



Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification



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ABSTRACT

The clarification step represents, in fruit juices industries, a bottleneck process because residual phenols cause severe haze formation affecting juice quality and impairing customers acceptance. An enzymatic step can be efficiently integrated in the process, and use of immobilized enzymes entails an economical advantage. In this work, covalent immobilization of recombinant POXA1b laccase from *Pleurotus ostreatus* on epoxy activated poly(methacrylate) beads was optimized thanks to a Response Surface Methodologies approach. Through regression analysis the process was well fitted by a quadratic polynomial equation ($R^2 = 0.9367$, adjusted $R^2 = 0.8226$) under which laccase activity reached $2000 \pm 100 \text{ U g}^{-1}$ of beads, with an immobilization efficiency of 98%. The immobilized biocatalyst was characterized and then tested in fruit juice clarification reaching up to 45% phenol reduction, without affecting health-effective flavanones content. Furthermore, laccase treated juice displays an improved sensory profile, due to the reduction of vinyl guaiacol, a potent off-flavor possessing a peppery/spicy aroma.

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1. Introduction

According to the “European fruit juice association” (www.aijn.org), EU fruit juice and nectars consumption is forecast to stand at 10.3 billion liters by 2017. The ongoing consumer health and wellness trend in many countries offers strong potential for increasing this market, due to the well-established beneficial effects associated to fruit juices consumption (Bharate & Bharate, 2014). Polyphenols in fruit juices are a natural source of antioxidants, and are responsible of the reported health benefits (Agcam, Akyildiz, & Akdemir Evrendilek, 2014). However, the same compounds are also the main factors involved in maderization process causing turbidity, color intensification, aroma and flavor alteration and formation of haze or sediments, affecting final product shelf life and consumer perception (Pezzella, Guarino, & Piscitelli, 2015). In order to reduce the impact of this phenomenon on the beverage products and to stabilize fruit juices, industries commonly use clarification processes through physical–chemical adsorbents and/or filtration technology. One disadvantage of these

techniques is that processed juices are not always stable, but rather tend to produce pronounced haze and enzymatic and non-enzymatic browning, caused by reactive phenolic compounds that cannot be removed (Friedman, 1996). Research has been concentrated, in recent years, to find effective and economical ways to reduce this phenomenon, with enzymes continuously gaining importance. Among enzymes useful within this process, several authors have proposed the use of laccases as stabilizing agents, due to their ability to oxidize most of the phenols present in juices (Neifar et al., 2011 and Gassara-Chatti et al., 2013) causing their polymerization and subsequent ease of removal.

Immobilization provides an excellent base for enzymes exploitation by increasing their reusability, enhancing their structural and catalytic stability in different environmental conditions, and reducing product inhibition (Sheldon, 2007). Apart from being affordable, immobilization generates continuous economic operations, automation, high investment/capacity ratio and recovery of product with greater purity (D'Souza, 1998). Several methods are used for enzyme immobilization and various factors influence the performance of the immobilized enzymes (Pezzella, Russo, Marzocchella, Salatino, & Sannia, 2014). The choice of the most suited method and the chemical nature of support material clearly depends on the application that enzyme is devoted to. Particularly, covalent binding is the most widely applied method in industrial

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applications due to several advantages including improved operational stability, robustness and reusability. The stabilization provided by covalent bonding is usually counterbalanced by partial enzyme deactivation. This negative effect can be mitigated by carefully optimizing the immobilization conditions in order to maximize the ratio between immobilized enzyme activity and activity of the primary enzyme solution.

In this work, covalent immobilization of the recombinant fungal laccase POXA1b (rPOXA1b) from the edible fungus *Pleurotus ostreatus* (Giardina et al., 1999) on epoxy activated poly(methacrylate) supports (Mateo et al., 2002 and Mateo et al., 2007) was investigated and the solid bio-catalyst was tested in fruit juices treatment. Laccases are widespread enzymes able to oxidize a wide range of phenolic substrates, using only molecular oxygen as cofactor and generating water as unique by-product. Due to its high redox potential and belonging to an enzymatic system of an edible fungus (Macellaro, Pezzella, Cicatiello, Sannia, & Piscitelli, 2014), rPOXA1b laccase has a great potential exploitability in food and beverage industries. The successful application of laccases in these fields require production of high amounts at reduced costs (Osma, Toca-Herrera, & Rodríguez-Couto, 2010). Concomitantly to strategies for their recombinant overexpression in suitable hosts, several approaches have been adopted along with optimization of immobilization processes to achieve affordable and reusable enzymatic system (Di Cosimo, McAuliffe, Pouloseb, & Bohlmannb, 2013; Durán, Rosa, D'Annibale, & Gianfreda, 2002; Jesionowski, Zdzarta, & Krajewska, 2014 and Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007). In this work an empirical modeling technique, named Response Surface Methodology (RSM), was used to optimize laccase immobilization yield. In RSM statistically designed experimental models are carried out to evaluate the relationship between a set of controllable experimental factors and observed results, thus to identify the optimum conditions for a multivariable system. We adopted one of the most common design, the Box Behnken (Box & Behnken, 1960), that reduces the number of experimental trials for its application and resulted in high efficiency of modeling of multiple parameters and their interactions (Costa Ferreira et al., 2007).

Stability and catalytic parameters of immobilized laccase were also assessed in comparison with the soluble counterpart. The solid optimized biocatalyst was exploited in the clarification of raw orange juice investigating the effect of laccase treatment on juice phenolic composition.

2. Materials and methods

2.1. Materials

Recombinant POXA1b laccase from *P. ostreatus* expressed in the eukaryotic host *Pichia pastoris* was provided by Biopox srl (Italy). Epoxy activated poly(methacrylate) beads (Sepabeads EC-HFA) were purchased from Resindion srl (Italy). All reagents were purchased from Sigma–Aldrich Corp. (St. Louis, MO) unless otherwise specified.

2.2. Fruit juice extraction

Mature ripened orange (*Citrus sinensis*), pomegranate (*Punica granatum*), apricot (*Prunus armeniaca*), peach (*Prunus persica*), cherry (*Prunus avium*) and apple (*Malus domestica*) fruits were obtained from a major market in Naples, Italy. The oranges were washed and peeled. The juice was extracted using a domestic juice extractor. The pomegranate fruit was peeled and the skin covering seeds was removed. The remaining part were homogenized by blender (Waring, USA) and centrifuged at 10,200×g for 5 min.

The pellet was discarded. The apricot, peach and cherry were pitted and homogenized by Waring blender. The apple was peeled and homogenized. All the juices were immediately processed after the extraction.

2.3. Enzyme immobilization

Immobilization reaction was performed incubating a variable quantity of poly(methacrylate) beads with 30 mL of 20,000 U L⁻¹ rPOXA1b laccase solution (1000 U g⁻¹ of protein) in 5 × 10⁻² M of different buffers (sodium citrate for pH 3, sodium phosphate for pH 6, Tris–HCl for pH 9) under magnetic stirrer (200 rpm) for 1 h at room temperature. Afterwards the supernatant was decanted and the biocatalyst particles washed with 30 mL of 5 × 10⁻² M phosphate buffer for 5 min for 3 times. Laccase activity was measured in the rinsing solution to calculate the immobilization yield (Y) as ratio between the total enzymatic IU presents in solution before (U_i) and after (U_f) immobilization reaction (%Y = [1 – (U_f/U_i)] × 100). Laccase activity (IU) was assayed by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (see enzyme assay, Section 2.5). The biocatalyst was stored at 4 °C in the phosphate buffer with 5 × 10⁻³ M glycine to saturate possible not reacted sites.

2.4. Experimental design and statistical analysis

The RSM was set up through a Box Behnken design choosing three independent variables (quantity of beads, pH and temperature) selected on the basis of preliminary experiments and the related experimental domain was fixed for each variable (Table 1). The ratio between the amount of enzyme and beads was varied keeping constant the volume and concentration of laccase solution and varying the quantity of beads (expressed in grams). A total of 15 or more experimental sets were carried out for each run.

Data obtained through the experimental matrix were computed for the determinations of regression coefficient of the second order multiple regression model:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{j=1}^k \sum_{i=1}^{j-1} \beta_{ij} x_i x_j$$

where Y is the predicted response variable, β_0 , β_i , β_{ii} and β_{ij} are regression coefficient of the model, x_i , x_j represent the independent variables in the form of actual value. The analysis of regression and

Table 1

Experimental Box Behnken design on the selected independent factors. For each factor three values (levels) were selected: high, low and average level.

	g of beads ^a	pH ^b	Temperature ^c (°C)
1	Average	Average	Average
2	High	Average	High
3	Low	Low	Average
4	Average	High	Low
5	Low	Average	Low
6	Average	Low	Low
7	Average	High	High
8	High	Average	Low
9	Average	Average	Average
10	Low	High	Average
11	Low	Average	High
12	Average	Average	Average
13	Average	Low	High
14	High	High	Average
15	High	Low	Average

^a Selected levels for g of beads are 1, 4.5, 8 for low, average, and high, respectively.

^b Selected levels for pH are 3, 6, 9 for low, average, and high, respectively.

^c Selected levels for temperature are 4, 22, 40 °C for low, average, and high, respectively.

variance was performed by Minitab 16 (Minitab Inc., LEAD Technologies).

2.5. Enzyme assay

Laccase activity was assayed at 25 °C by monitoring the oxidation of ABTS at 420 nm ($\epsilon_{420} = 36 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The assay mixture contained $2 \times 10^{-3} \text{ M}$ ABTS in $1 \times 10^{-1} \text{ M}$ sodium citrate buffer, pH 3.0. Laccase activity towards 2,6-dimethoxyphenol (DMP) was assayed in a mixture containing $1 \times 10^{-3} \text{ M}$ DMP in McIlvaine's citrate phosphate buffer adjusted to pH 5.0. Oxidation of DMP was followed by an absorbance increase at 477 nm ($\epsilon_{477} = 14.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

Immobilized enzyme activity was assayed incubating 1 mg of beads in 1 mL of substrate in the corresponding reaction buffer. The activity was determined by measuring, every 30 s, change in absorbance and following the reaction for 2 min. Enzymatic units were expressed as U g^{-1} of beads.

2.6. Characterization of free and immobilized laccase

2.6.1. Topographical characterization

Scanning electron microscope (SEM) analysis of epoxy activated poly(methacrylate) beads, before and after the laccase grafting step, were achieved using SEM Ultra Plus (Zeiss, Germany) with FEG (field emission gun) source operated at 10 kV. SEM samples were sputtered with gold through Sputter coater HR 208 (Cressington, England), achieving a gold layer thickness of 20 nm.

2.6.2. Determination of kinetic parameters

Michaelis–Menten constants K_M values were estimated for free and immobilized laccases using the software GraphPad Prism (GraphPad Software, USA; <http://www.graphpad.com/>) on a wide range of substrate concentrations ($5 \times 10^{-5} - 3 \times 10^{-3} \text{ M}$) through the following equation:

$$V = \frac{V_{\max}[S]}{K_M + [S]}$$

where V is the velocity of the reaction, $V_{\max}$ is the maximum velocity of the reaction and $[S]$ is the concentration of the substrate.

Enzyme activity was expressed in international units (IU).

2.6.3. Effect of pH and temperature

Laccase activity (free or immobilized) as a function of pH was assayed using ABTS and DMP as substrates in McIlvaine buffers (pH 2.0–8.0) at room temperature. The effect of temperature on laccase activity towards ABTS was evaluated in the temperature range of 25–85 °C in $5 \times 10^{-2} \text{ M}$ sodium phosphate buffer adjusted to pH 6.0. The activity was assayed as previously described.

2.6.4. Stability at pH and temperature

pH stability was evaluated by incubating enzymes in $5 \times 10^{-2} \text{ M}$ citrate buffer pH 3, $5 \times 10^{-2} \text{ M}$ phosphate buffer pH 6 and $5 \times 10^{-2} \text{ M}$ Tris–HCl pH 9 at 25 °C up to 65 days. Thermal stability was determined incubating free and immobilized laccases at the selected temperatures (25 °C, 55 °C, 65 °C) in $5 \times 10^{-2} \text{ M}$ phosphate buffer pH 6 until day 40. Residual laccase activity was assayed at room temperature in standard conditions with ABTS. The half-life ($t_{1/2}$) at different temperatures or pH is referred to the time corresponding to the 50% of residual activity. This value was extrapolated from the tendency curve related of enzyme deactivation in each condition.

2.6.5. Storage stability

For testing the storage stability of enzymes, free and immobilized laccase in $5 \times 10^{-2} \text{ M}$ sodium phosphate buffer pH 6.5 were stored at 4 °C for 6 months. Remaining laccase activity was assayed at room temperature in reference conditions at different times.

2.6.6. Reusability

Several consecutive oxidative cycles were performed in standard condition. At the end of each oxidation cycle, the immobilized laccase was washed three times with $5 \times 10^{-2} \text{ M}$ phosphate buffer pH 6.5 for 5 min under magnetic stirrer (200 rpm) and gravity filtered on a gauze. The procedure was then repeated with a fresh aliquot of substrate.

2.7. Fruit juices treatment

4 mL of fresh fruits juice were incubated with 0.5 g of immobilized enzymes (2000 U g^{-1}) for 1 h at room temperature in continuous stirring. The juices were then decanted and analyzed for phenol content. Controls were performed incubating 0.5 g of epoxy activated poly(methacrylate) beads in 4 mL of fruit juice in the same conditions.

Ultrafiltered samples were obtained treating 4 mL of fruit juice on 10,000 Dalton cut-off membrane on an amicon device (Merck Millipore Corporation, Italy).

2.8. Extraction and determination of total phenolic compounds

The extraction procedure was carried out with 0.5 mL juice/methanol (1:1) for 30 min at –20 °C. The sample was centrifuged for 30 min at $23,900 \times g$ and the supernatant recovered was assayed.

The total phenolic content of fruit was determined by using Folin–Ciocalteu assay (Singleton & Rossi, 1965). Gallic acid stock solution, (1 mg mL^{-1}) and standard concentrations of 0, 10, 25, 50, 100, 250 and $500 \mu\text{g mL}^{-1}$ were prepared in deionized water. The Folin–Ciocalteu procedure consisted of transferring 50 μL standard or sample into a 4–5 mL borosilicate tube, followed by additions of 430 μL H_2O and 20 μL Folin–Ciocalteu reagent. After mixing the samples, 50 μL 20% sodium carbonate and 450 μL H_2O were added. The sample mixtures were allowed to stand for 1 h at room temperature. The absorbance was measured at 725 nm. The phenolic content of samples was measured against the gallic acid (GA) calibration standard (0–500 ppm). Phenol reduction was estimated as percentage respect to untreated samples.

2.9. Mass spectrometry analyses

In order to obtain a molecular investigation of species occurring in the different samples, aliquots of methanolic extracts from fruit juice were submitted to mass spectral analyses by using both GCMS and LCMS/MS techniques.

2.9.1. GC–MS analysis

Aliquots of 1 μL of each methanolic extracts were directly analyzed using a GC–MS equipped with a 5975 MSD quadrupole mass spectrometer (Agilent technologies, USA) and a gas chromatograph 7820A (Agilent technologies, USA) by using a ZB-5MS fused silica capillary column (30 m length, 0.25 mm ID, 0.25 μm Film Thickness) from Phenomenex, (USA). The injection temperature was 250 °C. During analyses, the oven temperature was increased from 60 °C to 300 °C at $10 \text{ }^\circ\text{C min}^{-1}$ and held at 300 °C for 10 min. Electron Ionization mass spectra were recorded by continuous quadrupole scanning at 70 eV ionization energy, in the mass range 50–600 m/z . Each specie was interpreted on the basis of electron impact spectra (NIST 2011 library, Scientific Instrument Services, USA, and Analyst Software, SCIEX, Canada).

2.9.2. LC-MS/MS analysis

Methanolic extracts were dried under vacuum and resuspended in 200 μL of 0.1% formic acid. The resulting samples were filtrated using cellulose acetate spin filters (0.22 μm) from Agilent and 1 μL of each sample was analyzed by nanoLC Chip MS/MS, using a CHIP MS 6520 QTOF equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies, USA). After loading, the samples were first concentrated and washed at 4 $\mu\text{L min}^{-1}$ in 40 nL enrichment column (Agilent Technologies chip, USA), with 0.1% formic acid as eluent, and then fractionated on a C18 reverse-phase capillary column (75 $\mu\text{m} \times 43 \text{ mm}$ in the Agilent Technologies chip, USA) at flow rate of 400 nL/min with a linear gradient of eluent B (0.1% formic acid in 95% ACN) in A (0.1% formic acid in 2% ACN) from 7% to 60% in 50 min. Mono charged analytes were selected and analyzed using data-dependent acquisition of one MS scan (mass range from 100 to 1500 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Collision energy (CE) applied during fragmentation is calculated by the sequent empirical equations: $\text{CE} = 4\text{V}/100 \text{ Da} - 2.4\text{V}$. Raw data from nanoLC-MS/MS were analyzed using Qualitative Analysis software (Agilent MassHunter Workstation Software, version B.02.00, USA).

2.10. Statistical analysis

Those analysis not concerning RSM represent the mean of triplicate measurements and are expressed as mean $\pm$ S.D. (standard deviation of the mean). One-way analysis of variance (ANOVA) followed by the unpaired Student's *t* test or Tukey's test was used. $p < 0.05$ was considered statistically significant, unless otherwise specified.

3. Results and discussions

3.1. Immobilization of laccase on Sepabeads EC-HFA

Applicability at industrial scale of an enzyme is dependent on its performances as well as on its manufacturing costs, which have to be conformed to commercial and industrial demands. In this context, design of suitable immobilization process and its optimization in operating conditions are of utter importance. In the present work covalent immobilization of rPOXA1b laccase on epoxy activated poly(methacrylate) beads was chosen as a promising method to provide a useful catalytic system for several applications (Mateo et al., 2007). A RSM approach was applied to define process parameters improving laccase immobilization through the characteristic three major steps: performing statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Box Behnken design was performed selecting the range of levels variation for each factor on the basis of empirical and theoretical consideration concerning enzyme stability and volumetric constrain. As a fact, a pH range from 3 to 9 was chosen, avoiding extreme pH values that could negatively affect enzyme stability (Miele et al., 2010). Moreover, the quantity of poly(methacrylate) beads usable for this process is limited by the necessity of incubating them in an enzymatic solution properly covering their surface. Laccase immobilization yield was considered as response variable and it was evaluated as ratio between the total enzymatic IU presents in solution before and after immobilization reaction ($\%Y = [1 - (U_f/U_i)] * 100$). According to this design, 15 runs, replicated three times, were performed and experimental data (Table 2 column named "Yield") were obtained. Results obtained after running the trials of the Box Behnken design were fitted to a factorial equation to explain the dependence of lac-

Table 2

(A) experimental conditions of the experimental design for laccase immobilization and the corresponding experimental responses. (B) Analysis of variance (ANOVA) for the fitted quadratic polynomial model of laccase immobilization.

	g of beads	pH	Temperature	Yield (%)			
(A)							
1	4.5	6	22	70			
2	8	6	40	74			
3	1	3	22	13			
4	4.5	9	4	70			
5	1	6	4	45			
6	4.5	3	4	24			
7	4.5	9	40	50			
8	8	6	4	94			
9	4.5	6	22	70			
10	1	9	22	45			
11	1	6	40	36			
12	4.5	6	22	47			
13	4.5	3	40	23			
14	8	9	22	97			
15	8	3	22	20			
Source		DF	Seq Ss	Adj SS	Adj MS	F value	Prob > F
(B)							
Regression model		9	9177.07	9177.07	1019.67	8.21	0.016
Linear		3	7117.50	7117.50	2372.50	19.11	0.004
g of beads enzyme/beads		1	2664.50	2664.50	2664.50	21.46	0.006
pH		1	4140.50	4140.50	4140.50	33.36	0.002
Temperature		1	312.50	312.50	312.50	2.52	0.173
Square		3	1432.82	1432.82	477.61	3.85	0.091
g of beads enzyme/beads * g of beads enzyme/beads		1	22.02	3.39	3.39	0.03	0.875
pH * pH		1	1406.79	1410.01	1410.01	11.36	0.020
Temperature * Temperature		1	4.01	4.01	4.01	0.03	0.864
Interaction		3	626.75	626.75	208.92	1.68	0.285
g of beads enzyme/ beads * pH		1	506.25	506.25	506.25	4.08	0.099
g of beads enzyme/ beads * Temperature		1	30.25	30.25	30.25	0.24	0.642
pH * Temperature		1	90.25	90.25	90.25	0.73	0.433
Residual		5	620.67	620.67	124.13		
Lack of fit		3	268.00	268.00	89.33	0.51	0.716
Pure error		2	352.67	352.67	176.33		
Total		14	9797.73				
R ² = 93.67%			R ² adj. = 82.26%				

DF – (Total degrees of freedom) are the amount of information data estimates.

Sequential SS (Sequential Sum of Squares) are the numerators of the linear F-statistic.

Adjusted SS – (Adjusted sums of squares) are measures of variation for different components of the model. The order of the predictors in the model does not affect the calculation of the adjusted sum of squares.

Adjusted MS – (Adjusted mean squares) measure how much variation a term or a model explains, assuming that all other terms are in the model, regardless of the order they were entered.

F value – probability distribution value used in the ANOVA.

case immobilization yield on the designed variables as shown in the equation:

$$Y = -64.1346 - 0.958050 * \text{g of beads} + 30.7526 * \text{pH} - 2.17130 * \text{pH} * \text{pH} + 1.07143 * \text{g of beads} * \text{pH}$$

The second-order regression equation provided the levels of immobilized laccase as a function of parameters which can be presented in terms of coded factors. The factors can affect the results in different ways and are integrated in the equation as "linear" (g of beads * pH), "square" (pH * pH) or "interacting" (g of beads * pH) coefficients. The statistical significance of regression equation was checked by *F*-test, and the analysis of variance (ANOVA) is shown in Table 2B.

The Model *F*-value found of 8.21 corresponding to *p*-value 0.016 implies that the model is significant. The value of the determination coefficient R^2 calculated from the quadratic regression model

was 0.9367, while the value of the adjusted R^2 was 0.8226 indicating high degree of correlation between the observed and predicted values. Thus, the regression model provides a good explanation of the relationship between independent variables and the response (Yield). Other values reported in the ANOVA Table 2B are used by the software to calculate the p -value for a term and the R^2 statistic. Usually, interpretation of results is based on the p -values, the R^2 statistic and the adjusted R^2 statistic instead of the degrees of freedom, the sums of squares and the adjusted mean squares.

The lack-of-fit test measures the failure of the model to represent data in the experimental domain, at points which are not included in the regression. This test is desired to be non-significant to support the model (Montgomery, 2001). In this study, the lack of fit F -value of 0.51 with p -value 0.716 implies that the lack of fit is not significant.

All these results suggest that the quadratic model is statistically significant for the response, and therefore it can be used for further analysis. The 3D response surface plots (Fig. 1) were obtained by plotting the response (yield) on the Z -axis against any two variables while keeping the third variable at its average level. Based on the above analysis, in the range of our research, high pH values and high quantity of enzyme positively affects laccase immobilization, while temperature seems not to affect immobilization yield in the range of the tested conditions.

The maximum laccase immobilization yield of 100% was predicted at the following optimum conditions: 8 g of beads for 30 ml of 20,000 U L⁻¹ rPOXA1b solution, in a buffer at pH 9, incubating at 4 °C. Another three experiment sets were carried out to confirm the prediction. An average of 98 ± 5% yield was achieved, theoretically corresponding to an immobilization of 75 U g⁻¹ of beads, thus indicating an excellent fit with the predicted value. Notably, we reached 2000 ± 100 U g⁻¹ of beads with a remarkable increase of enzyme specific activity respect to that of the free counterpart of more than 25 folds. This effect has been already reported in other works (Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013), and it is probably due to 3D structure variations after covalent attachment on a solid support. Thus, the process could facilitate a conformational rearrangement of the active site, i.e. unveiling the binding pocket, that positively affects the catalytic properties.

As expected, when the immobilization process was performed at room temperature, similar yields (96%) were obtained, thus providing the conditions for a less expensive procedure.

3.2. Immobilized enzymatic system characterization

3.2.1. Morphological characterization

Morphologies of beads surface were investigated using Scanning Electron Microscope before and after immobilization procedure. As shown in SEM micrographs (Fig. 2B), surface of the treated beads was looked like a mesh, and showed very compact structures, revealing an increase of surface roughness upon enzyme loading respect to the not-treated ones (Fig. 2A).

3.2.2. Kinetic analysis

Apparent K_M constants were determined for the immobilized enzyme against two different laccase substrates, ABTS and DMP, and compared with the free counterpart (Supplementary material, Fig. S1). An opposite effect was observed for the two tested substrates. K_M value vs ABTS of the immobilized enzyme (0.032 ± 0.006 mmol l⁻¹) is lower (p -value < 0.05) in respect to that observed for the soluble rPOXA1b (0.063 ± 0.004 mmol l⁻¹). Conversely, a slight increase (p -value < 0.05) in the K_M value vs DMP was observed for the solid catalyst (0.227 ± 0.030 mmol l⁻¹) respect to the free enzyme (0.160 ± 0.010 mmol l⁻¹). Expected phenomena occurring during enzyme immobilization —such as elec-

trostatic and partitioning effects in the immobilized enzyme microenvironments, substrate mass transfer effects and/or changes in enzyme conformation— may differently affect properties of immobilized catalyst in a substrate dependent manner (steric hindrance, reaction mechanism etc.) (Pezzella et al., 2014).

3.2.3. Temperature and pH activity profiles

The effect of the temperature on the activity of free and immobilized laccases was determined in the range 25–85 °C. Both catalysts display a maximum at 75 °C, although free rPOXA1b retains most of its activity in a wider temperature range when compared with the immobilized enzyme (Supplementary material, Fig. S2). A reduced flexibility, caused for example by the occurrence of multi-point attachments, may explain the observed results.

Enzyme immobilization determines a shift in the optimal pH (from 4 to 5) towards ABTS along with a reduced enzyme activity at more acidic pHs (Supplementary material, Fig. S3). On the other hand, free and immobilized laccase show a similar pH activity profile against DMP.

3.2.4. Thermostability and pH stability

Enzyme thermostability was evaluated incubating free and immobilized rPOXA1b at selected temperatures (Supplementary material, Fig. S4). The immobilized enzyme displays and increased stability at all tested temperatures with a twofold increase in the $t_{1/2}$ values (Table 3). pH stability was investigated at values 3, 6 and 9 (Table 3 and Supplementary material, Fig. S5). The solid catalyst exhibits an enhanced stability at all pH values (p value < 0.05, for each value of immobilized vs free enzyme), further boosting rPOXA1b peculiar stability at alkaline pH (Miele et al., 2010) and improving its performances at acidic pH.

3.2.5. Immobilized biocatalyst stability

Laccase immobilized on Sepabeads was efficiently stored at 4 °C in 5 × 10⁻² M phosphate buffer pH 6.5 preserving almost 95% of its activity after 4 months and about 90% after 6 months. Under the same storage conditions, soluble laccase shows a 50% drop in activity after six months.

As far as enzyme reusability, immobilized enzyme retains 67% of initial activity after ten cycles of ABTS oxidation at room temperature (Supplementary material, Fig. S6). These performances, having a great influence on process economics, are superior than those previously reported, showing 50% residual activity under similar conditions (Liu et al., 2012 and Rekuć, Bryjak, Szymańska, & Jazębski, 2009).

3.3. Fruit juice treatment with immobilized laccase

The effectiveness of immobilized laccase in fruit juices treatment was investigated choosing orange juice as a model. Raw orange juice was incubated for 1 h at room temperature with the solid catalyst and the total phenol content analyzed in comparison with that of an ultra-filtered sample. Ultra-filtered juices are not always stable, but rather tend to produce pronounced subsequent haze, caused by reactive phenolics compounds that cannot be retained by the ultra-filtration membrane (Neifar et al., 2011). Laccase treatment generates up to 45% reduction, whereas after ultra-filtration, only a 15% phenols decrement was measured. No phenol reduction was observed in the control tests (fruit juice incubated with the poly(methacrylate) solid support without enzyme). Thus, it is possible to rule out a possible effect due to endogenous polyphenol oxidase activities.

The solid catalyst can be reused up to three times in juice treatment without losing efficiency.

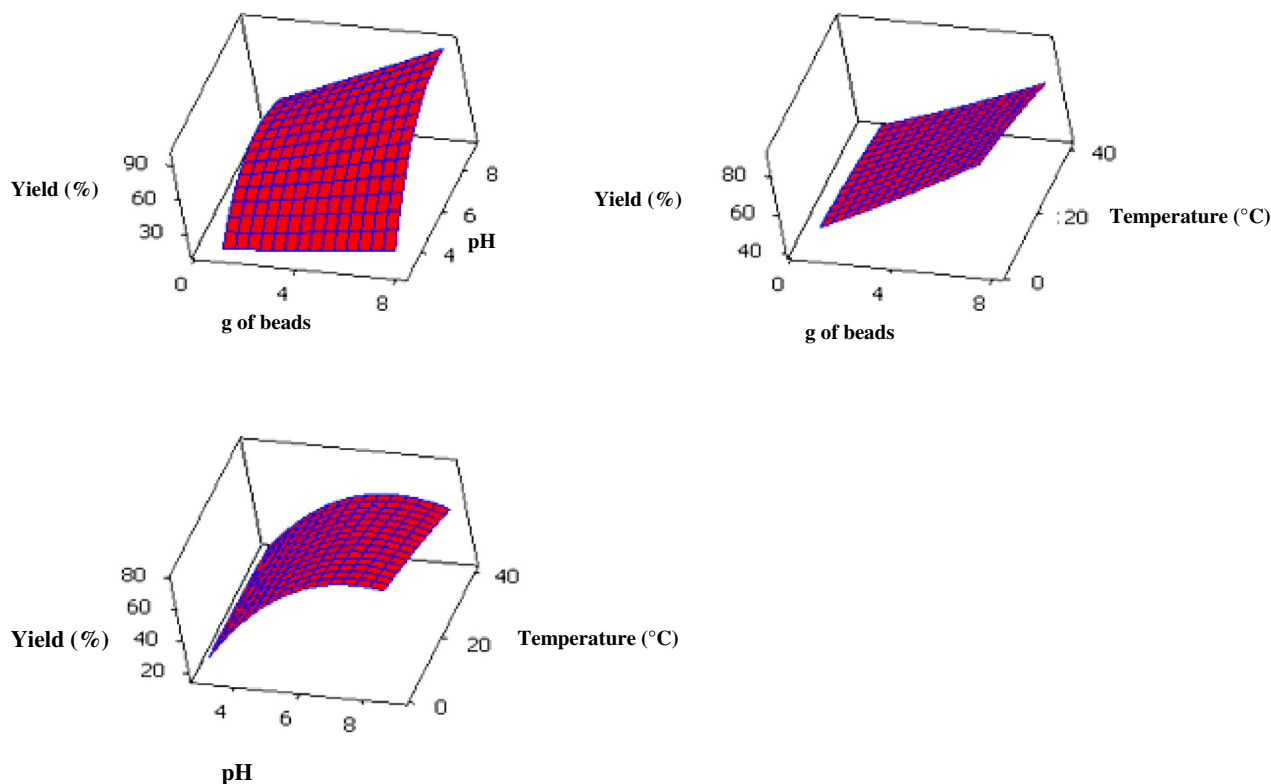


Fig. 1. 3D surface plot for the laccase yield immobilization as a function of temperature, pH and enzyme/beads ratio.

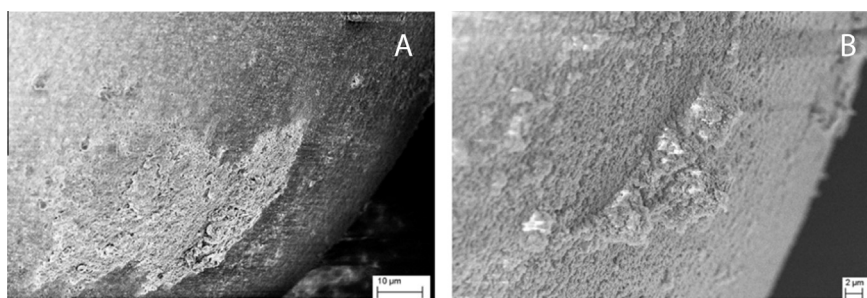


Fig. 2. Sem image analysis of epoxy activated poly(methacrylate) beads before, (A) and after (B) laccase immobilization.

Table 3

Temperature and pH stability profiles of (A) free and (B) immobilized laccase expressed as half-life, ABTS was used as the substrate for the enzyme assay. Each value represents the mean of triplicate measurements and varies from the mean by not more than 10%.

	$t_{1/2}$ temperature (h)			$t_{1/2}$ pH (day)		
	25 °C	55 °C	65 °C	pH 3	pH 6	pH 9
Free rPOXA1b	7.1	3.5	0.8	2.9	6.8	23.1
Immobilized rPOXA1b	16.3	7.8	1.5	5.7	16.0	63.7

3.3.1. Compounds identification

Citrus phenolics have been subject of increased interest in the last few years because their presence contributes to the sensory quality of fruit and juice, affecting color, bitterness, astringency, antioxidant activity and flavor (Sousa, da Rocha, Cardoso, Silva, & Zanoni, 2004). Sample aliquots of treated and not-treated raw orange juice were analyzed by LC–MSMS mass spectrometry to evaluate flavanones content. The total ion current chromatograms (TIC) showed a similar behavior. No decrease in flavanones contents could be appreciated in the LC–MSMS analyses (Supplemen-

tary material, Fig. S7). Thus, laccase treatment does not affect these health-effective molecules (Silva et al., 2014), probably due to a peculiar affinity towards other species present in the orange juice, such as phenols, that could compete for or avoid the oxidation of flavanones. This finding is unexpected if compared to the natural or induced oxidation process occurring in other fruit beverages like grape juice and wine, where the flavanones oxidation plays a prominent role in the formation of brown pigments (Shahidi & Naczki, 2003). Moreover, mass spectrometry analyses were used to evaluate the laccase treatment effect on phenolic content. Phenol standard mixture, containing coumaric acid, caffeic acid, synapinic acid, ferulic acid, vanillic acid and syringic acid (Barberis et al., 2014) was analyzed by GC–MS in order to set up the best chromatographic and mass spectral conditions (Supplementary material, Fig. S8). Mass spectral analyses were carried out on laccase treated orange juices. Commercial orange juice was used as control. The species were identified on the basis of electron impact mass spectra showing a general decrease in phenol content after laccase treatment. In particular, while caffeic acid seems to be unaffected by laccase treatment, the TIC showed the

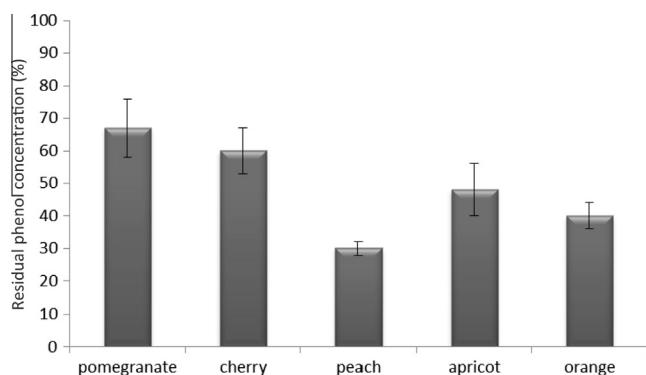


Fig. 3. The reduction effect of phenol content in different fruit juices after immobilized laccase incubation. The values are expressed in percentage of residual phenol concentration. Standard deviations derive from three independent replicates.

decrease of coumaric and ferulic acid. Moreover, in the laccase treated juice, the chromatograms showed an intense reduction of vinylguaiacol, a degradation product of ferulic acid. Vinylguaiacol is described as possessing a peppery/spicy aroma and is considered a potent off-flavor (Naim, Striem, Kanner, & Peleg, 1988). Thus, it can be conveyed that laccase treatment improves orange juice sensory profile and extends its shelf life.

Furthermore, in order to evaluate the versatility of the developed catalytic system, immobilized laccases were tested on different raw fruit juices. As reported in Fig. 3 the immobilized laccase system is able to sensitively reduce the phenol content of several fruit juices at a level comparable with that achieved for the orange juice.

4. Conclusion

Optimal conditions for laccase immobilization were set-up through a RSM approach using the Box Behnken design. Laccase based juice treatment allowed to reach up to 45% phenol reduction. The health-effective flavanones molecules are not affected by the treatment. Furthermore, laccase treated juice displays an improved sensory profile, due to the reduction of vinyl guaiacol, a potent off-flavor possessing a peppery/spicy aroma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.10.074>.

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