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Production of levan using immobilized *Bacillus licheniformis* levansucrase

Imen Dahech*, Rania Bredai, Karima Srih
Biochemistry laboratory, Faculty of sciences of sfax, PB 802, 3018 Sfax, (TUNISIA)
E-mail : imenbiologie@yahoo.fr

ABSTRACT

Bacillus licheniformis levansucrase was immobilized on different carriers using different immobilization methods including physical adsorption, covalent binding, ionic binding and entrapment. The most efficient immobilization was achieved by chitosan (10%) using glutaraldehyde as a bifunctional agent. This method gave an immobilization yield of 83% and a levansucrase activity recovery of 97%. The immobilized enzyme exhibited a shift in the optimal pH from 5.5 to 7.0, but the optimal temperature of activity was not affected. The immobilized enzyme retained about 50% of its initial catalytic activity even after being used during 5 successive uses. The main product synthesized was levan which could be used for important applications.

KEYWORDS

Bacillus licheniformis;
Levansucrase;
Immobilization;
Levan.

INTRODUCTION

Levansucrase (EC 2.4.1.10) is a β-D fructosyltransferase enzyme and its activity is characterized by the formation of free fructose, oligosaccharides and polysaccharides[1]. Microbial levans are produced from sucrose-based substrate by transfructosylation reaction of levansucrase by a variety of microorganisms[2,3]. Potential applications of levan have also been proposed as an emulsifier, formulation aid, stabilizer and thickener, surface-finishing agent, encapsulating agent, and carrier for flavor and fragrances[4]. It has been reported to have a number of biological functions such as the promotion of infection and necrosis, tumor inhibition, tumor stimulation, and increasing the permeability of cells to cytotoxic agents[5]. Fructooligosaccharides (FOS) possess a number of desirable characteristics such as low calories, no cariogenicity, and safety for diabetics and bifidus-stimulating functionality[6,7].

For industrial application, immobilization of biocatalysts such as enzymes or living cells on inert supports is a very appealing approach because it offers several advantages over free-cell fermentation in that it can facilitate product isolation and biocatalyst reutilization. From the commercial point of view the enzyme immobilization is a very important approach to facilitate the enzyme reuse. In addition, this approach may help to enhance catalytic activity and prolong catalytic life of the biocatalysts[8]. Enzymes may be immobilized by various methods, which may be broadly classified as physical, where weak interactions between the support and the enzyme occur, and chemical, where covalent bonds are formed with the enzyme[9]. Enzyme

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immobilization can be classified into five broad categories: physical adsorption[10], entrapment in a matrix[11], ionic binding[12], covalent binding[13] and cross-linking[14].

The enzyme immobilization on a solid support can offer several advantages, including repeated enzyme usage, ease of product separation, improvement of enzyme stability, and continuous operation in packed bed reactors[15]. Also, enzyme immobilization frequently results in improved thermal stability or resistance to shear inactivation[16]. The covalent immobilization of enzymes can be achieved in various ways, depending on the carrier on which the bonding is intended. Among the most commonly known carriers, gelatin and chitosan are often preferred. Both of them present high levels of biocompatibility and non-toxicity. Gelatin is a naturally occurring polymer produced by the partial hydrolysis of collagen. The reactive groups present in gelatin are primarily hydroxyl, carboxyl and amino functions. It is available world wide at low cost. It has the biological properties of collagen[17]. Chitosan has been known as an ideal support for enzyme immobilization because of its biocompatibility, biodegradability and anti-bacterial property[18,19].

Generally, levansucrase immobilization can be carried out by different methods, i.e. adsorption, covalent binding and gel entrapment. Only few reports have been published on the immobilization of levansucrase[20].

This work aimed to investigate the immobilization of *Bacillus licheniformis* levansucrase using different methods of immobilization including, physical adsorption, covalent binding and entrapment. The changes of the characteristic features of the enzyme brought about by immobilization were studied and stability of the immobilized enzyme has been compared to those of the free enzyme.

MATERIALS AND METHODS

Chemicals

Chitosan, chitin, glutaraldehyde (GA) and acrylamide were obtained from Sigma. The gelatin was provided by Amersham. Mono-Q-sepharose was purchased from Pharmacia.

Microorganism and culture conditions

The present study reports on a newly isolated thermophilic bacterial strain from Tunisian thermal source and identified as *Bacillus licheniformis*[21]. The strain was cultivated in a liquid medium containing sucrose 200 g/l, Na_2HPO_4 3.5 g/l, NaH_2PO_4 0.8 g/l, $MgSO_4$ 0.2 g/l, $NaNO_3$ 3.5 g/l and yeast extract 5.0 g/l. Cultivation was made in 500 ml flasks, each containing 100 ml of culture medium. The inoculum (6%, v/v), was transferred to the culture medium and the flasks were incubated at 40°C for 36 h and at an agitation rate of 200 rpm.

Enzyme production

The culture medium was centrifuged (5000 rpm) for 15 min in a cooling centrifuge. The clear supernatant was concentrated and fractioned at 70% ammonium sulfate according to standard precipitation methods. The pellet was suspended in the minimum volume of phosphate buffer (20 mM, pH 6.5), and centrifuged again. The supernatant was dialyzed against 3 changes of phosphate buffer (20 mM, pH 6.5) at 4°C for overnight to remove the ammonium salt. Dialysis was carried out using a cellulose dialysis membrane (220 mm in diameter) with a cut off point of 10 kDa. This partially purified enzyme (specific activity 15.62 U/mg protein) was used for the preparation of the immobilized enzyme.

Levansucrase assay

This was done according to the method of Yanase et al, (1991)[22] with some modification. 0.5 ml of culture filtrate was incubated with 1 ml of 20% sucrose and 1ml 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.

Immobilization methods

The partially purified levansucrase was immobilized by different methods on different carriers. Immobilization steps and enzyme storage were carried out at 4°C. Supernatants and washing volumes were pooled after each step and the non immobilized activity was

determined.

Ionic binding (Mono-Q-sepharose)

1 g of DEAE-Sepharose resin was washed twice with a 50 Mm phosphate buffer pH 7.0 and centrifuged for 2 min at 4600×*g*. The resin was mixed with 0.5 ml of the enzyme preparation (45 U/ml) and 1.5 ml phosphate buffer during 12 h under agitation. The mixture was then washed twice with the same buffer and centrifuged for 2 min at 4600×*g*.

Inclusion in polyacrylamide

The immobilization in polyacrylamide gel was achieved by mixing 3 mL of a solution of acrylamide and bis acrylamide (30:1), 4 ml water, 2 ml of 100 mM Tris–HCl buffer pH 7.0 and 1ml enzyme (67 U/ml) and the polymerization was achieved by the addition of 100 µl ammonium persulphate and 6µl TEMED. The gel film was polymerized at 4°C on a surface of 5cm×5cm and cut into small blocks (1cm×1cm). The gel pieces were washed twice with 25 mM phosphate buffer pH 7 before use.

Covalent binding by glutaraldehyde to chitosan, chitin and gelatin

Chitosan (0.5 g) was dissolved in 50 ml of HCl 0.1 M containing 2.5% (v/v) glutaraldehyde 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 separated by centrifugation (10 min at 4000×*g*) and washed with distilled water to eliminate the glutaraldehyde excess. The wet chitosan was mixed with 0.5 ml of the enzyme solution and stirred for a night at 4°C. The unbound enzyme was removed by washing with distilled water until no protein or activity was detected[23].

Chitin (0.5 g) was shaken with 5 ml 2.5% (v/v) glutaraldehyde. Chitin was then collected by centrifugation (10 min at 4000×*g*) and washed with distilled water to remove the glutaraldehyde excess. The wet chitin was mixed with 0.5 ml of the enzyme solution for a night at 4°C. The unbound enzyme was removed by washing with distilled water as described early.

The gelatin powder (5-10%, w/v) used for immobilization of the levansucrase enzyme was swelled in 5 ml potassium phosphate buffer 50 mM pH 7.0 and heated at 50°C for 10 min till complete solubilization of gelatin. The mixture was cooled and the enzyme was added (67 U/ml). After mixing of enzyme, the required amount of organic cross-linker (0.6%, w/v), glutaraldehyde was added. The mixture was stirred constantly and poured on a 5cm×5cm glass plate to prepare a thin film of the enzyme. The film was stored at 4°C for complete cross-linking. The immobilized enzyme film was thoroughly washed with 50 mM phosphate buffer pH 7.0 and cut into small blocks (1cm×1cm) before being eventually used in subsequent experiments.

The immobilization yield was expressed in the equation below:

Immobilization yield = (A – B) / A × 100

And the activity yield was defined according to the following expression:

Activity yield = C / A × 100

Various parameters were used in the immobilization estimation: where (A) is the total enzyme activity used for immobilization, (B) is the unbound enzyme activity, (A-B) the theoretical immobilized enzyme activity, and (C) is the obtained immobilized enzyme activity. The total enzyme activity is the total number of units added to the support during the immobilization reaction. The non-immobilized activity is the number of units in filtrates and washing volumes after immobilization; and the immobilized activity is the number of units detected in the support after immobilization and washing.

Properties of the free and immobilized levansucrase

Effect of pH

The effect of pH on the free and immobilized levansucrase was studied using sodium acetate buffer (pH 4-6, 50 mM), potassium phosphate buffer (pH 6-8, 50 mM) and glycin buffer (pH 8-10, 50 mM).

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.

Optimum temperature

The optimum temperature for levansucrase activity

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was determined by incubating the enzyme in 0.05 M phosphate buffer pH 7.0 at different temperature (30-50°C). In each case, the substrate was pre-incubated at the required temperature before the addition of the enzyme.

Thermostability

The free enzyme as well as the immobilized one were incubated in phosphate buffer 50 mM pH 7.0 at different temperature (40-50°C) for different periods of time. The residual levansucrase activities were measured according to the standard assay method.

Operational stability

It was performed with 1.0 g of immobilized levansucrase (wet) containing about 30 U of the enzyme. The immobilized 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.

Continuous synthesis of levan by levansucrase immobilized on chitosan

The immobilized enzyme (0.1 g/g of support) was incubated in batch for 45 h at 40°C in the presence of 20% of sucrose that was pre-dissolved in 20 mM acetate buffer, pH 5.6. Aliquots were withdrawn at various time intervals. The sugars in the samples were measured by the DNS method[24].

All the experiments were repeated three times, and the results were reproducible.

RESULTS AND DISCUSSION

Effect of immobilization methods and carriers on levansucrase activity

In the present study, *Bacillus licheniformis* levansucrase was immobilized on different carriers using different methods of immobilization including, physical adsorption, covalent binding, ionic binding and entrapment.

The data for the immobilization of the extracellular levansucrase by covalent binding (TABLE 1) indicated a high immobilization yield (97% and 83% of activity and immobilization yields, respectively) with chitosan through a spacer groups (gluteraldehyde). The good loading efficiency for the immobilization by covalent binding could be due to the formation of stable crosslinking between the carrier and the enzyme through spacer groups (gluteraldehyde) molecule. The decrease of activity with the other carriers could be attributed to diffusional limitation of the substrate and product[25].

TABLE 1 : Levansucrase immobilization and activity yields on different matrixes. DEAE-Sephadex, Amberlite, Duolite, Fluorosil, chitin, gelatine, polyacrylamide, chitosan

Type of resin	Polyacrylamide	Q-Sepharose	Chitin	Gelatin	Chitosan
Activity yield (%)	81.0	86.36	87.6	90.0	97.0
Immobilization yield (%)	45.4	78.0	72.0	31.8	83.0

The yield of enzyme activity depends on the nature of the carrier used for immobilization. Jang et al (2001)[26] reported that the immobilization of Fructosyltransferase (TFase) enzyme from *Bacillus cereus* by ionic binding on DEAE-cellulose-53 showed the highest immobilization yield (75%). Mostafa (2006)[27] obtained approximately the same immobilization yield (76.29%) for FTFase from Zymomonas mobilis on titanium activated magnetite. On the contrary it was reported that the immobilized FTFase prepared by physical adsorption on polyvinyl alcohol (PVA) had the highest immobilization yield (71.42%).

Characterization of the immobilized preparation

pH optimum and stability

To further characterize the immobilized preparation, pH and temperature dependence of enzymatic activities was investigated. Figure 1 shows the activity of free and immobilized levansucrase at different pH values. The data indicate that the immobilized enzyme exhibited a shift of pH from 5.5 to 7 when compared to the free enzyme. An improvement of pH stability upon immobilization was also observed (Figure 2). Study of the pH stability showed that the immobilization process protected the levansucrase from alkaline and severe

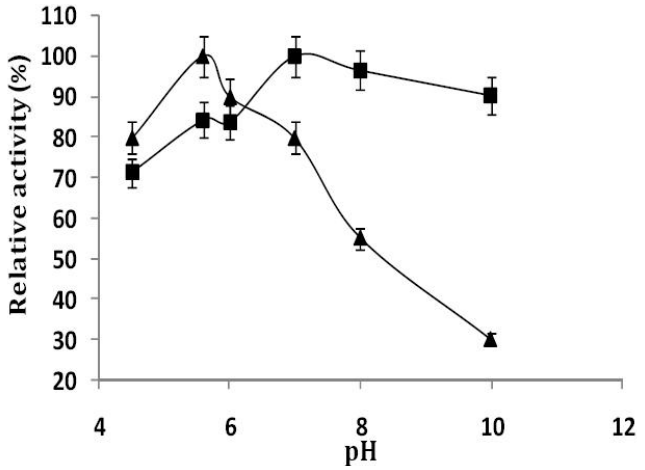

Figure 1 : Effect of pH on free and chitosan -immobilized levansucrase. Values reported in the figure are the means of determination performed in triplicate. Free enzyme (▲); immobilized enzyme (■)

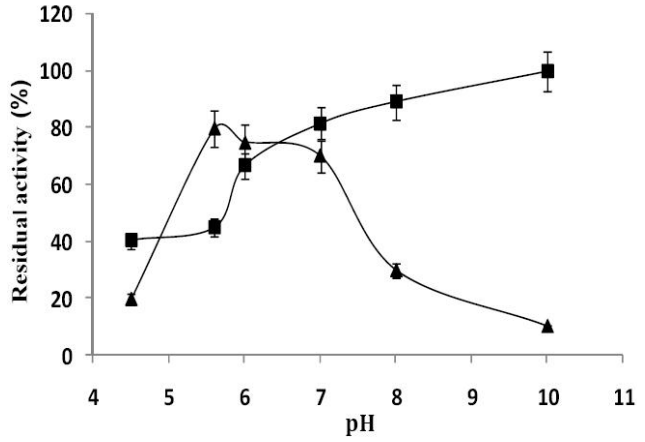

Figure 2 : Stability of pH on free and chitosan immobilized levansucrase. Free enzyme (▲); immobilized enzyme (■)

acidic media. Similar result was reported by Bryjac (2003)[28].

Effect of temperature on immobilized enzyme activity and thermostability

The free and immobilized *B. licheniformis* levansucrases assayed at temperatures ranging from 30 to 55°C (Figure 3). Both the free and immobilized levansucrase enzyme had an optimum temperature at 40-45°C, whereas the immobilized enzyme was more stable and retained 80% of its activity after 4 h incubation in the range of 30-50°C (Figure 4). The increase in the thermostability of the immobilized levansucrase could be attributed to the fact that the latter is less susceptible to conformational changes caused by temperature after immobilization into chitosan system while the quaternary

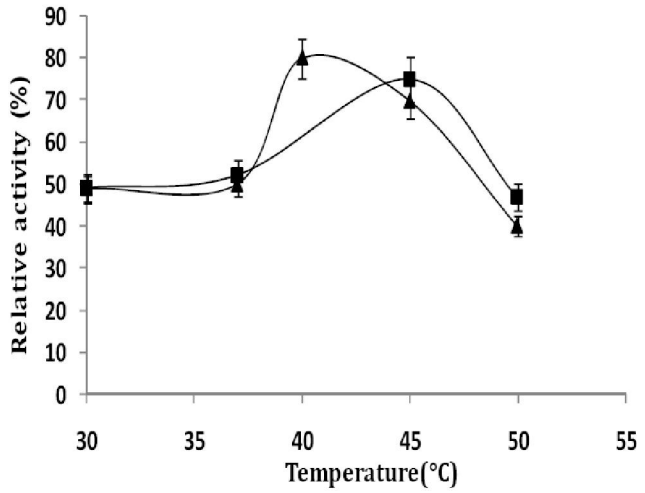

Figure 3 : Effect of temperature on free and immobilized levansucrase. Values reported in the figure are the means of determination performed in triplicate. Free enzyme (▲); immobilized enzyme (■)

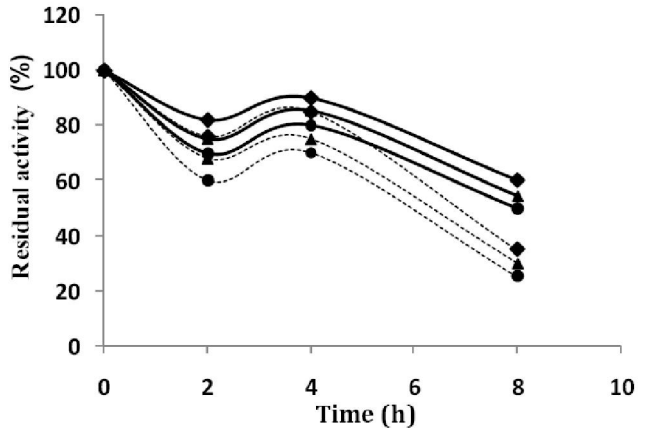

Figure 4 : Thermostability of the free and immobilized enzyme. Free and chitosan immobilized enzyme at 37 (▲); 45 (■) and 50°C (•). Residual enzyme activity was determined in the periodically withdrawn samples using sucrose as substrate. Free levansucrase (—); chitosan-immobilized levansucrase (…..)

structure of the free enzyme can be easily disaggregated[29].

Reusability of the immobilized enzyme

The operational stability of the immobilized extracellular partially purified levansucrase was evaluated in repeated batch process (Figure 5). The performance of the immobilized enzyme indicated the durability of the catalytic activity in repeated use of 5 cycles. It was reported by Jang et al (2001)[30] that the immobilized levansucrase of *Zymomonas mobilis* expressed in *Escherichia coli* retained 61% of the original activity after five repeated uses.

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Production of levan

Attempts were made to produce levan by the free and immobilized levansucrases. After 10 h of incubation, the percentage of levan synthesis by the immobilized enzyme increased and exceeded that of the free one (Figure 6). This could be explained by the better thermostability of the immobilized enzyme. The percentage of levan synthesis by the immobilized enzyme reached 40 % after 10 h of incubation.

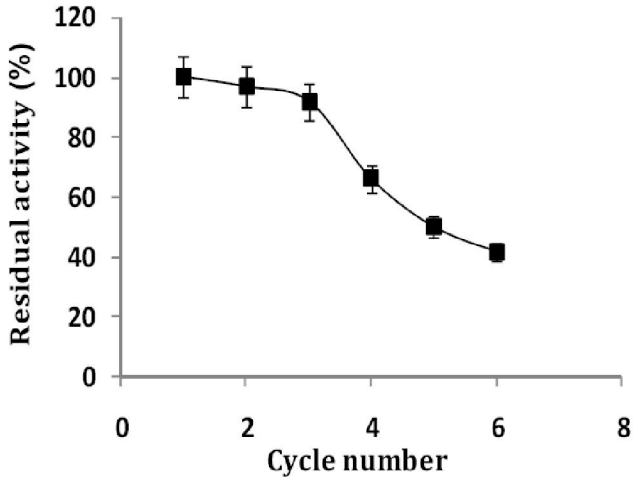

Figure 5 : Cycle number of the immobilized enzyme. The immobilized preparation was reused consecutively for 6 cycles

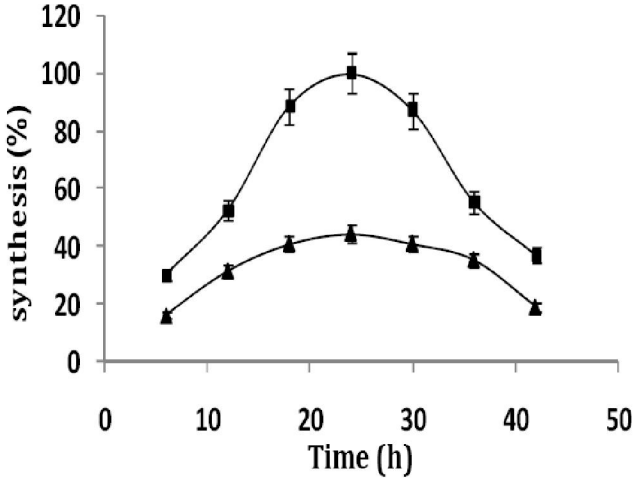

Figure 6 : Time course of levan production by *B. licheniformis* levansucrase; Free levansucrase (▲); chitosan-immobilized levansucrase (■)

CONCLUSION

The overall performance of the immobilized extracellular partially purified levansucrase stability is rather promising than the free enzyme. Thus, it suggests that *Bacillus licheniformis* extracellular partially purified levansucrase immobilized on chitosan by covalent binding is suitable for practical application. Thus, the immobilized levansucrase described here can be advantageously applied to produce levan which can be used as a valuable food additive.

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