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Urea Degradation in Some White Wines by Immobilized Acid Urease in a Stirred Bioreactor

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A purified acid urease preparation was covalently immobilized onto either Eupergit C 250 L or glutaraldehyde-cross-linked chitosan-derivative beads (i.e., Chitopearls BCW-3003 and BCW-3010). The kinetics of urea degradation in two target Italian white (i.e., *Grechetto* and *Sauvignon Blanc*) wines, as well as in a model wine solution, by using the above Eupergit C 250 L-, BCW-3003-, or BCW-3010-based biocatalysts, was confirmed to be of the pseudofirst order with respect to the urea concentration in the liquid bulk and not limited by urea mass transfer. In *Grechetto* and *Sauvignon Blanc* wines, the corresponding kinetic rate constants were quite similar, being about 7, 18, or 17% of that observed for free enzyme in the model wine solution, respectively. Owing to their minor sensitivity to the phenolic content of the wines tested, the chitosan-based biocatalysts might be potentially employable in the make up of packed-bed cartridges to continuously remove urea from commercial wines.

KEYWORDS: Chitopearls; enzyme and activity coupling yields; Eupergit C 250 L; free or immobilized acid urease; pseudofirst-order kinetic constant rate; stirred bioreactor; urea degradation kinetics; white wines

INTRODUCTION

Ethyl carbamate (urethane, EC) is intrinsically present in all fermented foods and beverages, being produced by the reaction between urea and ethanol (*1*). When administrated in high doses in animal tests, EC has revealed potential carcinogenic activity (*2*). Thus, there is a great deal of concern to reduce EC levels in food products, and for instance, in wines a safe level of 15 μg/L has been so far recommended (*3*).

Among the several preventive actions to reduce EC levels that were issued by the U.S. Food and Drug Administration, the hydrolysis of urea to NH_3 and CO_2 by acid urease (urea amidohydrolase, E.C.3.5.1.5) appears to be a suitable process to prevent EC formation (*3*). Among ureases, acid ones are a different subgroup with the optimal pH values in the range 2.0−4.5, these being produced by intestinal (*Lactobacillus*, *Streptococcus*, *Escherichia*, *Morganella*, and *Bifidobacterium*) and soil (*Arthrobacter mobiliz*) bacteria (*4*). More specifically, acid ureases from *Lactobacillus* sp. are presently commercially available in either soluble or insoluble form (*4*) and are used to degrade urea in some acidic alcoholic beverages, such as sake (*5*), and wine (*6*−*8*). In the case of the latter, the process efficacy varies with wine variety, wine content of inhibitory factors (i.e., in ranking fluoride, malate, ethanol, and phenolic compounds), and treatment conditions (*6*−*8*).

Enzyme immobilization has been recommended to facilitate enzyme reuse and improve stability and/or resistance to shear or inhibitory compound inactivation. The literature about urease immobilization is copious and dates back to about two decades (*9*). So far, several support materials, such as polyacrylonitrile (PAN) (*5*), chitosan derivatives (*5*, *10*, *11*), Eupergit C 250 L (*12*), and nylon beads, sepharose gels, silica gels, and gelatin films coated on cellulose acetate membranes (*13*), have been applied to bind acid urease. Since 1988, urea removal from sake by PAN-immobilized acid urease has been used by many Japanese companies (*5*).

In previous work (*11*, *12*), a purified acid urease preparation was covalently immobilized onto well-known commercial carriers, i.e., Eupergit C 250 L (*14*, *15*) and chitosan-based materials (*16*, *17*). When using glutaraldehyde-activated Chitopearls of different size (*11*), the specific activity (A_{Bw}) of immobilized acid urease decreased from ca. 300 to 70 IU g^{-1} wet support (ws) as the bead average radius (R) increased from 0.07 to 1.1 mm (*11*). Moreover, A_{Bw} reduced less than 5% after preservation in the wet form at 4 °C for 150−170 days, this practically agreeing with the storage stability of the chitosan-based biocatalysts prepared by Matsumoto (*5*). On the contrary, the Eupergit-based biocatalyst was by far not only less active (19 IU g^{-1} ws) but also less stable. In fact, its average activity reduced to 68 ± 15% of the initial one after 34 days (*12*).

In a model wine solution, corresponding to the central point of a composite design experiment carried out previously (*7*), in the either absence or presence of high-inhibitory grape seeds tannins (*8*), the kinetics of urea degradation in a stirred bioreactor resulted to be of the pseudofirst order with respect to the urea concentration in the liquid-bulk, independent of the support materials used to bind acid urease (*11*, *12*). Moreover, at the maximum level of grape seed tannins tested (374 ± 2 mg/L of gallic acid equivalent), the apparent pseudofirst-order kinetic rate constant reduced to no more than 58 (± 9)% of that pertaining to free acid urease, this proving quite a higher protective action

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Table 1. Main Characteristics of the Porous Matrices Used in This Work, as Extracted from the Corresponding Manufacturer's Leaflet or Literature (*18*, *19*) or Determined Experimentally Here

parameter	Eupergit C250L	BCW-3003	BCW-3010	unit
manufacturer	Röhm GmbH (Darmstadt, D)	Wako Chemicals GmbH (Neuss, D)		
bead size	180	177−420	840−1190	μm
average bead radius (R)	90	150	508	μm
specific surface area (a_p)	33333	20101	5911	m^{-1}
bead porosity (θ)	0.6 [18]	0.83 [19]	0.83 [19]	
bead tortuosity factor (τ)	1.0 [18]	1.0 [18]	1.0 [18]	
particle density (ρ_B)	1136 [19]	1136 [19]	1136 [19]	g/L ws
water fraction of wet matrix (x_{Bw})	80 ± 1	91 ± 1	86 ± 1	% w/w

against such compounds for the chitosan-based biocatalysts toward either free or Eupergit C 250 L-immobilized acid urease (*11*).

The main objective of this work was to compare the catalytic performance of acid urease immobilized on three previously selected support materials (i.e., Eupergit C 250 L, Chitopearls BCW-3003, and -3010) in two target Italian white wines, high in acidity and medium-bodied, produced from the *Grechetto* and *Sauvignon Blanc* grapes in the Umbria region of Italy.

MATERIALS AND METHODS

Raw Materials. A single lot (ref no. 5870734) of the commercial preparation Nagapsin, donated by Nagase Europa GmbH (Duesseldorf, Germany), was used. It consisted of a soluble powder, approximately composed of 96% (w/w) lactose and 4% (w/w) purified acid urease from *Lactobacillus fermentum*, to be stored at 4 °C. Its specific activity was approximately constant (642 ± 38 IU g^{-1}) throughout the experimentation, where one International Unit (IU) corresponds to the amount of powder that liberates 1 μmol min^{-1} of ammonia from urea at 20 °C, once it is dissolved in a standard reaction mixture composed of 0.1 M sodium-acetate buffer (pH 4.0) enriched with urea (83.33 mmol/L).

Eupergit C 250 L is an epoxy-(oxirane) activated macroporous support (*14*, *15*) that was kindly provided by Röhm GmbH (Darmstadt, Germany). The water content (x_{Bw}) of the beads as such or after 24-h of swelling in 0.05 M potassium phosphate buffer at pH 7.0 (KPB7), as determined by using the oven dry method at 105 °C for 24 h, was found to be 3.3 (± 0.2) or 84 (± 3)% (w/w), respectively.

Two chitosan-derivative beads, such as Chitopearl BCW-3003 (Wako catalog number 308-02071) and BCW-3010 (catalog number 302-02091), were obtained from Wako Chemicals GmbH (Neuss, Germany).

Table 1 lists the main characteristics of the support matrixes used, as extracted from the corresponding manufacturer's leaflet or the literature (*18*, *19*), or determined experimentally here.

An aqueous solution of glutaraldehyde (GA) at 25% (w/v) (Sigma-Aldrich, St. Louis, MO, USA) was 10 times diluted with 0.05 M potassium acetate buffer at pH 5.0 (KAB5) and used as the support-activating reagent solution.

A model wine solution representing the central point of the composite design described previously (*7*) was prepared by dissolving constant amounts of urea (1 mmol/L), tartaric (5 g/L), malic (2.5 g/L) and lactic (1.75 g/L) acids, potassium metabisulfite (0.2 g/L), and ethanol (13% v/v) in deionized water and then adjusting the resulting pH to 3.50. All reagents were of analytical grade.

Two commercial Italian white wines, produced in the Umbria region of Italy by *Sauvignon Blanc* or *Grechetto* grapes (2008 vintage) in the Monrubio winery (Monterubiaglio di Castel Viscardo, Terni, Italy), were spiked with urea (1 mmol/L) before being submitted to immobilized acid urease tests.

Enzyme Immobilization Procedure. Two different enzyme immobilization techniques were used. The first one consisted of a single step, that is, the direct binding of the acid urease moiety to the oxirane group (*20*), while the second one consisted of two consecutive steps (*21*), that is, the activation reaction between chitosan and gluteraldehyde and its subsequent attachment to the primary amine groups of acid urease.

A mass (m_{Bw} = 1.204 g) of wet Eupergit C 250 L beads and 70 mL of 0.05 M potassium phosphate buffer at pH 7.0 (KPB7), enriched with 24.0 g/L of Nagapsin and preconditioned at 20 °C, were charged into a 150-mL Pyrex flask, equipped with a portable, 40-mm marine-type propeller mixer IKA (mod. EUROSTAR) rotating at 250 rev min^{-1} that was mounted vertically on center with baffles at the wall. After incubation for 48 h, the biocatalyst was collected by vacuum filtration using a glass filter (1.2-μm Whatman GF/C disk) and washed twice with 50 mL of KPB7. All filtrates were collected and diluted with KPB7 to a final volume of 0.5 L.

The wet beads were then soaked in an aqueous solution containing 75 mmol/L glycine at 4 °C for 20 min (*22*) and finally washed with KPB7, as reported before (*12*). For both Chitopearls, the covalent binding method, previously described (*11*), was used.

The wet Chitopearls (m_{Bw}∼1.2 g) were washed with distilled water (50 mL), collected by vacuum filtration, as reported above, and rinsed with 50 mL of 0.05 M potassium acetate buffer at pH 5.0 (KAB5). Then, they were suspended into 30 mL of the 2.5% (w/v) GA solution in KAB5 so as to ensure a glutaraldehyde-to-chitosan ratio of 0.625 g g^{-1} (*11*). After 2 h of treatment at 20 °C, the GA-activated beads were thoroughly washed 3 times with deionized water (10 mL), filtered as reported above, and transferred to 70 mL of KAB5 containing 24.0 g/L of Nagapsin. The coupling reaction was allowed to proceed in a rotary shaker at 100 rev min^{-1} and 20 °C for 48 h. After incubation, the beads were decanted, thus allowing most of the clear immobilizing solution to be removed using a pipet, diluted with deionized water (10−15 mL), collected by vacuum filtration as reported above, and rinsed twice with 50 mL of KAB5. All filtrates were collected and diluted with KAB5 to a final volume of 0.5 L.

Until use, all of the above biocatalysts were stored in KPB7 enriched with 2% (v/v) isopropanol and 0.5 g/L ethyl parabene at 4 °C to avoid microbial contamination (*12*).

Determination of Bound Enzyme. The protein concentration in all solutions was determined according to the method by Lowry et al. (*23*) using the Total Protein Kit (Sigma, Saint Louis, Missouri, USA) and the associated protein standard solution (containing 100 g/L of bovine serum albumin, BSA) diluted to vary the BSA content in the range of 0 to 1 g/L. Thus, the enzyme concentration was expressed in g/L of BSA equivalent (BSAE).

The amount of bound protein was indirectly assessed by subtracting the amount of protein in the filtrate and washing solutions from the amount of protein present in the immobilizing solution. Protein loading was defined as the amount of bound protein per gram of wet support.

Enzyme Activity Assay. The acid urease activity for the immobilizing (A_{E0}) and filtrate (A_{Ef}) solutions was assayed by charging a 25-mL beaker containing a 10-mm magnetic stirrer with the following liquids: 5.65 mL of 0.1 M acetate buffer (pH 4.0), 5 mL of the same buffer containing 11 g/L of urea, and 0.35 mL of the sample to be tested.

The activity of any immobilized enzyme was assessed by weighing about 50 mg of any biocatalyst in a 25-mL beaker containing a 10-mm magnetic stirrer and consecutively adding 12 mL of 0.1 M acetate buffer (pH 4.0) and 10 mL of the same buffer containing 11 g/L of urea. In all cases, the resulting reaction mixture was stirred at 400 rev min^{-1} and incubated in a water bath at 20 °C for 10 min.

The urea concentration in the final reaction mixtures was determined using the enzymatic kit K-URAMR (Megazyme International Ireland Ltd., Wicklow, Ireland). The enzymatic assay was described by Kerscher and Ziegenhorn (*24*), and the procedure is available online (http://secure.megazyme.com/downloads/en/data/K-URAMR.pdf, accessed March 9, 2010).

The specific activity of the immobilizing (A_{E0}) and filtrate (A_{Ef}) solutions or any immobilized biocatalyst was estimated by dividing the

micromoles of ammonia formed per minute by the corresponding concentration of protein or wet biocatalyst.

Finally, the efficiency of immobilization was evaluated in terms of the enzyme coupling yield (ζ_E) by dividing the amount of bound protein to the support of choice by that of the enzymatic protein initially present in the immobilizing solution.

Urea Degradation Kinetics by Free or Immobilized Acid Urease. To assess the time course of the hydrolytic process under study, 80 mL of a real or model wine, preconditioned at 20 °C, was poured into a 100-mL rubber-capped flask, precharged with given amounts of free or immobilized acid urease. Each flask was immersed in a water bath to keep the reaction temperature at 20 ± 0.2 °C, using thermostat model F3 (Haake, Karlsruhe, Germany), and placed over a magnetic multistirrer model Multistirrer 15 (Velp Scientifica, Milan, Italy) to ensure a constant stirring level (100 rev min^{-1}). Several samples (1 mL) were withdrawn from any flask for as long as 7 h and were diluted with deionized water at room temperature before being assayed for ammonium and urea by using the K-URAMR kit (Megazyme International Ireland Ltd., Wicklow, Ireland).

Any interference between the enzymatic kit and tannins present in the real wine samples was limited by diluting each sample with an equal volume of an aqueous solution containing 1 g/L of polyvinylpolypyrrolidone. After intense mixing at 1800 rev min^{-1} for 1 min using a Vortex IKA MS1 minishaker (IKA-Werke GmbH, Staufen, Germany), the mixture was centrifuged at 10,000 rev min^{-1} (8720*g*) for 3 min via Centrifugette 4204 (ALC Apparecchi per Laboratori Chimici, Milan, Italy). Such a procedure was newly repeated, once 0.5 mL of the first supernatant had been collected. Finally, an aliquot (0.2 mL) of the second supernatant was enzymatically assayed for ammonium and urea.

The kinetics of urea hydrolysis catalyzed by free acid urease in the real and model wines was also assessed by setting the initial concentration of Nagapsin to about 1.2 g/L, this being equivalent to an enzymatic protein content of about 47.5 mg/L BSAE. On the contrary, the kinetics of urea hydrolysis catalyzed by immobilized acid urease in the two real wines was determined by using the three biocatalysts prepared in this work. Depending on their corresponding protein loading, their concentration was varied so as to keep the immobilized enzymatic protein concentration (E_i) dispersed in the liquid phase about constant (47 ± 3 mg/L BSAE) during each kinetic test.

Wine Analyses. The wine samples assayed were stored in 0.75-L bottles at 10 (± 1) °C in the dark before testing. The ethanol volumetric fraction, pH, and titrable and volatile acidities, as well as contents of glycerol, reducing sugars, total extract, ash, total SO_2, urea, and phenolic compounds, and wine density, were determined by using the OIV analytical methods (*25*). The overall content of phenolic compounds (P) was colorimetrically assessed at 700 nm using the Folin−Ciocalteu reagent and expressed as gallic acid equivalent (GAE) by referring to a calibration curve valid for a gallic acid concentration range of 0−30 mg/L (*26*). The kinematic viscosity (ν_L) at 20.5 ± 0.1 °C of the two wine samples was determined by using a capillary number 50 Cannon-Fenske viscometer (*27*) and then converted into the dynamic viscosity (μ_L).

All spectrophotometric measurements were carried out using a Lambda25 spectrophotometer (Perkin-Elmer Inc., Wellesley, MA) and quartz cells with a 1-cm path length. The concentrations of tartaric, L-malic, L-lactic, and citric acids were determined by high-pressure liquid chromatography (HPLC-DAD) (*28*) using an apparatus provided by Dionex Corp. (Sunnyvale, CA), consisting of a P680A pump coupled to a PDA-100 diode array detector and controlled by Chromeleon software. The column was a Nova-Pak C_{18}, 250 × 4 mm, 4 μm, protected by a guard column packed with the same material (Waters Corp., Milford, MA). Both columns were thermostatically controlled at 30 °C. The chromatographic conditions, as well as peak detection, identification, and quantification, were carried out as described by Cane (*28*). All analytical data are shown in **Table 2**.

Modeling of Urea Degradation in a Stirred Tank Bioreactor. In previous work (*12*), it was assumed that immobilized acid urease exhibited the same pseudofirst-order kinetic model of free enzyme, especially when S_L was by far smaller than the Michaelis−Menten constant of the free enzyme (*7*). Thus, the urea degradation rate compared to the unit volume of immobilized acid urease (r_{Si}) was expressed as follows:

$$r_{Si} = k_i S_L \tag{1}$$

Table 2. Mean Values and Standard Deviations of the Main Chemico-Physical Analyses for the Commercial Italian White Wines Investigated in this Work

wine type	Sauvignon Blanc	Grechetto	
parameter	value	value	unit
alcohol content	12.7 ± 0.6	12.9 ± 0.6	% v/v
pH	3.48 ± 0.04	3.27 ± 0.04	
total acidity	5.5 ± 0.3	5.5 ± 0.3	g[a]/L
volatile acidity	0.18 ± 0.02	0.21 ± 0.02	g[b]/L
glycerol	5.7 ± 0.3	5.1 ± 0.3	g/L
reducing sugars	0.66 ± 0.07	1.7 ± 0.2	g/L
total extract	21.8 ± 1.7	20.1 ± 1.6	g/L
ash	2.7 ± 0.2	2.2 ± 0.2	mg/L
overall SO_2	102 ± 8	95 ± 7	mg/L
urea	nd[c]	nd[c]	mmol/L
ammonium	5.0 ± 0.4	11 ± 1	mg/L
total phenolics	364 ± 24	450 ± 29	mg/L GAE
tartaric acid	3.1 ± 0.2	3.6 ± 0.2	g/L
L-malic acid	2.2 ± 0.2	1.5 ± 0.1	g/L
L-lactic acid	0.04 ± 0.00	nd	g/L
citric acid	0.22 ± 0.03	0.16 ± 0.02	g/L
density at 20.5 ± 0.2 °C	992 ± 3	990 ± 3	g/L
viscosity at 20.5 ± 0.1 °C	1.61 ± 0.01	1.56 ± 0.01	mPa s

[a] As tartaric acid equivalent. [b] As acetic acid equivalent. [c] nd, not detectable.

with

$$k_i = k'_i \rho_B Y_{P/B} \tag{2}$$

where k_i is the urea degradation pseudofirst-order kinetic rate constant for immobilized enzyme, k'_i its specific value, ρ_B the biocatalyst density, $Y_{P/B}$ the protein loading, and S_L the reagent concentration in the liquid bulk.

When using a perfectly mixed bioreactor, charged with a volume (V_L) of a solution with an initial concentration of urea (S_{L0}) and inoculated with a prefixed amount (m_{Bw}) of wet biocatalyst in the form of almost spherical beads with an average radius (R), the unsteady-state material balance for urea and its initial condition may be written as follows:

$$-\frac{dS_L}{dt} = k_L a_S (S_L - S_R) = \Omega v_S k_i S_L \tag{3}$$

$$S_L = S_{L0} \text{ for } t = 0 \tag{4}$$

where a_S and v_S are the overall biocatalyst surface and volume the per unit volume of liquid phase, respectively, k_L is the mass transfer coefficient in the liquid phase, S_R the reagent concentration at the biocatalyst surface, and Ω the effectiveness factor for a spherical biocatalyst in the presence of the external film transport resistance. This factor depends on the biocatalyst effectiveness factor (η), which is a function of the Thiele modulus for a pseudofirst-order reaction (Φ) and Biot number (Bi). All of these parameters may be estimated using the basic relationships extracted from Bailey and Ollis (*29*) and Satterfield and Sherwood (*30*), and reported previously (*11*, *12*).

In a stirred bioreactor, the mass transfer coefficient in the liquid phase (k_L) can be estimated by resorting to the following well-known dimensionless correlation (*29*):

$$Sh = \frac{2RK_L}{D_s} = 2 + 0.31(ScGr)^{1/3} \tag{5}$$

where Sh, Sc, and Gr are the Sherwood, Schmidt, and Grashof numbers, respectively (*29*). In particular, the urea diffusivity in the bulk liquid (D_S) was estimated as equal to 1.29×10^{-9} m^2 s^{-1} using the Wilke and Chang method (*31*), whereas that in the biocatalyst (D_{Se}) was calculated as suggested by Satterfield and Sherwood (*30*):

$$D_{Se} = \theta/\tau D_S \tag{6}$$

where θ and τ are the bead porosity and tortuosity factor, respectively (see **Table 1**).

Table 3. Experimental Results and Specific Activities per Unit Mass of Wet (ws) Support of Acid Urease Immobilized on Eupergit C 250 L and Some GA-Activated Chitosan Beads of Different Size

parameter		Eupergit C 250 L	Chitopearl BCW-3003	Chitopearl BCW-3010	unit
water fraction of wet biocatalyst	x_{BW}	80 ± 1	75 ± 5	79 ± 3	% w/w
protein conc. in the immobilizing solution	c_{P0}	0.978 ± 0.019	0.950 ± 0.024	0.913 ± 0.004	g/L BSAE
immobilising solution activity	A_{E0}	16.8 ± 0.2	16.2 ± 0.2	15.8 ± 0.2	IU mg^{-1} BSAE
protein concentration in the filtrate	c_{Pf}	0.106 ± 0.001	0.047 ± 0.001	0.093 ± 0.004	g/L BSAE
protein loading	$Y_{P/B}$	12.75	36.0	14.4	mg BSAE g^{-1} ws
specific immobilized enzyme activity	A_{Bw}	10.3 ± 0.1	260 ± 3	127 ± 4	IU g^{-1} ws
enzyme coupling yield	ζ_E	22.4	64.9	27.1	%

Table 4. Mean Values and Standard Deviations of the Apparent Urea Degradation Pseudofirst-Order Kinetic Rate Constant (k_{aj}) in Model, *Grechetto*, and *Sauvignan Blanc* Wines at 20 °C When Using Different Concentrations of the Commercial Acid Urease Preparation (C_n) or Wet Biocatalysts (C_{bw}) in the Liquid Bulk

wine sample	parameter	free enzyme	Eupergit C 250 L	BCW-3003	BCW-3010	unit
model wine	c_{Bw} or c_N	1.25	6.25	1.26	3.13	g/L
	k_{aj}	0.74 ± 0.02 (r^2 = 0.994)	0.18 ± 0.01 (r^2 = 0.986)	0.43 ± 0.02 (r^2 = 0.991)	0.45 ± 0.01 (r^2 = 0.998)	h^{-1}
Grechetto wine	c_{Bw} or c_N	1.20	3.53	1.26	3.13	g/L
	k_{aj}	0.23 ± 0.01 (r^2 = 0.996)	0.06 ± 0.01 (r^2 = 0.800)	0.122 ± 0.002 (r^2 = 0.998)	0.120 ± 0.006 (r^2 = 0.983)	h^{-1}
Sauvignon Blanc wine	c_{Bw} or c_N	1.20	3.53	1.25	3.13	g/L
	k_{aj}	0.149 ± 0.007 (r^2 = 0.983)	0.051 ± 0.007 (r^2 = 0. 883)	0.139 ± 0.005 (r^2 = 0.994)	0.135 ± 0.005 (r^2 = 0.987)	h^{-1}

Equation 3 can be integrated with its corresponding initial condition, eq 4, thus yielding:

$$\ln\left(\frac{S_L}{S_{L0}}\right) = -\int_0^t \Omega c_{Bw} Y_{P/B} k'_i dt \quad (7)$$

where the product of c_{Bw} by $Y_{P/B}$ will coincide with the free (E_f) or immobilized (E_i) enzymatic protein concentration dissolved or dispersed in the liquid phase, respectively.

Once the natural logarithm of the ratio between the current (S_L) and initial (S_{L0}) urea concentrations in stirred bioreactors using either free or immobilized enzyme is found to vary about linearly with the reaction time (t) and the overall effectiveness factor (Ω) is intrinsically or approximately unitary, the apparent slope of such a plot is expected to be proportional to E_f or E_i via the specific pseudofirst-order kinetic rate constant relative to the free (k'_f) or immobilized (k'_i) enzyme (*12*).

RESULTS AND DISCUSSION

Enzyme Binding and Activity Coupling Yields. For any of the support matrix used, **Table 3** reports the main experimental results, that is, the protein concentrations in the immobilizing solution (c_{P0}) and overall filtrate (c_{Pf}), protein loading ($Y_{P/B}$), immobilized acid urease activity per unit mass of wet support (A_{Bw}), and enzyme coupling yield (ζ_E).

It is worthy of note that acid urease, as immobilized onto Chitopearls BCW-3003 or BCW-3010, was characterized by a specific activity and a protein loading of 260 ± 3 or 127 ± 4 IU g^{-1} ws and 36 or 14.4 mg BSAE g^{-1} ws, respectively. These parameters resulted in being quite close to those obtained previously (*11*), thus allowing the preparation procedure for these biocatalysts to be regarded as quite reproducible. On the contrary, the specific activity of the Eupergit C 250 L-based biocatalysts, prepared in this work (~10 IU g^{-1} ws), was found to be slightly smaller than that (19 ± 3 IU g^{-1} ws) pertaining to the same biocatalysts attained earlier (*11*, *12*).

Finally, the enzyme coupling yield (ζ_E) was about 65% for the smaller chitosan derivative beads BCW-3003, but reduced to 27 or 22% for the larger BCW-3010 or smaller Eupergit C 250 L particles, respectively.

Urea Degradation Rate by Free or Immobilized Acid Urease. By using a stirred bioreactor and the same operating conditions (S_{L0} = 1 mmol/L, E_i~47 mg/L BSAE) used previously to study the immobilized enzyme activity in model wine solutions (*11*, *12*), it was possible to determine the time course of urea degradation in two target Italian white wines, that is, *Grechetto* (G) and *Sauvignon Blanc* (SB) wines. Their main chemico-physical characteristics are shown in **Table 2**.

Depending on the different protein loadings of the three biocatalysts (BCW-3003, BCW-3010, or Eupergit C 250 L) used, their concentration (c_{Bw}) in any wine sample under testing was varied as shown in **Table 4**. In this way, it was possible to keep the immobilized enzymatic protein concentration ($E_i = c_{Bw} Y_{P/B}$) dispersed in the liquid phase approximately constant (47 ± 3 mg/L BSAE) in all tests.

Figure 1 shows the semilogarithmic plot of the ratio between the current (S_L) and initial (S_{L0}) concentrations of urea against time (t) for the two real white wines of concern when using free or immobilized acid urease. In all cases, such plots were approximately linear.

By accounting for eq 7 and assuming the overall effectiveness factor (Ω) as practically unitary, use of the least-squares method yielded the average values and standard deviations of the experimental pseudofirst-order kinetic rate constant (k_{ai}) for any wine sample and biocatalyst examined, as listed in **Table 4**.

The apparent pseudofirst-order kinetic rate constant (k_{af}) for urea degradation in *Grechetto*, *Sauvignan Blanc*, and model wines at 20 °C by free acid urease was also reported in **Table 4**.

As reported previously (*11*, *12*), it was possible to confirm that whatever the biocatalyst used the contribution of the external and/or internal diffusion resistances to the overall substrate reaction was negligible. In fact, for any of the three biocatalysts used, both the effectiveness factors (Ω) and (η) were estimated as practically unitary. Despite the fact that these estimates were referred to biocatalysts with a unitary tortuosity factor (τ) (*18*), these would still hold even if τ was as great as 7, the typical τ values for industrial catalysts ranging from 1 to 7 (*30*). In fact, for τ = 7 the loss in both effectiveness factors varied from as small as 0.01 to 0.4% for the range of bead size used.

In previous work (*11*), the apparent pseudofirst-order kinetic rate constant (k_{af}) for free acid urease in the model wine solution

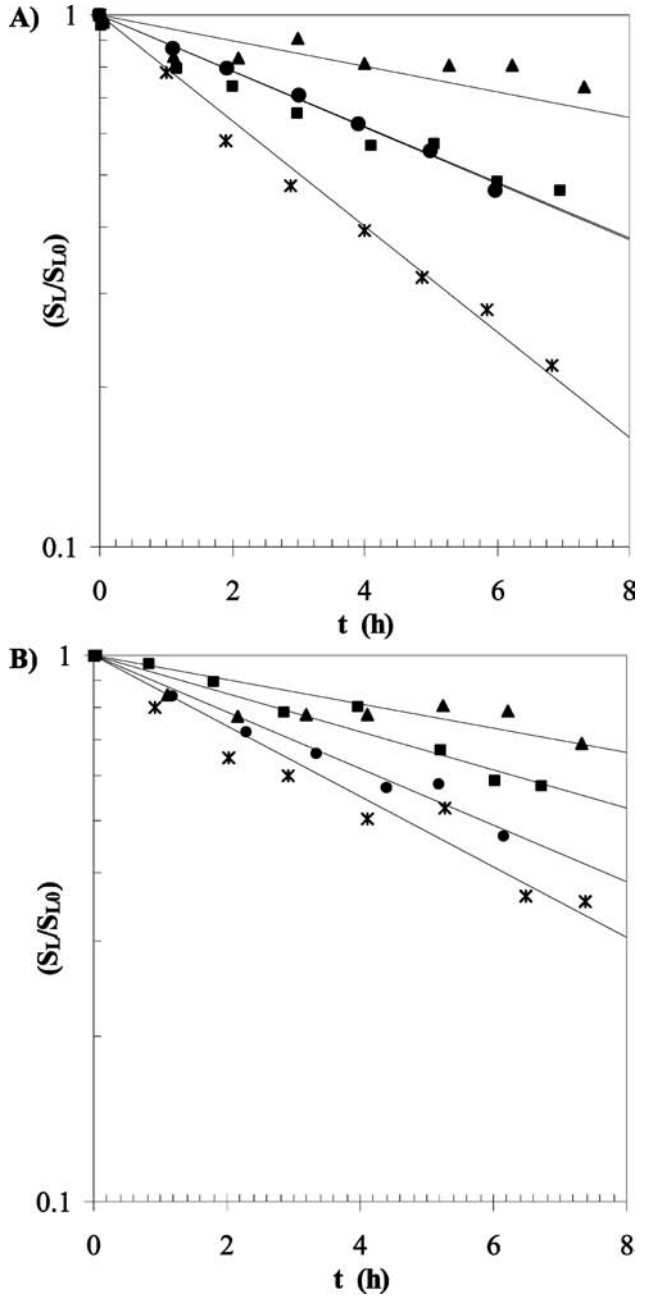

Figure 1. Time course of urea degradation at 20 °C for *Grechetto* (**A**) and *Sauvignon Blanc* (**B**) wines when using free enzyme (*, c_N = 1.2 g/L Nagapsin) or immobilized enzyme onto different matrixes (BCW-3003: ●, c_{Bw}= 1.25. BCW-3010: ■, c_{Bw}= 3.13. Eupergit C 250 L: ▲, c_{Bw}= 3.53 g/L wet carrier). Semilogarithmic plot of the ratio between the current and initial urea concentrations (S_L/S_{L0}) against time (t).

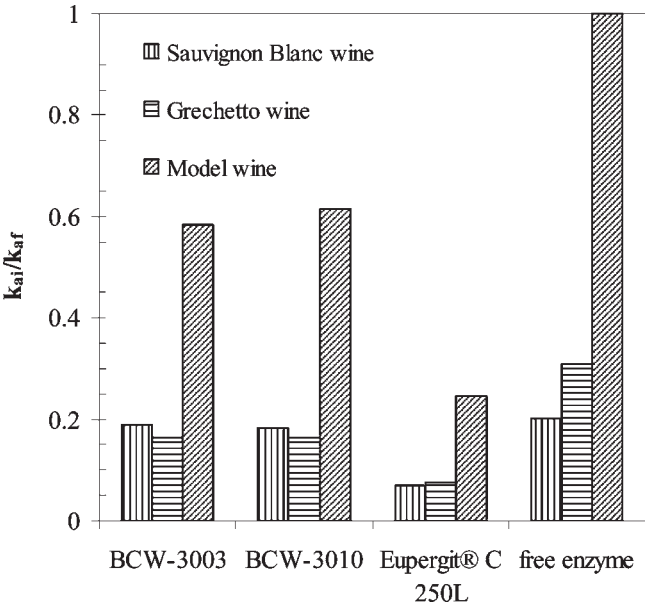

Figure 2. Experimental apparent pseudofirst-order kinetic rate constants for free acid urease (k_{af}) or enzyme immobilized (k_{ai}) onto different matrices (BCW-3003, BCW-3010, and Eupergit C 250 L) in *Grechetto*, *Sauvignon Blanc*, and model wines as compared to the k_{af} value pertaining to free acid urease in the model wine solution.

was found to be linearly related to the corresponding free enzymatic protein concentration (E_f) dissolved in the bulk medium, thus yielding a specific pseudofirst-order kinetic rate constant (k'_f) equal to 17 (± 1) L h^{-1} g^{-1} (r^2 = 0.984).

In these experiments, E_f was equal to 48 ± 1 mg/L BSAE, and this should have involved a k_{af} value of 0.82 (± 0.02) h^{-1}, that differed by about 10% from that (0.74 ± 0.02 h^{-1}) experimentally estimated (**Table 4**).

By referring the experimental k_{aj} values to the above k_{af} value, it was possible to directly compare the performance of the three biocatalysts tested in the model and real wines, as shown in **Figure 2**.

When using free acid urease, the apparent pseudofirst-order kinetic rate constant in *Grechetto* and *Sauvignon Blanc* wines was reduced to 30 or 20% of that observed in the model wine solution owing to their different composition and especially to their diversely higher phenolic content (**Table 2**).

When using acid urease immobilized onto Chitopearls BCW-3003 and BCW-3010 or Eupergit C 250 L, the apparent pseudofirst-order kinetic rate constant in *Grechetto* and *Sauvignon Blanc* wines was reduced to about 18 and 17% or 7% of that in the model wine solution for free enzyme (k_{af}), respectively. This confirmed that the biocatalysts obtained from Chitopearls BCW-3003 and BCW-3010 were more than twice less sensitive to the wine phenolic compounds than the Eupergit-based catalysts.

To explain such a peculiar behavior of the two white wines assayed, the purified acid urease preparation used here was assumed to behave approximately as the whole cell acid urease preparation (i.e., Enzeco Acid Urease, Enzyme Development Corp., New York) used previously (*7*). By using an initial urea concentration of 1 mmol/L throughout all the experimental design, the effects of the main inhibitory wine components (i.e., malic and lactic acids, potassium metabisulfite, and ethanol), as well as pH, on the specific pseudofirst-order kinetic rate constant for free acid urease (k'_f) in different model wine solutions were reconstructed by using the second-order canonical regression developed previously (*7*), that is eqs 8−14 in ref *8*. Then, to account for the inhibitory effect of the phenolic compounds (P) present in the real wines under testing, the effective specific pseudofirst-order kinetic rate constant (k'_{fe}) was guessed by resorting to the competitive inhibition model formerly proved, that is eq 15 in ref *8*, where the equilibrium constant (K_P) of the reaction between the enzyme and the total phenolic inhibitors was assumed as coincident with that (21.1 ± 0.5 mg/L GAE) pertaining to some white and rosé Italian wines (*8*). Despite the difference in the composition of the real wines assayed, the estimated k'_{fe} value (0.264 L h^{-1} g^{-1}) in *Sauvignon Blanc* wine was quite close to that calculated (0.254 L h^{-1} g^{-1}) in *Grechetto* one, thus corroborating the overall results shown in **Figure 2**.

Thus, the use of eqs 8−15 in ref *8* may be recommended as a short-cut procedure to roughly assess the kinetic response of free

acid urease in wines of different composition and figure out the likely effectiveness of the wine treatment by immobilized enzyme.

Concluding Remarks. The immobilization of acid urease onto glutaraldehyde-activated chitosan-derivative beads rather than Eupergit C 250 L gave rise to quite effective biocatalysts, their loss in activity being as small as 5% of the initial activity even after 150−170 days of storage at 4 °C (*11*).

For all the biocatalysts tested, the kinetics of urea degradation in two target real wines using a stirred bioreactor was of the pseudofirst order with respect to urea concentration, in agreement with previous findings in real and model wines using killed cell preparations (*6*−*8*, *12*, *32*), and was not controlled by urea external and intraparticle mass transfer. Owing to the peculiar phenolic content of *Grechetto* or *Sauvignon Blanc* wine, the apparent pseudofirst-order urea degradation kinetic rate constant for acid urease immobilized onto Eupergit C 250 L-, BCW-3003-, or BCW-3010-particles at E_i ~constant was reduced to about 7, 18, or 17% of that observed in the model wine solution for free enzyme, respectively (**Table 4**).

By accounting for their intrinsic positive characteristics of being resistant to the compression stress exerted by a column bed (*5*), sterilizable, nontoxic, biocompatible and biodegradable (*33*), and for being more than twice less sensitive to the wine phenolic content than the Eupergit-based biocatalysts, the chitosan-based catalysts seem to be potentially employable in the make up of packed-bed cartridges to continuously remove urea not only from sake (*5*) but also from commercial wines.

Wine treatment by using the commercially available killed cell acid urease preparations generally involves very low urea degradation rates because of the presence of numerous inhibitory components in real wines. In fact, by referring to the Enzeco Acid Urease preparation previously used (*7*, *8*), the maximum dose allowable was equivalent to quite a low enzymatic protein concentration (11.0 ± 0.6 mg/L BSAE) and yielded a low urea degradation rate (*12*). To accelerate the process, it would be necessary to resort to doses by far higher than the maximum allowable one (i.e., 75 mg/L) for wine treatment (*34*). Thus, strictly speaking, the wine detoxification trials performed here with free acid urease (see * symbols in **Figure 1**) could not be practically exploited since the dissolved enzymatic protein concentration (E_f) was about four times greater than the maximum allowable one. On the contrary, the wine treatment tests by using immobilized acid urease allowed the present regulation to be circumvented because the immobilized enzyme was insoluble in wine. Further increase in the experimentally observed urea degradation rates might be achieved by just enhancing the biocatalyst concentration (c_{Bw}) in the agitated bioreactor or alternatively by resorting to specifically designed packed-bed cartridges.

Further work will be directed to assess the operational performance and stability of a laboratory-scale packed-bed bioreactor to detoxify real wines and assess its economic feasibility.

NOTATION

GLOSSARY

A_{Bw} specific activity of the immobilized biocatalyst per unit mass of wet support (IU g^{-1} wet support)

A_{E0} acid urease activity in the immobilizing solution per unit mass of protein (IU g^{-1} BSAE)

A_{Ef} acid urease activity in the filtrate per unit mass of protein (IU g^{-1} BSAE)

a_p specific surface per unit volume for the biocatalyst (= $3/R$, m^{-1})

a_S overall biocatalyst surface per unit volume of liquid phase (= $a_p c_{Bw}/\rho_B$, m^{-1})

Bi Biot number (= $k_L R/D_{Se}$, dimensionless)

c_{Bw} wet biocatalyst concentration (g/L)

c_N concentration of Nagapsin preparation (g/L)

c_{P0} protein concentration in the immobilizing solution (mg/L BSAE)

c_{Pf} protein concentrations in the filtrate (mg/L BSAE)

D_S diffusivity for urea in the bulk liquid ($m^2 s^{-1}$)

D_{Se} effective diffusion coefficient for urea in the biocatalyst ($m^2 s^{-1}$)

E_f concentration of free enzymatic protein dissolved in the liquid bulk (mg/L BSAE)

E_i concentration of immobilized enzymatic protein dispersed in the liquid bulk (mg/L BSAE)

Gr Grashof number (= $8R^3\rho_L|\rho_L - \rho_B|g/(\mu_L)^2$, dimensionless)

j generic index used to refer to free (f) or immobilized (i) enzyme

K_P phenolic compound inhibition constant (mg/L GAE)

k_{aj} urea degradation apparent pseudofirst-order kinetic rate constant for free or immobilized enzyme (= Ωk_j; h^{-1})

k_j urea degradation pseudofirst-order kinetic rate constant for free or immobilized enzyme (h^{-1})

k'_j specific urea degradation pseudofirst-order kinetic rate constant for free or immobilized enzyme (L h^{-1} g^{-1})

k_L mass transfer coefficient in the liquid phase (m s^{-1})

m_{Bw} mass of wet carrier (g)

P concentration of phenolic compounds (mg/L GAE)

R average bead radius (m)

r^2 coefficient of determination (dimensionless)

r_{Si} urea degradation rate for immobilized enzyme referred to the unit volume of biocatalyst (mmol L^{-1} h^{-1})

S_L urea concentration in the liquid bulk (mmol/L)

S_R urea concentration at the biocatalyst surface (mmol/L)

Sc Schmidt number (= $\mu_L/(\rho_L D_S)$, dimensionless)

Sh Sherwood number (= $2Rk_L/D_S$, dimensionless)

t reaction time (h)

V_L liquid volume (L)

v_S overall biocatalyst volume the per unit volume of liquid phase (= c_{Bw}/ρ_B, dimensionless)

x_{Bw} water fraction of wet matrix or biocatalyst (g g^{-1})

$Y_{P/B}$ protein loading (g bound protein g^{-1} wet support)

Greek Symbols

ζ_E enzyme coupling yield (dimensionless)

η effectiveness factor for a spherical biocatalyst in the absence of the external film transport resistance {= $(3/\Phi)[1/\tanh(\Phi) - 1/\Phi]$, dimensionless}

θ biocatalyst porosity (dimensionless)

μ_L liquid viscosity (Pa s)

ν_L liquid kinematic viscosity (= μ_L/ρ_L, $m^2 s^{-1}$)

ρ_B biocatalyst density (g/L wet support)

ρ_L liquid density (g/L)

τ biocatalyst tortuosity factor (dimensionless)

Φ Thiele modulus for pseudofirst-order kinetics (= $R(k_i/D_{Se})^{1/2}$, dimensionless)

Ω effectiveness factor for a spherical biocatalyst in the presence of the external film transport resistance {= $\eta/[1 + (\eta\,\Phi^2)/(3Bi)]$, dimensionless}

Subscript

e	effective
f	referred to free enzyme
i	referred to immobilized enzyme
0	initial

LITERATURE CITED

(1) Ough, C. S.; Crowell, E. A.; Mooney, L. A. Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea, and ammonia effects of fortification on intracellular and extracellular precursors. *Am. J. Enol. Vitic.* **1988**, *39*, 243–249.

(2) Schlatter, J.; Lutz, W. K. The carcinogenic potential of ethyl carbamate (urethane): risk assessment at human dietary exposure levels. *Food Chem. Toxicol.* **1990**, *28*, 205–211.

(3) Butzke, C. E.; Bisson, L. F. *Ethyl Carbamate Preventative Action Manual*; Food and Drug Administration, Center for Food Safety and Applied Nutrition. http://www.cfsan.fda.gov/~frf/ecaction.html.

(4) Krajewska, B.; Ureases, I. Functional, catalytic and kinetic properties: a review. *J. Mol. Catal. B: Enzym.* **2009**, *59*, 9–21.

(5) Matsumoto, K. Removal of urea from alcoholic beverages by immobilised acid urease. *Bioprocess Technol.* **1993**, *16*, 255–273.

(6) Trioli, G.; Ough, C. S. Causes for inhibition of an acid urease from *Lactobacillus fermentus*. *Am. J. Enol. Vitic.* **1989**, *40*, 245–52.

(7) Fidaleo, M.; Esti, M.; Moresi, M. Assessment of urea degradation rate in model wine solutions by acid urease from *Lactobacillus fermentum*. *J. Agric. Food Chem.* **2006**, *54*, 6226–6235.

(8) Esti, M.; Fidaleo, M.; Moresi, M.; Tamborra, P. Modeling of urea degradation in white and rosé wines by acid urease. *J. Agric. Food Chem.* **2007**, *55*, 2590–2596.

(9) Krajewska, B. Ureases II. Properties and their customizing by enzyme immobilizations: a review. *J. Mol. Catal. B: Enzym.* **2009**, *59*, 22–40.

(10) Zhou, J.; Chen, S.; Wang, J. Research on orientedly immobilised urease via Concanavalin A. *Chin. J. Biotechnol.* **2008**, *24*, 617–621.

(11) Andrich, L.; Esti, M.; Moresi, M. Urea degradation kinetics in model wine solutions by acid urease immobilised onto chitosan-derivative beads of different sizes. *Enzyme Microb. Technol.* **2010**, *46*, 397–405.

(12) Andrich, L.; Esti, M.; Moresi, M. Urea degradation in model wine solutions by free or immobilized acid urease in a stirred bioreactor. *J. Agric. Food Chem.* **2009**, *57*, 3533–3542.

(13) Selvamurugan, C.; Lavanya, A.; Sivasankar, B. A comparative study on immobilisation of urease on different matrices. *J. Sci. Ind. Res.* **2007**, *66*, 655–659.

(14) Katchalski-Kazir, E.; Kraemer, D. M. Eupergit® C, a carrier for immobilization of enzymes of industrial potential. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 157–176.

(15) Boller, T.; Meier, C.; Menzler, S. Eupergit oxirane acrylic beads: how to make enzymes fit for biocatalysis. *Org. Process Res. Dev.* **2002**, *6*, 509–519.

(16) Krajewska, B. Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme Microb. Technol.* **2004**, *35*, 126–139.

(17) Honarkar, H.; Barikani, M. Applications of biopolymers I: chitosan. *Monatsh. Chem.* **2009**, *140*, 1403–1420.

(18) Spiess, A.; Schlothauer, R.-C.; Hinrichs, J.; Scheidat, B.; Kasche, V. pH gradients in immobilized amidases and their influence on rates and yields of β-lactam hydrolysis. *Biotechnol. Bioeng.* **1999**, *62*, 267–277.

(19) Tagatsuji, W.; Yoshida, H. Adsorption of organic acids on polyaminated highly porous chitosan: Equilibria. *Ind. Eng. Res.* **1998**, *37*, 1300–1309.

(20) Knezevic, Z.; Milosavic, N.; Bezbradica, D.; Jakovljevic, Z.; Prodanovic, R. Immobilization of lipase from *Candida rugosa* on Eupergit® C supports by covalent attachment. *Biochem. Eng. J.* **2006**, *30*, 269–278.

(21) Kayastha, A. M.; Srivastava, P. K. Pigeonpea (*Cajanus cajan* L.) urease immobilized on glutaraldehyde-activated chitosan beads and its analytical applications. *Appl. Biochem. Biotechnol.* **2001**, *96*, 41–53.

(22) Oliveira, M. I. P.; Pimentel, M. C.; Montenegro, M.C.B.S.M.; Araújo, A. N.; Pimentel, M. F.; Lins da Silva, V. *L*-glutamate determination in food samples by flow-injection analysis. *Anal. Chim. Acta* **2001**, *448*, 207–213.

(23) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(24) Kerscher, L.; Ziegenhorn, J. Urea. *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H. U., Ed.; Vol. 8; VCH Publishers Ltd.: Cambridge, UK, 1990; pp 444−453.

(25) Office International de la Vigne et du Vin. Recueil des méthodes internationaux d'analyse des vins et des mouts. OIV, Paris, **1990**.

(26) Di Stefano, R.; Cravero, M. C.; Gentilini, N. Methods for wine polyphenol study. *L'enotecnico* **1989**, *25*, 83–89.

(27) ASTM. Standard Method of Test for Kinetic Viscosity (ASTM D445-IP 71). *ASTM Standards: Electrical Insulating Materials: Part 29*, American Society for Testing and Materials, Baltimore, MD, 1964; pp 312−363.

(28) Cane, P. Quality control of wine by HPLC: determination of organic acids. *L'Enotecnico* **1990**, *1*, 69–72.

(29) Bailey, J. E.; Ollis, D. F. *Biochemical Engineering Fundamentals*, 2nd ed.; McGraw-Hill: New York, 1986; pp 204−227.

(30) Satterfield, C. N.; Sherwood, T. K. *The Role of Diffusion in Catalysis*; Addison-Wesley Publshing Co.: Reading, MA, 1963; p 47.

(31) Treybal, R. E. *Mass Transfer Operations*, 2nd ed.; McGraw-Hill: New York, 1968.

(32) Kodama, S; Yotsuzuka, F. Acid urease: reduction of ethyl carbamate formation in sherry under simulated baking conditions. *J. Food Sci.* **1996**, *61*, 304–307.

(33) Chen, J. P.; Chiu, S. H. Preparation and characterization of urease immobilized onto porous chitosan beads for urea hydrolysis. *Bioprocess Eng.* **1999**, *21*, 323–330.

(34) Bertrand, A. Utilizzazione dell'ureasi nel vino. In *Prodotti di trattamento ed ausiliari di eleborazione dei mosti e dei vini*; Vivas, N., Ed.; Eno-one srl: Reggio Emilia, Italy, 2003; pp 171−192.

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