Immobilization and properties of *Bacillus circulans* levansucrase

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Abstract

Levansucrase from a local isolate of *Bacillus circulans* was immobilized on wool adopting the physical adsorption technique. The relative activity of the immobilized enzyme was 55.20 % per g carrier and the specific activity (calculated on bound protein basis) was 78.12% of the free (soluble) enzyme. Compared to the free enzyme, the immobilized form revealed a higher optimal reaction temperature, low activation energy, higher K_m (Michaelis constant) and lowers V_{max} (maximal reaction rate). The same enzyme preparation exhibited thermal stability and resistance to chemical denaturation.

Key words: *B. circulans*, Levan, Immobilization, Levansucrase, Enzyme properties.

Introduction

Fructose-oligosaccharides (FOS) possess a number of desirable characteristics such as low calories, no cariogenicity, and safety for diabetics and bifidus-stimulating functionality (RoberFroid and Delzenne 1998, Bouhnik et al. 1999). Commercially available mixtures of FOS are either enzymatically synthesized by reverse β-fructosidase reaction (Hidaka *et al.*, 1986) or hydrolyzed from plant inulin (Vogel 1993). Both types contain β- (2-2) - glycosidic linkages between the fructose moieties. Another promising possibility to produce FOS with different molecular weights is the enzymatic synthesis by bacterial levansucrase [sucrose 2,6 β- D- fructan 6 B-O- fructosyltransferase, (FT), EC 2.4.1.10] from different types of bacteria. Generally, the microbial fructosyltransferase acts on sucrose by cleaving the β-(2-1)-linkage, transferring the fructosyl moiety to a suitable acceptor (e.g. another sucrose molecule), and also releasing glucose. This activity leads to the production of FOS or levan as a polymer.

From the commercial point of view the enzyme immobilization is a very important approach to facilitate the enzyme reuse. However, the immobilization process may or may not change the properties of the free enzyme (Clark 1994).

Different reports denote the immobilization of levansucrase using different immobilization techniques. Iizuka et al. (1993) reported the immobilization of *Bacillus natto* levansucrase on ceramic support; Marx et al. (1999) immobilized *Zymomonas mobilis* levansucrase by entrapment in calcium alginate beads. On the other hand, Bronshteyn et al. (2002) found that the immobilization of fructosyltransferase of *Streptococcus mutans* on hyroxyapatite induced the formation of stable enzymatically active complex.

In a previous communication (Abdel Fattah et.al. 2008) we have reported on the productivity of levansucrase as influenced by the immobilization and cultivation techniques.

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In continuation of this effort, the aim of this work is to deal with the immobilization of *B. circulans* levansucrase by physical adsorption on wool. The changes of the characteristic features of the enzyme brought about by immobilization were studied. The catalytic properties and stability of the immobilized enzyme have been compared to those of the free enzyme.

Materials and Methods

Microorganism

The levansucrase producing strain of *B. circulans* was obtained from the Culture of Collection of National Research Center, Dokki, Cairo, Egypt. It was maintained on potato dextrose agar slants at 4°C.

Enzyme Production

B. circulans was grown on the following medium (g/l): baker's yeast; 11.5, sucrose; 150, $MgSO_4$; 0.2, K_2HPO_4; 5.5. The pH was adjusted to 5.2 using sodium acetate buffer. Cultivation was made in 250 ml Erlenmeyer flasks, each containing 50 ml of the sterile medium.

The inoculum (2 % , v/v), was transferred to the culture medium and the flasks were incubated at 30°C for 72 h in shaking incubator at (50rpm). The culture medium was centrifuged (5000 rpm) for 15 min in a cooling centrifuge.

The clear supernatant was concentrated and fractioned at 50% ethanol concentration (v/v). This partially purified enzyme (specific activity 16.82 U/mg protein) was used for the preparation of the immobilized enzyme.

Enzyme Immobilization

One gram of crude wool was mixed with 2 ml of the enzyme solution (100 units in 0.2 M sodium acetate buffer pH 5.2). The mixture was incubated at 4°C overnight. The unbound enzyme was removed from the carrier by washing with the same buffer until no activity or soluble protein was detected in the washing solution.

Enzyme Assay

The enzyme solution (0.5 ml) in 0.2 M acetate buffer of pH 5.2 was incubated with 1 ml of 20% sucrose either in the free or immobilized form solution at 30°C for 15 min (Yanase et al. 1992). The produced reducing sugar was measured by glucose oxidase kits. One unit of enzyme activity was defined as the amount of enzyme that produces one μ mole glucose per min.

Protein Determination

The protein contents of the enzyme preparations were determined by the method of Lowry et al. (1951). The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein from the originally added protein.

Properties of the Free and Immobilized Levansucrase

Effect of pH

The effect of the pH value of free and immobilized levansucrase was studied using 0.2 M acetate buffer and 0.2 M potassium phosphate buffer respectively.

pH stability

The pH stability of the free and immobilized enzymes was examined after preincubating enzyme samples at 25 °C for 60 min at different pH values followed by adjusting the pH to the value of the standard assay system. The residual activity was assayed under the standard conditions.

Thermal Stability

Enzyme samples were incubated in 0.2 M acetate buffer at a designated temperature (35-60) °C for 15-60 min. The residual activity was assayed under the standard conditions.

Results and Discussion

Optimum pH

As shown in Figure 1, both the free and the immobilized enzymes exhibited different relative activities between the experimental pH ranges (pH 4.4 – 6.8). Both enzyme treatments were however acted optimally at pH values 5.2 and 5.6, respectively. On the other hand, at pH values less than 5.2 or more than 5.6, the activity % attained with the immobilized enzyme was relatively of higher magnitudes than that of the free enzyme. In a similar comment, Ivony et al. (1983) reported that the positively charged carriers (as in the present case) enhanced the activities of the enzyme adjusted to relatively lower pH values.
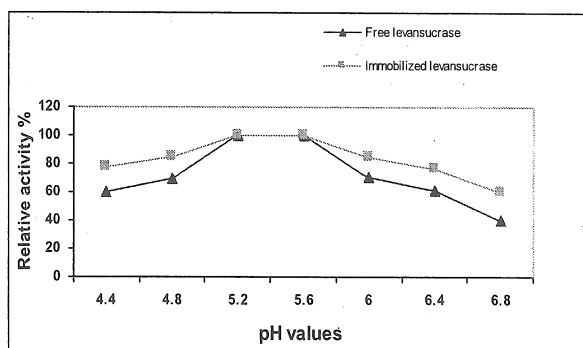

Figure 1. Effect of pH value on the activity of free and immobilized *B. circulans* levansucrase.

pH stability

The pH stability of the immobilized levansucrase was studied after 60 min incubation at 25 °C .The results illustrated in (Figure 2) showed that the immobilization process protected levansucrase from unfavourable changes in the pH value. The shifts in pH optimum for the catalytic activity after enzyme immobilization are mostly predetermined by the specific interaction between the enzyme structure and the support in a specific way and not by the character of the support in a single way. In general, different pH profiles of the immobilized levansucrase have been reported which depend on the applied immobilization methods and on the carriers used for enzyme immobilization (Jang et al. 2001, Platkova et al. 2005).
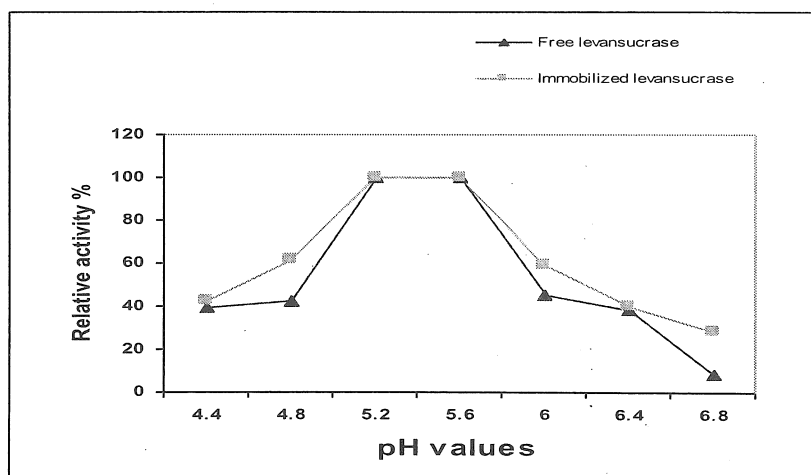

Figure 2. pH Stability of free and immobilized *B. circulans* levansucrase.

Optimum temperature.

Both the free and immobilized levansucrase enzyme had an optimum temperature at 35-40°C, whereas the immobilized enzyme exhibited more stability towards higher temperatures (Figure 3).
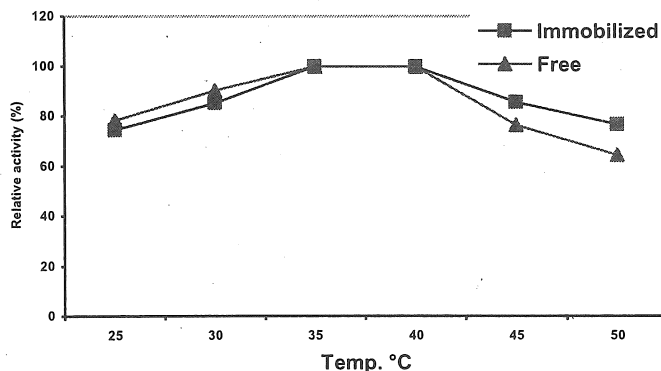

Figure 3. Effect of temperature on the activity of free and immobilized *B. circulans* levansucrase

The temperatures data were plotted in the form of Arrhenius plots (Figure 4). The slope of a logarithimic Arrhenius plots is related to the activation energy [Ea] for the molecule by the relation.

$$\text{Slope} = Ea/2.303 \text{ R} \quad \text{(Where R is the gas constant)}.$$

The plots for both the free and immobilized enzymes were linear and the calculated values of activation energy were equal to 5 and 4.55 Kcal/mol for the free and the immobilized enzyme, respectively. The lower value of the activation energy of the immobilized enzyme compared to the free enzyme may be attributed to the mass transfer limitation. Allenza et al. (1986) and Kitano et al. (1982) reported that the activation energies of the immobilized enzymes were lower because they considered the internal diffusion limitation is the rate limiting step. The decrease of the activation energy of immobilized amylase was also primarily reported (Kusano et al. 1989).
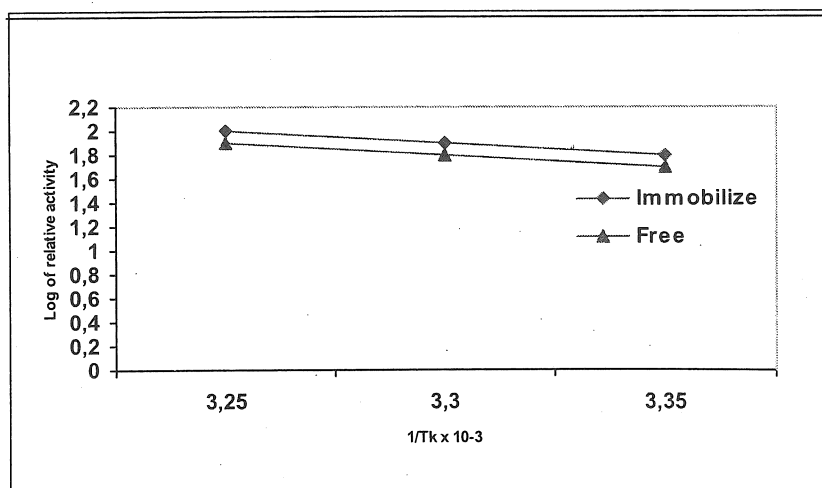

Figure 4. Arrhenius plots from the data presented in Figure 3.

Thermal stability

The rates of heat inactivation of the free and immobilized levansucrase were investigated in the temperature range between 40-60°C Figure 5.
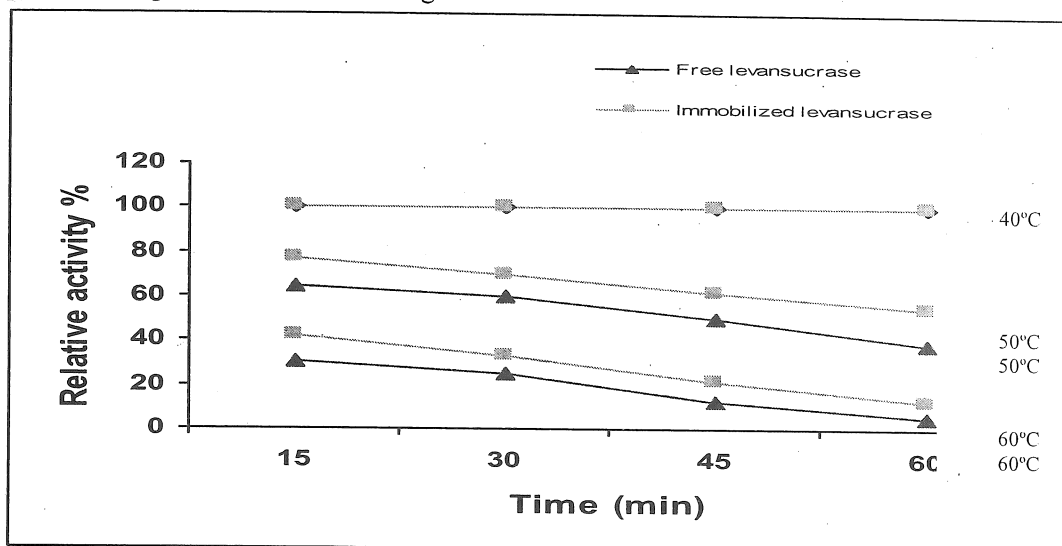

Figure 5. Heat inactivation of immobilized and free *B. circulans* levansucrase.
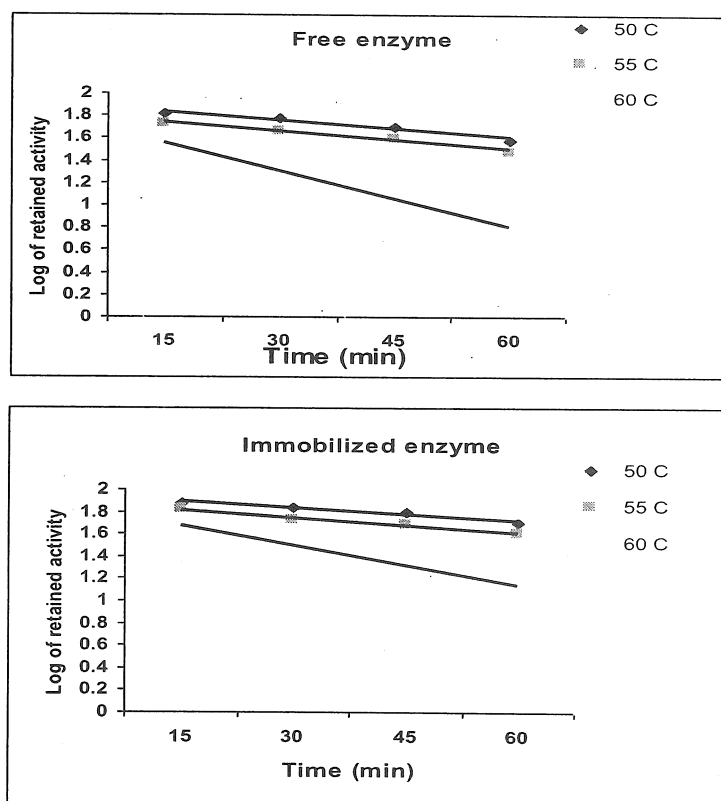

Figure 6. Log of activity retained as a function of time for free and immobilized *B. circulans* levansucrase.

In general, the immobilization process of the enzyme protected the enzyme against heat inactivation. For example, the calculated half-life value shows that the heat inactivation of the immobilized enzyme at 50, 55 and 60 °C was 256.7, 161.2 and 147.4 min respectively. Whereas at these temperatures the free enzyme was less stable (half-lives of 130.75, 115.5 and 103.4 min., respectively). In addition, the calculated values of deactivation constant rate at different temperatures indicated that thermal stability of the immobilized enzyme was superior to that of the free form. For example, the calculated deactivation rate constant at 60°C for the free enzyme was 6.7×10^{-3} min^{-1}, which is higher than that obtained with the immobilized enzyme 4.7×10^{-3} min^{-1} at the same temperature.

On plotting the logarithm of the activity retained against time, both the free and the immobilized enzyme gave straight-line plots, suggesting a first order reaction (Figure 6).

Substrate concentration

Linweaver-Burk plots of the free and immobilized levansucrase gave K_m values of 14.28 and 17.24 mg/ml respectively, with sucrose as a substrate (Figure 7). The reduction in affinity for the substrate on the immobilization of the enzyme may be due to conformational changes on immobilization and/or mass transfer effects. The V_{max} values (maximal reaction rate) of the immobilized enzyme were 166.67 U/mg proteins while that of the free form was 312.5 U/mg proteins.

Effect of some metal ions

The effects of various metal ions on the activity of the immobilized levansucrase as compared to their effects on the free enzyme were listed in table (1). The activity of both the free and the immobilized levansucrase proved to be unaffected in the presence of Ca^{2+} ions, while it was reduced markedly in Cu^{2+}, Hg^{2+}, Mn^{2+}, Fe^{3+} and Zn^{2+} treatments. However, it was observed that the inhibitory effects of these ions were less pronounced with the immobilized enzyme. This may be due to the protection of the immobilized enzyme by the carrier and this protection may result from structural changes in the enzyme molecule introduced by the immobilization procedure and consequently, lowering the accessibility of the inhibiting ions to the active site of the enzyme. These results coincided with those reported by (Tanaka et al. 1978, Chambert and Petit-Glarton 1984, and Ohtsuka et al. 1992).

Table 1. Effect of some metal ions on the activity of free and immobilized *B. circulas* levansucrase

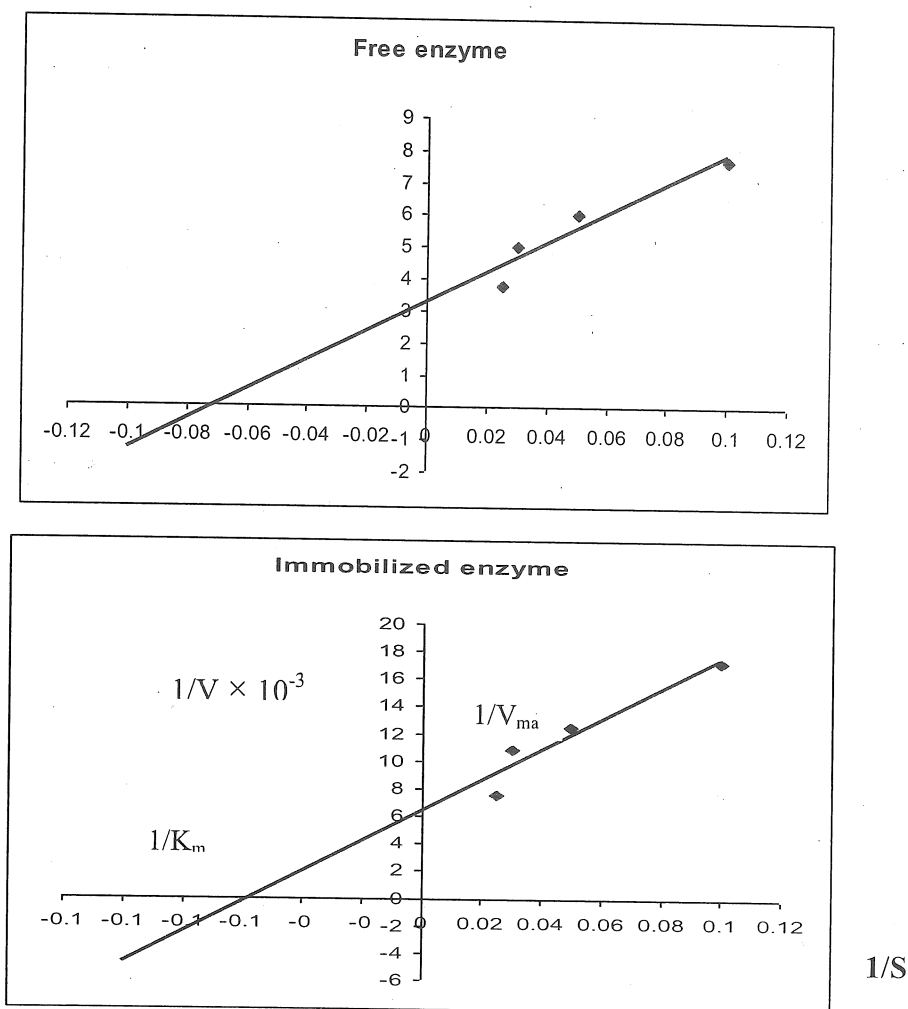| Metal Ion (Imµ /50 ml media)	Relative enzyme activity %	
	Immobilized	Free
Non	100	100
Cu^{2+} as $CuSO_4$	20.00	58.00
Hg^{2+} as $HgCl_2$	45.70	32.55
Mn^{2+} as $MnSO_4$	25.24	20.10
Ca^{2+} as $CaCl_2$	100	100
Fe^{3+} as $FeCl_3$	78.25	70.0
Zn^{2+} as $ZnSO_4$	30.32	25.00

Figure 7. Lineweaver-Burk plot for the free and immobilized *B.circulans* levansucrase.

Conclusion

The results revealed that the immobilized *B. circulans* levansucrase on wool showed resistance against thermal and chemical denaturation, increased tolerance to pH in a wide range and could be used in the production of levan. This provides a good efficiency for frucosylation to synthesise fructan polymers from sucrose (Rolla et al. 1983). The produced levan is one form of fructans offers a variety of industrial applications in the field of cosmetics, food and pharmaceutical products.

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