that acid invertase hydrolyzed β-fructouranosidase (glycoside links of the disaccharides) and also hydrolyzed other β-fructose containing oligosaccharides such as inulin from fructose residue. It was cleaving the terminal non reducing β-fructouranosidase residues.

The catalytic activity of the immobilized invertase was assessed using sucrose and inulin at different concentrations as substrate. The activities of the immobilized invertase toward sucrose and inulin were increased proportionally in a linear relationship with increase of concentration up to 2.5 and 5.0 mg /reaction mixture, respectively under the standard conditions at 50 °C (Figure 1). It seems that immobilized invertase followed Michaelis-Menten kinetics.

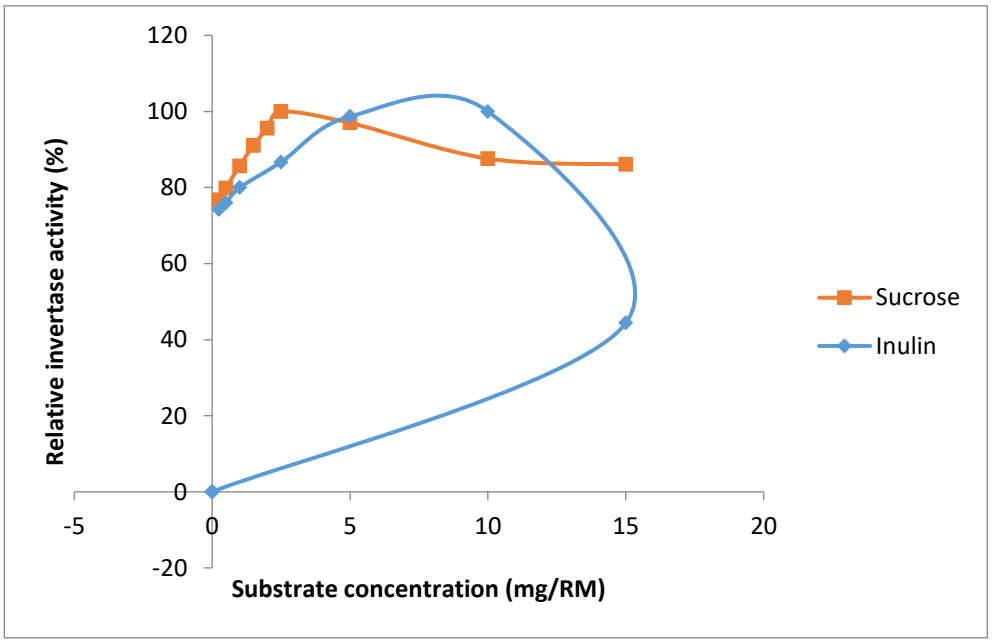

Fig. (1): Effect of different substrate concentrations on the immobilized invertase activity.

The K_m value of immobilized invertase toward sucrose was 0.0588 mg and V_{max} 285.7 U/mg at 50°C. The K_m value of the immobilized invertase also toward inulin was 0.05645 mg and V_{max} value was 113.6 U/mg (Figure 2). The immobilized invertase showed higher affinity and higher reaction velocity towards sucrose more than the corresponding free one (K_m 20.83 mg and V_{max} 3.7 U/mg) as determined previously [6]. The high porosity of calcium alginate gel could be increase substrate and product diffusion. Similar result was obtained by Mahmoud [21] who used wood saw dust waste as support for immobilization. In contrary, immobilization *Saccharomyces cerevisiae* invertase by entrapment in carrageenan gel beads showed lower affinity towards sucrose and lower reaction velocity than the free one [22]. Immobilized invertase on chemically activated poly (styrene-2-hydroxyethyl methacrylate) had higher K_m value and lower V_{max} than of free one [23]. Different result also was obtained by Algol *et al*. [24], Bayrumolu *et al*. [25] and Vu and Lee [26]. Variation of km values may be depend on the type of the support carrier and the immobilization method.

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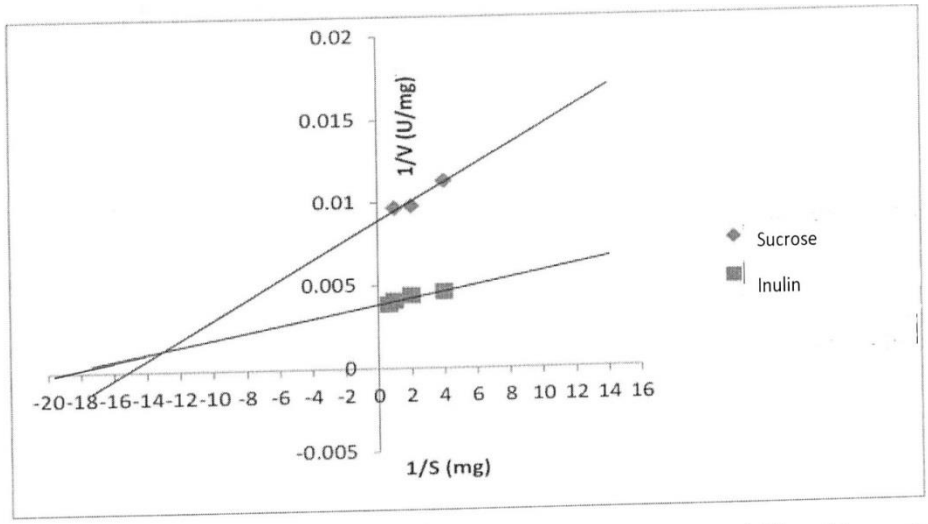

Figure (2) : Lineweaver - Burk plot's of the immobilized invertase

Immobilization of invertase in alginate with 5% glutaraldehyed as crosslinked preparation exhibited high resistance to inactivation upon exposure to temperature. It retained 100% of its activity when heated up to 65°C for 120 min. It retained 96 and 68.4 % of its initial activity when heated at 70 and 75°C for 120 min (Figure 3). Similar results was found by Ahmad *et al.* [27]. They reported that the immobilized invertase retained nearly all the activities when heated at 60°C without substrate even after 160 min. Mahmoud, [21] reported that immobilized invertase retained 100% of the activity after 1.0 h at 60°C. Immobilized *Saccharomyces cerevisiae* invertase showed high thermostability at 50°C for 24 h with retained 100% of its original activity [28]. While immobilized invertase in alginate retained 90 and 25% of its activity after heated at 50 and 60°C for 160 min [26&29]. The result reported by Danisman *et al*.[29], stated that immobilized invertase was stable at 50°C during a 90 min only retained 56 % of its activity at 60°C. High thermal stability of the prepared immobilized invertase represent valuable tools for a number of biotechnological processes.

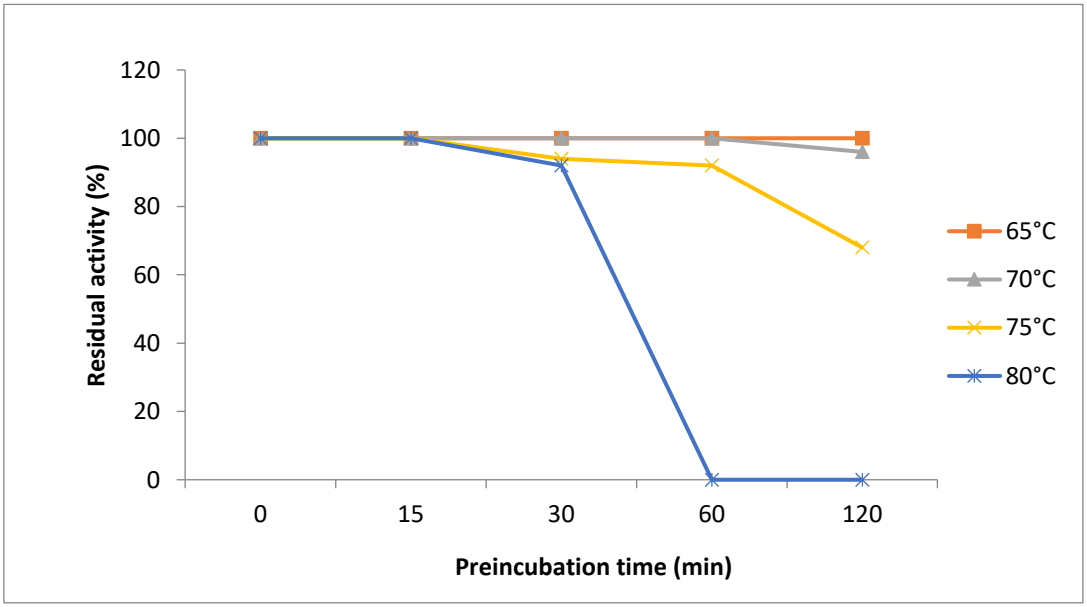

Fig. (3): Thermal- stability of the immobilized invertase.

- **Residual activity was expressed as a percentage of the initial activity without pre-incubation.**

Storage stability is a prominent factor for commercialization of an enzyme. Immobilized invertase retained 100% of its initial activity after stored in 0.1 M sodium acetate buffer, pH 5.0 at 11°C for 270 days

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(Figure 4). The corresponding free one showed less storage stability as it retained 88.6% of its activity after the same storage time period as mentioned by El-Sayed *et al*. [6] indicating a considerable enhanced of mobilization on stability. High stability of the immobilized enzymes was due to the physical protection by alginate gel. Approximately similar result had bee found by Mahmood [22], Gomez *et al*. [30] and Vu and Le [26] for invertase immobilized in carrageenan gel, on caboxymethyl cellulose modified chitin and alginate gel, respectively when storage for 12 weeks at 4°C. Our enzyme had highly storage stability higher than that found by Altinok et al. (2008). They reported that immobilized invertase retained 67 and 80 % of its initial activity after 30 and 60 days of storage at 4°C.

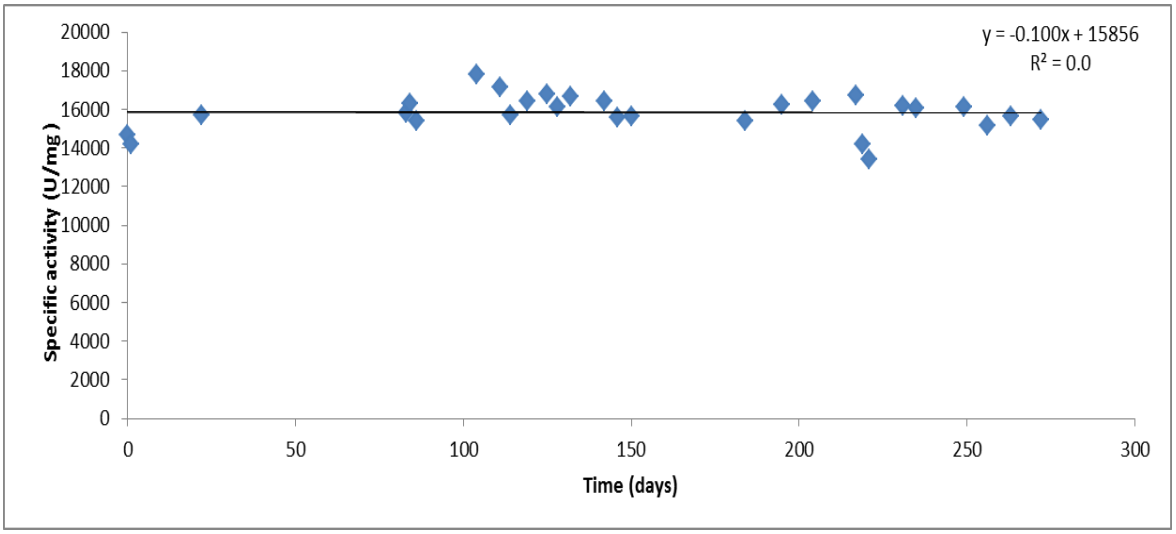

Fig. (4) Storage stability of the immobilized invertase stored in 0.1 M sodium acetate for 270 days.

Repeated use of the immobilized invertase beads for 15 times was studied. It could be reused 12 and 15 times and still remained active by 83.33 and 46.92% of its original activity, respectively (Figure 5). The decrease in the activity may be due to the progressive decay in mechanical resistance of the immobilization enzyme. Altinok *et al.* [23] reported that decrease of immobilized enzyme activity to 78 % retention of the initial activity was appeared after used 20 times.

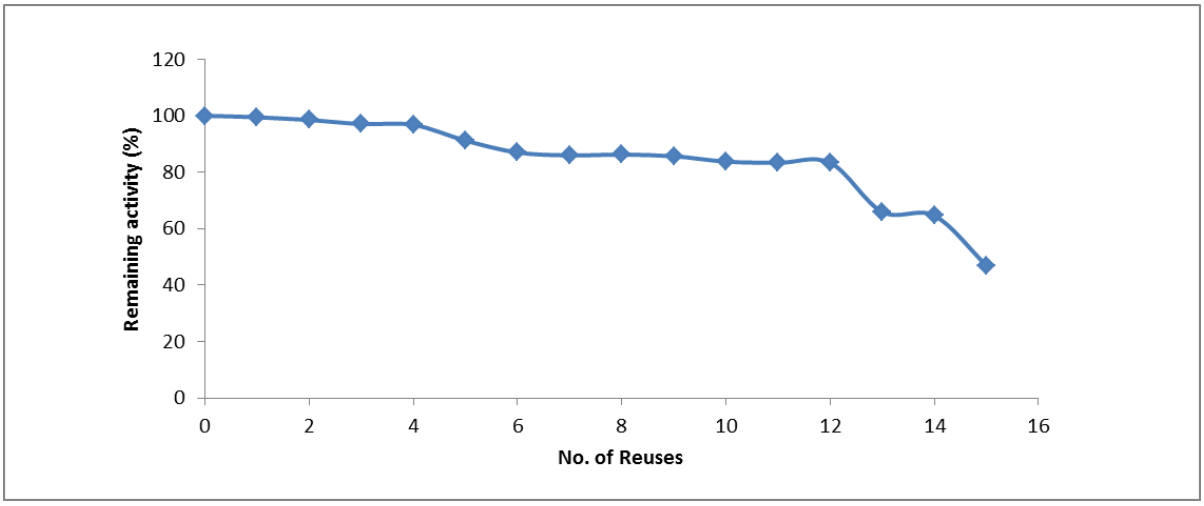

Fig. (5): Operational stability of the immobilized invertase during 15 batches (each batch of incubation time 40 min, pH 5.0 and at 50°C).

- **Relation activity was expressed as a percentage of the initial activity under the standard assay conditions.**

For maximal production of invert sugar from sucrose (Ss), optimal conditions for hydrolysis by the prepared immobilized invertase beads were applied. The production of invert sugar was maximized by

recycling the incubation of immobilized invertase bead for 15 repetitions. The immobilized invertase (0.02g bead) was successfully employed to produce 18.01mg invert sugar from sucrose in short time. The concentration of invert sugar, relative production and syrup saturation in the prepared hydrolysates produced were calculated. Invert sugar concentration was 18.01mg per total reaction mixture. Relative invert sugar production was 52.16 %.with high saturation.

The content of the prepared invert syrup was identified by TLC and comparing the coloured spots with the corresponding one of standards (Table1).Two visual spots of invert sugars were matched with both standard fructose and standard glucose. Thin layer chromatography analysis gave accurate confirmation for the presence of fructose and glucose [17].

Table (1): R_f value for TLC of invert sugar comparing with matching standards.

Sugars	Spots colour	R_f (scale R_f =1)	Standard matching
S_1	Main spots	0.17	Fructose
	Main spots	0.12	Glucose

The properties of the prepared immobilized invertase were compare with that of free one (Table 2). The immobilization impressively improved the thermal, storage stability and operational stability of invertase enzyme. It also exhibited lower K_m and higher V_{max} compare to the free one that estimated previously.

Table (2): Compare some properties of the free and immobilized invertase

Name	Results	
	*Free invertase	Immobilized invertase
Maximum activity at sucrose per reaction mixture	10 mg	2.5 mg
Maximum activity at inulin per reaction mixture	15 mg	5.0 mg
K_m toward sucrose **Vmax**	20.83 mg 3.7 U/mg	0.0625 mg 256.4 /mg
K_m toward inulin **Vmax**	----------	0.0588 mg 111.1U/mg
Thermostability	Maximum activity up to 60°C for 30 min, and 65°C for 15 min	Maximum activity up to 70°C for 120 min
Repeatability	----------	Reuses for 12 times with residual activity 83.33%
Storage stability	Retain 88.6 % of its initial activity.	Retain 100 % of its initial activity.
Invert syrup production from sucrose by 15 reuses of immobilized bead: **Yield** **Relative production** **Syrup saturation**	 ------- -------- --------	 18.01 mg 52.16 % 100 %

*Free invertase results were reported previously by El-Sayed *et al*. [6].

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CONCLUSION

Immobilization of invertase in alginate gel (low economic cost natural carrier) enhanced the activity and stability of the enzyme. Immobilized invertase hydrolyzed both α and β 1,2 glycosidic links. It showed high operational stability for 12 run, excellent thermostability and storage stability even after 270 days at 11°C. We also could used the prepared immobilized enzyme in preparation of invert syrup from sucrose by recycle methods. It could be easily separated and recovered from the reaction mixture. The amount of invert sugars produced from sucrose was 18.01 mg by using 0.02 g immobilized bead. The main biochemical properties of the prepared immobilized invertase were convenient for industrial applications that would not be feasible with a free one. It could be used for production of invert syrup.

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