Results in Chemistry 6 (2023) 101009

Contents lists available at ScienceDirect

Results in Chemistry

journal homepage: www.sciencedirect.com/journal/results-in-chemistry

Immobilization on biomimetic silica nanoparticles as a highly effective strategy for the stabilization of *Issatchenkia orientalis* β-glucosidase in wine conditions

Stefani de Ovalle, Beatriz M. Brena, Paula González-Pombo *

Área Bioquímica, Departamento de Biociencias, Facultad de Química, Universidad de la República, General Flores 2124, CC1157 Montevideo, Uruguay

ARTICLE INFO

Keywords:
Biomimetic nanoparticles
Enzyme stabilization
Enzyme immobilization
β-Glucosidases

ABSTRACT

β-Glucosidases (BGLs) are key enzymes for the hydrolysis of glycosidic aroma precursors of wines. However, BGLs produced by oenological yeasts are often inactive or unstable at typical wine pH values. In this work, different immobilization strategies on biomimetic NPs were tested to enhance the stability of *Issatchenkia orientalis* BGL and compared with traditional methods on agarose and Eupergit. Immobilization to biomimetic silica NPs by ionic interactions proved to be the most efficient method to achieve enzyme stabilization in model wine at pH 4.0. In particular, the subsequent glutaraldehyde crosslinking, achieved a high immobilization efficiency (71%) with an outstanding stabilization factor of 240-fold with respect to the soluble enzyme. This enzyme derivative preserved practically all the activity (98%) after15 days in model wine supplemented with Muscat glycosides, making possible its application for the release of aroma compounds relevant to the typicity of wine.

Introduction

β-Glucosidases (BGLs) are widely distributed among various organisms. In oenological environments they are present in grapes (Vitis vinifera), bacteria (mainly of the genus *Oenococcus*), filamentous fungi and yeasts, mostly in non-*Saccharomyces* and a few *Saccharomyces* species. They play a crucial role in the hydrolysis of non-volatile glycosidic aroma precursors in fruit juices, must, and wines [1]. Although the addition of commercial enzymes from fungal origin is a common practice in winemaking, the multiple enzyme activities from these preparations can lead to a loss of typicity [2]. To develop the varietal aroma profile of wines it is essential to select BGLs with specificity towards the target aroma precursors. However, most BGLs from oenological yeast show very limited activity during winemaking due to inhibition by ethanol and glucose, as well as poor activity and stability at wine pH values [3]. Therefore, the selection of strains producing BGLs with optimal activity and specificity for aroma development is a challenging task in enology [4–6]. The *Issatchenkia orientalis* BGL, isolated from Tempranillo grapes of Uruguay vineyards, exhibits promising specificity properties for oenology. The enzyme was active towards norisoprenoids, terpenes, and phenol-glycosides, giving rise to fruity, dried raisin, honey, and spice notes [7,8]. However, it is rapidly inactivated at the acidic pH of wine. In order for it to be suitable for oenological applications, it is necessary to improve its activity and stability under acidic conditions.

Immobilization onto solid supports is a simple and widely used alternative to improve the properties of biocatalysts, particularly for large-scale industrial processes. Among other advantages, enzyme immobilization has the potential to enhance enzyme stability, increase resistance to proteolysis, improve control of the process and biocatalyst reuse [9,10]. To obtain highly active and stable biocatalysts, new immobilization technologies and carrier materials have been developed such as biomimetic silica-based Nanoparticles (NPs) [11]. This technology is simple, rapid, inexpensive and uses non-toxic, mild chemistry [12]. Therefore, the main objective of the present work is to evaluate the efficacy of the biomimetic silica NPs, to improve *I. orientalis* BGL stability in stringent conditions. The results were compared with traditional immobilization methods on MANAE-agarose and Eupergit.

Materials

The enzyme substrate *p*-nitrophenyl-β-D-glucopyranoside (pNPG), tetramethyl orthosilicate (TMOS), cellulose membranes, polyethylenimine (PEI), glutaraldehyde and ethylenediamine (EDA) were

* Corresponding author.
E-mail address: pgonzale@fq.edu.uy (P. González-Pombo).

https://doi.org/10.1016/j.rechem.2023.101009
Received 23 March 2023; Accepted 12 June 2023
Available online 20 June 2023

supplied by Sigma-Aldrich (St. Louis, MO, USA). EUPERGIT C was kindly donated by Röhm Pharma (Darmstadt, Germany). Bradfords reagent was purchased from Bio-Rad laboratories (Richmond, CA, USA). PD-10 desalting column was purchased from GE Healthcare Chicago (Illinois). All other chemicals used were of reagent grade. *Issatchenkia orientalis* isolated from grape must was supplied by the Laboratorio de Enología (Facultad de Química, Montevideo- Uruguay). All other chemicals used were of reagent grade.

Methods

Culture conditions

The growth of *I. orientalis* strain was carried out for 72 h in modified YPG medium (25 g/L yeast extract, 1.0 g/L peptone and 8.0 mL/L glycerol). Cultures were carried out in Erlenmeyer flasks at 10% volume, at 28 °C and shaking at 180 rpm. The culture was centrifuged (5,000 xg) during 5 min at 4 °C and the BGL activity was determined in the supernatant [7].

Enzyme extract preparation

The culture supernatant was precipitated with ammonium sulfate (70% saturation) and centrifuged (5,000 xg) during 30 min at 4 °C. The resulting precipitate was dissolved in 0.1 M of sodium acetate buffer, pH 4.5 (activity buffer). The enzyme extract was dialyzed through cellulose membranes with a cut off of 14,000 Da. Finally, the enzyme extract was subsequently gel-filtered using a PD10 column and adjusted the desire pH conditions for immobilization (containing 0.1 EU/mL and 0.9 mg/mL of protein).

Protein and enzyme assay

Protein was quantified by Bradford method [13] and the BGL activity was determined spectrophotometrically using 10 mM of pNPG in 0.1 M sodium acetate buffer at pH 4.5 (activity buffer), at 23 °C. A volume of 100 μL of soluble or immobilized enzyme suspension were added to 1.25 mL of 10 mM pNPG as reported in [8]. One unit of enzyme activity (EU) was defined as the amount of enzyme that releases 1.0 μmol *p*-nitrophenol/min under the specified enzyme assay conditions.

Immobilization by entrapment in silica NPs

A silicic acid solution was prepared by hydrolyzing tetramethyl orthosilicate (TMOS, 1.0 M) in 1.0 mM hydrochloric acid. For entrapment, 0.25 mL of a 10 % v/v polyethylenimine in water, were mixed with 0.25 mL of hydrolyzed TMOS and 2.0 mL of the dialyzed enzyme extract at pH 8.0. The mixture was agitated for 10 min and centrifuged for 1 min at 1,300 xg.

Synthesis of silica NPs

Silica NPs were synthesized by mixing 1.0 mL of 0.1 M sodium phosphate buffer pH 8.0, with 0.25 mL of 10% v/v of PEI solution and 0.25 mL of hydrolyzed TMOS. The silica NPs were centrifuged for 1 min at 1,300 xg [14].

Ionic immobilization on silica NPs

A volume of 2.0 mL of the dialyzed extract at pH 4.5 and 1.0 g of silica NPs were incubated for 30 min, at 23 °C with soft shaking. The silica NPs after immobilization were centrifuged for 1 min at 1,300 xg [14].

Glutaraldehyde crosslinking

One gram of ionic NPs derivative was incubated with 4.0 mL of 0.5% v/v glutaraldehyde solution in 25 mM sodium phosphate buffer pH 7.0. and gently shaken for 1 h at 23 °C. After exhaustive washing with the same buffer the derivative was kept for 16 h at 4 °C. Subsequently, it was washed with activity buffer.

Covalent immobilization on silica NPs

One gram of silica NPs were incubated with 2.0 mL of a 5.0 % v/v of glutaraldehyde solution in 0.2 M sodium phosphate buffer pH 7.0. The resulting NPs were exhaustively washed. Two milliliters of the dialyzed enzyme extract in 25 mM sodium phosphate pH 7.0 were added to 1.0 g of activated silica NPs and gently shaken for 5 h at 23 °C. Subsequently, it was centrifuged for 1 min at 1,300 xg and the enzyme derivative obtained was washed with activity buffer.

Desorption of the enzyme

One gram of ionic NPs enzyme derivatives were incubated during 15 days at 23 °C in 10 mL of model wine at pH 4.0 (ethanol–water 12:88 v/v containing 3.5 g/L of tartaric acid, 2.5 g/L of malic acid and 60 mg/L of sodium metabisulfite). The released protein was measured in supernatants.

Covalent immobilization on epoxy-activated support

A fraction of the culture supernatant precipitated with ammonium sulfate (70% saturation) and centrifuged, was dissolved in 2.0 mL of 1.0 M sodium phosphate pH 7.0 (containing 0.1 EU/mL and 0.9 mg/mL of protein) and incubated with one gram of filter dried Eupergit C during 24 h at 4 °C.

The remaining epoxy groups were blocked with 3.0 M of glycine, pH 8.0, overnight at 4 °C and washed with distilled water followed by activity buffer.

Ionic immobilization on MANAE-agarose

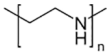
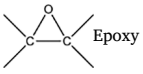
Glyoxyl-agarose, prepared as previously reported [15] was incubated with 1.0 M ethylenediamine solution at pH 10 [16]. The suspension was stirred for 2 h, filtered and washed sequentially with: 100 mM sodium acetate buffer at pH 4.0, sodium borate buffer at pH 9.0, and distilled water. One gram of filter-dried support was incubated with 2.0 mL of dialyzed enzyme extract in 10 mM sodium acetate pH 5.5 for 1 h at 23 °C and the enzyme derivative was washed with activity buffer.

Results and discussion

The *I. orientalis* strain used in this work was selected due to its high production of an extracellular β-glucosidase at pH 4.0 [7]. The Molecular weight of the enzyme is about 58 kDa and, like most glycosidases, it has an acidic isoelectric point (pI = 4.0). In addition to its interesting specificity properties, the enzyme displays a high tolerance to the typically high concentrations of glucose and ethanol of must and wines [7]. These exceptional characteristics would make it an attractive option in the winemaking industry. However, its low stability at acid pH (half-life at pH 4.0 = 1.5 h) limits its direct use in oenology, since the wine pH typically falls within the range of 2.5–4.0 [17].

In this work three different immobilization strategies were explored for optimizing the activity and acid pH stability of *I. orientalis* BGL, using the recently developed technology of biomimetic silica NPs (Table 1) [18]. The first strategy involves entrapping the enzyme within biomimetic silica NPs by incubating the enzyme in the mixture of TMOS and PEI (Entrapped-NPs). The second strategy involves chemically functionalizing the pre-formed surface of Silica NPs with glutaraldehyde

Table 1
Strategies and mechanisms of immobilization.

Support	Immobilization strategy	Reactive groups in the supports	Mechanism
Silica NPs	Entrapment (Entrapped-NPs)	———	The enzyme is added to the reactive mixture containing the necessary components to synthesize the support, remaining trapped
	Covalent (NPs-Glut)	Activation of the support with glutaraldehyde –N = CH–$(CH_2)_3$-CHO	Schiff base formation between exposed amino groups on the enzyme surface and aldehyde functional groups in the support
	Ionic (NPs-Ionic)	Functionalized with amino groups of polyethyleneimine 	Ionic interaction of negatively charged groups on the enzyme surface with amino groups in the support
	Ionic and glutaraldheyde crosslinking (NPs-Ionic-Glut)		In two stages: in the first, the enzyme is immobilized in the support in ionic form. The immobilized enzyme is then treated with a crosslinking agent (e.g., glutaraldehyde).
Derivatized Agarose (MANAE-Agarose)	Ionic (MANAE-Agarose)	Amino groups resulting from activation of agarose with glycidol and ethylenediamine –$NH3^+$	Ionic interaction of exposed negatively charged groups on the enzyme surface with positively groups in the support
Methacrylamide copolymer (Eupergit C)	Covalent (Epoxi-Eupergit C)	 Epoxy	In two steps: 1) hydrophobic adsorption of the enzyme to the support 2) covalent binding of the enzyme to the support, through exposed -SH, –OH, $–NH_2$ groups

(NPs-Glut) to enable covalent protein attachment to the aldehyde groups. The third strategy makes use of ionic interactions (NPs-Ionic) between the amino groups of the previously synthesized NPs from the PEI and the negatively charged groups on the enzyme [11]. To enhance the stability of the enzyme, the ionic-bound derivative was subsequently treated with glutaraldehyde (NPs-Ionic-Glut) to generate intra- and intermolecular enzyme-support crosslinks [19]. These immobilization strategies on silica-based NPs were compared with two traditional immobilization methods: ionic bonding to agarose-bound-ethylenediamine groups (MANAE-Agarose) and covalent binding to an epoxy-activated support (Epoxi-Eupergit C) (Table 1). In the Epoxi-Eupergit C derivative, the epoxy groups react with different nucleophilic groups present on the enzyme surface, forming multipoint covalent attachments which are known to promote conformational stability of enzymes [20].

The results from Table 2 show that the enzyme was efficiently immobilized with practically all the multiple methodologies and supports tested. Both covalent techniques (Epoxi-Eupergit C and NPs-Glut) showed very high activity yields (close to 80%) and almost all immobilized enzyme remained active (immobilization efficiency 96–99%). However, in the case of the Epoxi-Eupergit C derivative, blocking of remaining epoxy groups retained only 63% activity and there was no stabilization. On the other hand, the enzyme bound to glutaraldehyde-activated silica NPs showed a seven-fold increase in enzyme stability compared to the soluble enzyme.

The entrapment into silica NPs was the only approach that resulted in very low protein yields (10%) and expressed activity (7%). Most probably the entrapment conditions could be optimized by adjusting the concentrations and the ratio of TMOS to PEI. The TMOS plays a critical role in the structure of the silica NPs support and a higher concentration of PEI could enhance enzyme adsorption [11]. However, as described below, due to the outstanding activity and stability achieved through the NPs-ionic strategies, we have not prioritized further optimization of the Entrapped-NPs derivative.

Ionic adsorption proved to be the most effective method for immobilizing *I. orientalis* BGL, with activity yields ranging from 94% to 99% for MANAE-agarose and NPs-Ionic derivatives, respectively (Table 2). It appears that the acidic nature of the enzyme allowed for efficient immobilization at pH 4.5 onto silica NPs support. Even at 0.5 pH units above its pI, the enzyme retains sufficient negatively charged carboxylic groups to bind to the positively charged amino groups on the support. Practically all the enzyme bound to the MANAE-Agarose was active, showing the highest immobilization efficiency (98%). However, this derivative was not stabilized in model wine at pH 4.0. On the other hand, the BGL bound to NPs-Ionic support showed both a very high immobilization efficiency (84%) with an excellent stabilization factor of 64-fold. The high stabilization observed in biomimetic silica supports can be attributed to the nearby rigid environment, which reduces the flexibility of immobilized proteins and improves enzyme stability [18]. The subsequent crosslinking of the NPs-Ionic derivative with glutaraldehyde, used as a strategy to further enhance biocatalyst stability, decreased the immobilization efficiency to 71%. This result agrees with previous reports which show that the enzymatic activity may be affected by glutaraldehyde, due to a possible distortion of the enzyme structure [21]. Despite the 13% decrease in expressed activity, the remarkable stabilization factor of 240 far outweighs the loss.

When NPs-Ionic support was exposed to model wine at pH 4.0 for 15 days, it had a more relevant protein leakage (20%) than the derivative subsequently treated with glutaraldehyde (10%). While both derivatives showed some leakage, the most significant difference between them lies in the stability of the immobilized enzyme. As shown in Fig. 1, the remaining enzyme activity bound to the supports was gradually decreasing with the incubation time. After the 15-day period, a clear distinction was observed between the two derivatives. The NPs-Ionic derivative retained only 2% of its initial activity, whereas the NPs-Ionic-Glut derivative showed a much higher preservation rate of 48%. The results suggest that the glutaraldehyde crosslinking rigidifies the protein structure and strengthens the enzyme binding to the support, leading to a better preservation of the bound activity [21], even in the harsh environmental conditions of model wine. Consistently with the protective effect of the substrates on enzyme activity, the addition of wine glycosides resulted in significant improvements in stability. After a 15-day period, nearly all the expressed activity (98%) remained intact (Fig. 1). In the case of the most stable derivative (NPs-Ionic-Glut), the impact of Muscat wine glycosides was studied by de Ovalle et al., 2021. Those results suggest that the NPs-Ionic-Glut derivative shows potential in winemaking, to promote the liberation of the following specific aglycones: norisoprenoids (3-oxo-α-ionol, vomifoliol), terpenes (*cis*-8-hydroxylinalool) and phenols (4-vinylguaiacol), as previously reported

Table 2
Immobilization strategies of *I. orientalis* BGL and enzyme stabilization in model wine at pH 4.0.

Immobilization technique	Support	Activity Yield (%)	Expressed Activity (EU/g support)	Immobilization efficiency (%)	Protein yield (%)	Stability factor
Covalent	Methacrylamide activated with epoxy groups (Epoxi-Eupergit C)	79 ± 4	Prior to blocking: 0.1 After blocking: 0.06	Prior to blocking: 99 ± 2 After blocking: 63 ± 3	82 ± 3	1
	Silica NPS activated with glutaraldehyde (NPs-Glut)	80 ± 5	0.09	96 ± 5	83 ± 5	7
Trapping	Into sílica NPs (Entrapped-NPs)	7.0 ± 0.5	0.02	99 ± 1	10 ±0.1	–
Ionic	Agarose activated with glycidol and ethilendiamine (MANAE-Agarose)	94 ± 6	0.11	98 ± 3	80 ± 5	1
	Silica NPs (NH_3 groups) (NPs-Ionic)	99 ± 1	0.10	84 ± 3	84 ± 2	64
	Silica NPs crosslinked with glutaraldehyde(NPs-Ionic-Glut)	99 ± 1	0.09	71 ± 3	84 ± 2	240

Activity yield = [(applied EU– EU in percolate and washing)/applied EU] * 100.
Expressed activity = EU attached/ gram of support.
Immobilization Efficiency = [expressed EU/ (applied EU– EU in percolate and washing)] *100.
Protein yield = [(mg applied protein– mg of protein in percolate and washing)/ mg of applied protein]*100.
Stability factor in model wine at pH 4.0 at room temperature = half-life (t ½) of the immobilized derivative/ t ½ soluble enzyme.
Applied enzyme = 0.2 EU (1.8 mg protein) per gram of support.

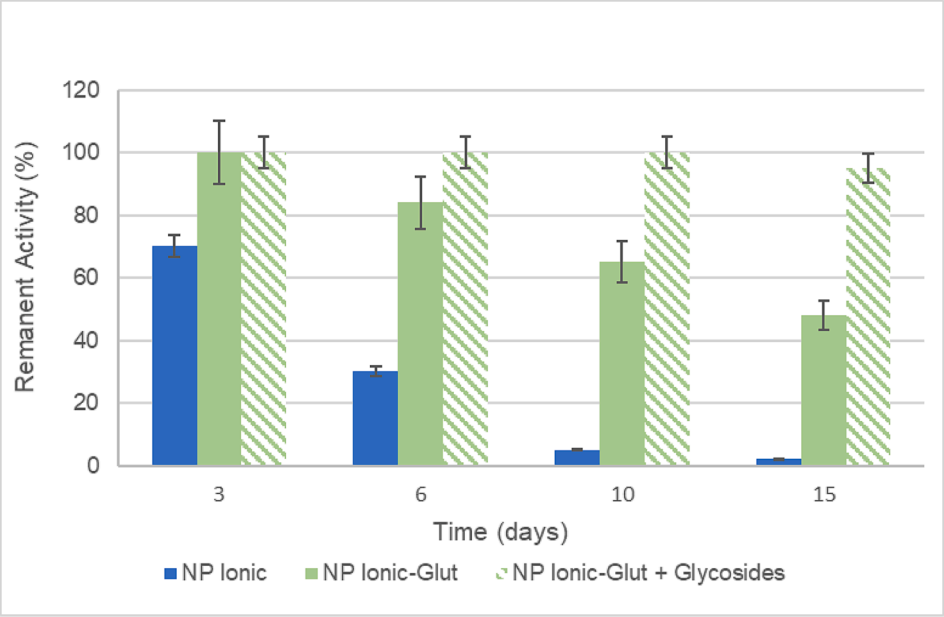


Fig. 1. Stability of β-glucosidase in model wine at pH pH 4.0 at room temperature.

[8].

Conclusions

In summary, immobilization to biomimetic silica NPs proved to be a very valuable strategy to achieve enzyme stabilization in model wine at pH 4.0. In particular, the enzyme immobilized through ionic interactions to the silica NPs and subsequently crosslinked with glutaraldehyde combines remarkable properties: high immobilization efficiency with an outstanding stabilization. The enzyme derivative displayed exceptional stability in the presence of Muscat wine glycosides, maintaining its activity for 15 days in model wine at pH 4.0. This remarkable feature makes it a promising candidate for applications in the wine industry, promoting the release of varietal aroma compounds.

CRediT authorship contribution statement

Stefani de Ovalle: Methodology, Investigation, Formal analysis. **Beatriz M. Brena:** Conceptualization, Project administration, Writing – review & editing. **Paula González-Pombo:** Supervision, Conceptualization, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work had the financial support of Program for the Development of Basic Sciences (PEDECIBA-Química) and Agencia Nacional de Investigación´ e Innovación´ (ANII), POS_NAC_2014_1_102369, Comisión Académica de Posgrado (CAP-UdelaR).

References

[1] J. Liu, X. Zhu, N. Ullah, Y. Tao, Aroma Glycosides in Grapes and Wine, J. Food Sci. 82 (2017) 248–259, https://doi.org/10.1111/1750-3841.13598.

[2] G. Spagna, R.N. Barbagallo, E. Greco, I. Manenti, P. Giorgio, A mixture of purified glycosidases from Aspergillus niger for oenological application immobilised by inclusion in chitosan gels, Enzyme Microb. Technol. 30 (2002) 80–89, https://doi.org/10.1016/S0141-0229(01)00455-0.

[3] R.N. Barbagallo, R. Palmeri, S. Fabiano, P. Rapisarda, G. Spagna, Characteristic of β-glucosidase from Sicilian blood oranges in relation to anthocyanin degradation, Enzyme Microb. Technol. 41 (2007) 570–575, https://doi.org/10.1016/j.enzmictec.2007.05.006.

[4] R.N. Barbagallo, G. Spagna, R. Palmeri, C. Restuccia, P. Giudici, Selection, characterization and comparison of β-glucosidase from mould and yeasts employable for enological applications, Enzyme Microb. Technol. 35 (2004) 58–66, https://doi.org/10.1016/j.enzmictec.2004.03.005.

[5] I. Belda, J. Ruiz, A. Esteban-Fernández, E. Navascués, D. Marquina, A. Santos, M. V. Moreno-Arribas, Microbial contribution to Wine aroma and its intended use for Wine quality improvement, Molecules. 22 (2017) 1–29, https://doi.org/10.3390/molecules22020189.

[6] H. Michlmayr, C. Schümann, P. Wurbs, N.M.B.B. da Silva, V. Rogl, K.D. Kulbe, A. M. del Hierro, A β-glucosidase from Oenococcus oeni ATCC BAA-1163 with potential for aroma release in wine: Cloning and expression in E. coli, World J. Microbiol. Biotechnol. 26 (2010) 1281–1289, https://doi.org/10.1007/s11274-009-0299-5.

[7] S. de Ovalle, B. Brena, L. Fariña, P. González-Pombo, Novel beta-glucosidase from *Issatchenkia orientalis* : Characterization and assessment for hydrolysis of muscat wine glycosides, Glob. J. Biochem. Biotechnol. 4 (2016) 174–183. ISSN:2465-7182/ http://www.globalscienceresearchjournals.org/.

[8] S. de Ovalle, B. Brena, P. González-Pombo, Influence of beta glucosidases from native yeast on the aroma of Muscat and Tannat wines, Food Chem. 346 (2021) 128899.

[9] B. Brena, P. González-Pombo, F. Batista-Viera, Immobilization of Enzymes and Cells, Immobil. Enzym. Cells Third Ed. Methods Mol. Biol. 1051 (2013) 15–31, https://doi.org/10.1007/978-1-62703-550-7.

[10] R. Fajardo-Ochoa, Escalante-Minakata, V. Ibarra-Junquera, Inmovilización De Células Y Enzimas, Acta Química Mex. 3 (2011) 42–56.

[11] D. Cazaban, A. Illanes, L. Wilson, L. Betancor, Bio-inspired silica lipase nanobiocatalysts for the synthesis of fatty acid methyl esters, Process Biochem. 74 (2018) 86–93, https://doi.org/10.1016/j.procbio.2018.07.004.

[12] J. Peijun, T. Huishan, X. Xu, W. Feng, Lipase Covalently Attached to Multiwalled Carbon Nanotubes as an Efficient Catalyst in Organic Solvent, AIChE J. 59 (2012) 215–228, https://doi.org/10.1002/aic.

[13] M.M. Bradford, A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/j.cj.2017.04.003.

[14] E. Jackson, M. Ferrari, C. Cuestas-Ayllon, R. Fernández-Pacheco, J. Perez-Carvajal, J.M. De La Fuente, V. Grazú, L. Betancor, Protein-templated biomimetic silica nanoparticles, Langmuir. 31 (2015) 3687–3695, https://doi.org/10.1021/la504978r.

[15] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida, J.M. Guisán, Preparation of activated supports containing low pK amino groups. A new tool for protein immobilization via the carboxyl coupling method, Enzyme Microb. Technol. 15 (1993) 546–550, https://doi.org/10.1016/0141-0229(93)90016-U.

[16] A. Baraldo Junior, D.G. Borges, P.W. Tardioli, C.S. Farinas, Characterization of β -Glucosidase Produced by Aspergillus niger under Solid-State Fermentation and Partially Purified Using MANAE-Agarose, Biotechnol. Res. Int. (2014 (2014)) 1–8, https://doi.org/10.1155/2014/317092.

[17] N. Iturmendi, D. Durán, M.R. Marín-Arroyo, Fining of red wines with gluten or yeast extract protein, Int. J. Food Sci. Technol. 45 (2010) 200–207, https://doi.org/10.1111/j.1365-2621.2009.02121.x.

[18] X. Song, Z. Jiang, L. Li, H. Wu, Immobilization of β-glucuronidase in lysozyme-induced biosilica particles to improve its stability, Front. Chem. Sci. Eng. 8 (2014) 353–361, https://doi.org/10.1007/s11705-014-1421-2.

[19] P.G. Vazquez-ortega, M.T. Alcaraz-fructuoso, J.A. Rojas-contreras, J. López-Miranda, R. Fernandez-Lafuente, Stabilization of dimeric β -glucosidase from Aspergillus niger via glutaraldehyde immobilization under different conditions, Enzyme Microb. Technol. 110 (2018) 38–45, https://doi.org/10.1016/j.enzmictec.2017.12.007.

[20] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, 40 (2007) 1451–1463. Doi: 10.1016/j.enzmictec.2007.01.018.

[21] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking, Biotechniques. 37 (2004) 790–802, https://doi.org/10.2144/3705A0790.