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IMMOBILISATION OF *Bacillus subtilis* NRC33a LEVANSUCRASE AND SOME STUDIES ON ITS PROPERTIES

M. A. Esawy[1,2*], D. A. R. Mahmoud[1] and A. F. A. Fattah[1]

[1]Department of Chemistry of Natural and Microbial Products,
National Research Center, Dokki, Cairo, Egypt.
[2]Laboratoire de Microbiologie IRD, IFR-BAIM, Universités de Provence
et de la Méditerranée, Phone: +(33) (4) 9182-8573, Fax: +(33) (4) 9182-8570,
ESIL case 925, 13288 Marseille Cedex 9, France.
E-mail: mona_esawy@hotmail.com, E-mail: hoda_esawy@yahoo.com

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Abstract - *Bacillus subtilis* NRC33a levansucrase was immobilised on different carriers using different immobilisation methods including physical adsorption, covalent binding, ionic binding and entrapment. The immobilised enzyme prepared by covalent binding on chitosan through 3% gluteraldehyde had the highest immobilization yield (81.51%). Therefore**,** it was used as a typical example for *Bacillus subtilis* NRC33a immobilised levansucrase and its properties were investigated. The time of the reaction and substrate concentration revealed that the activity of the immobilised enzyme was relatively lower than the free enzyme. The immobilised levansucrase showed a slight increase in activity compared with the free enzyme above 35°C. The activation energies were 6.62 and 9.27 kcal mol^{-1} for free and immobilised enzyme respectively. Although the thermal stability of the immobilised levansucrase was significantly improved in comparison to the free form, the deactivation energy of the immobilised enzyme was lower than that of the free enzyme. The half life of the immobilised and free levansucrase was also determined. The effect of different pH values reported that at acidic pH the activity of the immobilised levansucrase was higher than that of the free enzyme. The study of pH stability of free and immobilised levansucrase showed that the immobilisation process protected the enzyme from alkaline and severe acidic media. The effect of various metal ions showed that the free levansucrase was more sensitive to the inhibitory effect of the investigated substances. Immobilised levansucrase retained 51.13% after 14 repeated uses.
Keywords: Immobilisation; *Bacillus subtilis*; Levansucrase; Fructooligosaccharides.

INTRODUCTION

Immobilisation is very important for commercial uses, where the immobilised enzyme can be reused and easily removed from the hydrolysed mixture. The immobilisation process may or may not change the properties of the immobilised enzyme (Clarke, 1994).

Levansucrase (EC 2.4.1.10) is a ß-D-fructosyltransferase enzyme and its activity is characterised by the formation of free fructose, oligosaccharides and polysaccharides (Chambert et al, 1974). According to Hettwer (1995) levansucrase conducts three characteristic reactions: Synthesis of levan from sucrose by transfructosylation while releasing glucose, hydrolysis of levan to monosaccharides of fructose, exchange of [^{14}C] glucose in the reaction of fructose-2,1-glucose+ [^{14}C] glucose to fructose-2, 1-[^{14}C] gluose+glucose. Immobilisation of levansucrase has become a common goal for many workers in the microbiological and medical fields, due to the apparent significance of this group of enzymes in tumor therapy via exchanging the antitumor activity of leukocytes. Generally, levansucrase immobilisation

*To whom correspondence should be addressed

can be carried out by different methods, i.e. adsorption, covalent binding and gel entrapment. It was reported that the immobilisation of levansucrase from *Zymomonas mobilis* was done by different methods, like ionic binding, covalent binding, cross linking and matrix entrapment (Marx et al, 1999). The highest activity, stability, and immobilisation efficiency were in Ca-alginate beads. Also several researchers have introduced the immobilisation techniques of levansucrase using different immobilisation matrixes. It was found that the pure levansucrase from *Bacillus subtilis* was immobilised by covalent binding to glutraldehyde-activated amino silica and spherosil (Parlot and Mansen 1984). Immobilisation of levansucrase from *Bacillus natto* was prepared by dipping the honeycomp-shaped ceramic support in an enzyme solution (Iizuka et al, 1993). Finally, it was found that levansucrase immobilisation on calcium alginate gel strongly increased its polymerase activity (Chambert and Petit-Glatorn, 1993).

In the present study, *Bacillus subtilis* NRC33a levansucrase was immobilised on different carriers using different methods of immobilisation including, physical adsorption, covalent binding and entrapment. The immobilised enzyme performed by covalent binding on chitozan through a 3% glutraldehyde had the highest immobilisation yield .Therefore, this immobilised enzyme preparation was used as typical example for *Bacillus subtilis* NRC33a immobilised levansucrase and its properties was investigated. Chitosan has been known as an ideal support for enzyme immobilisation because of its hydrophilicity, biocompatibilty, biodegradibilty, and anti-bacterial property (Durate et al, 2002, Karim and Hashinaga, 2002).

EXPERIMENTAL

Microorganism and Growth Condition

Bacillus subtilis NRC33a was obtained from the Center of Cultures Collection of National Research Center (NRC). Nutrient agar medium was used for the culture maintenance and stock cultures. It had the following composition (g/L): peptone, 5.0; meat extract, 15.0; NaCl, 5.0; agar, 15.0; the pH was adjusted to 7.0. Unless otherwise specified, the following medium was used as culture medium for levansucrase production with some modification to obtain the optimized yield. This medium had the following composition: (g/L): Backers yeast, 20.0; sucrose, 200.0; $MgSO_4$, 0.15; K_2HPO_4, 5.0; completed by distilled water to L and the pH was 7.8 before autoclaving. The preparation of inoculum was done by adding 5 ml of sterile distilled water to 24-h old slants, which were scratched and transferred to each 300 ml Erlenmeyer flask. Cultivation of *Bacillus subtilis* NRC33a was carried out in 300 ml Erlenmeyer flasks. Each containing 100 ml of the cellular production and sterilized for 15 min at 121°C. The flasks were then inoculated with 5 ml of inoculum. The preparation of inoculum was done by adding 5 ml of sterile distilled water to 24-h old slants, which were scratched and transferred to each 300 ml Erlenmeyer flask. The flasks were incubated in a static incubator for 24 h at 30°C. The culture broth was then centrifuged in a refrigerated centrifuge (JANEKTZKI, K70, Germany) to separate the bacterial cells from the culture medium. The final pH, the biomass production and protein content were determined.

Assay of Levansucrase

This was done according to the method of (Yanase et al, 1991) with some modification. 0.5 ml of culture filtrate was incubated with 1 ml of 20% sucrose and 1 ml of 0.1 M acetate buffer at pH 5.2 was incubated at 30°C for 15 min. The reducing sugars produced were measured by glucose oxidase kits.

One unit of enzyme activity was defined as the amount of enzyme that produces reducing sugars equivalent to 1μmol of glucose per min.

The presence of levansucrase was confirmed by paper chromatography and HPLC (Abdel-Fattah et al 2005).

Determination of Protein

It was estimated according to the method of Lowry et al, (1951).

Fractional Precipitation with Acetone

Acetone (v/v %) was added slowly to the ice-cold enzyme solution until the required concentration of the acetone was reached. After removing the precipitated fraction by centrifugation in a refrigerated centrifuge, further acetone was added to the supernatent fluid and the process was repeated until the final concentration of acetone was reached (90%). The enzyme fractions obtained of 30, 40, 50, 60, 70, 80 and 90% concentrations of acetone were dried over anhydrous calcium chloride, under reduced pressure at room temperature and weighed. Each enzyme fraction was assayed for levansucrase activity and protein content.

Immobilisation Techniques

Physical Adsorption

It was carried out according to Woodward (1985). The carriers (1g) of chitosan, alumina, asbestos, and polyvinyl alcohol were incubated each with the 60% acetone enzyme fraction (100 U/g carrier) dissolved in 2.0 ml of 0.2 M acetate buffer (pH 5.2) at room temperature for 1 h. The unbound enzyme was removed from the carriers by washing three times with acetate buffer (pH 5.2).

Ionic Binding

It was carried out according to Woodward (1985). Whereas 1.0 g of each cation or anion exchangers Dowex 1-XB particle size 0.075-0.15 mesh, cellulose triacetate, DEAE-cellulose and sephadex A-50) was equilibrated with acetate buffer (0.2 M, pH 5.2), incubated with 2 ml of enzyme solution (100 U of the levansucrase).

Covalent Binding

It was carried out as follows, 1.0 g chitosan was shaken in 5ml 0.1M HCl containing 2, 3 or 5% (v/v) glutraldehyde (GA) for 2 h at 30°C. The solubilized chitosan was precipitated by addition of one ml of 0.1M NaOH. The precipitates were collected by filtration and washed with distilled water to remove the excess GA. The wet chitosan was mixed with 2.0 ml of enzyme solution (100 U levansucrase). After being shaken for h at 30°C, the unbound enzyme was removed by washing with distilled water.

Entrapment

Entrapment-in Ca-alginate beads was carried out according to Fraser and Bickerstaff (1997). Different concentrations of sodium alginate (1, 2, and 3%) were prepared by dissolving sodium alginate in distilled water. Then, 2 ml of the partially purified enzyme solution was mixed with 20 ml of each alginate solutions. The alginate-enzyme mixture was made into beads by dropping the alginate solution in 0.15 M $CaCl_2$ as cross- linking agent. The beads were left for further 20-30 min before collecting, washing with acetate buffer (0.2 M, pH 5.2) collecting and kept in the same buffer for a suitable time to remove the unbound enzyme. Entrapment in agar and agarose was carried out according to the method of Woodward (1985. Different concentrations of agar or agarose were prepared to give final concentrations of 2, 3 and 4% then the enzyme solution was added. After solidification it was cut into 1 mm^3 fragments and washed with acetate buffer (0.2 M at pH 5.2) to remove the unbound enzyme.

pH Stability and Thermal Stability

The pH stability of the free or immobilised enzymes was examined after pre-incubating the enzyme samples at 30°C for 60 min at different pHs, followed by adjusting the pH to the value of standard assay system. Then the residual activity was assayed under the standard conditions. The enzyme samples were incubated with acetate buffer (0.2 M, pH 5.2) at the design temperature (30-60°C) for 15 to 60 min and the residual activity was assayed under the standard conditions.

Operational Stability

It was performed with 1.0 g of immobilised levansucrase (wet) containing about 30 U of the enzyme. The immobilised form was incubated with 4 ml of sucrose substrate in acetate buffer (0.2 M, pH 5.2) at 30°C for 15 min. At the end of the reaction period, it was collected by centrifugation at 6000 rpm for 10 min, washed with distilled water and resuspended in 4 ml of freshly prepared substrate to start a new run. The supernatant was assayed for levansucrase activity.

Kinetic Studies

The Activation Energy (E_A)

It was determined from the slope of linear representation of Log (V_M) according to 1/T, resulting from the Arrhenius law:

$$\text{Log}(V_M) = \text{Cte} - E_A/(RT).$$

The enzyme activity (V_M) was expressed in Mol min^{-1}, the temperature (T) in degree Kelvin (K), the gas constant (R=1.987) in cal K^{-1} mol^{-1}, and the activation energy (E_A) in cal mol^{-1}.

The Deactivation Energy

Plotting activity data (Log of Ar (residual activity)/A_0 (initial activity) as a function of the time, the deactivation rate constant (k_d) was obtained. From Arrhenius equation:

$$k_d = k_{do} \exp(-E_d/RT)$$

Plotting ln of k_d as a function of the inverse of the absolute temperature, the energy of deactivation (E_d) is obtained as the product of the angular coefficient of the adjusted straight line times R, the universal gas constant.

Determination of the Half Life

Another important parameter related to enzyme stability is the enzyme half life ($t_{1/2}$) which corresponds to the time period necessary for the residual enzyme activity to decrease to 50% of its initial value. It can be calculated from the equation:

$$T_{1/2} = 0.693/k_d$$

Immobilisation Yield (U/g carrier)

It was calculated from the equation:
Immobilised enzyme (U/g carrier) = Enzyme added (U/g carrier) - Unbound enzyme (U/g carrier) (%).

RESULTS AND DISCUSSION

Bacillus subtilis NRC33a was a good producer of an inducible extracellular levansucrase, where the presence of levansucrase was confirmed by paper chromatography and HPLC. Fructose was shown to be the only component of the levan hydrolysate synthesized by levansucrase from *Bacillus subtilis* NRC33a. This result was coincided with those of Gross et al (1999) and Abdel-Fattah et al (2005).

In the present study, *Bacillus subtilis* NRC33a levansucrase was immobilised on different carriers using different methods of immobilisation including, physical adsorption, covalent binding, ionic binding and entrapment. The immobilisation yield is the key parameter, since it represents the general output of the efficiency of the immobilisation process (Brinbaum, 1994). The enzyme fraction precipitated at 60% acetone showed the highest specific levansucrase activity (32.87, U mg^{-1} protein) which represented 2.49 fold purification of that of the culture filtrate and was thus selected for preparation of the immobilised partially purified levansucrase. The immobilisation by ionic binding (Figure 1) showed high immobilisation yield was exhibited by the enzyme immobilised on DEAE-cellulose-53 (72.55%) and sephadex A-50 (55.11%). On the other hand, the immobilised enzyme on cellulose triacetate and Dowex-IXD showed low immobilisation yield. Jang et al (2001) reported that the immobilisation of FTFase enzyme from *Bacillus cereus* by ionic binding on DEAE-cellulose-53 showed the highest immobilisation yield (75%). Mostafa (2006) obtained approximately the same immobilisation yield (76.29 %) for FTFase from Zymomonas mobilis on titanium-activated magnetite. Also it was found that β-galactosidase immobilized on DEAE-agarose and PEI-Sepabeads showed highest immobilisation yield (Pessela et al, 2003). Immobilisation of levansucrase by physical adsorption (Figure 1) reported low immobilisation yield in all the used carriers (Alumina, Asbestos, Polyvinyl alcohol, Chitosan). This could be due to the weak binding between the carriers and the levansucrase. On the contrary it was reported that the immobilised FTFase prepared by physical adsorption on polyvinyl alcohol (PVA) had the highest immobilisation yield (71.42 %) (Mostafa 2006) The data for the immobilisation of the extracellular levansucrase by entrapment (Figure 2) suggested that gradual increase of the carrier concentration led to decrease in entrapping efficiency and the immobilisation yield. This could be due to the decrease of porosity and consequently to the limited diffusion. Similar observations were previously reported for the entrapped α-amylase (Abde-Naby; 1998, Mostafa, 2006).

The data for the immobilisation of the extracellular levansucrase by covalent binding (Figure 3) indicated a good immobilisation yield with chitosan through a spacer groups (gluteraldehyde) which showed good loading efficiency and good immobilisation yield (81.51%). The good loading efficiency for the immobilisation by covalent binding could be due to the formation of stable crosslinking between the carrier and the enzyme through spacer groups (gluteraldehyde) molecule. The results (Figure 3) showed that 3% gluteraldehyde crosslinking was found to be the best concentration to activate the support (chitosan) to give the highest yield of immobilised enzyme. It was reported that the amount of the immobilised glucose oxidase on chitosan was increased by increasing the length of the spacer groups (formaldehyde) (Yang et al 2004). On the contrary it was found that the best concentration of glutaraldehyde was 1% for ß-glactosidase activity immobilised on chitosan (Carrara and Rubiolo, 1994). The immobilised enzyme prepared by covalent binding on chitosan with 3% glutaraldehyde was used as a typical example for *Bacillus subtilis* NRC33a immobilised levansucrase and its properties were investigated.

Investigation of the effect of the reaction time and substrate concentration (data not showen) on the activity of the free and immobilised enzyme revealed a marginal decrease in activity (U/ml) compared to the free enzyme. Multiple fixation of the enzyme to matrix would also lead to a decrease in the activity due to the decrease in the catalytic activity (BicKerstaff, 1997).

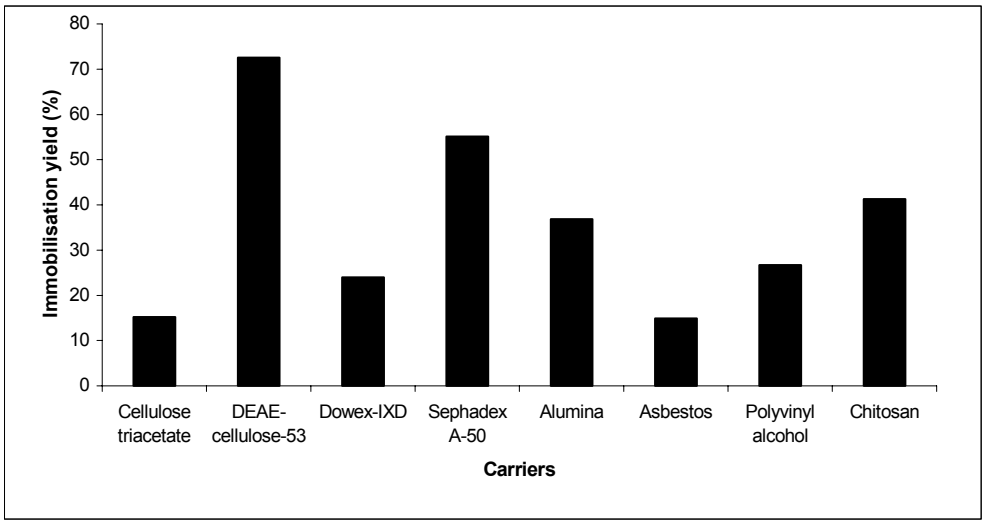


Figure 1: Immobilisation of the partially purified levansucrase from *Bacillus subtilis* NRC 33a by ionic binding and physical adsorption.

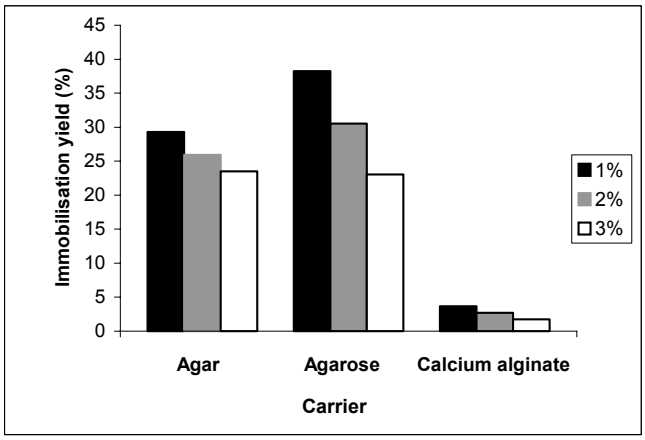


Figure 2: Immobilisation of the partially purified levansucrase from *Bacillus subtilis* NRC 33a by entrapment in agar, agarose and calcium-alginate.

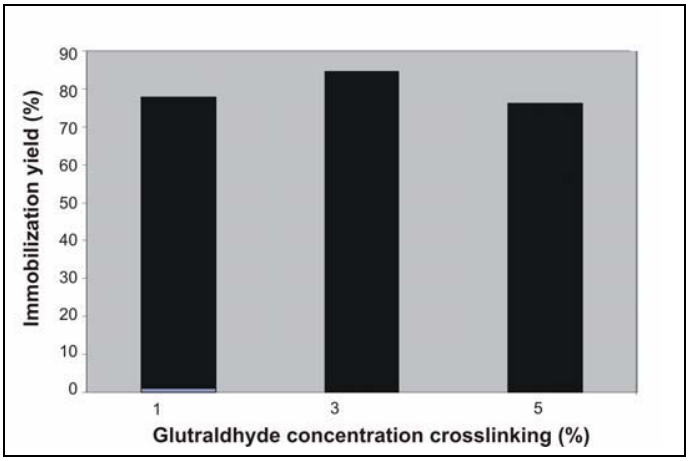


Figure 3: Immobilisation of the partially purified levansucrase from *Bacillus subtulis* NRC 33a by covalent binding with chitosan.

The results (Figure 4) showed that the free enzyme had higher activity (U/ml) than the immobilized enzyme up to 35°C after that the immobilised enzyme was more stable and showed a slight increase in activity. The immobilised enzymes by covalent binding (multiple point attachment) resulted in an increase of the enzyme rigidity, which is commonly reflected by increase in stability toward denaturation (Gottschalk and Jaenicke, 1991). The activation energy of the free and immobilised enzymes obtained from Arrhenius plots were 6.62 and 9.27 kcal /mol respectively (Figure 5). The higher values of activation obtained for the immobilised levansucrase indicated that the applied immobilisation procedures introduced changes in the structure of the enzyme molecule which impeded the enzyme catalyzed reaction (Krajewsk et al, 1990). Similar result was reported by (Gottschalk and Jaenicke 1991) whereas the activation energy of the free α-amylase was lower than the immobilised form. The investigation of the free and immobilised enzymes at different pHs (Figure 6) showed that the activity (U/ml) of the immobilised enzyme was similar to the free enzyme. It was observed that the immobilisation process -to some extent-protected levansucrase in the acidic range.(from pH 3.8 up to 4.6). It was observed that the immobilisation process protected FTFase in the acidic range (Mostafa, 2006).

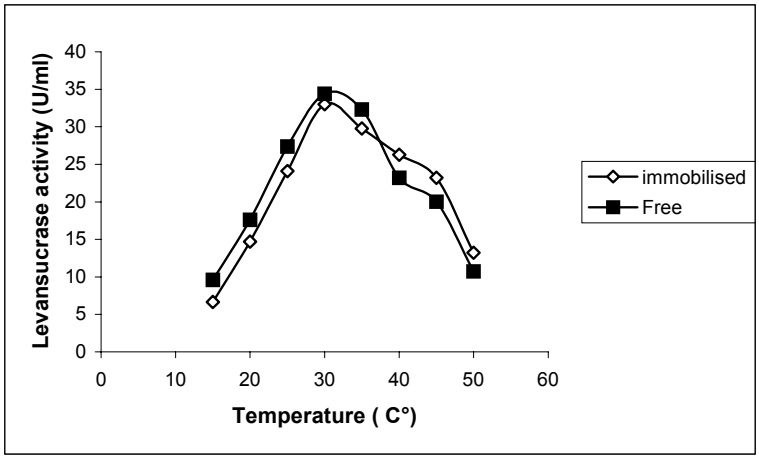


Figure 4: Effect of temperature on the activity of the free and immobilised levansucrase from *Bacillus subtilis* NRC 33a.

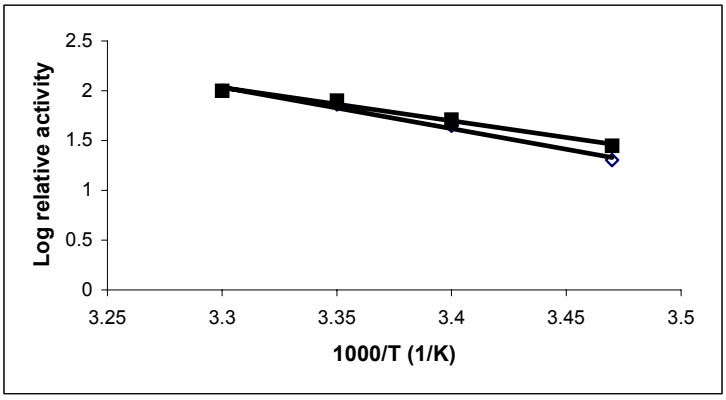


Figure 5: Arrhenius plot of activation rate constant of the free (■) and immobilised (□) levansucrase from *Bacillus subtilis* NRC33a.

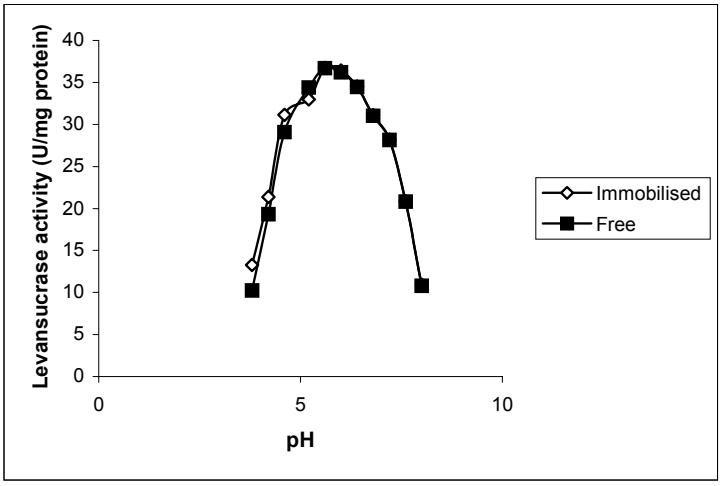
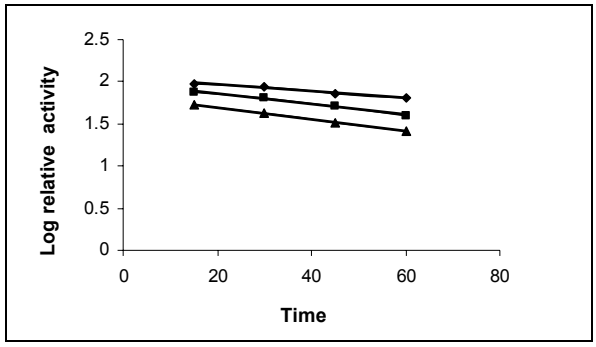


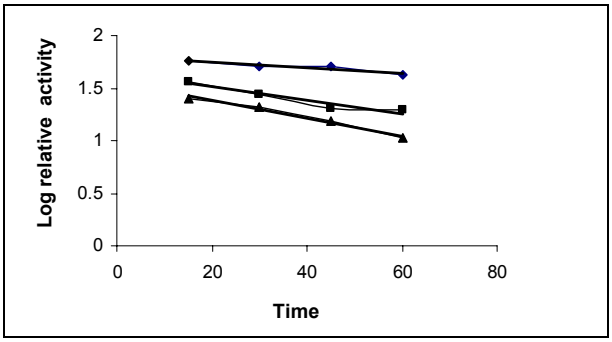
Figure 6: Effect of different pH values on the free and immobilised levansucrase from *Bacillus subtilis* NRC 33a.

The results in (Figure 7) showed that although the thermal stability of the immobilised levansucrase was significantly improved in comparison to the free form, the immobilised enzyme at 50°C lost the activity faster than the free form (15-60 min). This result could be explained that the rate constant of that part of the free enzyme that is still active, continues to increase with temperature (Tijskens et al, 2001). This interpreted the low value of the deactivation energy of the immobilised enzyme (14.1 kcal/mol) in comparison to the free enzyme (21.6 kcal/mol) at 50, 55, 60°C respectively (Figure. 7, 8). It was reported that E_d of the immobilised acid phosphatase is low compared to the free enzyme (Chang and Juang, 2004). On the contrary it found that the E_d of the immobilised invertase was higher than the free form (Bassetti et al, 2000). The half life were 231, 115.5, and 69.3 min for immobilised levansucrase at 50, 55 and 60°C respectively. The half life of the free enzyme were 161.6, 115.5 and 80.6 min at the same previous temperatures respectively.

Study of the pH stability (result not shown) showed that the immobilisation process protected the levansucrase from alkaline and severe acidic media. Similar result was reported by (Bryjac, 2003). The effect of various metal ions on the activity of the immobilised *Bacillus subtilis* NRC33a levansucrase as compared to the native enzyme (Table 1) exhibited a reverse relationship between most of the metal ions concentration and relative activity of the enzyme. The native enzyme was more sensitive to the inhibitory effect of the investigated substances. This may be due to the protection of the immobilised enzyme by the immobilisation matrix and hence the inhibiting reagents become less accessible to the enzyme active site (Akosy et al, 1998). The operational stability of the immobilised extracellular partially purified levansucrase was evaluated in repeated batch process (Figure 9). The performance of the immobilised enzyme indicated the durability of the catalytic activity in repeated use more than 14 cycles. The retained catalytic activity after being used for 14 cycles was 56% of the initial value of the immobilised enzyme. In this respect Marx et al (1999) showed that the immobilisation process of levansucrase of *Zymomonas mobilis on* Ca-alginate registered an operational stability of at least 600 h, during this time, only a negligible loss of activity could be observed. It was reported by (Jang et al, 2001) that the immobilised levansucrase of *Zymomonas mobilis* expressed in *Escherichia coli* retained 61% of the original activity after five repeated uses.



(a)



(b)

Figure 7: First-order plots of thermal inactivation of immobilised (a) and free (b) levansucrase

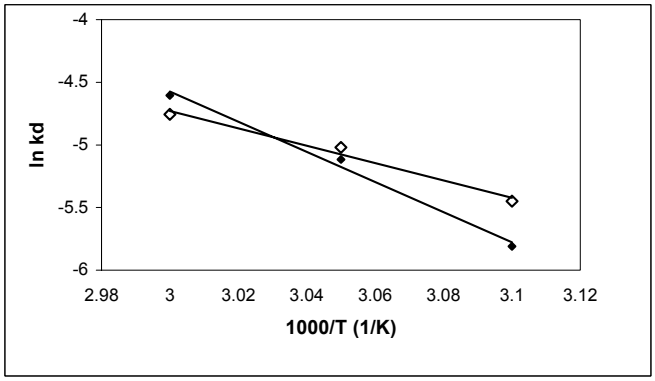


Figure 8: Arrhenius plot of deactivation rate constant of free (▪) and immobilised (▫) levansucrase from *Bacillus subtilis* NRC33a.

Table 1: Effect of the addition of different concentrations of some metal ions on the activity of the free and immobilised levansucrase.

Metal ion conc (Mm)	Relative activity of immobilized enzyme %			Relative activity of free enzyme %		
Metal salts	**0.1**	**1.0**	**10.0**	**0.1**	**1.0**	**10.0**
Control	**100**	**100**	**100**	**100**	**100**	**100**
$CuSO_4$	75	63	35	70	58	21
$FeCl_3$	92	79	65	87	71	60
$HgCl_2$	46	39	21	39	25	9
EDTA	100	100	100	100	100	100
SDS	89	79	52	86	73	48
Mn_2Cl	122	104	95	130	100	98
NaCl	80	71	50	74	60	45
AgCl	62	33	0	51	29	5
$MgSO_4$	100	99	88	100	93	79

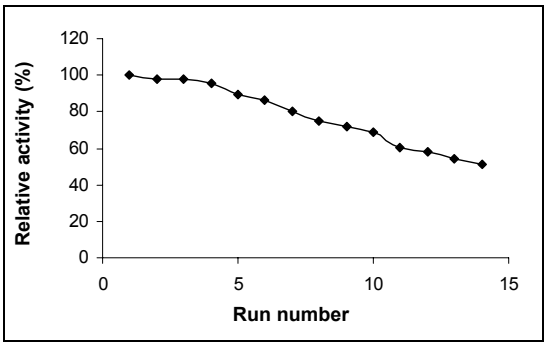


Figure 9: Operational stability of immobilised partially purified levansucrase.

CONCLUSIONS

The overall performance of the immobilised extracellular partially purified levansucrase stability is rather promising than the free enzyme. Thus, it suggests that *Bacillus subtilis* NRC33a extracellular partially purified levansucrase immobilised on chitosan by covalent binding is suitable for practical application.

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