Journal of Molecular Catalysis B: Enzymatic 73 (2011) 111–115

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Production of manno-oligosaccharides from locust bean gum using immobilized *Penicillium occitanis* mannanase

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ARTICLE INFO

Article history:
Received 5 March 2011
Received in revised form 29 June 2011
Accepted 17 August 2011
Available online 24 August 2011

Keywords:
β-Mannanase
Chitin
Immobilization
Stability
Manno-oligosaccharides

ABSTRACT

Mannanase was immobilized on chitin with glutaraldehyde by cross-linking reaction. The immobilization conditions and the characterization of immobilized enzyme were carried out. The immobilization yield and the mannanase activity recovery were 94.81% and 72.17%, respectively. The optimal mannanase activity shifted to lower pH after immobilization. However, the optimum temperature remained unchanged at 70 °C. The immobilized enzyme exhibited better thermal and pH stability than the free one. It also exhibited a high storage stability and retained 70% of its initial activity after 120 days. The main hydrolysis products yielded from locust bean gum were mannotriose and mannotetraose. The resulting manno-oligosaccharides could be used as a special nutrient for lactic bacteria.

1. Introduction

Endo-β-1,4 mannanases (E.C.3.2.1.78) are enzymes which hydrolyze the (1,4)-β-D-mannosidic linkages within the main chain of mannans and heteropolysaccharides consisting mainly of mannose; such as galactomannans or glucomannans, producing mannooligosaccharides [1].

β-Mannanases have been used to a great extent in various industrial processes. In fact, they are used in biobleaching of pulp and detergent industry [2–4], bioconversion of biomass wastes to fermentable sugars [5–7], and upgrading of animal feed stuff [4,8]. Mannanases could be also used to reduce the viscosity of coffee extracts [9,10]. Furthermore, they are employed in the preparation of mannooligosaccharides used as non-nutritional food additives for selective growth of human beneficial intestinal microflora (*bifido* bacteria and *lactobacilli*) [11,12].

The effective application of enzymes has often been restricted because of certain inconveniences, such as their non-reusability, instability and sensitivity to denaturation. These restrictions have actually been eased by the use of immobilized enzymes. Many advantages are gained through this technique including the enzyme reusability, the stability enhancement against the environmental effects, the easy recovery of biocatalyst from the finished product, simplifying the product purification process, providing opportunities for scaling-up and allowing the development of processes based on different reactor configurations and the reduction of operating costs [13,14].

Enzymes may be immobilized by a variety of methods, which may be broadly classified as physical, where weak interactions between the support and the enzyme exist, and chemical, where covalent bonds are formed with the enzyme [15]. Enzyme immobilization can be classified into five broad categories: physical adsorption [16], entrapment in a matrix [17], ionic binding [18], covalent binding [19] and cross-linking [20]. The latter involves the formation of covalent bonds between the support material and enzyme molecules through the employment of a bi- or multifunctional reagent.

Mannanases can be produced by various microorganisms, including bacteria, yeasts and fungi [21]. A filamentous fungus *Penicillium occitanis* (Pol 6) was previously described to produce three kinds of mannanase (ManI, ManII and ManIII). It was also shown that ManIII could be used to produce manno-oligosaccharides from locust bean gum and ivory nut mannan [22].

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doi:10.1016/j.molcatb.2011.08.007

The aim of this work was to investigate the immobilization of *P. occitanis* mannanase and to evaluate its hydrolytic efficiency for manno-oligosaccharides production.

2. Materials and methods

2.1. Chemicals

Chitin, Locust bean gum (LBG), Glutaraldehyde (GA), were obtained from Sigma. The carob seed flour from *Ceratonia siliqua* was obtained after grinding the seeds by a mill MOULINEX (particle size ≤1 mm).

2.2. Methods

2.2.1. Microorganisms and culture conditions

P. occitanis Pol6 was cultivated in a modified liquid Mandels medium [23]: KH_2PO_4, 2 g; $NaNO_3$, 5 g; $MgSO_4$, $7H_2O$, 0.3 g; $CaCl_2$, 0.3 g; yeast extract, 1 g; tween 80, 1 ml; water, 1 l and 2% carob seed flour. The pH value was adjusted to 5.5 and was supplemented with 1 ml of an oligoelements solution with $CoCl_2$, 2 g/l; $MnSO_4$. H_2O, 1.6 g/l; $ZnSO_4$. H_2O, 1.4 g/l; and $FeSO_4$. 7 H_2O, 5 g/l.

2.2.2. Production of mannanase in controlled fermentor

The enzyme production was carried out in a 30-l fermentor (Infors, Suisse) containing carob seed flour as substrate. The production medium was sterilized in situ at 121 °C for 30 min, cooled and inoculated with 7% of inoculum. The fermentor was operated at 30 °C, 250 rpm and 1.0 vvm aeration. The medium pH was maintained at 5.5 using sodium hydroxide (2 M) or orthophosphoric acid (2 M). Antifoam (Strictol 0.1%) was added when required. The feeding procedure of the fermenter with carob seed flour ($0.75\ g\ h^{-1}$) began after 3 days of batch culture. These optimized conditions were adapted for cellulase production on Avicel cellulose by *P.occitanis* Pol 6 [23].

After five days of fermentation, the crude broth was centrifuged for 10 min at 10,000 rpm and the supernatant was used as the crude enzyme for assay.

2.2.3. Preparation of partial purified mannanase

The crude extract of mannanase was precipitated by the addition of ammonium sulphate (60%) followed by centrifugation at 10,000 rpm at 4 °C for 30 min. The precipitates were dissolved in an appropriate volume of distilled water and dialyzed against water overnight at 4 °C.

2.2.4. Determination of β-mannanase activity and protein

β-Mannanase activity was determined by measuring the reducing sugar released from locust bean gum (LBG) according to the DNS method [24]. The assay mixture consisted of 0.5 ml LBG (0.5%, w/v) in 50 mM sodium acetate buffer pH 5.5 and 0.5 ml of diluted enzyme solution or a corresponding weighted amount of the immobilized enzyme. The reaction was incubated at 50 °C for 30 min. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of mannose per min under assay conditions.

Protein assay was determined by the method of Bradford [25] using bovine serum albumin as the standard protein. The amount of protein immobilized was estimated by subtracting the amount of protein determined in supernatant after immobilization from the total amount of protein used for immobilization.

All the assays were the means of three separate experiments.

2.2.5. Enzyme immobilization by cross-linking reaction

Chitin (0.5 g) was shaken with 5 ml glutaraldehyde at 2.5% (v/v), collected by centrifugation (10 min at 4000 rpm) and then washed several times with distilled water until the washings were free of glutaraldehyde. The wet chitin was mixed with 0.5 ml of the enzyme solution (87 U/ml) overnight at 4 °C. The unbound enzyme was removed by washing with distilled water. The immobilized β-mannanase was then dried for 24 h at 37 °C and stored at 4 °C [26].

The immobilization yield was expressed as follows:

$$\text{Immobilisation yield} = \frac{A-B}{A} \times 100$$

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

$$\text{Activity yield} = \frac{C}{A} \times 100$$

(A): the total enzyme activity used for immobilization; (B): the unbound enzyme activity; (A − B): the theoretical immobilized enzyme activity; and (C): 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 found in filtrates and washing volumes after immobilization; and the immobilized activity is the number of units detected in the support after immobilization and washing.

2.2.6. Properties of the free and immobilized mannanase: effect of pH and temperature on the activity and stability

The activity of the free and immobilized β-mannanase was assayed at different temperatures (40–70 °C) and pH values (from 2 to 10) using 50 mM citrate–phosphate buffer (pH 2–6), Tris–HCl buffer (pH 7–9) and glycine (pH 10).

The temperature effect on enzyme stability was checked at 40, 50, 60 and 70 °C during a period ranging from 30 min to 10 h. Both free and immobilized enzyme preparations (equal amounts in terms of proteins) were incubated in 50 mM citrate phosphate buffer pH 4 at the desired temperature. The residual activity was measured at specific time intervals under the standard assay conditions described above.

The free and the immobilized enzyme were incubated at various pH values at 50 °C for 4 h and their residual activities were determined at the optimum pH.

2.2.7. Thin-layer chromatography (TLC) of locust bean gum hydrolysis products

Locust bean gum (0.5%) was hydrolysed by both free and immobilized enzymes for 10 h at 70 °C and pH 4.0. Aliquots were withdrawn at various time intervals and the reaction was stopped by heating in boiling water for 5 min. The sugars in these hydrolysates were measured by DNS-method and analyzed by TLC which was carried out with a solvent system of Chloroform/acetic acid/water (6:7:1 by volume) using precoated silica gel plates (Merck). After developing the products, the sugar spots were visualized by spraying ethanol (95%) and H_2SO_4 (5%) and then drying the plates in an oven at 105 °C for 10 min.

3. Results and discussion

3.1. Immobilization of mannanases on chitin

The crude extract of *P. occitanis* mannanase was precipitated using 60% ammonium sulphate. The enzyme in the precipitate was dissolved in an appropriate volume of distilled water. It showed a specific activity of $12\ U\ mg^{-1}$ protein with a yield of 99.4% and 2.33 purification fold [22].

The β-mannanase was covalently coupled on chitin with glutaraldehyde by cross-linking reaction. Chitin showed a

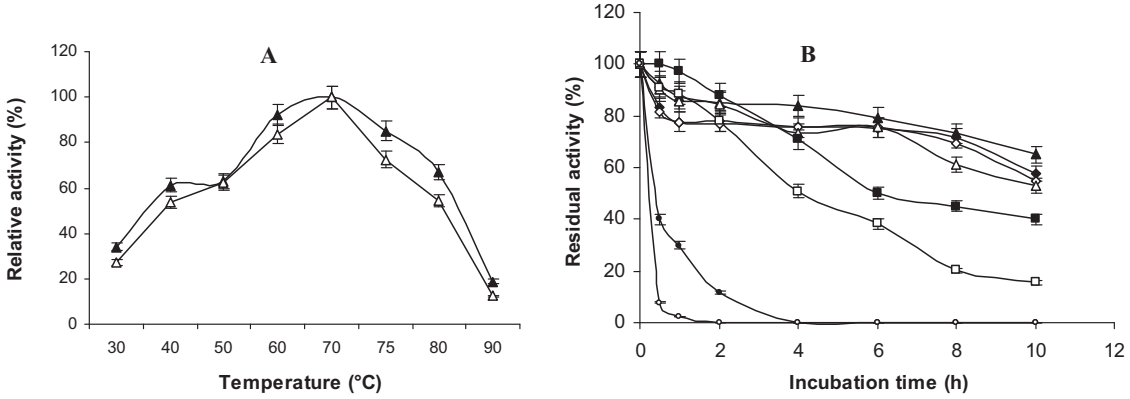

Fig. 1. Effect of temperature on the activity (Δ) (A) and on the stability of mannanase (B) at 40 °C (◊), 50 °C (Δ), 60 °C (□) and 70 °C (o). Empty symbols: free enzyme; filled symbols: immobilized enzyme.

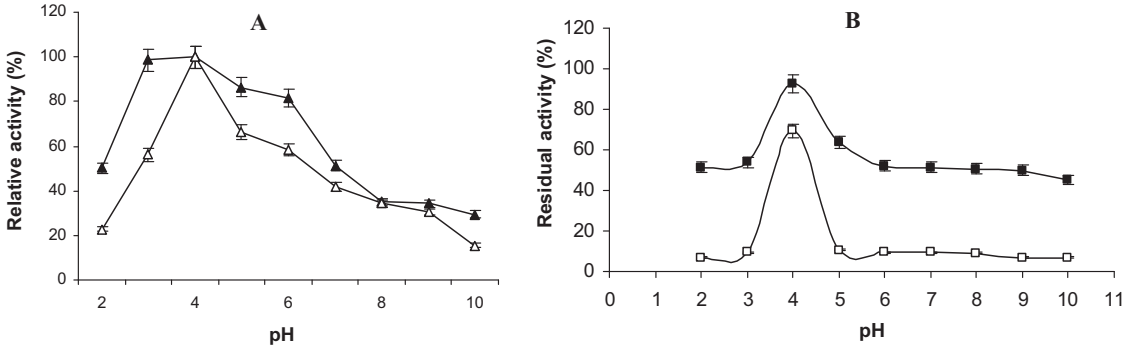

Fig. 2. Effect of pH on the activity (Δ) (A) and on the stability (□) of mannanase (B). Empty symbols: free enzyme; filled symbols: immobilized enzyme.

considerable bound enzyme activity (72.17%) and immobilization yield (94.81%) at 2.5% gluteraldehyde concentration and the amount of added enzyme was optimized to be 3 mg/ml (data not shown). This result was in agreement with other immobilized enzymes on chitin support when activated by glutaraldehyde such as α-L-arabinofuranosidase [27], gluco-amylases [28], pectinlyase [29], cellulases [30], chitosanase [31] and cyclodextrin glycosyltransferase [32]. In fact, the presence of the amino groups in chitin molecule provides a binding site for proteins. This can act as a solid support for preparation of the immobilized enzyme. Moreover Chitin is known by its hydrophilicity, biocompatibilty, biodegradibilty, and anti-bacterial property. It is also cheaper and the operation is much more convenient [15].

This good efficiency for the immobilization by covalent binding could be due to the formation of stable cross-linking between the carrier and the enzyme through glutaraldehyde [27]. In fact, the number of covalent bonds between the support and the enzyme depended on the support activation degree (concentration of aldehyde groups in the support surface) and on the concentration of amino groups in the enzyme molecule [31,33–35]. In addition, covalent binding through glutaraldehyde probably increased the carrier local surface area and, thus reduced the steric hindrance around the enzyme active site [36].

3.2. Characterization of the immobilized preparation

3.2.1. Effect of temperature on activity and thermostability

The temperature dependence of the free and immobilized β-mannanase activities was studied in 50 mM citrate phosphate buffer pH 4. The results indicated that both enzymes had a similar optimal temperature of 70 °C (Fig. 1A). However, the immobilized enzyme was more active and retained more than 70% of its relative

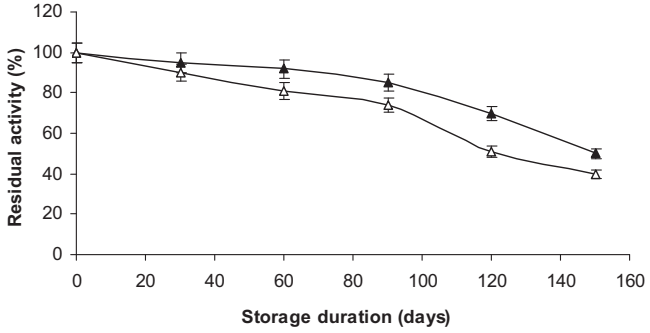

Fig. 3. Storage stability of mannanase (Δ) at 4 °C. Empty symbols: free enzyme; filled symbols: immobilized enzyme.

activity in the range of 60–80 °C. This result could be explained by the fact that the enzymes immobilized by cross-linking caused an increase of the enzyme rigidity, which is commonly reflected by an increase in the stability toward denaturation [37].

The temperature stabilities of the free and the immobilized β-mannanase were compared at temperatures ranging between 40 and 70 °C at pH 4 for 10 h (Fig. 1B). The immobilized enzyme was more stable than the free one, particularly when the temperature exceeded 50 °C. The immobilized β-mannanase, for instance, retained 50% of its activity at 60 °C after 6 h, while the free enzyme retained only 50% of its activity after 4 h under the same conditions. The immobilized and the free β-mannanase retained 40% and 7% of their activities, respectively, after 30 min of incubation at 70 °C. It was observed that the immobilization process on chitin protected the enzyme against heat inactivation. The increase in thermostability might arise from changing the conformational integrity of the

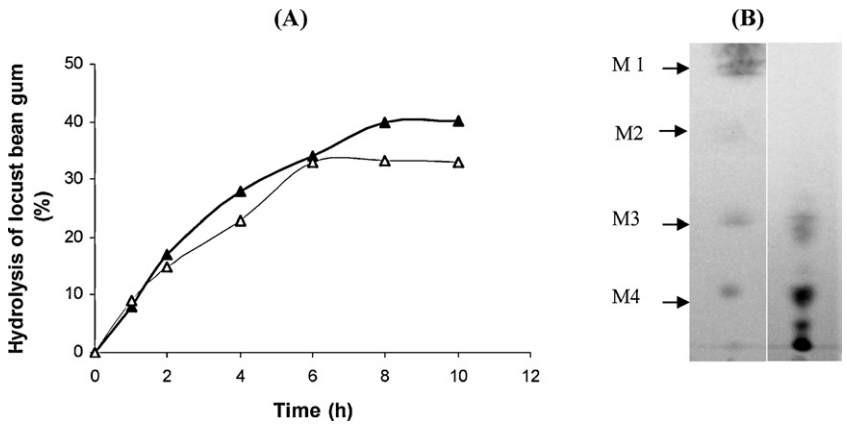

Fig. 4. Time course of the manno-oligosaccharide production (Δ) from locust bean gum by mannanase of *P. occitanis* (A). Empty symbols: free enzyme; Filled symbols: immobilized enzyme. TLC analysis of LBG hydrolysed by immobilized mannanase (B). Left panel: the standard mixture of sugars (M1: mannose, M2: mannobiose, M3: mannotriose and M4: mannotetraose); right panel: the LBG treated with mannanase.

enzyme structure by covalent bond formation via amino groups [35,38] or the existence of a local environment for the immobilized enzymes which is less damaging than the bulk solution conditions [39].

3.2.2. Effect of pH on activity and stability

As shown in Fig. 2A, the optimum pH of the immobilized enzyme shifted from 4 which was the optimum for free mannanase to a more acidic range (pH 3, 4). This slight change in pH could be attributed to the substantial difference in the ionic environment of the carrier around the enzyme active sites [40]. This phenomenon is usually observed when enzymes are covalently coupled to supports [41].

Compared to the free enzyme, the immobilized β-mannanase exhibited a higher activity at pH below 7 and lost only 13% and 18% of its activity at pH 5 and 6, respectively. However, this loss increased to 33% and 41% at pH 5 and 6, respectively. This characteristic is particularly required in paper and agro-industrial manufacturing [42].

The study of the pH stability showed that the immobilization process protected the mannanase from alkaline and severe acidic media (Fig. 2B). We observed that upon incubation for 4 h at 50 °C, the immobilized mannanase could act at different pH values, ranging from 2 to 9, displaying a residual activity of about 50%. This stabilizing effect in acidic and alkaline media could be attributed to the ability of the micro-environment, created between the support and the enzyme, to protect the latter from the denaturation caused by the change in pH and to make it more stable [42].

3.2.3. Storage stability of the immobilized mannanase

In order to investigate the industrial practicability of an immobilized enzyme process, the loss of enzyme activity at low temperature, known as storage stability is an important parameter to study. Chitin-immobilized mannanase was stored in a dry form at pH 7 and the activity was assayed periodically, during 150 days. As presented in Fig. 3, the immobilized mannanase was stable for a long period at 4 °C retaining 70% of its activity after 120 days of storage. Only 10% of the initial activity was lost after 60 days and the half-life was reached after 150 days of storage with respect to the free enzyme which showed half inactivation after 120 days. The results indicate that the immobilized enzyme had good storage stability. By holding the enzyme in a relatively fixed position, immobilization reduces the interaction between the enzyme molecules which contributes to deactivation by aggregation and to autolysis by proteolytic enzymes and therefore, creates a more rigid enzyme molecule [43]. A stable immobilized system and a long storage life are convenient for applications that would not be feasible with a soluble enzyme system.

3.2.4. Hydrolysis of locust bean gum

Attempts were made to produce manno-oligosaccharides from locust bean gum by a free and immobilized mannanase. The hydrolysis of the locust bean gum by the immobilized enzyme proceeded at the same speed as the free enzyme during the reaction on early stage (Fig. 4A). After 6 h, there was no further hydrolysis for the free enzyme, whereas hydrolysis continued up to 8 h with the immobilized enzyme. The final extent of the locust bean gum hydrolysis by the immobilized enzyme was $40 \pm 2\%$ higher than that by the free one ($33 \pm 1.5\%$). The difference in the free and immobilized performances possibly originated from their difference in thermal stability. It might be due to the fact that the enzyme was immobilized, which made it become more resistant than the free enzyme to the inhibition by released hydrolysed products.

The released sugars after 8 h of incubation by the immobilized enzyme were analyzed by TLC, using mainly mannose, mannobiose, mannotriose and mannotetraose as standards (Fig. 4B). During the hydrolysis reaction, the immobilized enzymes released mainly mannotriose and mannotetraose. Thus, the immobilized mannanase described here can be advantageously applied to produce manno-oligosaccharides which are proposed as a prebiotic [11,12].

4. Conclusion

In the present work, the immobilized mannanase with chitin as carrier using glutaraldehyde as cross-linking reagent showed significant advantages over the free enzyme. This technique exhibited high loading efficiency, an interesting storage stability and a good thermal and pH stability. Accordingly, it is suggested that mannansase immobilized on Chitin is suitable for the production of manno-oligosaccharides which can be used in food applications.

Acknowledgements

This research was financially supported in part by the Tunisian Ministry of Higher Education and Scientific Research. We express our gratitude to Prof. G. Tiraby (Cayla-InvivoGen) and Dr. H. Durand (Lallemand) for kindly supplying the *P. occitanis* (Pol 6) strain used in this work. We wish to extend our gratitude to Prof. Jamil Jaoua, founder and former head of the English Unit at the Sfax Faculty of Science for having proofread this paper.

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