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Urea Removal in Model Wine Solutions by Immobilized Acid Urease in a Stirred Bioreactor

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The kinetics of urea degradation in a model wine solution by acid urease, as such or immobilized on Eupergit® C 250 L and stabilised with glycine, was found to be of the pseudo-first order with respect to urea concentration with a specific pseudo-first order kinetic rate constant for free enzyme (k_{If}) about four times greater than that (k_{Ii}) pertaining to the immobilised counterpart. The reaction kinetics was estimated to be unaffected by the contribution of the external film and intraparticle diffusion mass transfer resistances.

1. 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 (Ough et al, 1988). Owing to its potential carcinogenic activity when administered in high doses in animal tests, EC levels in food products are to be greatly reduced. (Schlatter and Lutz, 1990; Zimmerli and Schlatter, 1991).
The feasibility of acid urease application for the removal of urea from several type wines has been extensively demonstrated, even if its effectiveness depends on the type of wine, content of some inhibiting factors (i.e., in order of importance, fluoride, malate, ethanol, and phenolic compounds), and usage conditions (Butzke and Bisson, 1996).
Immobilisation of acid urease on various matrices, such as polyacrylonitrile (PAN) and chitosan derivatives (Matsumoto, 1993; Zhou *et al*, 2008), nylon beads, sepharose gel, silica gel, and gelatine film coated on cellulose acetate membrane (Selvamurugan *et al*, 2007), has the potential advantages of significant cost savings owing to enzyme recycle through multiple cycles of batch-wise hydrolysis, improved stability or resistance to shear or inhibitory compound inactivation. Continuous urea removal from sake by immobilised acid urease on PAN has been applied by many companies in Japan since 1988 (Matsumoto, 1993).
The main aims of this work were to bind acid urease to a well known commercial epoxy-activated carrier, i.e. Eupergit® C 250L (Katchalski-Kazir and Kraemer, 2000), and to compare the kinetics of urea degradation in a wine model solution, when using a stirred bioreactor charged with soluble purified acid urease from *Lactobacillus fermentum*, as such or bound to Eupergit® C 250L.

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2. Materials and Methods

A commercial preparation Nagapsin® (ref. no. 2735159), 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 *L. fermentum*, with a specific activity of 766±7 IU g^{-1} at the moment of use, 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 Röhm GmbH (Darmstadt, Germany). The water content (x_{Bw}) of the support as such or after 2-h swelling in 0.05 M potassium phosphate buffer pH 7.0 (KPB7) increased from 0.6±0.1 to 81±1 % (w/w), respectively.

The performance of free or immobilised acid urease at 20°C was assessed in a model wine solution, that was prepared by dissolving constant amounts of urea (1 mol m^{-3}), tartaric (5 kg m^{-3}), malic (2.5 kg m^{-3}), and lactic (1.75 kg m^{-3}) acids, potassium metabisulphite (0.2 kg m^{-3}), and ethanol (13% v/v) in deionised water and then adjusting the resulting pH to 3.50 (Fidaleo *et al*, 2006). All reagents were of the analytical grade.

The direct enzyme binding on Eupergit® supports via oxirane groups was performed (Knezevic *et al*, 2006). About 600 mg of dry beads and 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 (mod. EUROSTAR) rotating at 250 rev min^{-1}, that was mounted vertically on centre with baffles at the wall. After about 24-h soaking, 72 cm^3 of KPB7 enriched with 16.01 kg m^{-3} of Nagapsin®, preconditioned at 20°C, were added while continuing mixing the dispersion. After incubation for 24 h, the biocatalyst was collected by vacuum filtration using a glass filter (0.45-μm Whatman GF/C disc), washed twice with 50 cm^3 of KPB7. All filtrates were collected and diluted with KPB7 to a final volume of 250 cm^3. After collecting about 50 mg of wet beads for determining the immobilised acid urease activity, the remaining wet beads were soaked in an aqueous solution containing 75 mol m^{-3} glycine at 4°C for 20 min (Oliveira *et al*, 2001), 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 suggested by the carrier manufacturer to avoid microbial contamination.

The protein concentration in all solutions tested was determined according to the method by Lowry *et al* (1951) using the Total Protein Kit (Sigma, Saint Louis, Missouri, USA) containing bovine serum albumin (BSA) as target protein.

The amount of bound protein was indirectly assessed by subtracting the amount of protein in the supernatant and washing solutions from the amount of protein present in the immobilising solution. This allowed the *protein loading* ($Y_{P/B}$) to be estimated as 25.9 g of bound protein per g dry support.

The acid urease activity in the immobilising solution, filtrate or immobilised enzyme was estimated by charging sequentially the following liquids in a 25-cm^3 beaker containing a 10-mm magnetic stirrer: 5 cm^3 of an aqueous solution at 5 kg m^{-3} of urea, 5.65 cm^3 of 0.1 M acetate buffer pH (4.0), 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 immobilized biocatalyst was estimated as 99±17 IU g^{-1} dry support or 3704±645 IU g^{-1} protein.

To assess the time course of the hydrolytic process under study, 80 cm^3 of the model wine solution, pre-conditioned at 20°C, were poured into a 100-cm^3 rubber-capped

flask, pre-charged with given amounts of free or immobilised acid urease. Each flask was immersed in a water bath to keep the reaction temperature at 20±0.2°C, using a thermostat, and placed over a magnetic multistirrer to assure a stirring level of 400 rev min^{-1}. Several samples (1 cm^3) were withdrawn from any flask for as long as 24 h and were diluted with deionised water at room temperature before being assayed for ammonium and urea by using the K-URAMR kit (Megazyme International Ireland Ltd, Wicklow, Ireland).
The kinetics of free acid urease in the model wine solution was also assessed by setting the initial concentration of Nagapsin® to 75, 300 and 820 g m^{-3}, this being corresponding to an enzyme content in the range of 4.3-46.5 g m^{-3} BSA equivalent. On the contrary, the kinetics of immobilised acid urease in the basic model wine solution was measured using the biocatalyst at three different levels, i.e. 3.8, 5.6 and 9.4 kg m^{-3} of wet carrier.

3. Results and Discussion

When using immobilized acid urease, it was assumed that enzyme coupling to Eupergit® supports did not affect the pseudo-first order kinetic model of free enzyme, especially when the urea concentration was by far smaller than the Michaelis-Menten constant of the free enzyme (Fidaleo *et al*, 2006). Thus, the urea degradation rate referred to the unit volume of immobilized acid urease (r_{Si}) was expressed as follows:

$$r_{Si} = k_{Ii}\, S \qquad (1)$$

with

$$k_{Ii} = k'_{Ii}\, \rho_B\, Y_{P/B} \qquad (2)$$

where k_{Ii} is the urea degradation pseudo-first order kinetic rate constant of the biocatalyst of concern, ρ_B the biocatalyst density, $Y_{P/B}$ the protein loading and k'_{Ii} the specific pseudo-first order kinetic rate constant relative to immobilised enzyme [expressed in m^3 (kg protein)$^{-1}$ h^{-1})].
When using a perfectly mixed bioreactor, charged with a volume (V_L) of a model wine solution at 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=3/R), the unsteady-state material balance for the reagent S may be written as:

$$-\frac{dS_L}{dt} = k_L\, a_S\, (S_L - S_R) = \Omega\, v_S\, k_{Ii}\, S_L \qquad (3)$$

with

$$\Omega = \frac{\eta}{1 + \frac{\eta \Phi^2}{3\,Bi}} \qquad (4)$$

$$\eta = \frac{3}{\Phi}\left(\frac{1}{\tanh(\Phi)} - \frac{1}{\Phi}\right) \qquad (5)$$

$$\Phi = R\sqrt{\frac{k_{Ii}}{D_{Se}}} \qquad (6)$$

$$Bi = \frac{k_L\, R}{D_{Se}} \qquad (7)$$

$$a_S = a_p\, c_{Bd}/\rho_B \qquad (8)$$

$$v_S = c_{Bd}/\rho_B \qquad (9)$$

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} the mass transfer coefficient in the liquid phase and effective diffusion coefficient for the reagent S; S_R is the reagent concentration at the biocatalyst surface; c_{BW} [$=c_{Bd}/(1-x_{BW})$] the wet biocatalyst concentration; and a_S and v_S are the overall surface and volume for the biocatalyst per unit volume of liquid phase (Bailey and Ollis, 1986).

By accounting for the following initial condition:

$$S_L = S_{L0} \qquad \text{for } t = 0 \qquad (10)$$

Eq. (3) can be integrated, thus yielding:

$$\ln\left(\frac{S_L}{S_{L0}}\right) = \int_{S_{L0}}^{S_L} \frac{dS_L}{S_L} = -\int_0^t \Omega\ c_{Bd}\ Y_{P/B}\ k'_{Ii}\ dt \qquad (11)$$

The kinetics of free acid urease in the model wine solution was assessed at different concentrations of Nagapsin® in the range of 75 to 820 g m^{-3}, this corresponding to an enzyme content (E_f) in the range of 4.3-46.5 g m^{-3} BSA equivalent, as shown by the open symbols in Fig. 1.

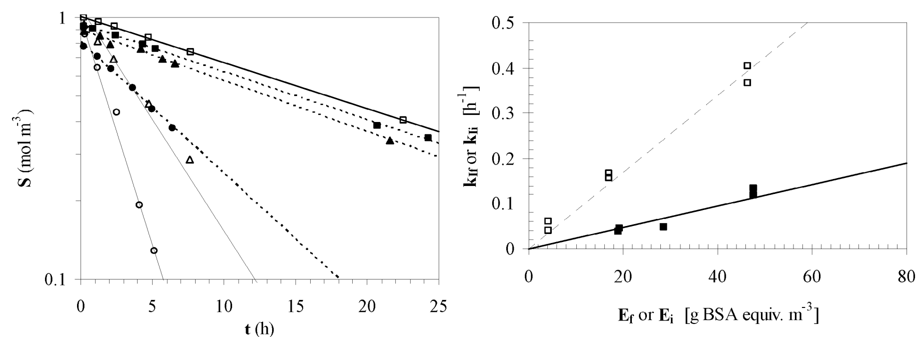

Figure 1 Time course of urea degradation at 20°C for a model wine solution when using different concentrations of free (□, c_N=75; △, c_N=300; ○, c_N=820 g m^{-3} Nagapsin®) or immobilised (■, c_{Bw}= 3.8; ▲, c_{Bw}= 5.7; ●, c_{Bw}= 9.4 kg m^{-3} wet carrier) enzyme.

Figure 2 Effect of free (E_f: open symbols) or immobilised (E_i: closed symbols) enzymatic protein concentration in the model wine solution essayed 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.

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 at the right-hand side of Eq. (11) to be regarded as a 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, while k'_{Ii} is equivalent to the specific pseudo-first order kinetic rate constant relative to the free enzyme (k'_{If}). In fact, by plotting each slope of the above plots *versus* its corresponding free enzymatic protein concentration (E_f) (Fig. 2), 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:

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

The kinetics of immobilised acid urease in the basic model wine solution was assessed at three different concentrations of the wet carrier.

The closed symbols in Fig. 1 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 ($\Omega\ c_{Bd}\ Y_{P/B}\ k'_{Ii}$) – see Eq. (11) - may be approximately regarded as a constant.

Owing to the small particle size used, the overall effectiveness factor (Ω) was preliminary assumed as inherently unitary, while the product of c_{Bd} by $Y_{P/B}$ was replaced by the enzymatic protein concentration (E_i) dispersed in the liquid phase. Thus, by referring to the closed symbols in Fig. 2 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}):

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

To check for the contribution of the external film and/or intraparticle diffusion resistances to the overall substrate reaction, independent estimates 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 immobilised enzyme, were carried out by resorting to well known literature relationships (Bailey and Ollis, 1986; Gómez de Segura et al, 2004; Satterfield and Sherwood, 1963; Spieß et al, 1999; Treybal, 1968) on the assumption that the density and viscosity at 20°C of the model wine solution coincided with those of pure water (Weast, 1982/83) and the wet biocatalyst concentration (c_{Bw}) was set to 10 kg m^{-3}. Both the estimated values of the 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. Thus, the overall urea degradation rate resulted to be controlled by the reaction kinetics, being negligible the contribution of the external film and intraparticle mass-transfer resistances.

In conclusion, since urea degradation by acid urease in real wines is limited by the presence of several inhibitory compounds, and the current EU regulatory restricts the maximum allowable concentration for killed cell commercial preparations to 75 g m^{-3} (Bertrand, 2003), the only way to accelerate this detoxification process would be to increase the concentration of the biocatalyst tested here in the wine lot to be treated in a stirred tank. Recovery of all the biocatalyst from the urea-exhausted wine by filtration would cause significant cost savings owing to multiple enzyme recycles in consecutive batch trials. 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.

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