HYDROLYSIS OF SUCROSE BY IMMOBILIZED *Saccharomyces cerevisiae* INVERTASE

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ABSTRACT

Invertase from *Saccharomyces cerevisiae* was immobilized by entrapment in carrageenan gel beads and used for sucrose hydrolysis. The immobilization efficiency was 88% when the concentration of the added enzyme was 100 unit/ml of gel and dropped to 49% upon increasing the added enzyme concentration to 500 U/ml. The immobilized enzyme remained fully active after 12 weeks of storage at 4°C. The hydrolytic activity of the enzyme was found to be influenced by temperature and pH. The preferred hydrolysis temperature was 55 °C for both the free and immobilized forms. The optimum pH was 4.5 for the free enzyme and was 4.5 – 5.5 for the immobilized enzyme. K_m values for the free and immobilized invertase were 63.4 and 87.8 mM, respectively. V_{max} values for free and immobilized invertase were calculated as 31.4 and 24 $mM.min^{-1}$, respectively indicating lower affinity by the immobilized enzyme for its substrate and lower reaction velocity. In a continuous process using packed bed column, hydrolysis percentages of 84, 29 and 18% were obtained by using flow rates of 0.25, 1 and 2 bv/hr, respectively. On the contrary, the specific productivity was enhanced by increasing the flow rate in the column.

INTRODUCTION

The enzyme invertase (β-D-fructofuranosidase, E.C. 3.2.1.26) catalyzes sucrose hydrolysis producing an equimolar mixture of glucose and fructose named inverted sugar which represents a commercially attractive product and has been extensively used in food industries due to its sweetness and liquid form. The yeast *Saccharomyces cerevisiae* is used in the production of this enzyme (Mazi *et al*., 2006; Mahmoud, 2007; Vu and Lee, 2008). Sucrose hydrolysis can be accomplished either by hydrochloric acid or by invertase with a notorious advantage for the enzymatic over the acid process in terms of energy economy, environmental safety and low formation of by-products. However, to match the low cost of the acid process, the invertase must be used in an immobilized form (Tomotani and Vitolo, 2006). The bioprocess of invert sugar production, as any other biotechnological process, is very complex due to the number of variables involved. Hence a balanced environment with optimum temperature, pH, agitation and flow rate is necessary for a good productivity (Blanch and Clark, 1997).

The enzyme immobilization technology offers technical and economical advantages such as repetitive use, easy removal of the enzyme from the reaction medium, longer half-life of the enzyme and the ability to the adaptation in the preferred continuous processes such as packed or fluidized bed reactors, in which it is possible to use higher enzyme dosage per volume of reactor than in the soluble enzyme process (Segura-Ceniceros *et al*., 2006). The main advantage of a continuous process over a batch process is the stabilization of operational

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conditions, simpler quality control and ease of automation which leads to reduce the overall production costs (Arica and Bayramoglu, 2006; Emregul *et al*., 2006).

In the present study, the immobilization of *Saccharomyces cerevisiae* invertase was achieved by entrapment within carrageenan gel beads. The catalytic performance of the immobilized enzyme at various temperatures, pH values, flow rates and sucrose concentrations were studied using batch and continuous processes.

MATERIALS AND METHODS

Materials: *Saccharomyces cerevisiae* invertase enzyme (46 units/mg) and carrageenan were purchased from Sigma Chemical Co. Sucrose was from Merck Chemical Co. All other chemicals were of analytical grade.

Immobilization procedure: Immobilization was carried out using the method of Iborra *et al*. (1997). Five ml of the enzyme solution (2500 units in citrate-phosphate buffer, 0.1M, pH 5) were mixed with 20 ml of carrageenan solution (3%) at 4 °C. This solution was then dropped into a stirred cold solution of potassium chloride (0.3 M) using a syringe with needle. The resulting beads were kept in solution for 10 minutes after which they were filtered off, washed with sterile water and stored at 4 °C.

Effect of invertase concentration on specific activity and immobilization efficiency: The immobilization procedure was carried out by using variable concentrations of the enzyme ranging from 100-500 U/ml of beads, then the activity of the immobilized enzyme was measured.

Effect of temperature and pH on sucrose hydrolysis: The effect of temperature on sucrose hydrolysis by the free and immobilized invertases was investigated in the range of 30-70 °C at pH 5. The dependence of sucrose hydrolysis degree upon pH was examined in the pH range of 3-7 using citrate-phosphate buffer at 45 °C.

Hydrolysis of sucrose using batch process: Sucrose solution (5%) was prepared in citrate-phosphate buffer (0.05M, pH 5). Hydrolysis was carried out at 45 °C in 100-ml conical flasks containing 20 ml of the solution and provided with a magnetic stirrer. Hydrolysis was initiated by the addition of 230 units of the enzyme as a solution or as carrageenan beads containing the same amount of the enzyme. After 15 minutes, samples were withdrawn and the total reducing sugars (TRS) produced were measured by spectrophotometric method using 3,5-dinitrosalicylic acid reagent (Miller, 1959).

Hydrolysis of sucrose using continuous process: The experiment was carried out in a packed bed reactor. A jacketed glass column (1.6x11 cm) was used. The carrageenan beads, containing the immobilized invertase were suspended in citrate-phosphate buffer solution (0.05 M, pH 5) and poured into the column. The column temperature was controlled at 45 °C by circulating water in the jacket. Sucrose solution, of 50% concentration was fed continuously into the column at flow rates of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 bed volume/hour (bv/hr). After steady state had been attained, aliquot samples were collected from the outlet solution and measured for the concentration of total reducing sugars (TRS).

Hydrolysis measurement: The hydrolysis percentage was calculated using the following formula: Hydrolysis (%) = $TRS/S_o \times 100$

TRS is the total reducing sugar (glucose + fructose) in mmole, S_o is the initial sucrose concentration in mmole/liter.

Determination of Kinetic Parameters: K_m and V_{max} values of the free and immobilized invertase were determined by Lineweaver-Burk plot using various substrate concentrations (0.029-0.29 M) in citrate-phosphate buffer (0.05 M, pH 5) at 45 °C.

Storage stability: The free and immobilized enzymes were kept in citrate-phosphate buffer (0.05 M, pH 5) at 4 °C and their activities were measured at one week intervals up to 12 weeks.

RESULTS AND DISCUSSION

Immobilization yield: The ratio of the activity of the immobilized invertase to the activity of the free invertase was found to be 88%. The immobilized enzyme was active for 42 days without remarkable drop in activity. Approximately full activity was retained after 16 batch reactions.

Effect of invertase concentration on the specific activity and immobilization efficiency: Specific activity, which is defined as the total enzyme units retained per ml of gel beads, was increased upon increasing the amount of the added enzyme in the gel matrix (Fig. 1). The relationship did not follow the first order kinetics. Total retained activities of 88, 207 and 245 U/ ml were retained when the added enzyme concentrations were 100, 300 and 500 U/ ml, respectively.

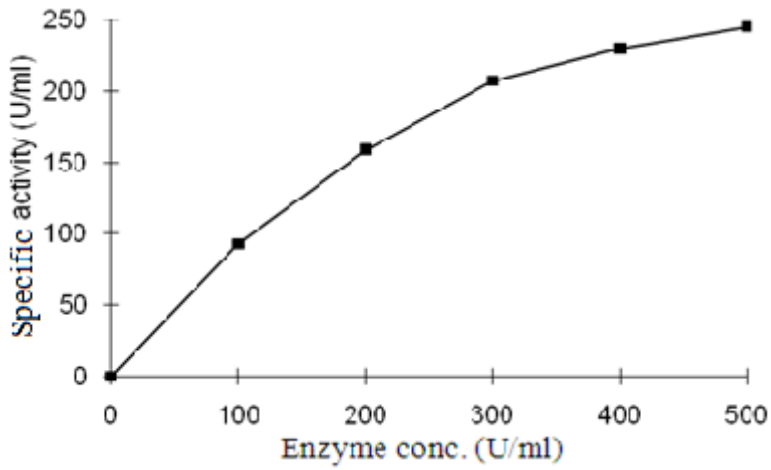

Fig. (1) : Effect of enzyme concentration on specific activity of immobilized invertase.

Another method could be used to express the relationship between the amount of the added enzyme and the retained activity by plotting the added enzyme concentration and the immobilization efficiency, which refers to the ratio of the immobilized enzyme activity to the free enzyme activity. This relationship was found to be reverse proportional (Fig. 2).

Immobilization efficiencies of 88, 69, and 49% were obtained with added enzyme concentrations of 100, 300, and 500 U/100ml, respectively. This decrease might be caused by the diffusional restriction in bead membrane so that the activity of the immobilized enzyme was not fully utilized (Chang *et al*., 1998) and

to the inhibitory effect of the hydrolysis products, glucose and fructose, when their concentrations increase inside the gel beads.

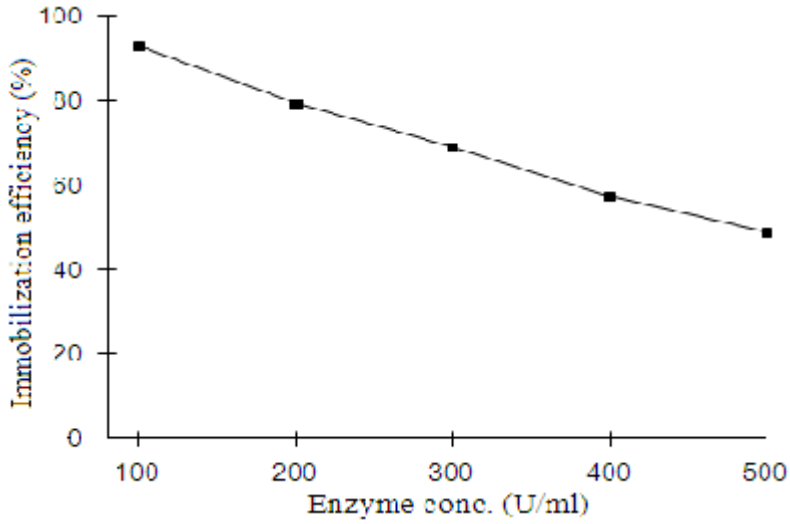

Fig. (2): Effect of enzyme concentration on Immobilization efficiency.

Storage stability: Storage stability is a prominent factor for commercialization of an enzyme. Immobilized invertase showed excellent storage stability compared to the free enzyme (Fig. 3). After 12 weeks of storage at 4 °C, 93% of the initial

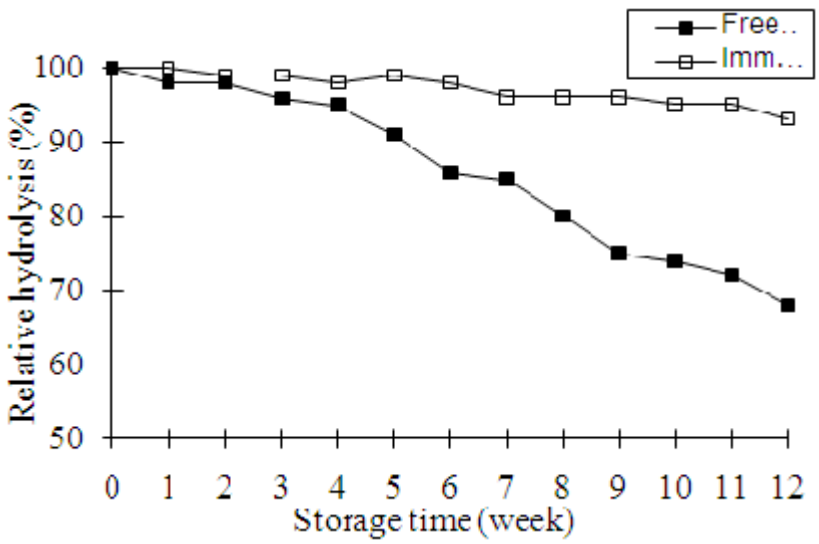

Fig. (3): Storage stability of free and immobilized invertase.

activity was retained in the immobilized enzyme. The free enzyme showed less storage stability and retained 68% of the initial activity during the same storage time period indicating a considerable enhancement of immobilization on stability. It has been argued that this higher stability of the immobilized enzyme was due to the physical protection of carrageenan gel. Approximately similar result has been

reported by Gomez *et al*. (2006) for invertase immobilized on carboxymethyl-cellulose modified chitin.

Effect of temperature on sucrose hydrolysis: Maximum hydrolysis, for both free and immobilized forms of invertase, was achieved at 55 °C with a good activity in the range of 40-60 °C. At lower temperatures, the reaction proceeded slowly and at higher temperatures, thermal deactivation of the enzyme became faster (Fig. 4). The decrease in activity may be attributed to a dramatic change in the structure of the enzyme that hindered the availability of the active sites, with a possible denaturation of the enzyme itself (Kouassi *et al*., 2005). Optimum temperature did not affected by immobilization but the immobilized enzyme had a relatively wider high activity range which suggests a higher thermal stability of the immobilized invertase. The enhancement of enzyme stabilization upon immobilization may be caused by the existence of a local environment for the immobilized enzymes which is less damaging than bulk solution conditions (Bailey and Ollis, 1986). Most microbial invertases have optimum temperatures in the range of 45-60 °C (Bergamasco *et al*., 2000; David, 2004; Ribeiro and Vitolo, 2005). Optimum temperature is a function of the enzyme source and experimental conditions such as the substrate concentration which has a remarkable protection effect against thermal denaturation. Wiseman and Woodward (1975) had recorded optimum temperatures of *Saccharomyces cerevisiae* invertase at 55 and 65 °C for sucrose concentrations of 2 and 60%, respectively.

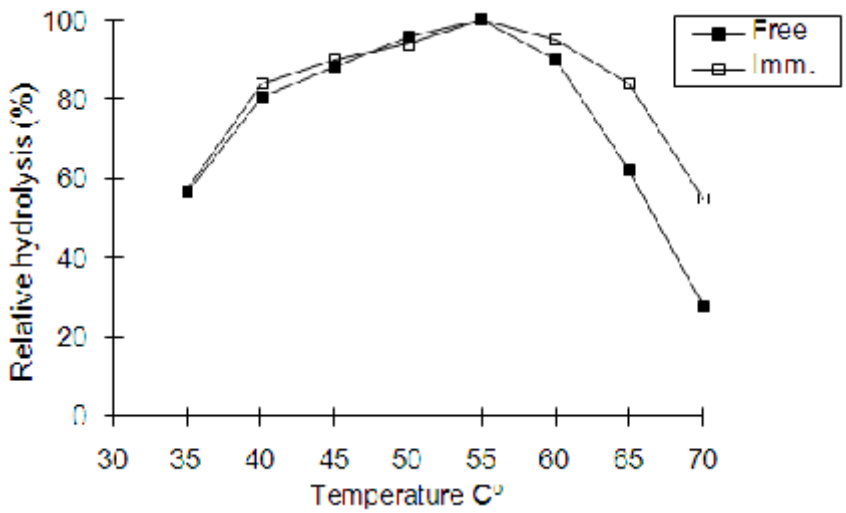

Fig. (4): Effect of temperature on sucrose hydrolysis by free and immobilized invertase from *Saccharomyces cerevisiae*.

Effect of pH on sucrose hydrolysis: Sucrose inversion was found to be influenced by the pH. Optimum pH of the free enzyme activity was 4.5. The immobilized enzyme showed better tolerance to the variation of solution pH. Its optimum pH was in the range of 4.5-5.5 with a wider range of activity. Out of the range of 4-6, the hydrolysis rate was clearly decreased (Fig. 5).

To explain the effect of pH on enzymatic activity. Kang *et al*. (1994) stated that enzymes possess multimeric structures at different pH values and the ability of

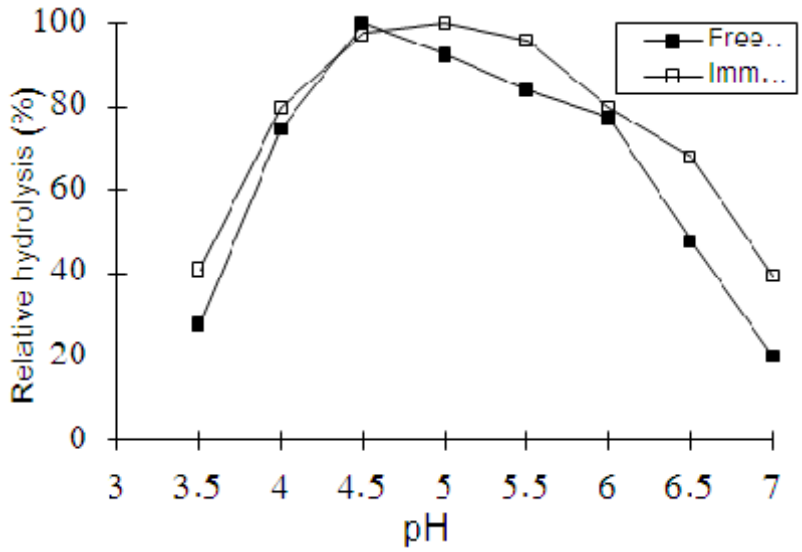

Fig. (5): Effect of pH on sucrose hydrolysis by free and immobilized invertase from *Saccharomyces cerevisiae*.

amino acids at the active sites to interact with the substrates depends on their electrostatic state. The decrease in activity at certain pH range shows that the enzyme faces some limitations. If the pH is not appropriate, the charge on one or all of the required amino acids is such that the substrate can neither bind nor react properly. The difference in the effect of pH on the activity between the free and immobilized forms may be resulted from the difference in the pH of the solution from the pH at the immobilized enzyme microenvironment owing to partition effects and diffusional limitations that may alter the hydrogen ion concentration in the immobilized enzyme vicinity. The same observation was reported by other investigators (Tanriseven and Dogan, 2001; Mahmoud, 2007). The main consequence of this difference is a shift in the immobilized enzyme pH activity profile with respect to the free enzyme (Bergamasco *et al*., 2000).

Kinetic parameters: The effect of substrate concentration on the free and immobilized invertase activity was studied using various concentrations of sucrose ranging from 30 to 200 mM and plotted according to Lineweaver-Burk. As shown in the Figure 6, the K_m values for the free and immobilized invertase were 63.4 and 87.8 mM, respectively. V_{max} values for free and immobilized invertase were calculated as 31.4 and 24 $mM.min^{-1}$, respectively. The K_m value of the immobilized enzyme was higher than that of the free invertase while V_{max} was lower. It has been found that when an enzyme is immobilized by entrapment in a gel such as carrageenan or alginate, the K_m of the immobilized enzyme increases and the V_{max} decreases. This indicates the decreased affinity by the immobilized enzyme for its substrate and lower reaction velocity which may be due to the inhibitory effect of

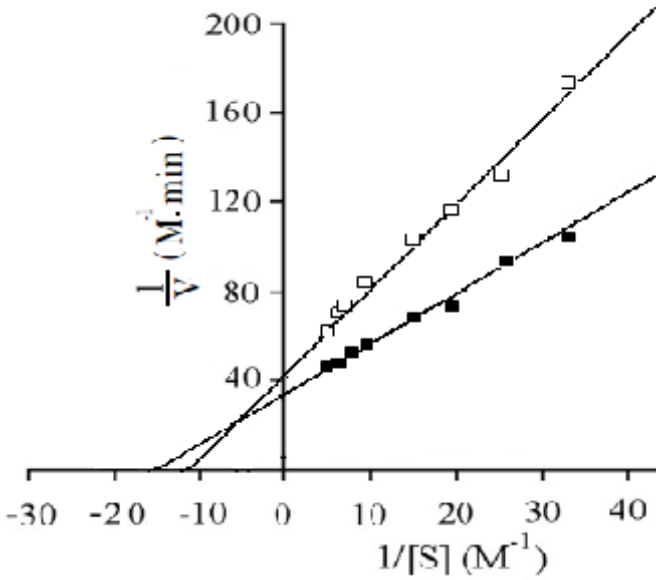

Fig. (6): Lineweaver-Burk plot of free (■) and immobilized (⊏) invertase.

the hydrolysis products (glucose and fructose), the difficulty for the substrate to access the active site of the enzyme and lower transporting of the substrate and products into and out the gel beads (Akgol *et al*., 2001; Bayramolu *et al*., 2003; Vu and Lee, 2008). However, a different result was obtained by Mahmoud (2007) who used wood saw dust waste as support for immobilization.

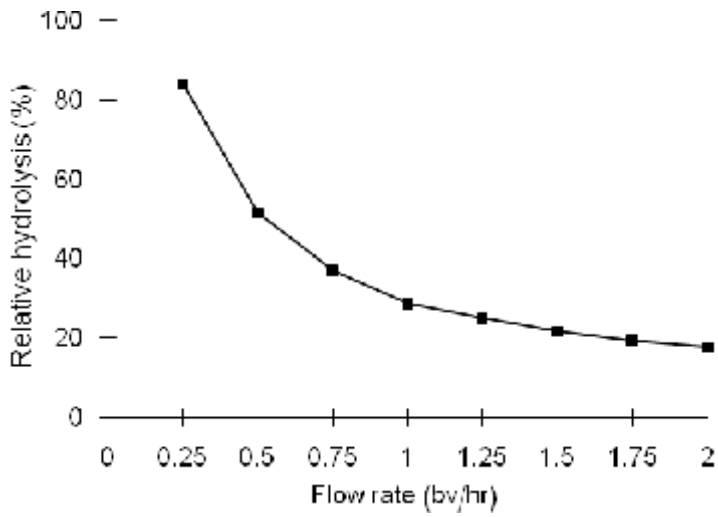

Fig. (7): Effect of flow rate on hydrolysis degree (bv = 10ml, sucrose conc. = 50%)

Effect of flow rate on hydrolysis degree: A temperature-controlled glass chromatographic column was used for this purpose. As shown in the Figure 7**,** the conversion degree of sucrose was decreased upon increasing its flow rate throughout the column. Hydrolysis percentages of 84, 29 and 18% were obtained by using flow rates of 0.25, 1 and 2 bv/hr, respectively. The hydrolysis degree diminished as the feeding rate increased because low residence time reduces the

contact time between the enzyme and the substrate. It was noticed that the hydrolysis of relatively high concentrated sucrose solutions needs a flow rate and other reactor conditions that give a high hydrolysis rate, otherwise the residual sucrose may crystallizes and degrades syrup quality (Serna-Saldivar and Rito-Palomares, 2005). In spite of achieving a high hydrolysis degree (84%) when using a slow flow rate of the substrate throughout the column (0.25 bv/hr), the obtained specific productivity was only 105 gm of hydrolyzed sucrose per one liter of immobilized enzyme per hour which raised to 177 gm/l/hr when the hydrolysis degree was 18% which obtained by using a relatively fast flow rate (2bv/hr) (Fig.8).

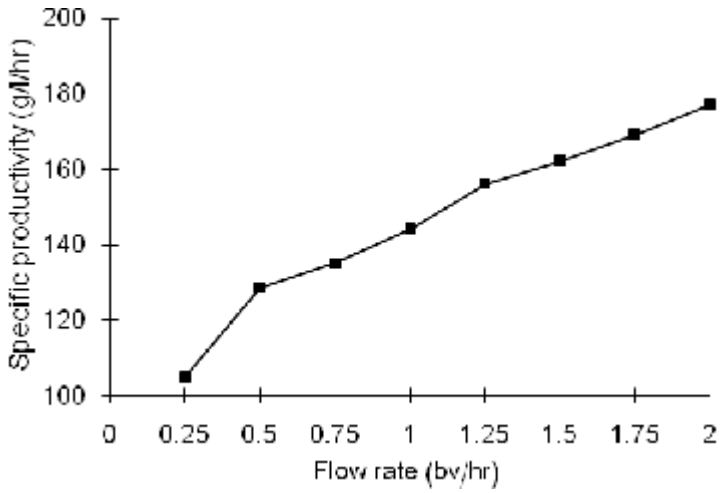

Fig. (8): Effect of flow rate on specific productivity of immobilized invertase.

This may be due to the inhibitory effect of the hydrolysis products (glucose and fructose) on the immobilized invertase upon using the slow flow rate.

تحلل السكروز باستخدام إنفرتيز خميرة *Saccharomyces cerevisiae* المقيد

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الخلاصة

تم تقييد إنزيم الإنفرتيز المنتج من خميرة *Saccharomyce cerevisiae* بطريقة الحجز في حبيبات هلام الكاراجينان واستخدم الإنزيم المقيد في عملية تحلل السكروز. بلغت كفاءة عملية التقييد ٨٨٪ عند استخدام تركيز ١٠٠ وحدة إنزيمية\ مل من الهلام وانخفضت إلى ٤٩٪ عند زيادة تركيز الإنزيم إلى ٥٠٠ وحدة\مل. كان الإنزيم المقيد أكثر ثباتا" من الإنزيم الحر خلال الخزن بدرجة حرارة ٤مْ لمدة ١٢ أسبوعا".

تأثرت كفاءة الإنزيم في تحلل السكروز بدرجة الحرارة وقيمة الأس الهيدروجيني. بلغت درجة الحرارة المثلى للتحلل ٥٥مْ وفي شكلي الإنزيم الحر والمقيد. أما الأس الهيدروجيني الأمثل لعمل الإنزيم الحر فكان ٤,٥ وللإنزيم المقيد ٤,٥ - ٥,٥. بلغت قيم ثابت ميكيلس للإنزيم الحر والمقيد ٦٣,٤ و ٨٧,٨ ملليمولر وعلى التوالي. فيما بلغت قيم السرعة القصوى للإنزيم الحر والمقيد ٣١,٤ و٢٤ ملليمول\ دقيقة, وعلى التوالي مما يشير إلى انخفاض كل من ألفة الإنزيم تجاه مادة الركيزة وسرعة التفاعل الإنزيمي نتيجة التقييد. استخدمت الطريقة المستمرة في التحلل لدراسة تأثير سرعة سريان محلول السكروز في كفاءة التحلل وقد أمكن الحصول على درجات تحلل مقدارها ٨٤ و ٢٩ و ١٨٪ باستخدام سرعات سريان مقدارها ٠,٢٥

و ١ و ٢ حجم العمود\ ساعة, وعلى التوالي. كما ارتفعت الإنتاجية النوعية للإنزيم المقيد بزيادة سرعة سريان المادة الركيزة في العمود.

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