# Immobilization of *Leuconostoc-paramesenteroides* Dextransucrase Enzyme and Characterization of its Enzyme Properties

Amal M. Hashem*[,1], Mona A. El-Refaei[1], Hasan M. Gebril[2] and Ahmed F. Abdel-Fattah[1]

[1]*Department of Natural and Microbial Products Chemistry, National Research Centre, Dokki, Cairo, Egypt*

[2]*Faculty of science, Ain Shams University, Cairo, Egypt*

**Abstract:** Dextransucrase from *Leuconostoc-paramesenteroides* was immobilized using different immobilization techniques. Entrapment in calcium alginate (2%) proved to be the most suitable technique (27.6% yields). The operational stability of the immobilized was retained 100% until 11 cycle, with decreasing of 70%, of the retained activity at 13 cycles. The specific activity of the free was compared to that of the immobilized enzyme.

The optimum temperature of the free enzyme was 65°C were as it was 70°C with the immobilized enzyme. The specific activity of the immobilized was higher than that of the free enzyme at pH 4. 100% of the specific activity was retained due to the thermal stability of the immobilized enzyme after heat treatment for 60 minutes at 60°C. The activation energy (EA) of the immobilized enzyme was lower than that of the free enzyme ($E_A$= 10.3 and 12.13 Kcal/mol respectively).

The calculated half-lives of the free enzyme at 40, 50, 60 and 70 were 15.0, 4.68, 4.68 and 4.0 min respectively which were lower than those of immobilized enzyme i.e. 401, 385, 295 and 42 min, respectively.

**Keywords:** Dextransucrase, enzyme immobilization, calcium alginate*, Leuconostoc.*

## INTRODUCTION

Many techniques for immobilization of enzymes on different types of the support have been developed [1]. The physical structure and chemical composition of support can influence the micro-environment of the immobilized enzymes, and consequently their biological properties [2, 3].

Immobilization of dextransucrase *via* entrapment in alginate provided high and good operational stability. This result suggests that dextransucrase immobilization in alginate is due to unique supramolecular structure [4].

Entrapment in calcium alginate beads gave the best results in terms of immobilization yield and stability [5].

Immobilization of dextransucrase in alginate fibers resulted in 90% relative activity [6]. The immobilized enzyme retained activity for ten batch reactions without a decrease in activity. Immobilization of dextransucrase in alginate fibers is superior to that in alginate beads in terms of immobilization yield and repetitive use [6].

Immobilization of dextransucrase from *Leuconostoc mesenteroides* PCSIR-4 on alginate is optimized for application in the biosynthesis of dextran from sucrose. Properties of dextransucrase were less affected by immobilization on alginate beads from soluble enzyme. Highest activities of both soluble and immobilized dextransucrase were found to be at 35°C and pH 5.0. Substrate maximum for immobilized enzyme changed from 125 mg/ml to 200 mg/ml. Incubation time for enzyme-substrate reaction for maximum enzyme activity was increased from 15 minutes to 60 minutes in case of immobilized enzyme. Maximum stability of immobilized dextransucrase was achieved at 25°C [7].

In this work, different immobilized dextransucrase enzyme preparations were made and investigated the relative performances such as the reusability. The properties of the immobilized and free enzymes also were compared

## MATERIALS AND METHODS

### Microorganism

*Leuconostoc paramesenteroides* isolated from sugar cane was identified by Microanalytical Centre at the Faculty of Sciences, Cairo University.

### Culture Media

Maintenance and preservation of *Leuconostoc* on different media were conducted by three serial transfers on medium A, and then transferred to medium B [8].

Medium A: Deep liver broth contains per 100 ml of distilled water: 10 ml of liver extract, 0.5 g Difco yeast extract, 1 g Difco tryptone, 0.2 g dipotasium hydrogen

*Address corresponding to this author at the Department of Natural and Microbial Products Chemistry, National Research Centre, Dokki, Cairo, Egypt; Tel: +0020223690986; E-mail: Amal_mhashem@yahoo.com

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phosphate and 0.5 g glucose. The pH was adjusted to 7.4.

A few liver particles were placed in each 16 × 150 mm rimless test tube and sterilized at 121°C for 15min.

Medium B: It had the same composition as the maintenance medium A except that glucose is replaced by sucrose (10%). Agar (2%) was added while liver particles were omitted.

**Dextransucrase Production**

The culture medium for enzyme production was defined as follows (g/L):

Sucrose, 100; yeast extract 2.5; magnesium sulfate heptahydrate, 0.2; dipotassium hydrogen phosphate 5.0.

The phosphate was sterilized separately and was added aseptically to the cold, sterile solution of the other ingredients. The initial pH of the sterile mixture was about 7.0. The culture was incubated at 25°C in static incubator for 24 hrs.

**Dextransucrase Activity**

Dextransucrase activity was determined by measuring the initial rate of fructose production using the dinitrosalicylic acid method (DNS method). Reaction were carried out at 30°C in 20 mM acetate buffer (pH 5.4), sucrose 100g/L. Exactly 100 µl of an enzyme solution was added to 500 µl of a solution of sucrose (400g/L), 200 µl sodium acetate buffer (pH 5.4) and 200 µl $CaCl_2$ (0.5 g/L).

The reaction mixture without dextransucrase was incubated for 5 min in a thermostated stirred tank reactor at 30°C. The reaction was started by the addition of dextransucrase, for 10 min, 100 µl samples were removed from the reaction mixture and added to 100 µl of reagent (DNS). The reaction was immediately stopped because of the high pH of the reagent. The tubes of samples and the tubes of standards were placed in a double boiler for approximately 5min so that the colorimetric reaction took place. After 15 min incubation on ice, samples were mixed with 1.5 ml of water and absorbance was measured at 540 nm. Experiments were carried out in triplicate. Standard was prepared as fructose. A control sample was prepared and treated similarly using boiled enzyme samples [9].

The international unit/ml=1 mol of fructose produced per minute per ml.

The protein determination was carried out using the method of Lowry *et al*., [10].

**Preparation of the Crude Enzyme**

Enzyme immobilization was performed with an enzyme preparation obtained by subjecting the culture filtrate to ultrafiltration after pretreatment with dextranase to remove the contaminated dextran. The ultrafiltrate was then lyophilized and used in the present part of work.

**Immobilization Method**

***Physical Adsorption***

This was carried out by using 1 g of different carriers (chitosan, alumina and asbestos) and 2 ml of the enzyme solution containing 76 U/g carriers [11].

***Ionic Binding***

This was carried out as follow: One gram of each of the cation or anion exchanger (Dowex 1-XB particle size 0.075-0.15 mesh, DEAE-cellulose) was equilibrated with acetate buffer (0.02 M pH 5.4) incubated with 2 ml of enzyme solution (76 U/g carrier) and washed with acetate buffer (pH 5.4) three times [11].

***Covalent Binding***

This was carried out as follow: One gram chitosan was submerged in 5 ml 0.1 M NaOH containing 2, 3 or 5% (v/v) glutraldehyde (GA) for 2 h at 30°C. The solubilized chitosan was precipitated by the addition of 1 ml of 0.1 M NaOH. The precipitate was collected by filtration and washed with distilled water to remove the excess GA. The wet chitosan was mixed with 2 ml of enzyme solution (76 U of dextransucrase). After being submerged for 1 h at 30°C, the unbound enzyme was removed by washing with distilled water.

***Entrapment***

The enzyme solution was entrapped with 1%, 2%, 3% and 4% calcium alginate gel. Different concentrations of agar were prepared to give a final concentration of 1%, 2% and 3% and then 2 ml of the crude enzyme (76 U) was added to each of these concentrations. After solidification, the mixture was cut into 1 – $mm^3$ fragments and washed with acetate buffer (0.02 M, pH 5.4) to remove the unbound enzyme [12].

**Some Properties of Free and Alginate Immobilized Dextransucrase Enzyme**

***Effect of Substrate Concentration (Sucrose)***

The effect of substrate concentrations on the free and immobilized enzyme was studied using different concentrations of sucrose as substrate ranging from 0.01 g – 0.3 g/ reaction mixture.

**Effect of Temperature of the Reaction**

In this experiment the effect of temperature of the reaction on dextransucrase activity was studied using different temperatures ranging from 20 to 80°C.

***Effect of pH Value of the Reaction***

The effect of different pH values ranging from 4 to 7 on the activity of free and immobilized dextransucrase was investigated using acetate buffer (0.02 M), the reaction was conducted for 10 min at 30°C.

***Thermal Stability at pH 5.4***

In these experiments, the immobilized and free dextransucrase were preheated at different temperatures (40 – 70°C) with different incubation periods (5 – 60 minutes) for each temperature followed by measuring the residual activity. In each case, controls were also carried out without heat treatment.

***Activation Energy (Ea)***

It was determined by plotting the log of the relative activity of the assayed temperature against 1/T (Kelvin) of the free and immobilized samples.

Ea = slop × 2.303 R (gas constant = 1.976).

Half-life and deactivation constant rate:

It was determined by plotting the log of the relative activity against time according to the following equation:

Half-life = 0.693/ slop.

Deactivation energy = slope of the straight line.

**Operational Stability of Dextransucrase Immobilized on Calcium Alginate**

In this experiment, 16.7 U immobilized enzyme was incubated with 8 ml sucrose (40% w/v), 3.2 ml $CaCl_2$ (0.05%) and 3.2 ml sodium acetate buffer (0.02 M) for 10 min, the immobilized enzyme was then collected by decantation, washed with the buffer and resuspended in a freshly prepared substrate to start new run. The operational stability of the immobilized enzyme was evaluated in repeated batch process.

***Immobilization Parameters***

The immobilization yield (IY) was calculated according to Eq. (1):

$$IY\% = [I / A – B] \times 100 \quad (1)$$

Were A is the activity of the enzyme added to the immobilization solution, B is the activity of the unbound enzyme, and I is the activity of the immobilized enzyme.

Relative activity % = activity of sample /activity of control x 100

Specific enzyme activity SEA = activity of sample / protein content.

**RESULTS AND DISCUSSION**

The lyophilized enzyme was immobilized with different carriers using different methods of immobilization. The efficiency of the enzyme immobilization was evaluated by different parameters including the retained enzyme activity, specific activity of the immobilized enzyme, and loading efficiency (immobilized enzyme / g carrier). The immobilization yield is also a key parameter since it represents the general output and efficiency of the immobilization process. As such calcium alginate proved to be the most suitable carrier (Table **1**), as it gave the highest immobilization yield (27.6%). Tanriseven and Dogan [6] previously reported that the only successful method for immobilization of dextransucrase was entrapment in alginate beads. Our results are also similar to those reported by many authors who obtained high immobilization yields [5, 13]. Also Qader *et al.,* [7] reported that immobilization of dextransucrase *via* entrapment in alginate provided high and good operation stability.

**Effect of Substrate Concentrations (Sucrose) on Dextransucrase Activity**

The investigation of the effect of the substrate concentration on the free and immobilized enzymes registered a low specific activity for the immobilized enzyme when compared with that of the free enzyme (Figure **1**). This drop in the specific activity (at 0.05 g substrate/reaction) after immobilization is a common phenomenon and could be attributed to diffusion

**Table 1. Immobilization *Leuconostoic paramesenteriodes* Dextransucrase**

| Methods of immobilization | Carrier | Unbound enzyme (U/g carrier) B | Immobilized enzyme (U/g carrier) J | Immobilized yield $\left(\frac{I}{A-B}\right)x100$ |
|---|---|---|---|---|
| Ionise binding | DEAE- Cellulose | 45.6 | 5 | 16.6 |
| | Dowex- IXD | 76 | / | / |
| Physical adsorption | Alumina | 48 | 4.4 | 15.7 |
| | Asbestos | / | / | / |
| | Chitosan | 35 | 4.3 | 10.5 |
| Entrapment | Agar 1% | 76 | / | / |
| | Agar 2% | 76 | / | / |
| | Agar 3% | 76 | / | / |
| | Calciunalginate 1% | 15.7 | 16.7 | 27.6 |
| | Calciunalginate 2% | 15.7 | 16.7 | 27.6 |
| | Calciunalginate 3% | 15.7 | 16.7 | 27.6 |
| | Calciunalginate 4% | 15.7 | 16.7 | 27.6 |

A= 76U/g carrier (added enzyme).

limitation of the substrate products to and from the immobilization matrix which causes problems not found with free enzymes [14]. This diffusion process often results in lower concentration of product at the enzyme active site than in the bulk solution [15]. In addition multiple fixation of the enzyme to matrix would also lead to decrease in the specific activity owing to the decrease in the flexibility of the enzyme molecule which is commonly reflected by a decrease in the catalytic activity [12]. However, it was found that no measurable changes occur in the specific enzyme activities of free and immobilized enzyme at substrate concentration of 0.2 g / reaction.

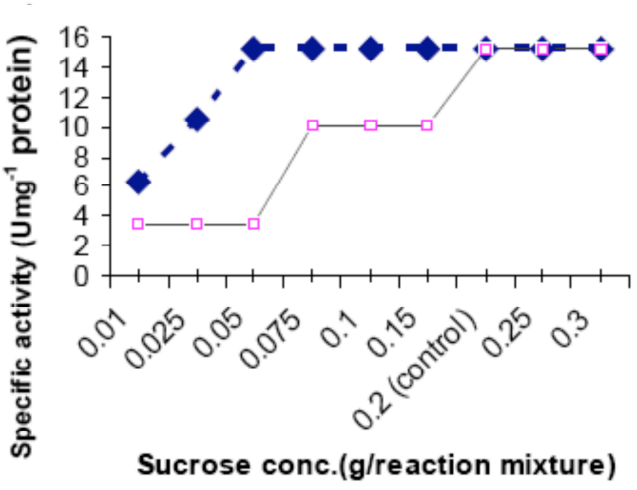



**Figure 1:** Effect of different substrate concentrations on free and immobilized *Leuconostoc paramesenteroides* dextransucrase activity.

Free (■) and immobilized (□) dextransucrase samples were added to a reaction mixture containing different concentrations of sucrose (0.01-0.3 gm / reaction mixture), the reaction mixture was incubated at 30°C for 10 min with shaking, then the specific dextransucrase activity was determined.

## Effect of Temperature on Activity of Free and Immobilized Enzyme

The maximal dextransucrase enzyme activities of both forms of enzyme were obtained at 25°C (Figure **2**). They gave the same specific activities up to 65°C. At 70°C and higher, the immobilized enzyme was more active than the free enzyme. This may presumably be due to protection of immobilization process against temperature. When the log of relative activity was plotted against temperature in the form of Arhenians plots, the plots for the immobilized and free enzyme were linear and the calculated values for the activation energies (Ea) were 10.3 and 12.13 kcal / mol respectively, the lower value of the activation energy of the immobilized enzyme compared to the free enzyme may be attributed to the mass transfer limitations, in addition Kitano *et al*., [16] and Allenza *et al.,* [2] reported that the activation energies of the immobilized enzymes were lower because the internal diffusion limitations is the rate limiting step.

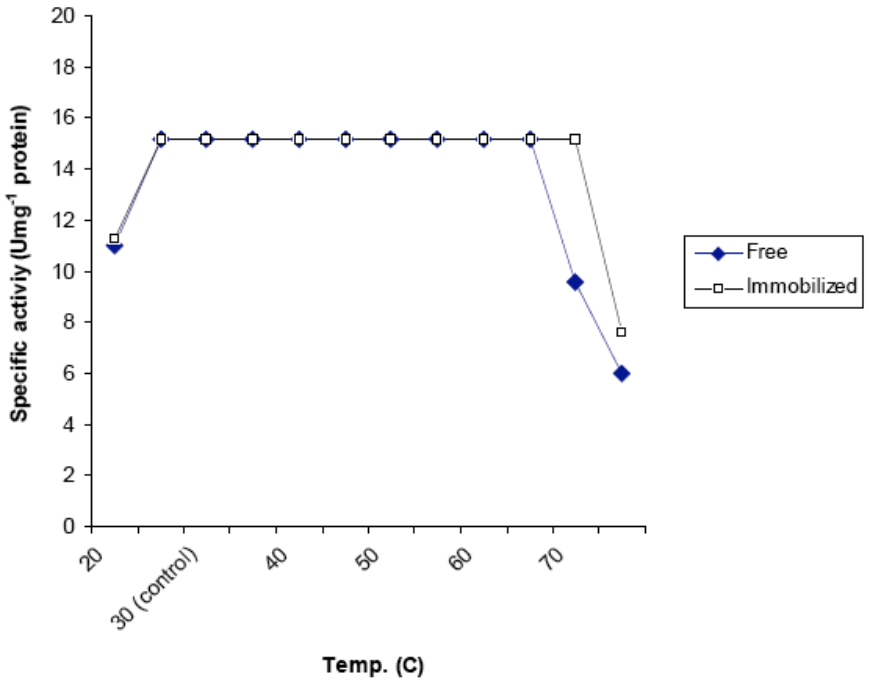



**Figure 2:** Effect of temperature on free and immobilized *Leuconostoc paramesenteroides* dextransucrase activity.
Free (□) and immobilized (■) dextransucrase samples were added to a reaction mixture containing 0.2 gm sucrose, at pH 5.4 (acetate buffer).
The reaction mixture was incubated at different temperatures for 10 min with shaking, then the specific activity of dextransucrase activity was determined.

## Effect of pH of the Reaction

The investigation of the free and immobilized enzymes at different pH showed that the specific activity of the immobilized enzyme in the acidic and neutral range was higher than that of the free one (Figure **3**). These results are similar to that reported by Kaboli and Reilly [17] and Qader *et al*., [7]. The specific

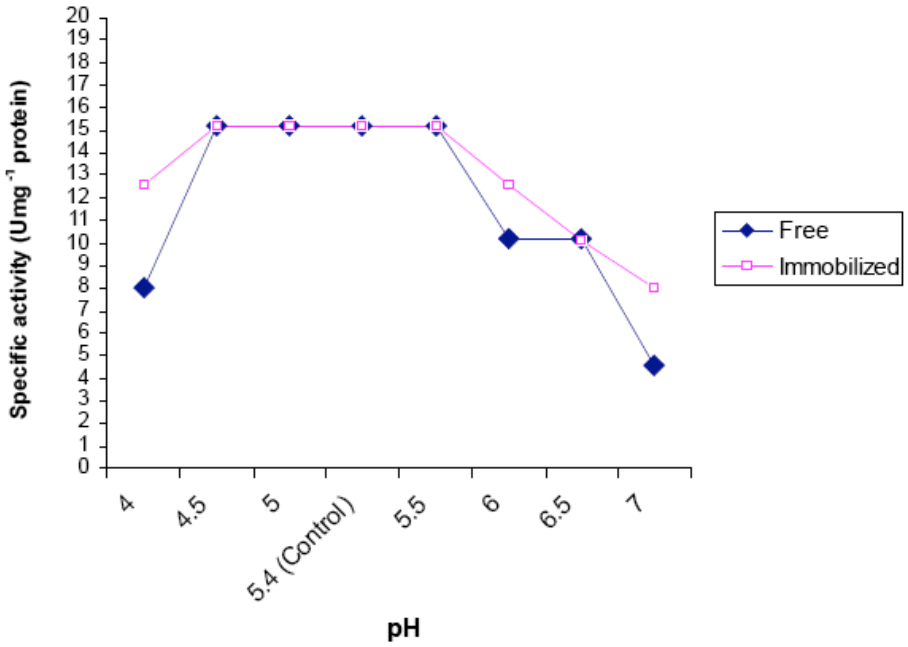
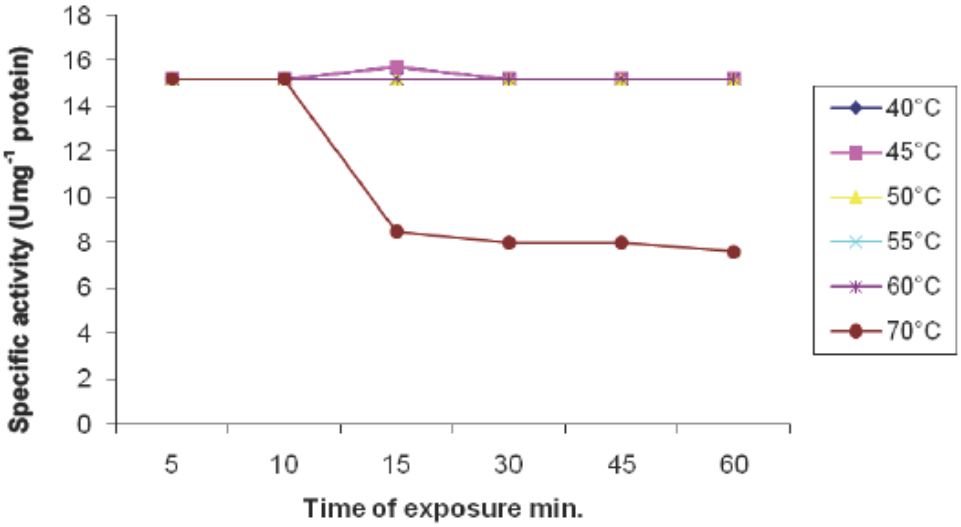



**Figure 3:** Effect of different pH values on free and immobilized *Leuconostoc paramesenteroides* dextransucrase activity.
Free (■) and immobilized (□) Dextransucrase samples were incubated at different pH values for 10 min, with shaking, and the specific activity was calculated.

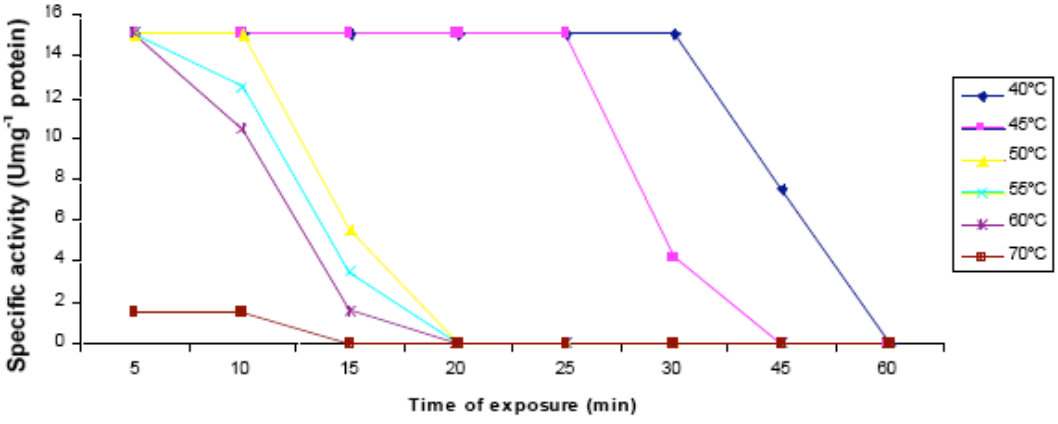
activity was higher in immobilized enzyme (12.6 U / mg) than that of free enzyme (8 U / mg). At pH 4.5 – 5.5 the specific activity was stable and the same in immobilized and free enzyme (15.2 U / mg protein) but at pH 6.5 – 7 the specific activity of the immobilized enzyme was higher (8 U / mg protein) than that of the free enzyme (4.6 U / mg). PH is known to promote changes in the partial configuration and activity of an enzyme [14] and also this was explained usually be conversion of the ionic microenvironment of the enzyme resulting in the binding of the enzyme and microenvironment or the chemical nature of the support [15].

## Thermal Stability

The immobilized enzyme retained 100% of its activity at 40, 50 and 60°C during all the pre-incubation periods tested (Figure **4a**). After 1h at 70°C, the immobilized enzyme retained 50% of its original activity, whereas the free one retained only 50% after 45 min at 40°C and lost 90% of its activity after 15 min at 60°C (Figure **4b**). Thermal stabilization has previously been reported for alginate-entrapped enzyme [7]. The better stability of an immobilized enzyme compared with the free form could be explained by the dextransucrase location inside the support (molecular confinement, which often occurs




**a**




**b**

**Figure 4: a).** Thermal stability of immobilized *L*. paramesenteroides dextransucrase.
Dextransucrase samples were pre-incubated at 40°C (♦), 45°C (■), 50°C (▲), 55 °C (x), 60°C (∗), 70°C (•) for 5, 10, 15, 20, 30, 45 and 60 minutes.
Note: pre-incubation of the immobilized enzyme at 40°C to 60°C gave 100% relative activity after incubation at 5, 10, 15, 30, 45 and 60 min, so the equal specific activities curves are overlapped.
**b).** Thermal stability of free *L. paramesenteroides* dextransucrase.
Dextransucrase samples were pre-incubated at 40°C (♦), 45°C (■), 50°C (▲), 55 °C (x), 60°C (∗), 70°C (•) for 5, 10, 15, 20, 30, 45 and 60 minutes.

**Table 2. Activation Energy, Half-Life, and Deactivation Rate Constant for Free and Immobilized *Leuconostoic parameseneteroides* Dextransucrase**

| Kinetic parameter | Free enzyme | Immobilized enzyme |
|---|---|---|
| Activation energy (Kcal/mol) | 12.13 | 10.3 |
| Half life (min) at | | |
| 40°C | 15.00 | 401.0 |
| 50°C | 4.68 | 385.0 |
| 60°C | 4.68 | 295.0 |
| 70°C | 4.00 | 42.0 |
| **Deactivation rate constant ($min^{-1}$) at** | | |
| 40°C | $62\times10^{-3}$ | $2\times10^{-3}$ |
| 50°C | $2\times10^{-1}$ | $2\times10^{-3}$ |
| 60°C | $2\times10^{-1}$ | $2.1\times10^{-3}$ |
| 70°C | $2.5\times10^{-1}$ | $2.2\times10^{-3}$ |

during the entrapment process), where the enzyme is protected against alterations of the microenvironment [18].

When the log of the relative activity was plotted against time, at temperatures causing inactivation (40, 50, 60 and 70°C), both the free and immobilized enzymes gave a straight line, meaning that the thermal inactivation process for both corresponded to the theoretical curves of a first-order reaction.

The calculated values for the half-life of the free and immobilized enzymes at different temperatures are represented in Table **2**. The results showed that the immobilized enzyme was more thermostable than the free one; for example the calculated half-lives of the free enzyme at 40, 50, 60 and 70°C were 15, 4.68, 4.68 and 4 min, respectively, which were all, lower than those of the immobilized enzyme. Our results were similar to those reported for thermal inactivation for immobilized amylases [19] and immobilized lipase [20].

## Operational Stability of Immobilized *Leuconostoc parameseneteroides* Dextransucrase

The main advantage of immobilizing an enzyme is that it allows repeated use which is important in the case of expensive enzymes. In this study, the stability of the immobilized system was assessed by reusing the immobilized Leuconostoc parameseneteroides dextransucrase for 11 cycles and the retained activity was 100% and after the 13$^{th}$ cycle the retained activity was 30% of the initial value of the immobilized enzyme (Figure **5**). In this respect, it was reported by Alcalde *et al*., [5] that dextransucrase immobilized in alginate beads retained its activity after seven cycles whereas the formation of the dextran resulted in a progressive swelling of the beads leading to the disruption of

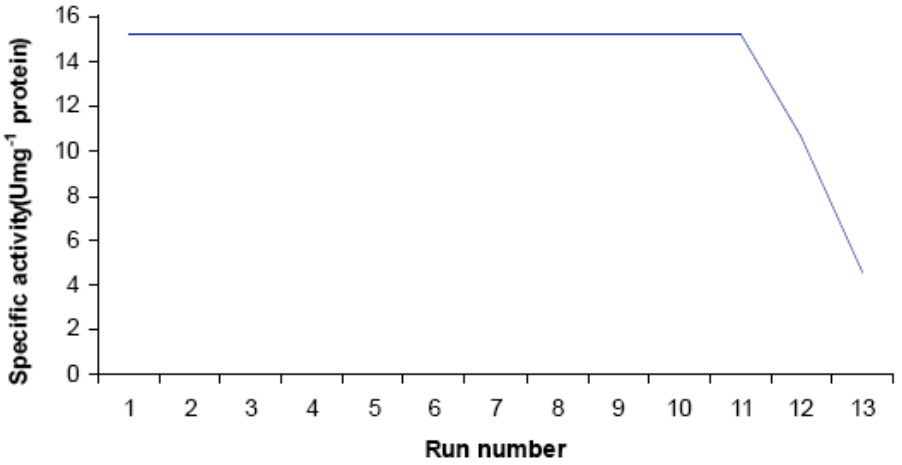



**Figure 5:** Operational stability of immobilized dextransucrase from *L.parameseneteroides* on calcium alginate.

beads. Gupta and Prabhu [13] found that 22% of the initial activity was lost after the six batch reaction. It was found that dextransucrase activity in beads decreased with each of the batch experiments, resulting in 55% loss in the activity after the seventh batch experiment [21]. This decrease was explained by transport limitations within the beads due to the formation of dextran. Dextransucrase immobilized in alginate beads decreased in each batch resulting in a 58% loss after the tenth batch reaction [6].

One of the most explored alternatives to immobilize dextransucrase is its entrapment in alginate beads [4, 22, and 23]. Although this method is normally used for the immobilization of whole cells or parts of cells (because globular proteins are too small in comparison with the size of alginate pores), it is convenient for dextransucrase, since the dextran layer covering the protein surface prevents leakage of the enzyme through the pores of the matrix [24, 25].

One of the most application of immobilized dextransucrase producing different molecular weight of dextran and also oligosaccharide.

Thus, it was demonstrated that immobilizing dextransucrase by entrapment in alginate was advantageous as regards the catalytic properties and stability. The stability of the immobilized dextransucrase was improved when compared with that of the free one.

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