Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Optimization of encapsulation of a microbial laccase enzymatic extract using selected matrices

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ARTICLE INFO

Keywords:
Laccase
Encapsulation
Encapsulation efficiency
Enzyme stability
Re-usability

ABSTRACT

The encapsulation of the laccase extract, obtained from *Coriolus hirsutus*, was investigated using selected matrices, including alginate, alginate-silica and silica sol-gel. Two methods, M1 and M2, were carried out for the hybridization of alginate with silica. The experimental findings indicated that the incorporation of silica into alginate, using M2, resulted by a higher (70%) encapsulation efficiency (EE) for the laccase extract as compared to that for the alginate (59%) alone. Furthermore, the encapsulation of the laccase extract in sol-gel resulted in an enhancement of its catalytic activity, with a concomitant increase (90%) in the EE. The alginate and sol-gel matrices also enhanced the laccase catalytic efficiency as compared to that for the free one, with a k_{cat} of 89.9, 63.7 and 56.9 min^{-1}, respectively. The protein release profiles indicated that the laccase was more effectively confined in the sol-gel and alginate-silica M2 as compared to that for the alginate and alginate-silica M1. In addition, the laccase extract in sol-gel exhibited an enhanced re-usability, with a relative residual enzyme activity of 82.7%, after 5 continuous reaction cycles. The alginate, alginate-silica M2 and sol-gel matrices demonstrated complete storage stability at 4 °C, without any loss in laccase activity after 6 weeks of storage.

1. Introduction

Laccases (EC 1.10.3.2) are multi-copper proteins that catalyze the oxidation of various aromatic and non-aromatic compounds, using molecular oxygen as a co-factor [1]. The occurrence of laccases in nature is abundant, where they have been found in several species of fungi, bacteria and higher plants; laccases are mainly associated with delignification, pigment formation and detoxification [2]. Even though laccases show a wide range of substrate specificity, their use in various industrial applications was often hindered by their high production costs and poor stability, as well as difficulties in their recovery and re-usability [2,3]. Nevertheless, such limitations could be overcome by the use of suitable methods of immobilization, which are aimed at a more efficient and economical use of the enzymes [4]. The immobilization of laccase has been reported extensively, using different methods and supports [5,6], where the encapsulation of enzymes in a semi-permeable matrix may be considered as one of the most appropriate approaches, as it offers relatively mild process conditions and little or no effect to their native structure [7].

Among the various matrices used for the encapsulation of laccase, alginate is one of the most commonly employed biopolymers, owing to its mild gelling properties and non-toxicity; however, the encapsulation in alginate gels is mostly associated with a lower stability and an uncontrolled porosity [8]. In order to develop more robust and stable matrices, the incorporation of silica into alginate has been investigated for the encapsulation of biomolecules [8,9].

Recently, Mohidem and Mat [10] reported that the encapsulation of laccase in inorganic silica gels has been linked to an enhancement of its catalytic efficiency. In addition, the silica sol-gel matrices offer several other advantages, including non-toxicity, improved enzyme stability, optical transparency, enhanced re-usability and storage stability, as well as the possibility of tuning gel porosity [11,12].

The overall objective of this study was to investigate the encapsulation of an enzymatic laccase extract, from *Coriolus hirsutus*, in selected matrices, including alginate, alginate-silica and silica sol-gel. The matrices were assessed in terms of their encapsulation efficiency and residual laccase activity. In addition, the kinetic parameters, protein leakage, re-suability and storage stability of the entrapped laccase were also investigated.

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https://doi.org/10.1016/j.procbio.2017.11.011
Received 8 August 2017; Received in revised form 7 November 2017; Accepted 17 November 2017

2. Materials and methods

2.1. Materials

Coriolus hirsutus (MYA-828) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) via Cedarlane Labs (Burlington, ON). Ferulic acid, bovine serum albumin (BSA), tetraethyl orthosilicate (TEOS) (98% purity) and calcium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol was purchased from Commercial Alcohols Inc. (Branchville, QC). Sodium alginate and all organic reagents of HPLC grade or higher were obtained from Fischer Scientific (Fair Lawn, N.J.). Syringes and needles were purchased from BD Syringes and Needles (Mississauga, ON). All reagents and buffers were prepared in deionized water, using Milli Q plus (Millipore).

2.2. Production of Coriolus hirsutus biomass

Coriolus hirsutus was maintained on malt agar media plates and incubated at 20 °C as per the ATCC protocol. The production of the *Coriolus hirsutus* biomass was carried out according to the procedure described by Taqi [13]. The fermentation was initiated by the inoculation of the *C. hirsutus* on malt agar and used for the pre-culture. The pre-culture was incubated at 28 °C for 7 days, at 160 rpm following which; it was poured into fresh media and incubated under the same conditions for a period of 6 days.

2.3. Recovery and enrichment of the laccase enzymatic extract

The recovery and enrichment of the enzyme was performed as per Taqi [13]. Briefly, the mycelium pellets were filtered and laccase was recovered by ultra-filtration using a Prep/Scale TFF cartridge (Millipore) with polyethersulfone membrane of 10 kDa cut-off filter and a pressure of 10 psi. All protocols were performed at 4 °C and the enriched enzymatic extract was lyophilized in the presence of mannitol (2.5% w/v) and stored at −80 °C for further analysis.

2.4. Encapsulation of the laccase enzymatic extract in selected matrices

2.4.1. Encapsulation of the laccase extract in alginate matrix

The encapsulation of laccase extract in alginate was carried out according to the procedure described by Bou-Mitri [14], with certain modifications. The enzymatic laccase suspension (1 mg/mL protein) was prepared in glycine-sodium hydroxide buffer (0.2 M, pH 9.5) and mixed with an alginate solution of 1.5% (w/v), to a final protein to alginate ratio of 1:15 (w/w). The mixture was dropped into the calcium chloride solution (0.1 M) following which, the calcium alginate beads formed were hardened for 1 h at 4 °C, under continuous stirring. Subsequently, the encapsulated laccase was recovered by suction filtration on Whatman filter paper (No. 4), and washed with deionized water. The beads were then washed with acetone, dried using a gentle stream of nitrogen and stored at −20 °C for further assays. The filtered calcium chloride and the washing solutions were collected for protein determination.

2.4.2. Encapsulation of the laccase extract in alginate-silica matrix

Two methods were used to encapsulate laccase in alginate-silica matrix. The first method (M1) was carried out as described by Heichai-Segal et al. [15]. The calcium alginate beads containing laccase were prepared as described previously; however, the beads were recovered after 5 min and immediately added to a solution of hexane and the silica precursor (TEOS) (v/v). The incubation was carried out overnight at 4 °C, to allow the diffusion of silica precursor into the alginate gel, forming alginate-silica hybrids. The alginate-silica beads were then filtered and washed thoroughly to remove any unreacted silica.

The second method (M2) was performed as per the protocol outlined by Xu et al. [16], with certain modifications. The method involved the incubation of calcium alginate beads in a pre-hydrolyzed silica sol, which was prepared by the ultrasonication and vigorous stirring of TEOS (11 mmol), deionised water (22 mmol) and 0.04 M hydrochloric acid (HCl) (0.004 mmol), until the formation of a clear solution. The prepared alginate beads were recovered as described for M1 following which, they were added to the pre-hydrolyzed sol for 15 min. The cured beads were later recovered, washed and re-suspended in the calcium chloride solution for further hardening. All supernatants and washing solutions were collected for protein determination.

2.4.3. Encapsulation of the laccase extract in silica sol-gel matrix

The silica sol-gel encapsulation of the laccase extract was carried out as described by Mohidem and Mat [10,17], with modifications. A pre-hydrolyzed sol of TEOS, deionised water and HCl was prepared, as described previously. Laccase extract (1 mg protein/mL of silica sol) was suspended in potassium phosphate buffer (0.01 M, pH 6.0) and added to the silica sol. In addition, this was followed by an immediate addition of the potassium phosphate buffer (0.4 M, pH 7.0) to facilitate gelation and was continuously agitated, until the onset of gelation (1–3 min). The resulting gel was then washed, recovered and lyophilized at −42 °C for 6 h and stored under refrigeration to maintain its catalytic activity. All washing solutions were collected to analyze their protein content.

2.5. Protein determination

The protein content of the free enzyme extract and washing solutions was determined according to a modification to the Lowry method [18], using bovine serum albumin (BSA) as a standard for the calibration curve.

2.6. Enzyme activity of the encapsulated laccase extract

The enzyme activity assay for both free and encapsulated laccase was carried out according to Bou-Mitri [14]. A sample of free or encapsulated laccase extract was suspended in sodium acetate buffer (0.1 M, pH 5.0), and the enzyme activity was measured following the oxidation of ferulic acid (30 mM) as a substrate. The decrease in the absorbance at 320 nm was measured spectrophotometrically (Beckman Instruments Inc., San Raman, CA). The specific activity was defined as μmol of converted ferulic acid/mg protein/min at selected time intervals (0–10 min). The enzymatic bioconversion was carried out at 50 °C under continuous stirring at 150 rpm. All laccase assays were performed in triplicate trials and were run simultaneously with blank reaction containing everything except the enzyme.

The Michaelis-Menten parameters, K_m and V_{max} of the free and encapsulated laccase extract were determined from the Lineweaver-Burk plots and the catalytic efficiency, k_{cat} was calculated from the ratio of V_{max}/K_m. Plots were constructed by measuring laccase activity within a range of ferulic acid concentrations (0–50 mM). All activity assays were conducted by means of triplicate trials, under the standard conditions, described earlier.

2.7. Characterization of the encapsulated laccase enzymatic extract

2.7.1. Encapsulation efficiency (EE) and relative (%) of residual laccase activity

The encapsulation efficiency (EE) for the alginate and alginate-silica beads was quantified by the difference between the total initial added protein to the pre-gel solution, and the sum of total released protein into the calcium chloride bath and the washing solutions. In contrast, the EE for the sol-gel matrix was defined as the difference between the total added initial protein to the silica sol and the determined protein in the recovered washing solutions, respectively. The encapsulation efficiency (%) and the residual enzyme activity (%) were calculated as follow: