Food Hydrocolloids 61 (2016) 191–200

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Chitosan beads from microbial and animal sources as enzyme supports for wine application

Ilaria Benucci [a], Claudio Lombardelli [a], Ilaria Cacciotti [b, *], Katia Liburdi [a], Francesca Nanni [c], Marco Esti [a]

[a] Dept. for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Via S. Camillo de Lellis, 01100 Viterbo, Italy
[b] Dept. of Engineering, University of Rome "Niccolò Cusano", INSTM RU, Via Don Carlo Gnocchi, 3, 00166 Rome, Italy
[c] Dept. of Enterprise Engineering, University of Rome "Tor Vergata", INSTM RU "Rome-Tor Vergata", Via del Politecnico, 1, 00133 Rome, Italy

ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form
12 May 2016
Accepted 14 May 2016
Available online 15 May 2016

Keywords:
Chitosan beads
Aspergillus niger
Stem bromelain
Enzyme immobilization
White wine
Packed-bed reactor

ABSTRACT

Chitosan beads from a microbial source, i.e. *Aspergillus niger* (An), were produced by precipitation method for the first time and compared with supports prepared from an animal source, i.e. shellfish derived chitosan with three different molecular weights (low (LMW), medium (MMW) and high (HMW)). The produced beads were used as enzyme carriers to be applied in a continuous packed-bed reactor (PBR) for white wine protein stabilization.

For this purpose, the beads were crosslinked with glutaraldehyde (GDH) and stem bromelain was immobilized on the carrier surface as a model enzyme, following two different procedures (i.e., cross-linking with glutaraldehyde (GDH) and direct linkage (DL)).

Drop-like beads with an average diameter of 3.0–3.5 mm and a moisture content of 86–94% were obtained. The morphology of the produced beads in dried state, in terms of shape and surface, was studied by means of a scanning electron microscope (SEM), evidencing the obtainment of nearly spherical or oval particles. The efficacy of the crosslinking procedure and of bromelain immobilization was demonstrated by means of SEM investigation and infrared spectroscopy (FT-IR) analysis, revealing a rougher surface. Various initial protein concentrations ranging between 0.45 and 18.00 mg_{BSAeq} mL^{-1} were tested in order to identify the optimal amount and to evaluate the influence of the initial concentration on the total protein loading.

Stem bromelain proved to be more active when immobilized by DL on An beads and was efficient in reducing white wine hazing potential continuously, as verified with a laboratory bench-scale PBR.

1. Introduction

Chitosan (CS), a cationic polymer combined by β 1-4 glicosidic linkage, is obtained from chitin, a major structural component of the invertebrates exoskeleton and of the fungi cell wall (Santos, Veiga, Pina, Podczeck, & Sousa, 2002). Commercially available CS is usually produced from shrimp and crabshell chitin by N-deacetylation, using strong alkali. There are various limitations in using this procedure industrially: (i) the seasonality in resource availability, (ii) the high processing costs, (iii) the complexity of the procedure, (iv) the varying physico-chemical characteristics of CS (White, Farina, & Fulton, 1979). Moreover, the presence of shrimp antigen as well as heavy metal contaminants (nickel and copper) in the final product could cause allergic reactions, as described by other authors (Tan, Tan, Wong, & Khor, 1996). In order to overcome these limitations, the production of CS from fungal mycelia is an alternative which enables to obtain a CS free of heavy metals and standardized in terms of acetylation degree, molecular weight, viscosity, charge distribution (Nwe, Furuike, & Tamura, 2010).

In the food industry CS appears to be a good support for enzyme immobilization (Shellfish Wastes; Talbert and Goddard, 2012), since it is non toxic, user-friendly, available in various forms (powder, gel, fibers and membranes) and demonstrating high protein affinity (Zappino et al., 2015). CS beads, which vary in molecular weight and deacetylation degree, generally prepared by the precipitation method, have been widely studied as carriers for enzyme immobilization (Biró, Németh, Sisak, Feczkó, & Gyenis, 2008; Krajewska, 2004).

* Corresponding author.
E-mail address: ilaria.cacciotti@unicusano.it (I. Cacciotti).

http://dx.doi.org/10.1016/j.foodhyd.2016.05.016
0268-005X/

In this work, CS beads from a microbial source, i.e. *Aspergillus niger* (An), were produced for the first time by precipitation method, and compared with CS beads prepared from shellfish CS powder with three different molecular weight polymers (i.e. low (LMW), medium (MMW) and high (HMW)). In fact, at present the European food law only permits the application of microbial CS from *Aspergillus niger* in the food industry (REG. EC No. 606/2009). CS membrane based on chitosan from *A. niger* (An) was obtained for the first time as immobilization support by Zappino et al. (2015).

The aim of this study was to produce biopolymeric enzyme carriers with optimal properties to be applied in a packed-bed reactor (PBR), for white wine protein stabilization. To date there is still a need to develop specific and effective methods alternative to the addition of fining agents (i.e. bentonite), which represents the common but unsustainable enological practice to achieve stable white wines. The enzymatic hydrolysis of wine proteins via proteases could represent a useful and specific tool to prevent haze formation in white wine, without affecting the organoleptic properties (Benucci et al., 2016). Among proteases, bromelain from pineapple stem, categorized as a food additive by the U.S. Food and Drug Administration and included in the list of substances generally recognized as safe (U.S. Food and Drug Administration, 2016), was tested.

In order to achieve this purpose, the obtained CS beads were used as supports for immobilizing pineapple stem bromelain using glutaraldehyde (GDH) as cross-linker, or by direct linkage. Their thermal properties were studied by differential scanning calorimetry (DSC) before and after crosslinking with GDH. The functional chemical groups were detected by infrared (FT-IR) spectroscopy measurements. Moreover, the morphology of the produced carriers was analyzed by means of a scanning electron microscope (SEM) before and after enzyme immobilization, in order to evaluate the influence of the surface topography on immobilization efficiency. The proteolytic activity of the biocatalysts was evaluated and the best biocatalyst was selected for white wine protein stabilization treatment in laboratory bench-scale PBR.

It is important to highlight that, to the best of our knowledge, microbial CS has not yet been used for the production of spherical supports to be applied in a continuous bioreactor.

2. Materials and methods

2.1. Materials

Stem bromelain (EC 3.4.22.32) was provided by Sigma Aldrich (Milan, Italy). Beads as supports for enzyme immobilization were produced using CS powders obtained from 2 different sources: i) microbial from *A. niger* (An; Lot#12121611L4; percentage of deacetylation: 70%, supplied by KitoZyme, Herstal, Belgium); ii) animal, consisting in shellfish polymers with three different molecular weights (i.e., low 50–190 kDa (LMW; Lot#MKBG3334V; percentage of deacetylation: 75%), medium 190–310 kDa (MMW; Lot#MKBH1108V; percentage of deacetylation: 75–85%) and high 310–375 kDa (HMW; Lot#MKBF9232V; percentage of deacetylation: 75%), supplied by Sigma Aldrich, Milan, Italy).

The selected synthetic tripeptide chromogenic substrate, Bz-Phe-Val-Arg-*p*-nitroaniline (pNA), was purchased from Bachem (Bubendorf, Switzerland). All the other chemicals were analytical grade (Sigma Aldrich, Milan, Italy). The unfined Sauvignon blanc wine (pH: 3.3; total acidity: 5.9 $g_{tartaric\ acid}\ L^{-1}$; L(−)-Malic acid: 0.9 g L^{-1}; ethanol 12.9% v/v; total SO_2 83 mg L^{-1}; free SO_2 13 mg L^{-1}; total phenols 141 $mg_{gallic\ acid\ eq}\ L^{-1}$), produced during the 2014 vintage, was supplied by the winery Casale del Giglio (Le Ferriere (LT), Italy).

2.2. Chitosan based supports preparation and characterization

CS beads were prepared according to the precipitation method (Biró et al., 2008). Animal (5 w/v %) and microbial ((15 w/v %) CS powders were dissolved in an aqueous solution of acetic acid (2 v/v %). The obtained solutions were added dropwise through a capillary into a gently stirred coagulation liquid (2 N sodium hydroxide and 26 v/v % ethanol). The obtained macrospheres were filtered and washed with distilled water until neutrality.

2.2.1. Moisture content

The moisture content was determined immediately after CS beads making. Approximately 10 g were dried at 90 °C for 24 h in a hot air oven (Memmert, Germany) for each CS carrier. The moisture content (MC, %) was calculated according to the Eq. (1), where m_d is the dried mass and m_w is the wet mass:

$$MC(\%) = \frac{m_w - m_d}{m_d} \times 100 \quad (1)$$

In order to obtain an average value, the experiment was repeated three times for each CS sample.

2.2.2. Spectroscopic characterization and morphology

Infrared spectra (Fourier Transform Infrared Spectroscopy, FTIR Perkin Elmer) were recorded in the region 400–4000 cm^{-1} using KBr pellets (1%wt/wt), spectral resolution of 4 cm^{-1}, scans number 32. The normalization of the acquired spectra was obtained by assigning a 100% value to the most intense peak.

The morphology of the CS beads was analyzed by observation at field-emission gun scanning electron microscope (FEG-SEM, Cambridge Leo Supra 35, Carl Zeiss), after drying at 40 °C for 48 h in an oven. The dried samples were analyzed after sputter-coating with gold under argon atmosphere (25 mA, 120 s).

2.2.3. Thermal properties

The thermal properties of the prepared beads, before and after crosslinking with GDH, were investigated by means of differential scanning calorimetry (DSC, TAInstruments Q2000) in the following conditions: sample weight ~5 mg, nitrogen atmosphere (N_2 flow rate 50 cc min^{-1}), range of temperature 20–400 °C, heating rate 10 °C/min.

2.3. Enzyme immobilization

Stem bromelain was immobilized on CS beads by applying two previously mentioned procedures (Benucci, Esti, Liburdi, & Garzillo, 2012): i) direct immobilization linkage (DL) which involves the protease carboxyl groups of Asp or Glu residues and the amino groups of the support (Benucci et al., 2012) and ii) cross-linking using glutaraldehyde (GDH) through the Schiff's base formation (Çetinus & Öztop, 2003).

The cross-linking procedure was performed by dipping 1 g of beads into 10 mL of glutaraldehyde solution 3% (v/v), maintaining the obtained suspension under constant agitation for 1 h at room temperature, and then profusely washing it with distilled water.

The coupling of the enzyme was carried out by immersing the unmodified or activated beads in stem bromelain solutions, which were prepared by adding appropriate amounts of enzyme powder to the immobilization buffer (tartaric acid/sodium tartrate 0.03 M, pH 3.2).

Different initial protein concentration in the range of 0.45–18.00 $mg_{BSAeq}\ mL^{-1}$ were tested. The components were shaken (150 rpm) overnight at 20 °C; the CS beads with immobilized bromelain were then filtered and washed thoroughly with 2 M NaCl in order to