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Urea Degradation in Model Wine Solutions by Free or Immobilized Acid Urease in a Stirred Bioreactor

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In this work, a purified acid urease preparation was covalently immobilized on Eupergit C 250 L and stabilized with glycine. Its average activity was found to be 69 ± 16% of the initial one after 34-day storage at 4 °C. The kinetics of urea degradation in a model wine solution by immobilized enzyme was confirmed to be of pseudo-first-order with respect to the urea concentration in the liquid bulk, its apparent pseudo-first-order kinetic rate constant (k_{li}) being about one-fourth of that (k_{lf}) pertaining to the free enzyme. In the operating conditions tested, the reaction kinetics was estimated as unaffected by the contribution of the external film and intraparticle diffusion mass transfer resistances. Because in the presence of the high-inhibitory tannins extracted from grape seeds in the range of 3−620 g of GAE m^{-3} the loss in k_{li} was quite smaller than that in k_{lf}, the biocatalyst tested here is likely to overcome the present limits to the application of free acid urease in wine treatment.

KEYWORDS: Enzyme and activity coupling yields; Eupergit C 250 L; free or immobilized acid urease; grape seed tannins; model wine solution; pseudo-first-order kinetic constant rate; stirred bioreactor; urea degradation kinetics

INTRODUCTION

Ethyl carbamate (urethane, EC) is a naturally occurring component in all fermented foods and beverages, being spontaneously produced by the reaction between urea and ethanol (*1*). Owing to its potential carcinogenic activity when administered in high doses in animal tests (*2*, *3*), EC levels in food products are to be greatly reduced. In particular, to minimize EC levels in wine, the U.S. Food and Drug Administration has so far recommended several precautionary actions, such as control of fertilization techniques, adjustment of nutrient contents in grape musts, use of suitable yeast and lactic acid bacteria strains, acid urease application, and control of storage conditions (*4*).

The feasibility of acid urease application for the removal of urea from several types of wines has been extensively demonstrated (*5*−*10*). However, the effectiveness of this treatment differed depending on the type of wine, the content of some inhibiting factors [i.e., in order of importance, fluoride, malate, ethanol, and phenolic compounds (*11*−*13*)], and usage conditions.

In previous work (*14*) the effects and interactions of the concentrations of malic (*M*) and lactic (*L*) acids and potassium metabisulfite (*K*), ethanol volumetric fraction (*E*), and pH on the specific activity of a commercial preparation of whole cell acid urease (Enzeco Acid Urease) in model wine solutions was assessed by performing a central composite design. The experimental responses were fitted by using a second-order polynomial reduced to its canonical form so as to identify the only statistically significant principal axes.

The kinetics of urea hydrolysis to ammonia and carbon dioxide by free urease (EC 3.5.1.5)

$$(NH_2)_2CO + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2 \qquad (1)$$

is generally described by means of a modified Michaelis−Menten reaction rate expression, which incorporates pH -dependent kinetics, substrate inhibition, and noncompetitive product inhibition by NH_4^+ (*15*)

$$r_{Sf} = \frac{v_{max} S_L}{\left(K_M + S_L + \frac{S_L^2}{K_S}\right)\left(1 + \frac{A_L}{K_A}\right)} \qquad (2)$$

where r_{Sf} is the urea degradation rate (expressed in mol h^{-1} m^{-3}) by free enzyme, v_{max} is its maximum value, K_M is the Michaelis−Menten constant, K_S and K_A are the substrate and product inhibitory constants, respectively, and S_L and A_L are the substrate and ammonium concentrations in the liquid bulk, respectively. More specifically, the Michaelis−Menten constant (K_M) was reported as practically independent of pH (*16*), its value for the acid urease from *L. fermentum* being equal to 3 ± 2 mol m^{-3} at pH 3 and 20 °C (*14*).

In model (*14*) and real (*17*) wines, r_{Sf} was found to vary quite linearly with S_L up to $S_L \approx K_M$, thus allowing its kinetic model to be reduced to the pseudo-first-order one

$$r_{Sf} = k_{If} S_L \qquad (3)$$

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with

$$k_{If} = k'_{If}E_f \quad (4)$$

where k_{If} is the pseudo-first-order kinetic rate constant, E_f the free enzymatic protein concentration, and k'_{If} the specific pseudo-first-order kinetic rate constant relative to the free enzyme.

Moreover, in model wine solutions k_{If} was found to be largely greater (*14*) than that pertaining to some real wines assayed by Kodama (*13*) and Trioli and Ough (*11*). It was also assumed that activity of free acid urease in real wines was competitively inhibited by phenolic compounds (*P*, the total phenolic content being expressed in g m^{-3} gallic acid equiv, GAE) as a result of their linking to the enzyme active site to form an enzyme−inhibitor complex (EP) (*17*). In this way, the effective pseudo-first-order kinetic rate constant (k_{Ife}) was estimated as

$$k_{Ife} = \frac{k_{If}}{1 + \frac{P}{K_{Pf}}} \quad (5)$$

where K_{Pf} is the equilibrium constant of the reaction between the free enzyme (E) and phenolic inhibitors (P). For the five Italian wines tested previously (*17*), this constant resulted to be equal to 21.1 ± 0.5 g of GAE m^{-3}, whereas it ranged from 15.7 to 44.7 g of GAE m^{-3} when the enzyme activity was checked using model wine solutions having the same pH, composition, and overall phenolic content (in the range of 100−850 g of GAE m^{-3}) of the real wines examined with a basic difference in the tannin sources (i.e., grape seeds or skins, respectively) (*17*).

These results confirmed previous findings by Trioli and Ough (*11*), who observed a greater reduction in the acid urease activity when the model wine samples were integrated with grape seed tannins with respect to those enriched with catechin in the range of 0−880 g of GAE m^{-3}. Thus, use of model wine solutions enriched with tannins extracted from grape seeds was suggested to assess preliminarily the technoeconomic feasibility of the acid urease hydrolytic process by free or immobilized acid urease (*17*).

Immobilization of acid urease on an inert carrier would have the potential advantages of significant cost savings (because it facilitates enzyme recycle through multiple cycles of batchwise hydrolysis), improved stability, or resistance to shear or inhibitory compound inactivation. Acid urease has been so far immobilized on various matrices, such as polyacrylonitrile (PAN) and chitosan derivatives (*16*, *18*), nylon beads, Sepharose gel, silica gel, and gelatin film coated on cellulose acetate membrane (*19*). Continuous urea removal from sake by immobilized acid urease on PAN has been applied by many companies in Japan since 1988 (*16*).

In the present work, acid urease was bound to a well-known commercial carrier, Eupergit C 250 L (*20*). In particular, this epoxy-activated immobilization support has been identified as one of the most useful carriers for covalent immobilization of a wide variety of different enzymes (i.e., penicillin amidase, EC 3.5.1.11; D-amino acid oxidase, EC 1.4.3.3; glutaryl-7-aminocephalosporanic acid acylase, EC 3.5.1.4; 5-acetylneuraminic acid aldolase, EC 4.1.3.3; and cytidine deaminase, EC 3.5.4.5) for industrial pharmaceutical applications (*20*, *21*) because of its ability to stabilize protein conformation by multipoint attachment (*20*−*22*).

Therefore, the main aims of this work were to compare the apparent pseudo-first-order kinetic rate constants of urea degradation in a wine model solution, corresponding to the central point of a composite design experiment previously carried out (*14*), in the absence or presence of grape seeds tannins, using a stirred bioreactor charged with soluble purified acid urease from *L. fermentum*, as such or bound to Eupergit C 250 L.

MATERIALS AND METHODS

Raw Materials. Two lots, 1 (ref no. 2735159) and 2 (ref no. 3707118), of the commercial preparation Nagapsin, both donated by Nagase Europa GmbH (Duesseldorf, Germany), were used. They consisted of soluble powders, approximately composed of 96% (w/w) lactose and 4% (w/w) purified acid urease from *L. fermentum*, to be stored at 4 °C. At the moment of their use, the corresponding average specific activities were 766 ± 7 or 420 ± 27 IU g^{-1}, respectively, where 1 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 (SRM) composed of 0.1 kmol m^{-3} sodium acetate buffer (pH 4.0) enriched with urea (83.33 mol m^{-3}).

Eupergit C 250 L is an epoxy (oxirane)-activated macroporous support with an average particle size of 180 μm that was kindly provided by Rohm GmbH (Darmstadt, Germany). The water content (x_{Bw}) of the beads as such or after 2 h of swelling in 0.05 M potassium phosphate buffer, pH 7.0 (KPB7), increased from 3.2 ± 0.4 to 84 ± 3% (w/w), respectively.

The performance of free or immobilized acid urease at 20 °C was assessed in a model wine solution representing the central point of the composite design previously described (*14*). This model solution was prepared by dissolving constant amounts of urea (60 g m^{-3}), tartaric (5 kg m^{-3}), malic (2.5 kg m^{-3}), and lactic (1.75 kg m^{-3}) acids, potassium metabisulfite (0.2 kg m^{-3}), and ethanol (13% v/v) in deionized water and then adjusting the resulting pH to 3.50. All reagents were of analytical grade.

An extract of grape seeds (Grap'tanPC), manufactured by Ferco Oenologie (Saint Montan, France), was used as a source of phenolic compounds for the model wine solution used. Its moisture and total phenolics contents were found to be 2.3 ± 0.3% (w/w) and 0.62 ± 0.01 g of GAE per g of extract, respectively.

To assess the inhibitory effect of tannins on immobilized acid urease, the above model wine solution was enriched with 5, 25, 150, 300, 500, or 1000 g m^{-3} of such an extract.

Enzyme Immobilization Procedure. Enzyme immobilization on Eupergit supports was carried out in accordance with the conventional method that involves the direct enzyme binding on polymers via oxirane groups (*23*). Two slightly different procedures were used. The first one was preliminarily used to prepare the immobilized beads and assess their storage stability, whereas the second one was used to prepare the biocatalysts for kinetic studies in a stirred bioreactor.

In the first procedure, unmodified Eupergit C 250 L beads (25 mg in each test) were charged in 10 cm^3 rubber-capped flasks and soaked in 2 cm^3 of KPB7 at 4 °C for 24 h before immobilization. Then, 3 cm^3 of the same buffer solution enriched with different amounts of the enzyme preparation (4−12 kg m^{-3}) was added. After gentle shaking three times a day, the dispersion was left to stand at 4 °C for 72 h. The biocatalyst was recovered by centrifugation (5000 rev min^{-1} for 3 min). After collection of the supernatant, the beads were newly suspended in KPB7 and centrifuged. All supernatants were collected and diluted with the above buffer solution to a constant final volume. A few lots of the biocatalyst were dispersed in 1 M NaCl, recovered by centrifugation, and then soaked in an aqueous solution containing 75 mol m^{-3} glycine at 4 °C for 20 min (*24*). Alternatively, other lots were soaked in the above buffer solution containing 5 kg m^{-3} bovine serum albumin (BSA) at room temperature for 24 h (*25*). The biocatalysts stabilized with glycine or BSA were

recovered by centrifugation, washed with KPB7, and newly centrifuged before their activity was assessed as reported below.

To avoid microbial contamination during storage at 4 °C, the biocatalysts were stored in KPB7 enriched with 2% (v/v) isopropanol and 0.5 kg m^{-3} ethyl parabene, as suggested by the carrier manufacturer. The storage stability of biocatalysts as such or stabilized was assessed after dispersion of the beads, as recovered by the aforementioned centrifugation−washing−centrifugation steps, in 25 cm^3 beakers containing 22 cm^3 of the above SRM for as long as 34 days.

The second procedure was used to prepare amounts of biocatalyst adequate for kinetic studies. To this end, 0.6−1.2 g of dry beads and 30−35 cm^3 of KPB7 at 20 °C were charged into a 150 cm^3 Pyrex flask, equipped with a portable, 40 mm marine-type propeller mixer IKA (model EUROSTAR) rotating at 250 rev min^{-1}, which was mounted vertically on center with baffles at the wall. After about 24 h of soaking, 72−100 cm^3 of KPB7 enriched with 16.0 or 24.0 kg m^{-3} of Nagapsin, preconditioned at 20 °C, was added while the dispersion continued to be mixed. After incubation for 24 or 48 h, the biocatalyst was collected by vacuum filtration using a glass filter (1.2 μm Whatman GF/C disk), washed twice with 50 cm^3 of KPB7. All filtrates were collected and diluted with KPB7 to a final volume of 250 cm^3.

The wet beads were soaked in an aqueous solution containing 75 mol m^{-3} glycine at 4 °C for 20 min (*24*), washed with KPB7, and stored at 4 °C in the wet state in KPB7 supplemented with 2% (v/v) isopropanol and 0.5 kg m^{-3} ethyl parabene, as reported above.

Determination of Bound Enzyme. The protein concentration (c_P) in all solutions was determined according to the method of Lowry et al. (*26*) using the Total Protein Kit (Sigma, St. Louis, MO) and the associated protein standard solution (containing 100 kg m^{-3} of BSA) diluted to vary the BSA content in the range from 0 to 1000 g m^{-3}.

The amount of bound protein (m_{Pb}) was indirectly assessed by subtracting the amount of protein in the supernatant and washing solutions ($c_{Pf}V_f$) from the amount of protein present in the immobilizing solution ($m_{P0} = c_{P0}V_0$):

$$m_{Pb} = (c_{P0}V_0) - (c_{Pf}V_f) \quad (6)$$

where V_0 and V_f are the volumes of the immobilizing solutions and supernatant and washing solutions, respectively, and c_{P0} and c_{Pf} are the protein concentrations in the corresponding solutions.

The *protein loading* was defined as the amount of bound protein (m_{Pb}) per gram of dry support (m_{Bd}) and calculated as

$$Y_{P/B} = m_{Pb}/m_{Bd} \quad \text{(g of bound protein/g of dry support)} \quad (7)$$

with

$$m_{Bd} = m_{Bw}(1 - x_{Bw}) \quad (8)$$

where m_{Bw} is the mass of wet carrier supplied.

Enzyme Activity Assay. The acid urease activity in the immobilizing solution (A_{E0}), supernatant or filtrate (A_{Ef}), or immobilized enzyme (A_{BEI}) per unit mass of protein was estimated as follows.

In a 25 cm^3 beaker containing a 10 mm magnetic stirrer the following liquids were added in sequence: 5.65 cm^3 of 0.1 M acetate buffer pH (4.0), 5 cm^3 of the same buffer containing 11 kg m^{-3} of urea, and 0.35 cm^3 of the sample to be tested. The resulting reaction mixture was agitated at 400 rev min^{-1} and incubated in a water bath at 20 °C for 10 min.

The specific activity of the immobilizing solution was estimated by dividing the ammonia formation rate (r_{A0}) by its corresponding protein content (c_{P0}):

$$A_{E0} = r_{A0}/c_{P0} \quad \text{(IU per gram of BSA equiv)} \quad (9)$$

The specific activity of the supernatant or filtrate solution, including the washing solutions, was estimated by dividing the ammonia formation rate (r_{Af}) by its corresponding protein concentration (c_{Pf}):

$$A_{Ef} = r_{Af}/c_{Pf} \quad \text{(IU per gram of BSA equiv)} \quad (10)$$

The specific activity of the immobilized biocatalyst was estimated by dividing the ammonia formation rate (r_{AB}) by the amount of dry biocatalyst (m_{Bd}) or bound protein (m_{Pb}) as

$$A_{Bi} = r_{AB}/m_{Bd} \quad \text{(IU per gram of dry support)} \quad (11)$$

$$A_{BEi} = A_{Bi}/Y_{P/B} \quad \text{(IU per gram of bound protein)} \quad (12)$$

The efficiency of immobilization was evaluated in terms of enzyme (ζ_E) and activity (ζ_A) coupling yields, which were estimated as follows:

$$\zeta_E = m_{Pb}/m_{P0} \quad (13)$$

$$\zeta_A = A_{BEi}/A_{E0} \quad (14)$$

Urea Degradation Kinetics by Free or Immobilized Acid Urease. To assess the time course of the hydrolytic process under study, 80 cm^3 of the model wine solution as such or enriched with Grap'tanPC, preconditioned at 20 °C, was poured into a 100 cm^3 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 a thermostat model F3 (Haake, Karlsruhe, Germany), and placed over a magnetic multistirrer model Multistirrer 15 (Velp Scientifica, Milan, Italy) to ensure two different stirring levels at 250 or 400 rev min^{-1}. Several samples (1 cm^3) were withdrawn from any flask for as long as 24−150 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 kits and grape seed tannins, if present in the model wine samples, was limited by diluting the samples with an equal volume of an aqueous solution containing polyvinylpyrrolidone (PVP) at the same concentration of the grape seed extract present in the sample to be assayed.

The kinetics of free acid urease in the model wine solution at zero tannin content ($P = 0$) was also assessed by setting the initial concentration of Nagapsin to 75, 300, and 820 g m^{-3}, these being equivalent to enzymatic protein contents in the range of 4.3−46.5 g m^{-3} of BSA equiv. On the contrary, the kinetics of immobilized acid urease in the basic model wine solution was measured using the biocatalyst prepared by the second procedure at three different levels, that is, 3.8, 5.6, and 9.4 kg m^{-3} of wet carrier. Further tests, using a constant concentration of 6.25 kg m^{-3} wet biocatalyst, were carried out to assess the effect of grape seed tannins on the kinetic response of immobilized acid urease.

RESULTS AND DISCUSSION

Storage Stability of Immobilized Acid Urease. The storage stability at 4 °C of soluble acid urease immobilized on Eupergit C 250 L, used as such or after stabilization with glycine or BSA, was assessed for as long as 34 days (data not shown). Despite a fast partial inactivation in the first 5−10 days, the immobilized biocatalyst as such or stabilized with glycine tended to recover its initial activity, especially when using immobilizing solutions containing as much as 8−12 kg m^{-3} of Nagapsin (lot 1). Moreover, it was impossible to corroborate the stabilizing effectiveness of BSA observed in the covalent immobilization of penicillin acylase from *Streptomyces lavendulae* on Eupergit C by Torres-Bacete et al. (*25*).

When the Nagapsin concentration in the immobilizing solution was further varied from 8 to 16 kg m^{-3}, the immobilized biocatalyst stabilized with glycine exhibited a sudden inactivation after just 5 days; however, it kept as much as 69 ± 16% of its initial activity over the following 30 days independent of the enzyme added per unit mass of support used (**Figure 1**). The initial abrupt inactivation might be a consequence of the heterogeneity of the immobilized biocatalyst with acid urease molecules differing in their orientation toward the bulk solvent (*27*).

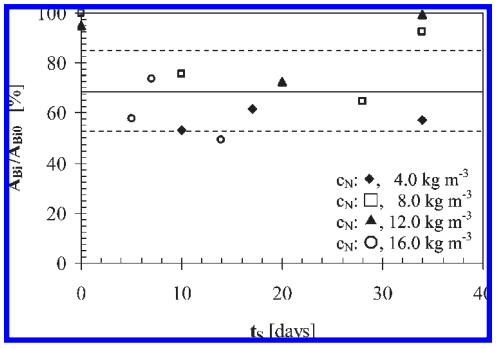

Figure 1. Effect of the concentration of Nagapsin preparation (c_N) in the immobilizing solution on the storage stability at 4 °C of soluble acid urease immobilized on 25 mg of Eupergit C 250 L and stabilized with glycine: percentage specific biocatalyst activity (A_{Bi}/A_{Bi0}) versus storage time (t_S).

Enzyme Binding and Activity Coupling Yields. Table 1 reports the experimental conditions used to immobilize conventionally acid urease on the above wet carrier and stabilize the biocatalyst with glycine, as well as the protein concentrations in the immobilizing solution (c_{P0}) and overall filtrate (c_{Pf}), protein bound on support (m_{Pb}), protein loading ($Y_{P/B}$), immobilized acid urease activity per unit mass of support (A_{Bi}) or protein bound (A_{BEi}), and enzyme (ζ_E) and activity (ζ_A) coupling yields.

In particular, when using lot 1 of Nagapsin, $Y_{P/B}$ (= 25.9 mg of BSA equiv per g of dry support) was found to be in good agreement with that pertaining to other enzymes bound to the same support, that is, lipase (39 mg g^{-1}) (*23*) or dextransucrase (23.1 mg g^{-1}) (*27*). However, when using lot 2 and the above immobilizing conditions, $Y_{P/B}$ declined to about 7 mg g^{-1}. By trial and errors, it was possible to improve $Y_{P/B}$ to 22.5 mg g^{-1} by increasing both the Nagapsin concentration in the immobilizing solution and the immobilization time (t_I) from 16 to 24 kg m^{-3} and from 24 to 48 h, respectively (**Table 1**). Under these circumstances, the enzyme (ζ_E) and activity (ζ_A) coupling yields did not differ, being approximately equal to 24 and 27%, respectively (**Table 1**).

Modeling of Urea Degradation in a Stirred Tank Bioreactor. When using immobilized acid urease, it is highly probable that enzyme coupling to the support of choice does not affect the pseudo-first-order kinetic model of free enzyme, especially in all applications where the urea concentration is by far smaller than the Michaelis−Menten constant of the free enzyme (*14*). In these circumstances, the urea degradation rate referred to the unit volume of immobilized acid urease (r_{Si}) may be expressed using the following modified form of eq 3

$$r_{Si} = k_{Ii} S \tag{15}$$

with

$$k_{Ii} = k'_{Ii} \rho_B Y_{P/B} \tag{16}$$

where k_{Ii} is the urea degradation pseudo-first-order kinetic rate constant of the biocatalyst of concern, ρ_B is the biocatalyst density, $Y_{P/B}$ is the *protein loading*, k'_{Ii} is the specific

Table 1. Experimental Conditions and Assessment of the Enzyme and Activity Coupling Yields for Lots 1 and 2 of Soluble Acid Urease as a Result of Direct Binding to Eupergit C 250 L Beads Followed by Stabilization with Glycine

		value		
parameter	symbol	lot 1	lot 2	unit
immobilisation time	t_i	24	48	h
mass of dry support	m_{Bd}	597.7	1161.8	mg
support soaking volume	V_S	35	30	cm^3
immobilizing solution volume	V_0	72	100	cm^3
Nagapsin concentration in the immobilizing solution	c_N	16.0	24.0	kg m^{-3}
protein concentration in the immobilizing solution	c_{P0}	881 ± 14	1058 ± 18	g m^{-3} BSA equiv
initial protein mass	m_{P0}	63 ± 1	106 ± 2	mg of BSA equiv
protein added per unit mass of support	$Y_{P/S}$	106	91	mg of BSA equiv g^{-1} of support
immobilizing solution activity	A_{E0}	13918 ± 119	9632 ± 957	IU g^{-1} of BSA equiv
filtrate volume	V_f	250	250	cm^3
protein concentration in the filtrate	c_{Pf}	192 ± 9	319 ± 6	g m^{-3} of BSA equiv
protein bound on support	m_{Pb}	15.5	26.1	mg of BSA equiv
protein loading	$Y_{P/B}$	25.9	22.5	mg of BSA equiv g^{-1} of support
specific immobilized enzyme activity	A_{Bi}	99 ± 17	61 ± 3	IU g^{-1} of dry support
	A_{BEi}	3816 ± 665	2713 ± 125	IU g^{-1} of BSA equiv
enzyme coupling yield	ζ_E	24.4	24.7	%
enzyme activity coupling yield	ζ_A	27 ± 5	28 ± 1	%

pseudo-first-order kinetic rate constant relative to immobilized enzyme, and S is the urea concentration in the liquid infiltrating into the bead pores.

When using a perfectly mixed bioreactor, charged with a volume (V_L) of the model wine solution with an initial concentration of urea S_{L0} and inoculated with a prefixed concentration (c_{Bd}) of dry biocatalyst in the form of almost spherical beads with an average radius R and specific surface per unit volume (a_p), the urea concentration profile within the spherical biocatalyst and through the liquid film adhering the external surface of the biocatalyst itself is sketched in **Figure 2**. In these circumstances, the overall surface (a_S) and volume (v_S) for the biocatalyst per unit volume of liquid phase may be estimated as

$$a_S = a_p c_{Bd}/\rho_B \tag{17}$$

$$v_S = c_{Bd}/\rho_B \tag{18}$$

The unsteady-state material balance for urea may be written as

$$S_L V_L|_t = S_L V_L|_{t+dt} + k_L a_S V_L (S_L - S_R)\, dt \tag{19}$$

with the boundary condition

$$k_L a_S V_L (S_L - S_R) = \Omega(k_{Ii} SL) v_S V_L \tag{20}$$

with

$$\Omega = \frac{\eta}{1 + \frac{\eta\Phi^2}{3Bi}} \tag{21}$$

$$\eta = \frac{3}{\Phi}\left(\frac{1}{\tan h(\Phi)} - \frac{1}{\Phi}\right) \tag{22}$$

$$\Phi = R\sqrt{\frac{k_{Ii}}{D_{Se}}} \tag{23}$$

$$Bi = \frac{k_L R}{D_{Se}} \tag{24}$$

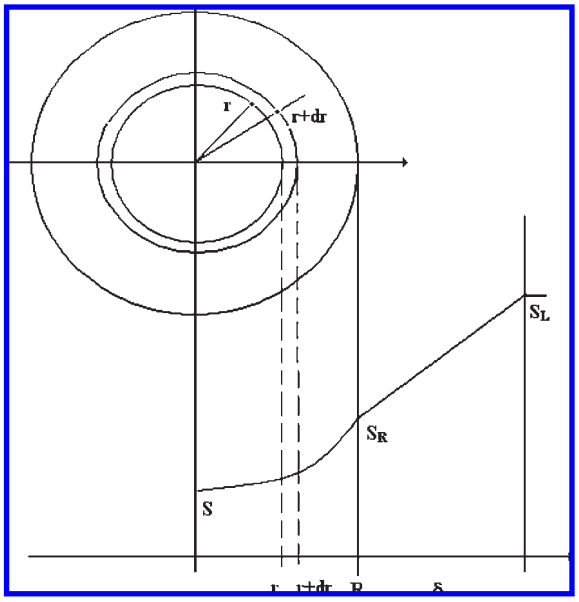

Figure 2. Substrate concentration profile within the spherical biocatalyst of average radius R and liquid film adhering to the external surface of the biocatalyst itself.

where Ω or η is the effectiveness factor for a spherical biocatalyst in the presence or absence of the external film transport resistance, Φ the Thiele modulus for pseudo-first-order kinetics, Bi the Biot number, which measures the ratio between the external film transport and intraparticle diffusion rates of the reagent of concern, k_L and D_{Se} are the mass transfer coefficient in the liquid phase and effective diffusion coefficient for urea, and S_R is the reagent concentration at the biocatalyst surface (*28*).

Equation 19 can be converted into the following first-order differential equation

$$-\frac{d\,S_L}{d\,t} = k_L a_S (S_L - S_R) = \Omega v_S k_{Ii} S_L \tag{25}$$

and integrated with the following initial condition:

$$S_L = S_{L0} \quad \text{for } t = 0 \tag{26}$$

By introducing eqs 16 and 18 into the right-hand side of eq 25 and integrating by variable separation, the following can be obtained:

$$\ln\left(\frac{S_L}{S_{L0}}\right) = \int_{S_{L0}}^{S_L} \frac{dS_L}{S_L} = -\int_0^t \Omega\, c_{Bd}\, Y_{P/B}\, k'_{Ii}\, dt \tag{27}$$

Finally, by referring to eqs 1 and 27, it would be possible to evaluate the instantaneous concentration of ammonium ions in the liquid bulk

$$A_L = A_{L0} + 2(S_{L0} - S_L) \tag{28}$$

where A_{L0} is the corresponding initial molar concentration of NH_4^+.

Urea Degradation Rate by Free Acid Urease. The kinetics of free acid urease in the model wine solution was assessed at different concentrations of Nagapsin (lot 1) in the range of 75−820 g m^{-3}, this being equivalent to a protein content (E_f) in the range of 4.3−46.5 g m^{-3} of BSA equiv, as shown by the open symbols in **Figure 3**.

It can be noted that the semilogarithmic plots of the dependent variable (S_L) against reaction time (t) were approximately linear, thus allowing the integrand function ($\Omega\ c_{Bd} Y_{E/B} k'_{Ii}$) in the integral in the right-hand side of eq 27 to be regarded as a practically constant function. Actually, in the case of free enzyme, the overall effectiveness factor (Ω) is intrinsically unitary, the product of c_{Bd} by $Y_{P/B}$ coincides with the free enzymatic protein concentration (E_f) dissolved in the liquid phase, whereas in accordance with eq 4 k'_{Ii} is equivalent to the specific pseudo-first-order kinetic rate constant relative to the free enzyme (k'_{If}). In fact, by plotting

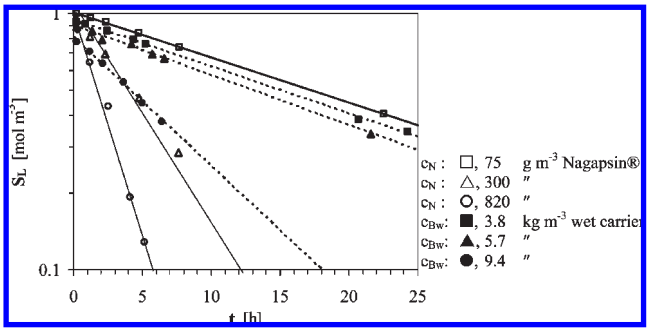

Figure 3. Time course of urea degradation at 20 °C for a model wine solution when using different concentrations of free (c_N) or immobilized (c_{Bw}) enzyme.

each slope of the above plots versus its corresponding free enzymatic protein concentration (E_f) (**Figure 4**), it was possible to estimate the specific pseudo-first-order kinetic rate constant relative to the free enzyme (k'_{If}) by means of the least-squares method:

$$k'_{If} = (8.5 \pm 0.3) \times 10^{-3} \quad m^3\ h^{-1}\ g^{-1}\ \text{of BSA equiv} \qquad (r^2 = 0.99)$$

Urea Degradation Rate by Immobilized Acid Urease. The kinetics of immobilized acid urease in the basic model wine solution was measured using the biocatalyst prepared using the second procedure and lot 1 enzyme preparation at three different levels, that is, 3.8, 5.6, and 9.4 kg m^{-3} of wet carrier.

The solid symbols in **Figure 3** show almost linear relationships between the natural logarithm of the current urea concentration (S_L) and time (t) for any c_{Bd} level tested, thus confirming that even in this case the integrand function ($\Omega c_{Bd} Y_{P/B} k'_{Ii}$) in the integral in the right-hand side of eq 27 may be approximately regarded as a constant.

Owing to the small particle size used, the overall effectiveness factor (Ω) was preliminarily assumed as inherently unitary, whereas the product of c_{Bd} by $Y_{P/B}$ was regarded as equal to the enzymatic protein concentration (E_i) dispersed in the liquid phase. Thus, by referring to the solid symbols in **Figure 4** and using the least-squares method, it was possible to assess the specific pseudo-first-order kinetic rate constant relative to the immobilized enzyme (k'_{Ii}):

$$k'_{Ii} = (2.4 \pm 0.2) \times 10^{-3} \quad m^3\ h^{-1}\ g^{-1}\ \text{of BSA equiv} \qquad (r^2 = 0.97)$$

To check for the contribution of the external film and/or intraparticle diffusion resistances to the overall substrate reaction, an independent estimate of the urea diffusivity in the bulk liquid (D_S) and in the biocatalyst (D_{Se}), as well as the mass transfer coefficient (k_L) in the case of immobilized enzyme, was carried out by resorting to well-known literature relationships (*27*−*31*) on the assumption that the density and viscosity at 20 °C of the model wine solution coincided with those of a typical white wine (*32*) having the same alcohol content, whereas the wet biocatalyst concentration (c_{Bw}) was set to 10 kg m^{-3}.

All estimates are listed in **Table 2**. It can be noted that the overall urea degradation rate was controlled by the reaction kinetics, the contribution of the external film and intraparticle mass-transfer resistances being negligible. In fact, both the estimated effectiveness factors for the biocatalyst used in the presence (Ω) or absence (η) of the external film transport resistance were practically unitary, in agreement with our preliminary assumption. Although such estimates were referred to a biocatalyst having a unitary tortuosity factor (τ), as extracted from Spiess et al. (*29*), they would still hold even if τ was as great as 7, the typical τ values for industrial catalysts ranging from 1 to 7 (*31*).

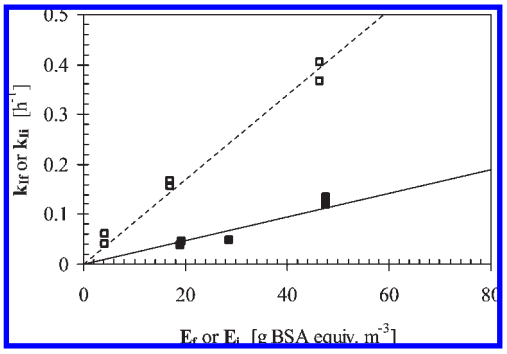

Figure 4. Effect of free (E_f, open symbols) or immobilized (E_i, solid symbols) enzymatic protein concentration in the model wine solution on the corresponding pseudo-first-order kinetic rate constant (k_{If} or k_{Ii}) of urea degradation at 20 °C by free or immobilized acid urease.

Table 2. Estimates of the Effectiveness Factors for Acid Urease Immobilized on Eupergit C250L in the Presence (Ω) or Absence (η) of the External Film Transport Resistance by Resorting to Well-Known Literature Relationships (*27*−*31*)

parameter	value	unit
average bead radius (R)	90	μm
bead porosity (θ)	0.6[a]	
bead tortuosity factor (τ)	1.0[a]	
particle density (ρ_B)	370[b]	kg/m^3
reaction temperature (T)	20	°C
model wine density (ρ_L)	998[c]	kg/m^3
model wine viscosity (μ_L)	0.00168[c]	Pas
urea diffusivity (D_S)[d]	1.29×10^{-9}	$m^2\ s^{-1}$
urea effective diffusivity ($D_{Se} = \theta/\tau D_S$)[e]	7.77×10^{-10}	$m^2\ s^{-1}$
wet biocatalyst concentration (c_{Bw})	10	kg/m^3
biocatalyst water fraction (x_{Bw})	0.84	g/g
dry biocatalyst concentration (c_{Bd})	1.6	kg/m^3
protein loading ($Y_{P/B}$)	25.9	g of BSA equiv kg^{-1} of dry support
specific pseudo-1st order kinetic rate constant (k'_{Ii})	0.0024	$m^3\ h^{-1}\ g^{-1}$ of BSA equiv
pseudo-first-order kinetic rate constant (k_{Ii})	0.099	h^{-1}
Schmidt number [$Sc = \mu_L/(\rho_L D_S)$]	1300	
Grashof number [$Gr = \frac{8R^3\rho_B(\rho_L - \rho_B)g}{\mu_L^2}$]	12.7	
Sherwood number [$Sh = 2 + 0.31(Sc\ Gr)^{1/3}$][f]	9.9	
mass transfer coefficient (k_L)	7.11×10^{-5}	m s^{-1}
Biot number [$Bi = Rk_L/D_e$]	8.2	
Thiele modulus (θ)	0.017	
effectiveness factor (η)	1.0000	
global effectiveness factor (Ω)	1.0000	

[a] Spiess et al. (*29*). [b] Gómez de Segura et al. (*27*). [c] Košmerl et al. (*32*). [d] Estimated by the Wilke and Chang method (*30*). [e] Satterfield and Sherwood (*31*). [f] Bailey and Ollis (*28*).

Effect of Stirring Rate on Urea Degradation Rate. To check further the negligible contribution of the external film resistance to the urea reaction rate, two further tests using free or immobilized acid urease were performed at low (250 rev min^{-1}) and high (400 rev min^{-1}) stirring rates under constant concentrations of free enzyme preparation (c_N = 1.0 kg m^{-3}) or wet biocatalyst (c_{Bw} = 9.4 kg m^{-3}), respectively.

The estimated pseudo-first-order kinetic rate constants for the free (k_{If}) and immobilized enzyme (k_{Ii}), each being obtained by the least-squares method and characterized by different variances (s_j^2) and degrees of freedom (δ_j), the latter being equal to the overall number of trials (n_j) minus 1, were compared with the classic inequality of the hypothesis test for means by resorting to the two-sided Student test for the confidence level of 0.005. In this way, it was possible to assess that the stirring rate effect on the generic k_{Ij} value was practically insignificant, at least over the experimental ranges tested. This result was further checked by estimating the enhancement in the pseudo-first-order kinetic rate constant as derived from the increase in the stirring level from 250 to 400 rev min^{-1} either for the free or immobilized enzyme, as shown in **Figure 5**.

Thus, the urea hydrolytic process in the model wine solution by free or immobilized acid urease was independent of the external and intraparticle mass transfer resistances, but controlled by the reaction kinetics only.

Effect of Grape Seed Tannins on Immobilized Acid Urease. In accordance with previous findings (*17*) assessing that the activity of a killed cell preparation, such as Enzeco Acid Urease, was greatly inhibited by the tannins extracted from grape seeds, the model wine solution of concern was enriched with 5−1000 g m^{-3} of the above tannin source to assess its inhibitory effect on the activity of acid urease immobilized on Eupergit C250 L. To this end, a second batch of biocatalyst was prepared using the second procedure and lot 2 enzyme preparation (**Table 1**) and added to the reaction mixture to ensure a constant wet biocatalyst concentration of about 6.25 kg m^{-3}.

Figure 6 shows a semilogarithmic plot of the ratio between the current and initial urea concentrations (S_L/S_{L0}) against time (t) for the model wine solution enriched with different amounts of the phenolic source of choice.

By fitting the natural logarithm of (S_L/S_{L0}) versus t via the least-squares method, it was possible to determine the average value and standard deviation of the experimental pseudofirst order kinetic rate constant (k_{Ii}) for immobilized acid urease as a function of P.

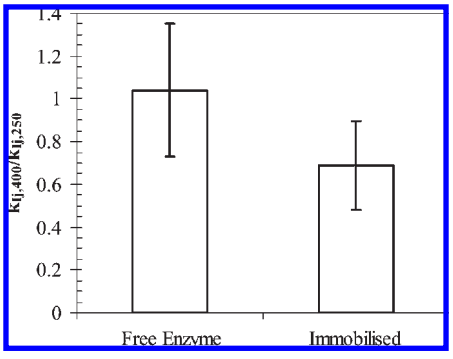

Figure 5. Enhancement in the pseudo-first-order kinetic rate constant ratio for the free (k_{If}) or immobilized (k_{Ii}) enzyme by increasing the stirring level from 250 to 400 rev min^{-1} under a constant concentration of free enzyme preparation (c_N = 1.0 kg m^{-3}) or wet biocatalyst (c_{Bw} = 9.4 kg m^{-3}).

Figure 7 compares the performances of these tests (■) to those carried out previously (*17*) using free enzymes either in model wines solutions enriched with tannins extracted from grape skins (△) or seeds (○) or in five real wines (□). More specifically, when using immobilized acid urease the current $k_{Ii,P}$ values were related to that observed in the model wine solution devoid of phenolics ($k_{Ii,P=0}$), whereas when using free enzymes the pseudo-first-order kinetic rate constant values ($k_{If,P}$) were similarly referred to that ($k_{If,P=0}$) pertaining to model wine solutions containing no phenolics, but with the same composition and pH of the corresponding real wines (*17*).

By resorting to the same competitive inhibition model (eq 5) previously used (*17*), it was possible to determine an inhibition constant (K_{Pi}) for the grape seed extract and the present biocatalyst of about 35.0 g of GAE m^{-3}, by minimizing the mean percentage error among the experimental and calculated kinetic rate constant ratios via a nonlinear fitting method.

The continuous line in **Figure 7** shows a quite satisfactory agreement between the experimental and calculated data,

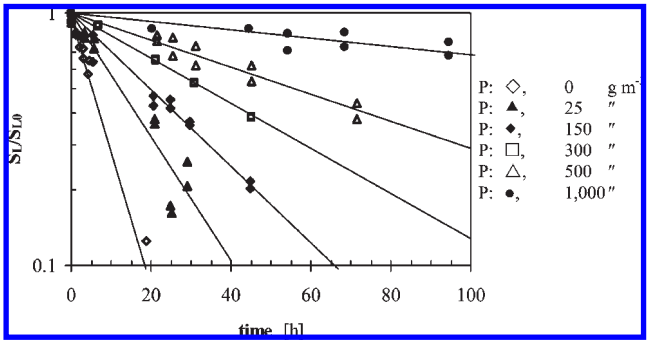

Figure 6. Semilogarithmic plot of the ratio between the current and initial urea concentrations (S_L/S_{L0}) against time (t) for the model wine solution enriched with different amounts of grape seeds extract (P) using a constant wet biocatalyst concentration (c_{Bw} = 6.25 kg m^{-3}). The continuous lines represent the least-squares regression lines.

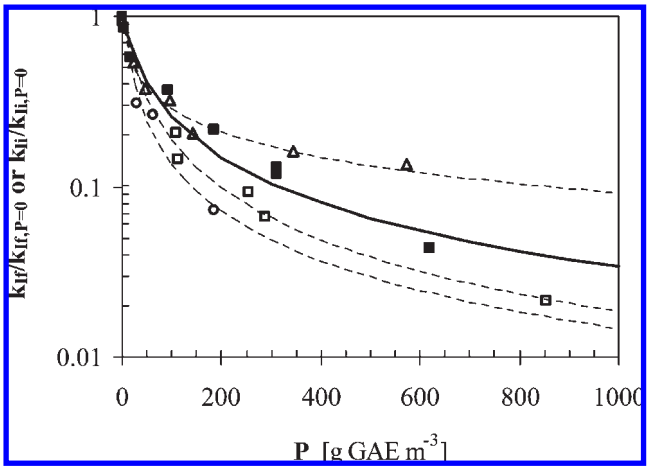

Figure 7. Effect of phenolic compound concentration (P) on the experimental pseudo-first-order kinetic rate constants for immobilized acid urease ($k_{Ij,P}$) in model wine solutions enriched with grape seed extract (■) or for free acid urease in real wines (□) or model wines enriched with grape skin (△) or seed (○) tannins (*17*), as referred to the kinetic rate constants for free ($k_{If,P=0}$) or immobilized ($k_{Ii,P=0}$) enzyme in model wines devoid of any phenolic compound. The broken and continuous lines were calculated using eq 5 with the phenolic compound inhibition constants K_{Pj} reported in the text.

their corresponding mean percentage error being about 18.7%.

The above K_P value was about 2 times greater than that (15.7 g of GAE m^{-3}) characterizing the inhibitory effect of grape seed extract on free acid urease (*17*), revealing quantitatively that enzyme immobilization attenuated the high-inhibitory effect of grape seed tannins toward free enzymes.

Concluding Remarks. The immobilization of acid urease on Eupergit C 250 L via its direct binding to the carrier oxirane groups (*23*), followed by stabilization with glycine (*24*), gave rise to a quite stable biocatalyst, because its residual activity was found to be as much as 69 ± 16% of its initial value even after 34 days of storage at 4 °C. This finding was independent of the enzyme preparation added per unit mass of support used. However, the observed protein loading ($Y_{P/B}$) appeared to be dependent on the activity of the original source of purified acid urease used.

The kinetics of urea degradation in a model wine solution by free purified acid urease was established to be of pseudo-first-order with respect to urea concentration, in agreement with previous findings in real and model wines using killed cell preparations (*11*, *13*, *14*, *17*). Such a mechanism was even corroborated when using purified acid urease immobilized on Eupergit C 250 L. More specifically, the pseudo-first-order kinetic rate constant relative to the free enzyme (k_{If}) was found to be about 4 times greater than that relative to the immobilized counterpart (k_{Ii}) under constant concentration of the enzymatic protein dissolved (E_f) or dispersed (E_i) in the liquid bulk.

Actually, k_{If} accounted for the effect of external resistance of urea transport from the bulk solution to the enzyme sites only, whereas k_{Ii} incorporated the effect of simultaneous film and interparticle mass-transfer resistances (*30*). However, by resorting to well-known literature relationships (*27*−*31*), the contribution of the external film and/or intraparticle diffusion resistances to the overall substrate reaction was found to negligible, the overall urea degradation rate being controlled by just the reaction kinetics.

Further testing allowed the activity of immobilized acid urease to be assessed in the presence of the high-inhibitory tannins extracted from grape seeds (*17*) in the range of 5−1000 g m^{-3}, thus establishing a protective action against such compounds of enzyme immobilization toward free enzymes.

Thus, use of immobilized acid urease may potentially overcome the present limits to the application of free acid urease in wine treatment.

The first limit refers to the maximum allowable concentration for killed cell commercial preparations, which is presently equal to 75 g m^{-3} (*6*). In the case of the Enzeco Acid Urease preparation previously used (*14*, *17*), this corresponds to a concentration of 11.0 ± 0.6 g of BSA equiv m^{-3}, which in the case of the soluble purified acid urease preparation used here was found to be associated with quite a low value of the pseudo-first-order kinetic rate constant (k_{If}), as shown in **Figure 4**.

The second one accounts for the fact that in real wines the presence of several inhibitory components may reduce the effective k_{If} values by a factor of 10−100 (*14*, *17*).

Therefore, the biocatalyst tested here may accelerate urea degradation in real wines by increasing simply its concentration in the wine lot to be treated in a stirred tank. Its recovery from the urea-exhausted wine by filtration not only may result in significant cost savings owing to multiple enzyme recycles in consecutive batch trials but may also avoid the bitter taste assayed in some wines treated with 50 g m^{-3} of the aforementioned killed cell preparation (*6*).

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

ABBREVIATIONS USED

Notation

A_{BEi}	specific activity of the immobilized biocatalyst per unit mass of bound protein (IU g^{-1} of bound protein)
A_{Bi}	specific activity of the immobilized biocatalyst per unit mass of dry support (IU g^{-1} of dry support)
A_{E0}	acid urease activity in the immobilizing solution per unit mass of protein (IU g^{-1} of BSA equiv)
A_{Ef}	acid urease activity in the supernatant or filtrate per unit mass of protein (IU g^{-1} of BSA equiv)
A_L	instantaneous concentration of ammonium ions in the liquid bulk (mol m^{-3})
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 (m^{-1})
Bi	Biot number, as defined by eq 24
c_{Bd}	concentration of dry biocatalyst (kg m^{-3})
c_{Bw}	wet biocatalyst concentration (kg m^{-3})
c_N	concentration of Nagapsin preparation in the immobilizing solution (kg m^{-3})
c_{P0}	protein concentration in the immobilizing solution (g of BSA equiv m^{-3})
c_{Pf}	protein concentrations in the supernatant/filtrate and washing solutions (g of BSA equiv m^{-3})
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_i	concentration of immobilized enzymatic protein dispersed in the liquid bulk (g of BSA equiv m^{-3})
E_f	concentration of free enzymatic protein dissolved in the liquid bulk (g of BSA equiv m^{-3})
Gr	Grashof number $[=8R^3\rho_L(\rho_L-\rho_B)g/\mu_L^2]$
K_A	product inhibitory constants (mol m^{-3})
K_M	Michaelis−Menten constant (mol m^{-3})
K_{Pj}	phenolic compound inhibition constant for free or immobilized enzyme (g of GAE m^{-3})
K_S	substrate inhibitory constant (mol m^{-3})
k_{If}	urea degradation pseudo-first-order kinetic rate constant for free enzyme (h^{-1})
k_{Ii}	urea degradation pseudo-first-order kinetic rate constant for immobilized enzyme (h^{-1})
$k_{Ij,P}$	pseudo-first-order kinetic rate constant for free or immobilized enzyme at a given concentration of phenolics enzyme (h^{-1})
k'_{If}	specific pseudo-first-order kinetic rate constant relative to the free enzyme (m^3 h^{-1} g^{-1} of BSA equiv)
k'_{Ii}	specific pseudo-first-order kinetic rate constant relative to immobilized enzyme (m^3 h^{-1} g^{-1} of BSA equiv)
k_L	mass transfer coefficient in the liquid phase (m s^{-1})
m_{Bd}	mass of dry carrier (g)
m_{Bw}	mass of wet carrier (g)

m_{Pb} amount of bound protein, as defined by eq 6 (mg of BSA equiv)
m_{P0} amount of protein in the immobilizing solution (mg of BSA equiv)
n_j overall number of trials
P concentration of phenolic compounds (g m^{-3} of gallic acid equiv, GAE)
R average bead radius (m)
r^2 coefficient of determination (dimensionless)
r_{A0} ammonia formation rate in the immobilizing solution (mmol m^{-3} min^{-1})
r_{AB} ammonia formation rate for immobilized enzyme (mmol m^{-3} min^{-1})
r_{Af} ammonia formation rate in the supernatant or filtrate solution (mmol m^{-3} min^{-1})
r_{Sf} urea degradation rate for free enzyme referred to the unit volume of liquid bulk (mol m^{-3} h^{-1})
r_{Si} urea degradation rate for immobilized enzyme referred to the unit volume of biocatalyst (mol m^{-3} h^{-1})
S urea concentration in the liquid penetrating the biocatalyst pores (mol m^{-3})
S_L urea concentration in the liquid bulk (mol m^{-3})
S_R urea concentration at the biocatalyst surface (mol m^{-3})
Sc Schmidt number [$=\mu_L/(\rho_L D_S)$]
Sh Sherwood number ($=2Rk_L/D_{Se}$)
s_j^2 generic variance
T reaction temperature (°C)
t reaction time (h)
t_I immobilization time (h)
t_S storage time (h)
V_0 volume of the immobilizing solutions (cm^3)
V_f volume of the supernatant/filtrate and washing solutions (cm^3)
V_L liquid volume (m^3)
v_{max} maximum urea degradation rate (mol h^{-1} m^{-3})
v_S overall biocatalyst volume the per unit volume of liquid phase (dimensionless)
x_{Bw} water fraction of wet biocatalyst (g g^{-1})
$Y_{P/B}$ protein loading (g of bound protein g^{-1} of dry support)
$Y_{P/S}$ protein added per unit mass of dry support (mg of BSA equiv g^{-1} of dry support)

Greek Symbols

δ_j degrees of freedom
ζ_A enzyme activity coupling yield, as defined by eq 14 (dimensionless)
ζ_E enzyme coupling yields, as defined by eq 13 (dimensionless)
η effectiveness factor for a spherical biocatalyst in the absence of the external film transport resistance, as defined by eq 22 (dimensionless)
η_L liquid viscosity (Pa s)
θ biocatalyst porosity (dimensionless)
ρ_B biocatalyst density (kg m^{-3})
ρ_L liquid density (kg m^{-3})
τ biocatalyst tortuosity (dimensionless)
Φ Thiele modulus for pseudo-first-order kinetics, as defined by Eq 23 (dimensionless)
Ω effectiveness factor for a spherical biocatalyst in the presence of the external film transport resistance, as defined by eq 21 (dimensionless)

Subscripts

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) Zimmerli, B.; Schlatter, J. Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. *Mutat. Res*. **1991**, *259*, 325–350.
(4) Butzke, C. E.; Bisson, L. F. Ethyl carbamate preventative action manual, Food and Drug Administration. Center for Food Safety and Applied Nutrition, published online at http://www.cfsan.fda.gov/~frf/ecaction.html, 1997.
(5) Kodama, S.; Yotsuzuka, F. Acid urease: reduction of ethyl carbamate formation in sherry under simulated baking conditions. *J. Food Sci*. **1996**, *61* (2), 304–307.
(6) Bertrand, A. Utilizzazione dell'ureasi nel vino. In *Prodotti di Trattamento ed Ausiliari di Elaborazione dei Mosti e dei Vini*; Vivas N., Ed.; Eno-one srl: Reggio Emilia, Italy, 2003; pp 171−192.
(7) Fujinawa, S.; Burns, G.; De La Teja, P. Application of acid urease to reduction of urea in commercial wines. *Am. J. Enol. Vitic*. **1990**, *41* (4), 350–354.
(8) Fujinawa, S.; Kodama, S.; Todoroki, H.; Suzuki, T. Trace urea determination in red wine and its degradation rate by acid urease. *Am. J. Enol. Vitic*. **1992**, *43* (4), 362–366.
(9) Fujinawa, S.; Todoroki, H.; Ohashi, N.; Toda, J.; Terasaki, M. Application of an acid urease to wine: determination of trace urea in wine. *J. Food Sci*. 1990, *55* (4), 1018−1022, 1038.
(10) Ough, C. S.; Trioli, G. Urea removal from wine by an acid urease. *Am. J. Enol. Vitic*. **1988**, *39* (4), 303–7.
(11) Trioli, G.; Ough, C. S. Causes for inhibition of an acid urease from *Lactobacillus fermentus*. *Am. J. Enol. Vitic*. **1989**, *40* (4), 245–52.
(12) Famuyiwa, O.; Ough, C. Modification of acid urease activity by fluoride ions and malic acid in wines. *Am. J. Enol. Vitic*. **1991**, *42*, 79–80.
(13) Kodama, S. Optimal conditions for effective use of acid urease in wine. *J. Food Sci*. **1996**, *61* (3), 548–552.
(14) 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.
(15) Moynihan, H. J.; Lee, C. K.; Clark, W.; Wang, N.-H. L. Urea hydrolysis by immobilized urease in a fixed-bed reactor: analysis and kinetic parameter estimation. *Biotechnol. Bioeng*. **1989**, *34*, 951–963.
(16) Matsumoto, K. Removal of urea from alcoholic beverages by immobilised acid urease. *Bioprocess Technol*. **1993**, *16* 255–273.
(17) 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.
(18) Zhou, J.; Chen, S.; Wang, J. Research on orientedly immobilised urease via concanavalin A. *Chin. J. Biotechnol*. **2008**, *24*, 617–621.
(19) Selvamurugan, C.; Lavanya, A.; Sivasankar, B. A comparative study on immobilisation of urease on different matrices. *J. Sci. Ind. Res*. **2007**, *66*, 655–659.
(20) Katchalski-Kazir, E.; Kraemer, D. M. Eupergit® C, a carrier for immobilization of enzymes of industrial potential. *J. Mol. Catal. B: Enzymatic* **2000**, *10*, 157–176.

(21) 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.
(22) Mateo, C.; Abian, O.; Fernadez-Lafuente, R. Increase in the conformational stability of enzymes immobilized on epoxy-activated supports by favouring additional multipoint attachment. *Enzyme Microb. Technol*. **2000**, *26*, 509–515.
(23) 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.
(24) 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.
(25) Torres-Bacete, J.; Arroyo, M.; Torres-Guzmán, R.; de la Mata, I.; Castillón, M. P.; Acebal, C. Covalent immobilization of penicillin acylase from *Streptomyces lavendulae*. *Biotechnol. Lett*. **2000**, *22*, 1011–1014.
(26) 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.
(27) Gómez de Segura, A.; Alcalde, M.; Yates, M.; Rojas-Cervantes, M. L.; López-Cortés, N.; Ballesteros, A.; Plou, F. J. Immobilization of dextransucrose from *Leuconostoc mesenteroides* NRRL B-512F on Eupergit C supports. *Biotechnol. Prog*. **2004**, *20*, 1414–1420.
(28) Bailey, J. E.; Ollis, D. F. *Biochemical Engineering Fundamentals*, 2nd ed.; McGraw-Hill Book: New York, 1986; pp 204−227.
(29) 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.
(30) Treybal, R. E. *Mass Transfer Operations*, 2nd ed.; McGraw-Hill Book: New York, 1968.
(31) Satterfield, C. N.; Sherwood T. K. *The Role of Diffusion in Catalysis*; Addison-Wesley Publishing: Reading, MA, 1963; p 47.
(32) Košmerl, T.; Abramovič, H.; Klofutar, C. The rheological properties of Slovenian wines. *J. Food Eng*. **2000**, *46*, 165–171.

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