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Assessment of Diffusion Limitations on the Performance of Immobilised Acid Urease Derivatives

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In this work, the specific activity of acid urease immobilised on Eupergit® C250L at different enzyme loadings $Y_{P/B}$ in the range of 48-170.5 mg BSAE/g dry support (ds) was satisfactorily reconstructed by using the simultaneous film and intraparticle diffusion and Michaelis-Menten kinetic reaction model. By referring to the intrinsic kinetic parameters of free acid urease, the statistically significant change in the apparent affinity of immobilised enzyme for urea was attributed to diffusion limitations only. When operating in a stirred bioreactor in the pseudo-first order regime, the specific activity of the biocatalyst was mainly restricted by its internal resistance, the corresponding effectiveness factor (η) reducing from 0.9 to 0.28 as $Y_{P/B}$ increased from 12.5 to 400.0 mg BSAE/g ds. The combined effect of internal and external mass-transfer resistances limited the advantages of using biocatalysts with enzyme loadings greater than 150 mg BSAE/g ds.

1. Introduction

The industrial-scale use of a relatively costly enzyme as a catalyst generally asks for its immobilization so as to make its recovery and reuse for long times economically feasible.
Nowadays, there are quite numerous immobilization protocols directed to improve the enzyme loading in immobilised enzymes so as to enhance their specific activity (Mateo et al., 2007; Grazù et al., 2010).
By fitting the experimental data (substrate reaction rate versus the limiting substrate concentration) via the Michaelis-Menten model, two independent parameters, called the apparent Michaelis-Menten constant (K_{Ma}) and maximum reaction rate (V'_{max}), are generally estimated, these being somewhat different from those characterising the native enzyme (Bailey and Ollis, 1986). Such a change in the kinetic parameters is mainly attributed to some structural changes in the enzyme introduced by the immobilization procedure, even if the lower accessibility of the substrate to immobilized enzyme should be accounted for.
One of the main problem associated with immobilised enzymes is the selection of their appropriate enzyme loading ($Y_{P/B}$). By increasing $Y_{P/B}$, the activity of the immobilized derivative increases whereas its effectiveness factor decreases. Thus, no definite rule is available to maximise the bioreactor performance, this being a complex combination of kinetic, chemico-physical, and mass transport parameters.
Previously (Bortone et al., 2012), immobilisation of acid urease on Eupergit® C250L was carried out using a phosphate buffer at pH 7.0 by varying the ionic strength and concentration of a commercial acid urease preparation. The resulting immobilised derivatives exhibited $Y_{P/B}$ values ranging from 48 to 171 mg bound enzyme per g dry support.
Aim of this work was to assess whether the change in the apparent affinity of immobilised enzyme for urea might be attributed to diffusion limitations instead of the structural changes in the enzyme as resulting from the immobilisation procedure, in order to select the most appropriate enzyme loading for the biocatalyst to be used in the bioreactor of concern.

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2. Materials and Methods

A single lot (ref. no. 3707118) of the commercial preparation Nagapsin, donated by Nagase Europa GmbH (Duesseldorf, Germany), was used. The soluble powder, approximately composed of 96% (w/w) lactose and 4% (w/w) purified acid urease from *Lactobacillus fermentum*, was stored at 4 °C. Its specific activity was equal to 601 ± 46 IU/g, where one International Unit (IU) corresponds to the amount of preparation capable of releasing 1 μmol/min of ammonia from urea at 20 °C in a standard reaction mixture composed of 0.1 M sodium-acetate buffer (pH 4.0) enriched with urea (83.33 mM) and ethanol (12.5% v/v).
Eupergit® C250L (lot no. B060519592), granted by Röhm GmbH (Darmstadt, Germany), was used as enzyme carrier, its main characteristics being the following: average bead diameter, d_P=175 μm; ratio of bead porosity (θ) and tortuosity factor (τ) equal to 0.67 (Tischer and Wedekind, 1992); particle density, ρ_B = 370 kg ds m^{-3} (Gómez de Segura et al., 2004); water mass fraction of beads as such, x_{Bd} = 3.6 ± 0.2 % w/w, or after swelling, x_{Bw} =75 ± 2% w/w.
The protein weight fraction of the Nagapsin preparation was estimated according to the Bradford protein assay (Bradford, 1976) and was equal to 3.3 ± 0.4 % (w/w), as reported previously (Bortone et al., 2012).
Urease concentration in all enzyme solutions was determined indirectly from ammonium ion formation rate measurements and expressed in mg of bovine serum albumin equivalent/L (BSAE).
A series of immobilised enzyme derivatives was prepared by incubating prefixed masses of dry beads in immobilising solutions consisting of a sodium phosphate buffer (pH 7.0) at different ionic strengths (0.05 - 1.0 M) and concentrations of the Nagapsin preparation (12 - 144 g/L) under gentle shaking at 25 °C for 48 h. Thereafter, the biocatalyst was collected by vacuum filtration using a glass microfiber filter (2.7-μm pore Whatman GF/D disk) and washed with 0.05 M sodium phosphate buffer (pH 7.0) and soaked in an aqueous solution containing 3 M glycine (pH 8.5) under gentle shaking at 25 °C for 24 h. Further details were given by Bortone et al. (2012). The stabilised beads were filtered, washed with the aforementioned buffer and stored in the wet state in a storage buffer consisting of the above buffer supplemented with 2% (v/v) isopropanol and 0.5 g/L ethyl parabene at 4 °C to avoid microbial contamination, as suggested by the carrier manufacturer. The protein loading ($Y_{P/B}$) was defined as the amount of bound enzyme per unit mass of dry support and expressed as mg of BSAE per g of dry support (ds).
For the free enzyme, the enzyme activity was assayed using 25-mL beakers immersed in a water bath placed over a magnetic multistirrer to keep the reaction temperature at 20.0 ± 0.2 °C by means of a thermostat. Each beaker was firstly charged with 11 mL of 0.1 M sodium acetate buffer (pH 4.0) enriched with ethanol 12.5 % (v/v) and different concentrations of urea in the range of 0.3 - 83.3 mM. After thermal equilibration, the hydrolytic reaction was started by adding a known volume of a solution containing the enzyme preparation. After 10 min, a sample (0.4 mL) was withdrawn to determine the ammonium ion content using the Spectroquant Ammonium reagent kit (Merck KGaA, Darmstadt, Germany). The specific enzyme activity (π_{AE}) of any solution was estimated by dividing the ammonium ion formation rate (r_A) by its corresponding free enzyme concentration (c_{PF}) and expressed in IU per g of BSAE.
For the immobilized biocatalysts, the enzyme activity was determined using 50-mL adjustable hanging bar spinner flasks (Bellco Glass, Inc., Vineland, NJ, USA) immersed in a water bath at 20.0 ± 0.2 °C. Each flask was charged with a known volume (39.00 - 43.98 mL) of the above reaction solution and kept under constant stirring speed (about 320 or 670 rpm) until its temperature was constant (20.0 ± 0.2 °C). Then, different amounts of wet beads were added to vary the biocatalyst concentration (c_{Bw}) in the range of 227 - 455 mg/L of wet support (ws). After a few minutes to reach the prefixed temperature, 0.020 - 5.0 mL of a concentrated solution of urea was added to vary the overall urea concentration from 0.3 to 83.3 mM and start the hydrolytic reaction. After 9.5 min, agitation was stopped and the beads allowed to sediment for 0.5 min so as to withdraw 0.4 mL of the supernatant for ammonium determination. The specific activity (π_{AB}) of any biocatalyst was estimated by dividing the ammonium ion formation rate (r'_A) by the dry biocatalyst concentration (c_{Bd}) and expressed as IU per g of dry support (ds).

3. Theoretical background

The hydrolysis of urea is catalyzed by ureases (urea amidohydrolases, EC 3.5.1.5) to yield carbon dioxide and ammonia as final products (Fidaleo and Lavecchia, 2003; Krajewska, 2009):

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

In previous work (Bortone et al., 2012), the ammonia formation rate (r_A) for free acid urease exhibited a typical saturation pattern as the urea concentration was increased from 0 to 83.3 mol m^{-3} and was therefore fitted by using the well-known Michaelis-Menten kinetic model:

$$r_A = \frac{V_{max}\, S_L}{K_M + S_L} \quad (2)$$

with

$$V_{max} = k_{cat}\, c_{Pf} \quad (3)$$

where V_{max} is the maximum ammonia formation kinetic rate constant; S_L is the bulk urea concentration; c_{Pf} is the bulk Bradford protein concentration for free enzyme; while K_M (=2.55 ± 0.14 mM) and k_{cat} (= 18.86 ± 0.34 IU/mg BSAE) are the intrinsic Michaelis constant and specific ammonia formation kinetic rate constant for free enzyme.
In the case of acid urease immobilised onto Eupergit® C250L in the same range of urea concentrations, the ammonia formation rate (r'_A) for immobilised acid urease referred to liquid volume was reconstructed by using the Michaelis-Menten kinetic model too:

$$r'_A = \frac{V'_{max}\, S_L}{K_{Ma} + S_L} \quad (4)$$

with

$$V'_{max} = k'_{cat}\, c_{Pi} \quad (5)$$

$$c_{Pi} = c_{Bd}\, Y_{P/B} \quad (6)$$

where V'_{max} is the maximum ammonia formation kinetic rate constant for immobilised enzyme, K_{Ma} or k'_{cat} the apparent Michaelis constant or specific ammonia formation kinetic rate constant for immobilised enzyme, c_{Pi} the bulk Bradford protein concentration for immobilised enzymes, c_{Bd} the bulk concentration of dry beads and $Y_{P/B}$ their corresponding enzyme loading. In the case of acid urease immobilised on Eupergit® C250L, k'_{cat} was found to be little affected by immobilisation and was set as equal to k_{cat}, while K_{Ma} was found to increase almost linearly from 2.55 ± 0.14 to 5.38 ± 0.87 mM at $Y_{P/B}$=170.5 mg BSAE/g ds (Bortone et al., 2012). As suggested by the short-cut method based on the observable Thiele modulus (Bailey and Ollis, 1986) and later confirmed by comparing the specific activity of two immobilised derivatives at a high enzyme loading (~126 mg BSAE/g ds), as such or ground to < 5 μm, with that of free enzyme (Bortone et al., 2012), the contribution of intraparticle diffusion was found to be predominant, while it practically vanished after bead grinding, the consequent specific activity approaching the value for free enzyme.
In this work, the change in the apparent affinity of immobilised enzyme for urea was not attributed to structural changes in the enzyme in consequence of the immobilisation procedure, but to diffusion limitations only, these reducing the substrate concentration in the vicinity of the immobilised enzyme. To this end, the specific ammonia formation rate (π_{AB}) for immobilised acid urease referred to liquid volume was reconstructed as follows:

$$\pi_{AB} = \Omega \frac{k'_{cat}\, Y_{P/B}\, S_L}{K_M + S_L} \quad (7)$$

with

$$\Omega = \frac{\eta}{\gamma} \frac{\gamma + \alpha}{1 + \alpha} \quad (8)$$

$$\gamma = \frac{S_L}{S_R} = 1 + \frac{\eta \Phi^2}{3\,\mathrm{Bi}\,(1 + 1/\alpha)} \quad (9)$$

$$\alpha = \frac{K_M}{S_R} \quad (10)$$

$$\Phi = R \sqrt{\frac{k'_{cat}\, \rho_p\, Y_{P/B}}{K_M\, D_{Se}}} \quad (11)$$

where Ω and η are the effectiveness factors for a spherical biocatalyst in the presence or absence of the external film transport resistance; Bi ($=k_L\ R/D_{Se}$) is the Biot number, which measures the ratio between the external film transport and intraparticle diffusion rates of the reagent of concern; k_L the mass transfer coefficient in the stagnant film surrounding the catalyst; S_L and S_R are the substrate concentrations in the liquid bulk and at the biocatalyst surface while γ is the corresponding ratio, as derived from the boundary condition at the bead surface; Φ is the Thiele modulus for Michaelis-Menten kinetics; α a dimensionless parameter ranging from 0 to ∞ as the reaction kinetics is of the zero- or first-order, respectively; D_S (=1.29 x 10^{-9} m^2 s^{-1} at 20 °C, Schwartzberg and Chao, 1982) is the diffusivity for urea in the bulk liquid; D_{Se} the effective diffusivity for urea in the biocatalyst depending, in this case, almost exclusively on the biocatalyst porosity and tortuosity factor (Bailes and Ollis, 1986). In particular, k_L was derived from the empirical regressions specifically developed for spinner flask vessels (Aunins et al., 1989) and for assessing mass transfer around spheres in agitated vessels (Asai et al., 1998), whereas the effectiveness factor (η) for Michaelis-Menten kinetics was estimated by resorting to the approximate expressions extracted from Moo-Young and Kobayashi (1972).

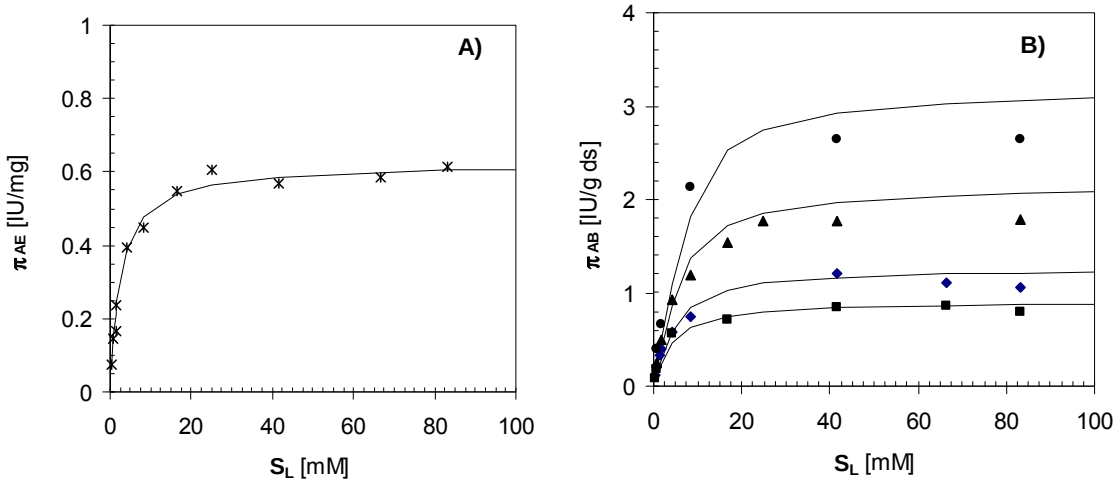

*Figure 1: Effect of urea concentration (S_L) on the specific ammonia formation rate of (**A**) free enzyme (π_{AE}: ✳) at a free enzyme concentration (c_{PF}) of 4.2 mg BSAE/L and (**B**) immobilised derivatives (π_{AB}) with different enzyme loadings and immobilised enzyme concentrations: ■, $Y_{P/B}$ = 48 mg BSAE/g ds and c_{Bd} = 0.11 mg BSAE/L; ◆, $Y_{P/B}$ = 67 mg BSAE/g ds and c_{Bd} = 0.11 mg BSAE/L; ◢, $Y_{P/B}$ = 114 mg BSAE/g ds and c_{Bd} = 0.055 mg BSAE/L; ●, $Y_{P/B}$ = 170.5 mg BSAE/g ds and c_{Bd} = 0.055 mg BSAE/L. The continuous lines were plotted as reported in the text.*

4. Results and discussion

Figure 1 shows the experimental values of the specific ammonia formation rate for free (π_{AE}) and immobilised (π_{AB}) acid urease referred to liquid volume against the urea concentration (S_L) in the range of 0 to 83.3 mol m^{-3}. The continuous line in Fig. 1A was plotted using Eq.s (2)-(3) with the aforementioned intrinsic kinetic parameters. Quite a good fitting capability was observed, the average percentage error among the experimental and calculated π_{AE} values being ~4.1%.

All the continuous lines in Fig. 1B were plotted using. Eq.s (7)-(11) on the assumption that the immobilisation procedure did not alter the intrinsic kinetic parameters of free acid urease. In particular, once estimated the mass transfer coefficient (k_L), for each bulk substrate concentration (S_L) of the medium where the biocatalyst had been dispersed, the numerical method of successive substitution was used to solve Eq. (9) to determine the substrate concentration at the biocatalyst surface (S_R) and thus calculate the external effectiveness factor Ω using Eq. (8).

As shown in Fig. 1B, the agreement among the experimental and calculated specific activities π_{AB} is apparently satisfactorily. Since the average percentage error was about 20%, almost of the same order of the coefficients of variations for the experimental data, it was possible to attribute the about linear effect of protein loading on the apparent Michaelis constant, previously observed (Bortone et al., 2012) to the

external and internal mass-transfer resistances to substrate diffusion in the operating conditions used in this work.

Thus, by referring to a series of biocatalysts characterised by enzyme loadings ranging from as low as 12.5 - 25 mg BASE/g ds (Andrich et al., 2009a-b, 2010) to as high as 400 mg BASE/g ds, the simultaneous film and intraparticle diffusion-reaction model described by Eq.s (7) – (11) was applied to assess their corresponding specific activity (π_{AB}), as well as the relative contribution of the stagnant film surrounding the biocatalyst and intraparticle substrate diffusion when using a stirred bioreactor charged with 0.5 kg m^{-3} of wet beads and a model solution enriched with 1 or 100 mM of urea .

As shown in Table 1, at S_L values by far greater than K_M, the reaction is zero order throughout the catalyst and this implies an internal effectiveness factor near to unity with a practically negligible contribution of the external film. Unfortunately, this case is of limited, if nil, practical value, real wines usually containing less than 1 mM of urea (Andrich et al., 2010; Esti et al., 2007). At such an S_L value, the reaction is of pseudo-first order and η reduced from 0.9 to 0.28 as $Y_{P/B}$ increased from 12.5 to 400.0 mg BSAE/g ds (Table 1). Moreover, the contribution of the external resistance yielded a further reduction of 3 to 25% in the overall effectiveness factor (Ω) in the above $Y_{P/B}$ range. Thus, the specific activity of the biocatalyst (π_{AB}) tended to smooth its initial linear increase with $Y_{P/B}$. For instance, π_{AB} about doubled from 60 to 110 IU/g ds as $Y_{P/B}$ was increased four times, that is from 100 to 400 mg BSAE/g ds. In all these cases, the overall effectiveness factor (Ω) was smaller than 0.42 (Fig. 2).

Table 1: Effect of the enzyme loading ($Y_{P/B}$) of a few acid urease derivatives on their specific activity (π_{AB}) as calculated using the simultaneous film and intraparticle diffusion-reaction model described in the text under the following assumptions: reaction temperature 20 °C; liquid density, ρ_L = 1000 kg m^{-3}, and viscosity, μ_L = 1 mPas; c_{Bw} = 0.5 kg m^{-3}; c_{Bd} = 0.12 kg m^{-3}; k_L = 1.28x10^{-4} m/s; Bi = 13.

Biocatalyst characteristics	**Value**						
Enzyme loading ($Y_{P/B}$, mg BSAE/g ds)	12.5	25.0	48.0	67.0	114.0	170.5	400.0
Immobilised enzyme concentration (c_{Bd} $Y_{P/B}$, g BSAE m^{-3})	1.5	3.0	5.8	8.1	13.8	20.7	48.5
Urea concentration (S_L, mM)				1.0			
Effectiveness factor due to intraparticle diffusion (η)	0.90	0.79	0.66	0.59	0.48	0.41	0.28
Effectiveness factor due to external and internal diffusion (Ω)	0.87	0.75	0.61	0.53	0.42	0.34	0.21
Specific ammonium ion formation rate (π_{AB}, IU/gm ds)	14	24	37	46	61	75	109
Urea concentration (S_L, mM)				100.0			
Effectiveness factor due to intraparticle diffusion (η)	1.00	0.99	0.99	0.99	0.99	0.99	0.98
Effectiveness factor due to external and internal diffusion (Ω)	1.00	0.99	0.99	0.99	0.99	0.98	0.98
Specific ammonium ion formation rate (π_{AB}, IU/gm ds)	56	111	212	295	501	748	1747

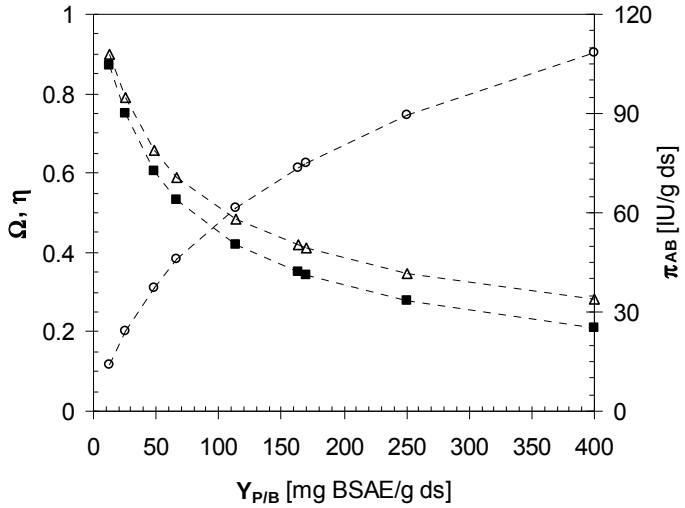

Figure 2: Estimated values of internal (η: △) and overall (Ω: ■) effectiveness factors and specific ammonium ion formation rate (π_{AB}: ○) for a few acid urease immobilised derivatives with different enzyme loading ($Y_{P/B}$) when operating in a stirred bioreactor charged with 0.5 kg of dry beads m^{-3} and a model solution containing 1 mM of urea.

5. Conclusions

The specific activity of several acid urease immobilised derivatives with enzyme loadings of 48 - 170.5 mg BSAE/g ds was satisfactorily reconstructed by using the simultaneous film and intraparticle diffusion and Michaelis-Menten kinetic reaction model. In this way, by referring to the intrinsic kinetic parameters of free acid urease, the change in the apparent affinity of immobilised enzyme for urea was attributed to diffusion limitations only, these reducing the urea concentration near the immobilised enzyme.

When operating in a stirred bioreactor in the pseudo-first order regime, the specific activity of the biocatalyst was greatly affected by its internal resistance, the corresponding effectiveness factor (η) reducing from 0.9 to 0.28 as $Y_{P/B}$ increased from 12.5 to 400.0 mg BSAE/g ds. In the same $Y_{P/B}$ range, the contribution of the external film or boundary layer further lowered the overall effectiveness factor (Ω) by 3 to 25% when setting the stirrer at the higher level used. In conclusion, there was a marginal benefit in the use of biocatalysts with enzyme loadings greater than 150 mg BSAE/g ds, since the combined effect of internal and external mass-transfer resistances made the overall effectiveness factor smaller than 40%.

Further work will be aimed at assessing the operational performance and stability of a laboratory-scale packed-bed cartridge to remove continuously urea from model and real wines.

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