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Production and in vitro evaluation of oligosaccharides generated from lichenan using immobilized *Penicillium occitanis* lichenase

Fatma Chaari[a], Lilia Belghith-Fendri[a], Semia Ellouz-Chaabouni[a,b,*]

[a] Unité Enzymes et Bioconversion, Biology Department, Sfax National School of Engineers, Sfax University, B.P 1173, 3038 Sfax Cedex, Tunisia
[b] Unité de Service Commun Bioréacteur Couplé à un Ultrafiltre, Sfax National School of Engineers, B.P. 1173, 3038 Sfax Cedex, Tunisia

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ABSTRACT

Lichenase from *Penicillium occitanis* Pol6 was immobilized on glutaraldehyde crosslinked chitosan–clay composite beads and used for lichenan hydrolysis. The immobilization yield and lichenase activity were 94.81 ± 4.7% and 72.17 ± 3.6% of initial activity, respectively. Optimum pH of the free and immobilized Lic-Pol6 was found to be between 3.0 and 4.0. Immobilized Lic-Pol6 was more active and retained more than 94% of its relative activity in the range of 40–80 °C. The immobilized Lic-Pol6 retained 63.7% of its activity after eight cycles of uses. It also exhibited high storage stability and had only 37% activity loses after 120 days at 4 °C. The generated oligosaccharides GG showed a high antioxidative capacity (175 μmol/ml α-tocopherol equivalents) at 10 mg/ml and an important DPPH radical-scavenging activity (IC50 = 2.5 ± 0.01 mg/ml). Furthermore, GG had a high antimicrobial activity against *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Salmonella thyphimirium*.

1. Introduction

The β-1,3-1,4-glucans are non-starchy polysaccharides found in the endosperm cell walls especially in oats and barley [1]. These polysaccharides are polymers of β-D-glucopyranose where about 30% of glucose residues are C (O) 3-linked and 70% are C (O) 4-linked. The endo-type enzymes hydrolyzing β-1,3-1,4-glucans can be grouped into four main categories according to the type of glycosidic linkage they cleave: β-1,3-1,4-glucanases (lichenases, EC 3.2.1.73), β-1,4-glucanases (cellulases, EC 3.2.1.4), β-1,3-glucanases (laminarinases, EC 3.2.1.39), and β-1,3(4)-glucanases (EC 3.2.1.6) [2–4].

Lichenases have received considerable attention thanks to their enzymatic functions and importance in industrial applications. For example, in the brewing industry, the addition of β-

1,3-1,4-Glucanases can reduce brewer mash viscosity and turbidity, increase extract yields, and produce high-quality brewing malt [5]. In animal feed industry, supplementation of these enzymes can increase β-glucan digestibility in feed stuffs, improve feed conversion efficiency, and reduce sanitary problems such as sticky droppings [6,7]. In laundry detergents, these enzymes are used to remove food and care product stain containing β-glucan [8]. Furthermore, they are employed in the preparation of oligosaccharides used as non-nutritional food additives for selective growth of human beneficial intestinal microflora (bifido bacteria and lactobacilli) [9]. In general, β-glucan oligosaccharides are produced by chemical or enzymatic hydrolysis of β-glucan [10]. Production of β-glucan oligosaccharides via hydrolysis of β-glucan by β-glucanase is difficult due to the low stability. To overcome these problems, immobilized enzymes have been used. 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 [11]. Also, enzyme immobilization frequently results in improved thermal stability or resistance to shear inactivation [12].

Chitosan is a natural polymer and has been widely used as a support material for enzyme immobilization [13–15]. Chitosan is characterized by an excellent hydrophilicity, high porosity and big adhesion area. It possesses hydroxyl (OH) and amino (NH2) groups, which link with enzymes easily [16]. It can be cross-linked with glutaraldehyde to prevent from dissolution in acidic solutions (pH < 2) [17]. Also glutaraldehyde crosslinking provides aldehyde groups in order to link to amine groups of the enzyme. Cross-linked chitosan beads have greater mechanical strength and are more applicable in biochemical engineering, but there are still operational defects such as bead density is too similar to water (leading to float easily)

* Corresponding author at: Unité Enzymes et Bioconversion, Biology Department, Sfax National School of Engineers, Sfax University, B.P 1173, 3038 Sfax Cedex, Tunisia. Tel.: +216 74675331; fax: +216 74275595.
E-mail address: Semia.Chaabouni@enis.rnu.tn (S. Ellouz-Chaabouni).

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and its texture is too soft. This would largely limit industrial applications of chitosan for this purpose [18,19]. The mentioned problems of chitosan beads can be improved in conjunction with other powders such as clays and activated carbon to increase its density and mechanical strengthen, and thus to extend its applications [20–22]. In this study chitosan–clay composite beads were prepared. The composite beads were crosslinked with glutaraldehyde and then lichenase was immobilized on these beads. The activity, thermal stability and recyclability of free and immobilized enzyme were characterized. Optimum temperature and pH, thermal and pH stability, re-uses and storage stability of free and immobilized lichenase were examined. Furthermore, immobilized lichenase was used for lichenan hydrolysis to produce physiologically active oligosaccharides.

2. Materials and methods

2.1. Materials

Nanoclay (average particle size ≤25 μm), chitosan, glutaraldehyde, were obtained from Sigma–Aldrich (St. Louis, USA). Oligosaccharides used as standards for TLC analysis, were obtained from Sigma. All other chemicals were used in analytical grade.

2.2. Microorganisms and culture conditions

Penicillium 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; $MnSO_4{\cdot}H_2O$, 1.6 g/l; $ZnSO_4{\cdot}H_2O$, 1.4 g/l; and $FeSO_4{\cdot}7H_2O$, 5 g/l.

2.3. Partial purification of lichenase from P. occitanis Pol6

Lichenase of *P. occitanis* Pol6 was purified as described by Chaari et al. [24]. The cell-free supernatant was incubated during 10 min at 50 °C. After rapid cooling, insoluble denatured proteins were removed by centrifugation during 15 min at 4000 rpm.

The sample was loaded on a column (10 cm × 1.6 cm) of Q-Sefinose previously equilibrated in 20 mM Tris–HCl buffer (pH 8.5) containing 0.01% Triton X-100. The column was washed with the same buffer to remove unbound proteins. Elution was carried out with a gradient of 0–1 M NaCl in the same buffer. Fractions of 4 ml were collected at a flow rate of 60 ml/h. Protein contents (Abs 280 nm) and lichenase activity were determined. Fractions with lichenase activity were collected and concentrated by lyophilisation. The concentrated sample was used as a partially purified enzyme for subsequent studies.

2.4. Enzymes assay

Lichenase activity was determined using 0. 5 ml of 1% lichenan dissolved in phosphate citrate buffer (50 mM, pH 5.0) to which 0.5 ml of the appropriately diluted enzyme solution was added. The release of reducing sugars after 30 min incubation at 50 °C was measured as glucose equivalents by the dinitrosalicylic acid method (DNS) [25]. One unit of β-glucanase was defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per min.

2.5. Activation of clay

Activation of clay was done as reported previously by Chang and Juang [19]. Fifty grams of the nanoclay were activated by refluxing with 250 ml of 1 M H_2SO_4 at 80 °C for 2 h in a round-bottom flask. The slurry was then air-cooled and filtered through a glass fiber. The filter cake was repeatedly washed with deionized water until the filtrate was neutral.

2.6. Preparation of chitosan–clay composite beads

Chitosan flakes (1 g) and activated clay (1 g) were dissolved in 1 M acetic acid (100 ml) and agitated with a disperser (IKA, Ultra-Turrax T25 basic) at 24,000 rpm for 10 min. The yielded viscous solution was placed in a vacuum dryer for 3 h to remove air bubbles, and then was sprayed drop-wise through a syringe, at a constant rate, into a neutralizing solution containing 15% NaOH and 95% ethanol in a volume ratio of 4:1. The beads were left in solution for 1 day. The prepared beads were washed with deionized water until the solution was neutral.

2.7. Crosslinking of chitosan–clay composite beads by glutaraldehyde

An aliquot of wet composite beads (0.05 g) were placed in a 100 ml vessel containing 50 ml of 1% glutaraldehyde. The solution was agitated at 150 rpm and 30 °C. After crosslinking for 2 h, the beads were washed thoroughly deionized water and stored at 4 °C in phosphate buffer (0.05 M, pH 7.0).

2.8. Immobilization of lichenase of P. occitanis (Lic-Pol6) on crosslinked chitosan–clay composite beads

0.05 g cross linked composite beads was contacted with 2 ml of 1 mg/ml Lic-Pol6 in a shaker for 4 h at 4 °C. Then the beads were filtered and the volume of filtrate recorded. The filtrate was collected and used for loading efficiency determination. After washing with phosphate buffer (0.05 M, pH 7.0) three times, the enzyme immobilized composite beads were stored at +4 °C for further use.

Activity immobilization yield represents per cent of the ratio of enzyme activity of the immobilized enzyme to the total units of soluble enzyme used for immobilization. This parameter, although theoretically of not much significance, is of practical interest and it should be as high as possible when industrial application is contemplated [26].

The immobilization yield (IY%) of enzyme is defined as follows:

$$\text{Immobilization yield} = (A - B)/A \times 100$$

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

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

As the equations above indicate, various parameters were used in the estimation of immobilization: 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 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.

2.9. Effect of pH and temperature on the activity and stability of the enzyme

The optimum pH of the free and immobilized Lic-Pol6 was determined at 50 °C by incubating enzymes at different pH (1.0–8.0) for 30 min. The pH stability of native and immobilized Lic-Pol6 was