



FACOLTÀ DI AGRARIA, Università degli Studi della Tuscia, Viterbo

Dipartimento di Scienze e Tecnologie Agroalimentari

(AGR/15)

CORSO DI DOTTORATO DI RICERCA

Biotechnologie degli Alimenti - XXII CICLO

TITOLO TESI DI DOTTORATO DI RICERCA

***MODEL AND REAL WINES DETOXIFICATION BY
FREE AND IMMOBILISED ACID UREASE
IN STIRRED BIOREACTORS***

TUTOR

Prof. Ing. Mauro Moresi

DOTTORANDO

Lucia Andrich

COORDINATORE

Prof. Marco Esti

Anno Accademico 2008-09

Ringraziamenti

Innanzitutto desidero ringraziare la mia famiglia per l'incoraggiamento e il sostegno dimostratomi in tutti questi anni, ringrazio tutti gli amici che mi sono stati vicini. Un grazie particolare vanno alla Sig.ra Grazia Franco, per la sua infinita disponibilità e pazienza, al Dott. Alessio Fardelli per la sua amicizia e disponibilità e al Dott. Marcello Fidaleo per la sua preziosa guida e amicizia che ha mi ha dimostrato durante tutto il mio dottorato e in particolare nel primo anno.

Un ultimo grazie, che viene dal profondo del mio cuore va ad una donna veramente speciale, che è stata una guida, un'ottima insegnante, ma soprattutto un'amica durante tutto il mio percorso di formazione universitaria, quindi grazie davvero alla Dott.ssa Angela Zinnai.

Contents

Abstract

Riassunto

Notation

Introduction.....p.1

Chapter 1 : Ethyl carbamate.....p.5

1.1 Introduction.....p. 7

1.2 Ethyl Carbamate: properties and uses.....p.7

1.3 Potential ethyl carbamate carcinogenicity p.8

1.4 Ethyl carbamate origin in wines.....p.13

1.5 Ethyl carbammate precursors.....p.14

1.6 Ethyl carbamate maximum allowed dosage.....p.16

**1.7 Ethyl Carbammate maximum allowed levels in alcoholic
beverages.....p.16**

1.8 Ethyl carbamate preventative action.....p.18

1.8.1. Viticulture.....p.18

1.8.1.1 Vineyard fertilization..... p.18

1.8.1.2 Cover Crops.....p.20

1.8.1.3 Cultivars and Rootstocks.....p.20

1.8.2. Juice Nutrient: status and additions.....p.21

1.8.3. Yeast Strains.....p.22

1.8.4. Lactic acid bacteria.....p.22

1.8.5. Ureases application.....p.23

1.8.6. *Sur Lie* aging.....p.23

1.8.7. Distillation and fortification.....p.24

1.8.8. Shipment and Storage.....p.25

Chapter 2: Ureases.....p.27

2.1 Ureases: function and brief story.....p.29

2.2 Enzyme a real efficient biological catalyst.....p.30

2.3 The enzymatic inhibitors.....	p.32
2.4 Ureases Sources.....	p.38
2.4.1 Ureases in plants.....	p.38
2.4.2 Ureolytic bacteria pathogenic to humans and animals.....	p.39
2.4.3 Acid ureases.....	p.40
2.5 Proteic structures of ureases.....	p.43
2.6 Urease catalyst properties.....	p.46
2.6.1 Proposed reaction mechanisms for urease-catalyzed urea hydrolysis.....	p.46
2.6.2 Kinetic properties of ureases and enzyme substrates.....	p.47
2.7 Urease inhibitors.....	p.49
Chapter 3: enzymatic reaction engineering.....	p.55
3.1 Free urease kinetic model.....	p.57
3.2 Free acid urease kinetics in real wine.....	p.65
3.3 The enzyme immobilization.....	p.67
3.3.1 Definition	p.67
3.3.2 The main enzymatic immobilization methods.....	p.69
3.4 Immobilised ureases and their applications.....	p.72
3.5 The carriers used in this PhD work.....	p.73
3.5.1 Eupergit [®] C.....	p.73
3.5.2 Chitosan.....	p.75
3.5.2.1. Solvent evaporation method.....	p.79
3.5.2.2. Neutralization method.....	p.79
3.5.2.3. Cross-linking method.....	p.80
3.5.2.4. Ionotropic gelation method (or coacervation).....	p.80
3.6 Immobilised acid urease kinetic model.....	p.83
Chapter 4:Materials and Methods.....	p.87
4.1 Raw materials.....	p.89
4.1.1 Nagapsin preparation.....	p.89
4.1.2 Supports.....	p.89

4.1.2.1 Eupergit® C 250 L.....	p.89
4.1.2.2. Chitopearl.....	p.89
4.1.3 Real wine assayed.....	p.90
4.2 Solution preparations.....	p.90
4.3 Analytical Methods.....	p.91
4.3.1 Ammonium ion.....	p.91
4.3.2 Urea.....	p.92
4.3.3 Raw protein.....	p.92
4.4 Procedures and operating conditions.....	p.93
4.4.1 Enzyme immobilisation on Eupergit® 250 L.....	p.93
4.4.2 Enzyme immobilisation on Chitopearls.....	p.95
4.4.3 Bound enzyme assessment.....	p.96
4.4.4 Enzyme activity assays.....	p.97
4.4.5 Enzyme storage stability.....	p.98
4.4.6 Urea degradation kinetics by free or immobilised acid urease.....	p.98
4.5 Wine Analyses.....	p.100
4.5.1 Alcohol content.....	p.100
4.5.2 pH.....	p.100
4.5.3 Total acidity.....	p.100
4.5.4 Volatile acidity.....	p.100
4.5.5 Glycerol.....	p.101
4.5.6 Ash	p.101
4.5.7 Overall SO ₂	p.102
4.5.8 Total phenolics.....	p.102
4.5.9 Total dried extract.....	p.103
4.5.10 Organic acids and reducing sugars.....	p.103
Chapter 5: Results and Discussion.....	p.105
5.1 Immobilisation of acid urease.....	p.107
5.1.1 Enzyme immobilisation onto Eupergit® C 250 L.....	p.107
5.1.2 Enzyme immobilisation onto chitosan-based biocatalysts beads.....	p.107

5.1.3 Effect of bead size.....	p.110
5.2 Storage stability of immobilised acid urease.....	p.113
5.2.1 Eupergit®-based biocatalysts.....	p.113
5.2.2 Chitoppearl-based biocatalysts.....	p.113
5.3 Urea degradation rate by free acid urease.....	p.116
5.4 Urea degradation rate by immobilised acid urease.....	p.117
5.4.1 Immobilised Acid Urease kinetics.....	p.117
5.4.2 Effectiveness factor estimation.....	p.119
5.5 Effect of grape seed tannins on immobilised	
enzyme kinetics.....	p.123
5.6 Urea degradation in real white wines by immobilised	
acid urease.....	p.125
5.6.1 Wine characteristics.....	p.125
5.6.2 Urea degradation in real wines by immobilised enzyme.....	p.126
5.6.3 Effectiveness factors of immobilised acid urease in real wines.....	p.129
Conclusions.....	p.135
References.....	p.141

Abstract

This PhD thesis research project was aimed at selecting the most appropriate immobilisation carrier (i.e., Eupergit® C 250 L and chitosan beads, named Chitopearls BCW-3001, BCW-3003, BCW-3010, and HP-3020) for acid urease in terms of the specific activity of immobilised enzyme under different operating conditions, such as immobilization time, enzyme- or activating reagent-to-carrier ratios, pH of the immobilization buffer, and storage stability for as long as 30-170 days. The performance of immobilised acid urease in model wine solutions with or without grape seed tannins and real wines was assessed in bench-top scale stirred bioreactors and mathematically described by resorting to a conventional diffusion-reaction model.

In particular, at the optimal glutaraldehyde-to-chitosan bead ratio ($Y_{GA/CHI}$) of 0.625 g g⁻¹, the specific activity (A_{Bi}) of acid urease immobilised covalently onto porous chitosan beads decreased from circa 300 to 70 IU g⁻¹ wet support as the bead average diameter (d_p) increased from 0.14 to 2.2 mm. Generally, A_{Bi} reduced less than 5% after preservation in the wet form at 4°C for 150-170 days. Only the biocatalyst prepared using the Chitopearl BCW-3001 lost about 40% of its initial activity. The activity of acid urease immobilised onto Eupergit® C 250L was smaller (19 ± 3 IU g⁻¹ ws) and unstable. In fact, in the same storage conditions A_{Bi} reduced to $68 \pm 15\%$ of the initial activity after about 34 days.

The kinetics of urea degradation in the model wines using these biocatalysts was of the pseudo-first order with respect to the urea concentration in the liquid bulk, the apparent pseudo-first order kinetic rate constant (k_{fi}) ranging from about two thirds to one fifth of that (k_{if}) pertaining to free acid urease. 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. When the model wine solution was enriched with the high-inhibitory tannins extracted from grape seeds, at the maximum level tested (374 ± 2 g GAE m⁻³) k_{fi} reduced to no more than $(58 \pm 9)\%$ of k_{if} , this proving quite a higher protective action against such compounds for the chitosan-based biocatalysts towards free or Eupergit® C 250 L-immobilised acid urease.

The kinetics of urea degradation in two target Italian white (i.e., *Grechetto* and *Sauvignon Blanc*) wines by using acid urease immobilised onto Eupergit® C 250 L or glutaraldehyde-crosslinked chitosan-derivatives beads (that is, Chitopearls BCW-3003

and BCW-3010), was confirmed to be of the pseudo-first 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 apparent pseudo-first order kinetic rate constants, when using Eupergit® C 250 L-, BCW-3003- or BCW-3010-based biocatalyst, were quite similar, even if they reduced to about 7, 18 or 17% of that observed for free enzyme in the model wine solution, respectively.

The chitosan-based biocatalysts resulted to be more than twice less sensitive to the phenolic content of the wines tested than the Eupergit® C 250 L-based ones, this making the former potentially employable in the make up of packed-bed cartridges to remove continuously urea from commercial wines.

Riassunto

Questa tesi di dottorato ha riguardato la selezione del supporto commerciale più appropriato (Eupergit® C 250 L e sfere di chitosano, denominate Chitopearls) per immobilizzare ureasi acida valutando l'attività specifica dell'enzima immobilizzato al variare di diversi parametri operativi, come il tempo d'immobilizzazione, i rapporti enzima:supporto ($Y_{P/B}$) e reagente attivante:supporto ($Y_{GA/CHI}$), il pH del tampone d'immobilizzazione e la stabilità operativa per 30 o 170 giorni.

La cinetica d'idrolisi dell'urea tramite i biocatalizzatori così ottenuti è stata determinata sia in soluzioni modello con o senza polifenoli estratti da vinaccioli, sia in vini reali in bioreattori ad agitazione meccanica da banco, descrivendola con il modello convenzionale di diffusione-reazione.

In particolare, in corrispondenza del rapporto ottimale $Y_{GA/CHI}=0.625 \text{ g g}^{-1}$, l'attività specifica (A_{Bi}) dell'ureasi acida immobilizzata covalentemente sulle Chitopearls decresceva da circa 300 a circa 70 IU g^{-1} di supporto umido (ws) all'aumentare del diametro medio (d_p) della particelle di supporto da 0,14 a 2,2 mm.

Per i biocatalizzatori conservati in soluzione acquosa a 4°C A_{Bi} si riduceva meno del 5% dopo 150-170 giorni. Solo il biocatalizzatore a base di Chitopearl BCW-3001 perdeva circa il 40% dell'attività iniziale. Per contro, l'attività ureasica del biocatalizzatore a base di Eupergit® C 250 L risultava non solo nettamente inferiore ($19\pm3 \text{ IU g}^{-1} \text{ ws}$), ma anche più instabile, in quanto nelle medesime condizioni di conservazione adottate per i biocatalizzatori immobilizzati su Chitopearls l'attività si riduceva al $68\pm15\%$ di quella iniziale dopo appena 34 giorni.

La cinetica d'idrolisi dell'urea nelle soluzioni di vino modello promossa da tutti i biocatalizzatori in esame è stata descritta con un modello di pseudo-primo ordine rispetto alla concentrazione di urea nel mezzo di reazione e da un costante cinetica apparente di pseudo-primo ordine (k_{fi}) pari al 66-20% di quella dell'enzima libero (k_{if}). Nelle condizioni operative esaminate, la cinetica di reazione non era limitata dalle resistenze al trasferimento di materia all'esterno e all'interno del biocatalizzatore.

In presenza di tannini estratti da vinaccioli ($374\pm2 \text{ g GAE m}^{-3}$), k_{fi} si riduceva al ($58\pm9\%$) di k_{if} e ciò dimostrava che i biocatalizzatori immobilizzati su chitosano esercitavano una migliore azione protettiva nei confronti di questi polifenoli rispetto all'enzima sia libero che immobilizzato su Eupergit C 250 L.

La cinetica di idrolisi dell'urea nei due vini bianchi italiani prescelti (*Grechetto* e *Sauvignon Blanc*) in presenza di ureasi acida, immobilizzata su Eupergit® C 250 L o su chitosano pre-attivato con gluteraldeide (Chitopearl BCW-3003 e BCW-3010), era anche essa descritta tramite un modello di pseudo-primo ordine rispetto alla concentrazione di urea ed anche in questo caso non era limitata dalle resistenze al trasporto di urea nel film esterno e all'interno del biocatalizzatore .

Nei vini *Grechetto* e *Sauvignon Blanc*, la costante cinetica apparente di pseudo-primo ordine relativa ai biocatalizzatori immobilizzati su Eupergit® C 250 L-, BCW-3003- o BCW-3010 era abbastanza simile, anche se si riduceva rispettivamente al 7, 18 o 17% del valore relativo all'enzima libero in vino modello.

I biocatalizzatori immobilizzati su chitosano sono risultati almeno due volte meno sensibili alla frazione polifenolica presente nei vini esaminati rispetto all'ureasi immobilizzata su Eupergit® C 250 L, il che permetterebbe di impiegarli per realizzare reattori a colonna impaccata per la detossificazione in continuo di vini chiarificati.

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). Because EC has shown a potential carcinogenic activity when administered in high doses in animal tests (Schlatter *et al.*, 1990), there is a great deal of interest in reducing EC levels in food products. In particular, a safe level of 15 mg m^{-3} has been so far recommended in wines (Butzke *et al.*, 1997).

Besides the numerous preventive actions to reduce EC levels issued by the U.S. Food and Drug Administration, the hydrolysis of urea to NH_3 and CO_2 by acid urease (E.C.3.5.1.5) seems to be a suitable way to avoid EC formation from such a precursor (Butzke *et al.*, 1997). However, the effectiveness of this treatment is highly dependent on the type of wine, content of inhibiting factors (that is, in order of importance, fluoride, malate, ethanol, and phenolic compounds), and usage conditions (Trioli *et al.*, 1989).

The kinetics of urea hydrolysis was described by a modified Michaelis-Menten reaction rate expression, incorporating pH dependent kinetics, substrate inhibition, and noncompetitive product inhibition by NH_4^+ (Moynihan *et al.*, 1989). In model and real wines for urea concentrations (S) less than 1 mol m^{-3} , acid urease kinetics was safely regarded of the pseudo-first order with respect to S (Esti *et al.*, 2007; Fidaleo *et al.*, 2006). Moreover, in model wine solutions the pseudo-first order kinetic rate constant was found to be largely greater (Fidaleo *et al.*, 2006) than that pertaining to some real wines, probably because of the competitive inhibition exerted by phenolic compounds (Esti *et al.*, 2007; Kodama, 1996; Trioli *et al.*, 1989).

Immobilisation of acid urease on inert carriers would have the potential advantages of significant cost savings, and improved stability or resistance to shear or inhibitory compound inactivation. Acid urease has been so far immobilized on various matrices (Matsumoto, 1993; Selvamurugan *et al.*, 2007; Zhou *et al.*, 2008). Continuous urea removal from sake by immobilised acid urease has been applied in Japan since 1988 (Matsumoto, 1993).

2. Mathematical Modelling

When using immobilized acid urease, it was assumed that enzyme coupling to the supports of choice did not affect the pseudo-first order kinetic model of free enzyme, especially when S 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:

$$r_{Si} = k_{ii} S, \quad k_{ii} = k'_{ii} \rho_B Y_{P/B} \quad (1)$$

where all the symbols are defined in the *Notation* section.

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 concentration (c_{Bw}) of wet biocatalyst in the form of almost spherical beads with an average diameter (d_p), the unsteady-state material balance for the reagent S and its initial condition may be written as:

$$-\frac{dS_L}{dt} = k_L a_S (S_L - S_R) = \Omega v_S k_{ii} S_L \quad (2)$$

$$S_L = S_{L0} \quad \text{for } t = 0 \quad (3)$$

Equation (2) can be integrated with its corresponding initial condition

$$\ln\left(\frac{S_L}{S_{L0}}\right) = -\int_0^t \Omega c_{Bw} Y_{P/B} k'_{ii} dt \quad (4)$$

Provided that the semilogarithmic plot of the ratio between the current (S_L) and initial (S_{L0}) concentrations of urea vs. reaction time (t) in stirred bioreactors is approximately linear. In these conditions, using free or immobilised enzyme, the product of c_{Bw} by $Y_{P/B}$ coincides with the free (E_f) or immobilised (E_i) enzymatic protein concentration dissolved or dispersed in the liquid

phase. If the overall effectiveness factor (Ω) is intrinsically or approximately unitary, the apparent slopes of $\ln(S_L/S_{L0})$ -vs.- t are directly proportional to E_f or E_i via the specific pseudo-first order kinetic rate constant relative to the free (k'_{if}) or immobilized (k'_{ii}) enzyme.

3. Material and Methods

A few lots of the commercial preparation Nagapsin[®] (Nagase Europa GmbH, Duesseldorf, Germany), consisting of 96% (w/w) lactose and 4% (w/w) purified acid urease from *Lactobacillus fermentum*, were used. Their specific activity was found to decay throughout the experimentation from 766 ± 7 to 420 ± 27 IU g⁻¹, respectively, where 1 IU corresponds to the amount of powder that liberates 1 μ mol min⁻¹ of ammonia from urea at 20°C, once it is dissolved in 0.1 kmol m⁻³ sodium-acetate buffer (pH 4.0) enriched with urea (83.33 mol m⁻³).

Two types of porous supports were used as listed in Table 1. 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 pH 5.0 (KAB5) and used as the support-activating reagent solution.

The performance of free or immobilised acid urease at 20°C was assessed in a model wine solution (Fidaleo *et al*, 2006), and two real Italian white *Grechetto* and *Sauvignon Blanc* wines. The model wine solution 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 3.5. All reagents were of the analytical grade. An extract of grape seeds (Grap'tan[®]PC: Ferco Oenologie, St. Montan, F), was used as a source of phenolic compounds, its moisture and total phenolics contents (P) being 2.3 ± 0.3 % (w/w) and 0.62 ± 0.01 g of gallic acid equivalent (GAE) per g of extract, respectively.

Enzyme immobilisation on Eupergit[®] supports are here summarised: 0.6-1.2 g of dry beads and 30-35 cm³ of KPB7 at 20°C were charged into a 150-cm³ Pyrex flask, equipped with a portable marine-type impeller rotating at 250 rev min⁻¹. After soaking for about 24 h, 72-100 cm³ of KPB7, enriched with 16.0 or 24.0 kg m⁻³ of Nagapsin and preconditioned at 20 °C, were added while the dispersion continued to be mixed. After incubation for 24 or 48 h, the biocatalyst was collected by vacuum filtration and washed twice with 50 cm³ of KPB7.

For Chitopearl BCW-3001, the two methods (*i.e.*, absorption cross-linkage, A, and covalent binding, B) described by Matsumoto (1993) were slightly modified and tested. Method B was then used for all other chitosan-derivative beads, all experimental conditions are summarised in Table 2.

Until use, all biocatalysts were stored in 0.05 M potassium phosphate buffer pH 7.0, enriched with 2% (v/v) isopropanol and 0.5 kg m⁻³ ethyl parabene, at 4°C to avoid microbial contamination (Andrich *et al*, 2009).

In both methods, all filtrates and washing solutions were collected and diluted with KAB5 to a constant final volume (250 or 500 cm³).

To assess the performance of free and immobilised acid urease in real wines, Nagapsin was newly immobilised onto Chitopearls (BCW-3003 and BCW-3010) using the method B and onto Eupergit[®], all the experimental conditions are reported in Table 3. The protein concentration in the immobilising solutions and overall filtrates was determined according to the method by Lowry *et al* (1951) using the Total Protein Kit (Sigma, St. Louis, MO, USA) and expressed as bovine serum albumin equivalent (BSAE).

The amount of bound protein (m_{pb}), protein loading ($Y_{p/B}$), specific activity (A_{BI}), and enzyme (ζ_E) and activity (ζ_A) coupling yields for any biocatalyst were estimated and reported in the Tables 2 and 3.

Table 1 Main characteristics of porous matrices used in this work.

Parameter	Eupergit® C250L	BCW-3001	BCW-3003	BCW-3010	HP-3020	Unit
Manufacturer	Röhm GmbH(Darmstadt, D)	Wako Chemicals GmbH (Neuss, D)				
Bead size	130-210	74-210	177-420	840-1190	1,900-2,500	µm
Average bead diameter (d_p)	180	142	300	1016	2,200	µm
Water fraction (x_w)	3.4±0.2	84.6±0.4	91.0±0.6	86±1	80±1	% w/w

Table 2 Experimental conditions and specific activities per unit mass of dry (ds) or wet (ws) support of acid urease immobilised on Eupergit® C 250 L (Andrich et al, 2009) or a few GA-activated chitosan beads of different size.

Parameter		Eupergit® C250L	Chitopearl BCW-3001	Chitopearl BCW-3003	Chitopearl BCW-3010	Chitopearl HP-3020	Unit
Mass of wet support	m_s	601.3	1200	1001.4	1200.4	1200.2	mg
Support soaking volume	V_s	35	0	0	0	0	cm ³
Immobilising solution volume	V_{E0}	72	70	70	70	70	cm ³
Nagapsin® conc. in the immob. sol.	C_N	16	24	24	24	24	kg m ⁻³
Protein conc. In the immob. sol.	C_{P0}	881±14	886±17	895±17	885±4	893±7	g BSAE m ⁻³
Initial protein mass	m_{P0}	63±1	62±1	63±1	62.0±0.3	62.5±0.5	mg BSAE
Prot. added per unit mass of ws	$Y_{P/S}$	105.5	52	52	52	52	mg BSAE g ⁻¹ ws
Immobilising solution activity	A_0	13.9±0.1	17.3± 0.1	15 ± 1	17.8 ± 0.1	19.8±0.4	IU mg ⁻¹ BSAE
Protein concentration in the filtrate	C_{Pf}	192±9	29.3±0.4	48 ± 1	48 ± 1	85 ± 3	g m ⁻³ BSAE
Filtrate volume	V_f	250	500	500	500	500	cm ³
Protein bound on support	m_{pb}	15.5	47.4	38.8	20.8	20.1	mg BSAE
Protein loading	$Y_{P/B}$	25.8	39.5	38.7	17.3	16.8	mg BSAE g ⁻¹ ws
Specific immobilised enz. activity	A_{Bd}	99±17	1494±1	1059±11	727±75	209±4	IU g ⁻¹ ds
Specific immobilised enz. Activity	A_{Bw}	19±3	308.2±0.1	260±3	151±16	71±1	IU g ⁻¹ ws
Immobilised enz. specific activity	A_{BP}	3816±665	7805±3	6725±69	8729±907	4239±79	IU g ⁻¹ BSAE
Enzyme coupling yield	ζ_E	24.4	76.4	61.9	33.6	32.2	%
Enzyme activity coupling yield	ζ_A	27±5	45±0.1	43.9±0.5	49±5	21.4±0.4	%

To assess the time course of the hydrolytic process, 80 cm³ of the model wine solution, as such or enriched with Grap'tan® PC and the two real wines were poured into a 100-cm³ rubber-capped flask, pre-charged with given amounts of free (0.3-1.5 kg m⁻³ Nagapsin®) or immobilised (1.25-9.4 kg m⁻³ of wet carrier) acid urease, under constant temperature (20°C) and different stirring (100 and 400 rev min⁻¹).

Samples (1cm³) were withdrawn from any flask for as long as 24-50 h and being assayed for ammonium and urea by using the K-URAMR kit (Megazyme Int. Ireland Ltd, Wicklow, IRL). Any interference between the enzymatic kit and grape seed tannins was limited by diluting any sample with an equal volume of an aqueous solution containing polyvinyl-pyrrolidone (PVP) at the same concentration of the grape seed extract present in the sample to be assayed.

The inhibitory effect of tannins on acid urease immobilised on Eupergit® C 250 L (~6.25 kg ws m⁻³) was assessed by enriching the model wine solution with 5-1000 g m⁻³ of Grap'tan® PC. Further testing at 300 g m⁻³ of seed extract were carried out using GA-activated chitosan beads at 1.2-3.1 kg ws m⁻³.

Table 3 Experimental conditions and specific activities per unit mass of dry (ds) or wet (ws) support of acid urease immobilised on Eupergit® C 250 L and some GA-activated chitosan beads of different size.

Parameter		Eupergit® C 250L	Chitopearl BCW-3003	Chitopearl BCW-3010	Unit
Mass of wet support	m_{BW}	1204.0	1200.7	1200.2	mg
Water fraction	x_{BW}	80 ± 1	75 ± 5	79 ± 3	% w/w
Immobilising solution volume	V_0	70	70	70	cm ³
Nagapsin® conc. in the immob. sol.	C_N	24	24	24	kg m ⁻³
Protein conc. in the immobilizing sol.	C_{p0}	978 ± 19	950 ± 24	913 ± 4	g BSAE m ⁻³
Initial protein mass	m_{p0}	68 ± 1	66 ± 2	63.9 ± 0.3	mg BSAE
Prot. Added per unit mass of ws	$Y_{p/S}$	57	55	53	mg BSAE g ⁻¹ ws
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}	106 ± 1	47 ± 1	93 ± 4	g m ⁻³ BSAE
Filtrate volume	V_f	500	500	500	cm ³
Protein bound on support	m_{pb}	15.3	43.2	17.3	mg BSAE
Protein loading	$Y_{p/B}$	12.75	36.0	14.4	mg BSAE g ⁻¹ ws
Specific immobilised enz. activity	A_{Bd}	51.3 ± 0.4	1059 ± 11	609 ± 20	IU g ⁻¹ ds
Specific immobilised enz. activity	A_{Bw}	10.3 ± 0.1	260 ± 3	127 ± 4	IU g ⁻¹ ws
Immobilised enz. specific activity	A_{BEi}	0.80 ± 0.01	7.24 ± 0.07	8.79 ± 0.29	IU mg ⁻¹ BSAE
Enzyme coupling yield	ζ_E	22.4	64.9	27.1	%
Enzyme activity coupling yield	ζ_A	4.8 ± 0.1	47.2 ± 0.5	56 ± 2	%

4. Results and Discussion

Enzyme bounding and activity coupling yields and storage stability of immobilised acid urease

Tables 2 and 3 reports the experimental conditions used to immobilise acid urease on the carriers used, as well as the protein concentrations in the immobilising solution (C_{p0}) and overall filtrate (C_{pf}), protein bound on support (m_{pb}), protein loading ($Y_{p/B}$), immobilised acid urease activity per unit mass of dry (A_{Bd}) or wet (A_{Bw}) support or protein bound (A_{BEi}), as well as enzyme (ζ_E) and activity (ζ_A) coupling yields.

When using Eupergit® C 250 L, the protein loading for the biocatalyst stabilised with glycine agreed with that obtained with other enzymes (Knezevic *et al*, 2006; Gómez de Segura *et al*, 2004). Despite a fast partial inactivation in the first 5 days, the biocatalyst maintained as much as $68 \pm 15\%$ of its initial activity over the following 30 days (Andrich *et al*, 2009).

Use of the absorption cross-linkage method to immobilise acid urease on Chitopearl BCW-3001 gave rise to biocatalysts with specific activities (A_{Bw}) decreasing from ~ 300 to 230 IU/g ws as $Y_{GA/CHI}$ was increased from 0 to 3 g of GA/g ws, whereas use of the GA-cross-linking method yielded a peak value in A_{Bw} of 320 ± 20 IU/g ws at $Y_{GA/CHI} = 0.625$ g/g, such an optimal ratio being also appropriate for the covalent bounding of chloro-peroxidase to aminopropyl-glass beads (Kadima *et al.*, 1990). Once the method B had been tested on the other Chitopearls at $Y_{GA/CHI} = 0.625$ g g⁻¹, the specific activity (A_{Bw}) tended to decrease almost linearly from 310 to 71 IU/g ws with the logarithm of the average bead diameter (Table 2).

For regarding the experimental conditions reported in the Table 3, the specific activity and a protein loading resulted to be quite near to those obtained previously (Table 2), 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 case (~ 10 IU g⁻¹ wet support, ws), was found to be slightly smaller than that (19 IU g⁻¹ ws) pertaining to the same biocatalysts attained earlier (Table 2).

Moreover, the enzyme coupling yield (ζ_E) was of 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. Finally, the activity coupling yield (ζ_A) was of more or less 50% for the beads of the BCW series, but it dropped to as little as 5% for the 180- μ m Eupergit® C 250 L particles.

The biocatalysts obtained from Chitopearl of the BCW and HP series exhibited less than 5% loss in A_{Bw} after preservation in the aforementioned buffer at 4°C for 62 days. Only the specific activity of the biocatalysts prepared using the Chitopearl BCW-3001 tended to reduce by about 40% after 62-day storage. However, throughout the following 78 days its residual specific activity was found to be approximately constant ($181 \pm 16 \text{ IU g}^{-1}$).

Urea degradation rate by free or immobilised acid urease

By using a stirred bioreactor, the kinetic analysis of free or immobilised acid urease in the model wine solution yielded a series of approximately linear semilogarithmic plots of (S_L/S_{L0}) against t , thus confirming previous assumptions (see Section 2). The slopes of such diagrams, expressing the apparent pseudo-first order kinetic rate constant (k_{ai}), resulted to be proportional to the free (E_f) or immobilised (E_i) enzymatic protein concentration dissolved or dispersed in the liquid phase.

When using Eupergit® C 250 L, the specific pseudo-first order kinetic rate constant relative to the free enzyme [$k'_{if} = (8.5 \pm 0.3) \times 10^{-3} \text{ m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSA}_e$] was estimated to be about 4 times greater than that relative to the immobilized counterpart [$k'_{ii} = (2.4 \pm 0.2) \times 10^{-3} \text{ m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSA}_e$] (Andrich *et al.*, 2009).

When using the chitosan beads, $k_{ai} [= -0.13 \ln(d_p) + 1.2]$ exhibited an almost linearly decreasing trend with $\ln(d_p)$, this agreeing with the aforementioned trend of A_{Bw} -vs.- $\ln(d_p)$. Moreover, k'_{if} was estimated to be 1.5-6 times greater than k'_{ii} , thus confirming the greater performance of the GA-activated Chitopearl of the BCW series with respect to Eupergit® C 250 L and Chitopearl HP-3020.

The semi-logarithmic 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 immobilised acid urease were approximately linear, in all cases, as shown in Fig.1.

To check for the contribution of the external and/or internal 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 (Baley & Ollis, 1986; Gómez de Segura *et al.*, 2004; Kosmerl *et al.*, 2000; Satterfield *et al.*, 1963; Spieß *et al.*, 1999;; Tagatsuji *et al.*, 1998; Treybal, 1968) on the assumption that the density and viscosity at 20°C of the model wine solution coincided with those of a typical white wine (Košmerl *et al.*, 2000) with the same alcohol content. For all the biocatalysts used, both the effectiveness factors (Ω) and (η) were estimated as practically unitary, despite Ω slightly decreased from 0.9999 to 0.9996 as d_p increased from 140 to 2,200 μm .

Also for the three biocatalysts used to removal urea in real wines, both the effectiveness factors (Ω) and (η) resulted have been estimated to be practically unitary, as established for the biocatalysts used to remove urea in model wine solution. Thus, whatever the biocatalyst used here, in a model wine solution and in real wines, the overall urea degradation rate was controlled by the reaction kinetics, being negligible the contribution of the external film and intraparticle mass-transfer resistances. Despite such estimates were referred to biocatalysts with a unitary tortuosity factor (τ), these would still hold even if τ was as great as 7, the typical τ values for industrial catalysts ranging from 1 to 7.

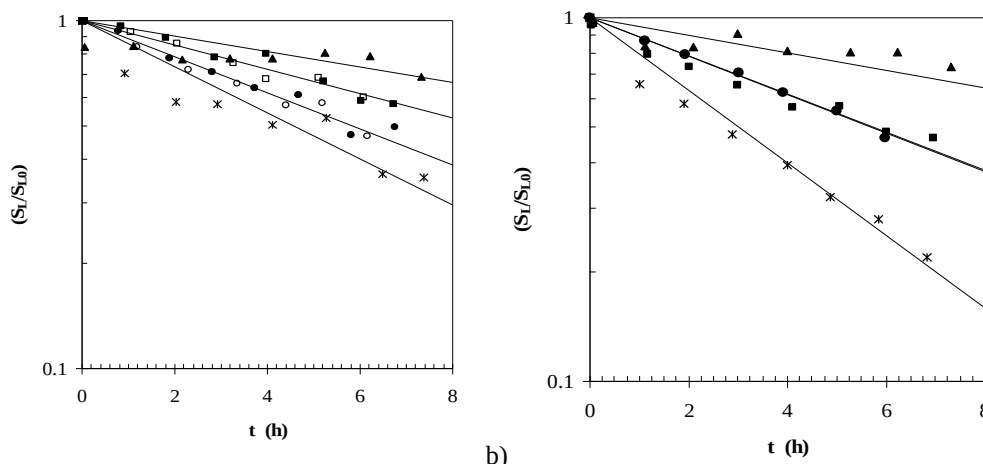


Figure 1 Time course of urea degradation at 20°C for Grechetto (a) and Sauvignon Blanc (b) wines when using free enzyme (*, $c_N=1.2 \text{ kg m}^{-3}$ Nagapsin®) or immobilised enzyme onto different matrices (BCW-3003: ○, ●, $c_{BW}=1.25$; BCW-3003: ■, □, $c_{BW}=3.13$; Eupergit® C 250 L: ▲, $c_{BW}=3.53 \text{ kg m}^{-3}$ wet carrier): Semi-logarithmic plot of the ratio between the current and initial urea concentrations (S_t/S_{L0}) against time (t).

Effect of grape seed tannins on immobilized acid urease kinetics

In accordance with previous findings (Esti *et al*, 2007) assessing that the activity of a killed cell preparation, such as Enzeco® Acid Urease, was by far inhibited by the tannins extracted from grape seeds, the model wine solution of concern was enriched with $5\text{--}1000 \text{ g m}^{-3}$ of the above tannin source to assess its inhibitory effect on the activity of acid urease immobilized on Eupergit® C250 L. The resulting almost linear $\ln(S_t/S_{L0})$ -vs.- t plots allowed the experimental pseudo-first order kinetic rate constant (k_{fi}) for immobilised acid urease to be determined as a function of P . In the circumstances, k'_{if} tended to reduce hyperbolically in the range of $0\text{--}600 \text{ g GAE m}^{-3}$ with an inhibition constant about two times greater than that characterizing the inhibitory effect of grape seed extract on free acid urease (Esti *et al*, 2007).

Fig. 2 shows the semi-logarithmic plots for the model wine solution as such or enriched with about 300 g m^{-3} of the phenolic source of choice when using the 4 chitosan beads tested at different concentrations so as to charge the stirred bioreactor with almost the same enzymatic protein content ($51\pm3 \text{ g BSAE m}^{-3}$). Use of the least squares method yielded the average value and standard deviation of the experimental k_{fi} values for such biocatalysts. At $P=187\pm1 \text{ g GAE m}^{-3}$, the k_{fi} values relative to Eupergit® or chitosan-beads reduced to 20% (Andrich *et al*, 2009) or $70\pm10\%$ of those pertaining to the model wine solution devoid of phenolics, respectively. Thus, enzyme immobilization on chitosan beads instead of Eupergit® ones attenuated quite efficiently the high-inhibitory effect of grape seed tannins towards free enzymes.

5. Conclusions and future perspectives

Despite enzymes (such as α -, β -, and gluco-amylases, cellulases, lipases, proteases, etc.) are largely used in agro-processing, immobilized enzyme bioprocesses are still restricted to the isomerisation of glucose, and hydrolysis of lactose, sucrose and acyl-L-amino acids, probably because of the typical misoneism of the food industry as coupled to the low profitability of a great number of food products.

Urea degradation by immobilized acid urease on Chitopearls appeared to be a suitable way to reduce EC levels in model wines for significant cost savings owing to multiple enzyme recycles in consecutive batch using a stirred tank reactor, for their reduced sensitivity to tannin inhibition, and for the possibility of overcoming the maximum allowable concentration for killed cell commercial preparations of 75 g m^{-3} with no after bitter taste (Bertrand, 2003).

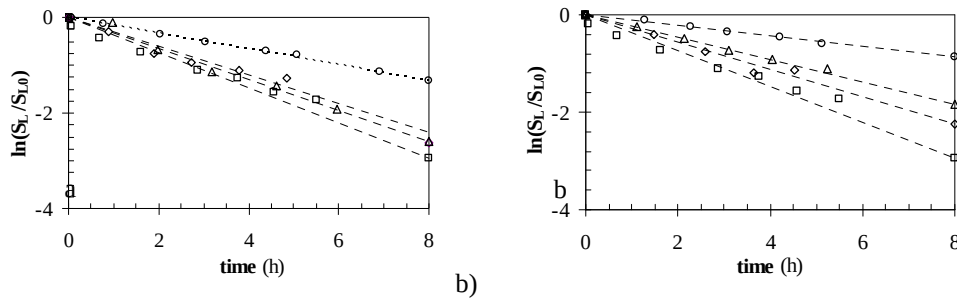


Figure 2 Time course of urea degradation at 20°C for a model wine solution as such (a) or enriched with Grap'tan® PC at $P \sim 187 \pm 1 \text{ g GAE m}^{-3}$ (b) when using different concentrations of biocatalysts prepared with Chitopearl BCW-3001 ($\square$, $c_{Bw} = 1.25 \text{ kg m}^{-3} \text{ ws}$), BCW-3003 ($\diamond$, $c_{Bw} = 1.26 \text{ kg m}^{-3} \text{ ws}$), BCW-3010 ($\triangle$, $c_{Bw} = 3.13 \text{ kg m}^{-3} \text{ ws}$), HP-3020 ($\circ$, $c_{Bw} = 3.13 \text{ kg m}^{-3} \text{ ws}$); Semi-logarithmic plot of the ratio between the current and initial urea concentrations (S_L/S_{L0}) against time (t). The continuous lines represent the least squares regression lines

The kinetics of urea degradation by immobilised ureases in two target Italian wines (that is *Grechetto* or *Sauvignon Blanc* wines) using a stirred bioreactor was of the pseudo-first order with respect to urea concentration, in agreement with previous findings in real and model wines using killed cell preparations (Bertrand, 2003; Esti *et al.*, 2007; Fidaleo *et al.*, 2006; Trioli *et al.*, 1989).

To increase the urea degradation rates achieved here, one needs to increase further the biocatalyst concentrations (c_{Bw}) in the agitated bioreactor or alternatively to resort to specifically designed packed-bed cartridges.

6. Nomenclature

A_{Bj} , specific activity of the immobilized biocatalyst per unit mass of dry or wet support or bound protein (IU g^{-1}); A_0 , enzyme activity in the immobilising solution ($\text{IU g}^{-1} \text{ BSA}_e$); a_p , specific surface per unit volume for the biocatalyst ($=6/d_p$, m^{-1}); a_s ($=a_p c_{Bd}/\rho_B$), overall biocatalyst surface per unit volume of liquid phase (m^{-1}); Bi ($=\frac{1}{2} d_p k_L/D_{Se}$), Biot number; c_{Bj} , concentration of dry or wet biocatalyst (kg m^{-3}); c_N , concentration of Nagapsin® in the immobilising solution (kg m^{-3}); c_{P0} , c_{Pf} , protein concentration in the immobilising and overall filtrate ($\text{g BSA}_{eq} \text{ m}^{-3}$); D_s , diffusivity for urea in the bulk liquid ($\text{m}^2 \text{ s}^{-1}$); D_{Se} , effective diffusion coefficient for urea in the biocatalyst ($\text{m}^2 \text{ s}^{-1}$); d_p , average bead diameter (m); E_j , concentration of free or immobilized enzymatic protein in the liquid bulk ($\text{g BSA}_e \text{ m}^{-3}$); Gr ($=d_p^3 \rho_L | \rho_B | g/\mu_L^2$), Grashof number; k_a , apparent pseudo-first order kinetic rate constant (h^{-1}); k_{ij} , urea degradation pseudo-first order kinetic rate constant for free or immobilized enzyme (h^{-1}); k'_{ij} , specific pseudo-1st order kinetic rate constant relative to the free or immobilised enzyme ($\text{m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSA}_e$); k_L , mass transfer coefficient in the liquid phase (m s^{-1}); m_{Bj} , mass of dry or wet carrier (g); m_{pb} ($=c_{P0}V_0 - c_{Pf}V_f$), amount of bound protein (mg BSA_e); m_{p0} , amount of protein in the immobilising solution (mg BSA_e); P , concentration of phenolic compounds (g GAE m^{-3}); r_{Si} , urea degradation rate for immobilized enzyme referred to the unit volume of biocatalyst ($\text{mol m}^{-3} \text{ h}^{-1}$); S_L , S_R , urea concentration in the liquid bulk or at the biocatalyst surface (mol m^{-3}); Sc ($=\mu_L/(\rho_L D_s)$), Schmidt number; Sh ($=d_p k_L/D_L = 2 + 0.3 (Gr Sc)^{1/3}$), Sherwood number; t , reaction time (h); V_0 , V_f , volume of the immobilising solutions and overall filtrate solutions (cm^3); V_L , liquid volume (m^3); V_s , support soaking volume (cm^3); v_s ($=c_{Bd}/\rho_B$), overall biocatalyst volume the per unit volume of liquid phase; x_{Bw} , water fraction of wet biocatalyst (g g^{-1}); $Y_{GA/CHI}$, glutaraldehyde-to-chitosan mass ratio (g g^{-1}); $Y_{p/B}$ ($=m_{pb}/m_{Bd}$), protein loading ($\text{g bound protein g}^{-1} \text{ dry or wet supp}$); $Y_{p/S}$, protein added per unit mass of wet support ($\text{mg BSA}_e \text{ g}^{-1} \text{ ws}$); ζ_A ($=A_{Bj}/A_0$), enzyme activity coupling yield; ζ_E ($=m_{pb}/m_{p0}$) enzyme coupling yield;

η ($=3/(\Phi(1/\tanh(\Phi)-1/\Phi))$), effectiveness factor for a spherical biocatalyst in the absence of the external film transport resistance (dimensionless); η_L , liquid viscosity (Pa s); ρ_B , ρ_L , biocatalyst or liquid density (kg m^{-3}); τ , biocatalyst tortuosity; Φ ($=\frac{1}{2} d_p \sqrt{k_{ij}/D_{Se}}$), Thiele modulus for pseudo-1st order kinetics; Ω ($=\eta/[1+\eta\Phi^2/(3 Bi)]$), effectiveness factor for a spherical biocatalyst in the presence of the external film transport resistance.

Subscripts: d, dry; f, free; I, immobilised; 0, initial; w, wet.

Notation

A	concentration of ammonium ions (mol m^{-3})
A_0	immobilising solution activity (IU mg^{-1} BSAE)
A_{B0}	initial specific activity of the immobilised biocatalyst per unit mass of wet support (IU g^{-1} wet support)
A_{Bd}	specific activity of the immobilized biocatalyst per unit mass of dry support (IU g^{-1} dry support)
A_{BEi}	specific activity of the immobilized biocatalyst per unit mass of bound protein (IU g^{-1} bound protein)
A_{Bd}	specific activity of the immobilized biocatalyst (IU g^{-1} dry support)
A_{Bi}	specific activity of the immobilized biocatalyst per unit mass of wet support (IU g^{-1} wet support)
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 immobilising 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_{EI}	acid urease activity in the biocatalyst per unit mass of enzyme (IU/g BSA equiv.)
A_L	instantaneous concentration of ammonium ions in the liquid bulk (mol m^{-3})
A_N	activity of Nagapsin [®] preparation (IU g^{-1})
a_p	specific surface per unit volume for the biocatalyst ($=6/d_p, \text{m}^{-1}$)
a_s	overall biocatalyst surface per unit volume of liquid phase ($=a_p c_{Bw}/\rho_B, \text{m}^{-1}$)
Bi	Biot number ($=k_L R/D_{Se}$)
BSA	bovine serum albumin
BSAE	bovine serum albumin equivalent;
c_B	concentration of biocatalyst (kg m^{-3})
c_{Bw}	wet biocatalyst concentration (kg m^{-3})
c_{E0}	enzyme concentration in the immobilising solution (g GAE m^{-3})
c_{Ef}	enzyme concentrations in the filtrate and washing solutions (g GAE m^{-3})
c_N	concentration of Nagapsin [®] preparation in the immobilising solution (kg m^{-3})

C_{P0}	protein concentration in the immobilising solution (g BSAE m ⁻³)
C_{Pf}	protein concentrations in the filtrate solutions (g BSAE m ⁻³)
D	impeller diameter (m)
d_p	average bead diameter (m)
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	ethanol volumetric fraction (% v/v)
E_0	initial enzyme concentration (g m ⁻³)
EC	ethyl carbamate
E_f	free enzyme concentration (g m ⁻³)
E_f	concentration of free enzymatic protein dissolved in the liquid bulk (g BSAE m ⁻³)
E_i	concentration of immobilized enzymatic protein dispersed in the liquid bulk (g BSAE m ⁻³)
f	referred to free enzyme
GA	glutaraldehyde
GAE	gallic acid equivalent
Gr	Grashof number $\left[\frac{d_B^3 \rho_L (\rho_B - \rho_L) g}{\mu_L^2} \right]$
i	referred to immobilized enzyme
IU	International unit
j	generic index used to refer to free (f) or immobilised (i) enzyme
K	concentration of potassium metabisulphite (kg m ⁻³)
K_A	product inhibitory constants
$KAB4$	sodium acetate buffer (pH 4.0)
$KAB5$	sodium acetate buffer (pH 5.0)
k_{aj}	urea degradation apparent pseudo-first order kinetic rate constant for free or immobilised enzyme ($= \Omega k_{ij}'$; h ⁻¹)
k_I	pseudo-first order kinetic rate constant for ammonia (m ³ g ⁻¹ min ⁻¹)
k_{Ie}	effective kinetic rate constant, as defined by Eq.(3.20) (m ³ g ⁻¹ min ⁻¹)
k_{If}	pseudo-first order kinetic rate constant relative to free enzyme (h ⁻¹)

k_{ij}	urea degradation pseudo-first order kinetic rate constant for free or immobilised enzyme (h^{-1})
$k_{ij,p}$	urea degradation pseudo-first order kinetic rate constant for free or immobilised enzyme at a given concentration of phenolics (h^{-1})
k'	ammonia formation kinetic rate constant (IU mg^{-1})
k'_{if}	specific pseudo-first order kinetic rate constant relative to the free enzyme ($\text{m}^3 \text{g}^{-1} \text{h}^{-1}$)
k'_{ii}	specific pseudo-first order kinetic rate constant relative to immobilised enzyme [$\text{m}^3 (\text{kg enzyme})^{-1} \text{h}^{-1}$]
k'_{ij}	specific urea degradation pseudo-first order kinetic rate constant for free or immobilised enzyme ($\text{m}^3 \text{h}^{-1} \text{g}^{-1} \text{BSAE}$)
k_L	mass transfer coefficient in the liquid phase (m s^{-1})
K_M	Michaelis-Menten constant (mol m^{-3})
K'_M	saturation constant (mol m^{-3})
K_P	phenolic compound inhibition constant (g GAE m^{-3})
KPB7	potassium phosphate buffer (pH 7.0)
K_{Pf}	equilibrium constant of the inhibitory reaction between free enzyme and total phenolics (g GAE m^{-3})
K_S	substrate inhibitory constant
L	concentration of lactic acid (kg m^{-3})
M	concentration of malic acid (kg m^{-3})
m_{Bd}	mass of dry carrier (g)
m_{Bw}	mass of wet carrier (g)
m_{Eb}	amount of bound enzyme (mg BSA equiv.)
m_i	overall number of experiments as defined in Table 3.1.
m_{p0}	amount of protein in the immobilising solution (mg BSAE)
m_{pb}	amount of bound protein, as defined by Eq. (4.4) (mg BSAE)
ms	mass of wet support (mg)
N	stirring rate (rev. min^{-1})
n_j	overall number of trials
P	concentration of total phenolic compounds (g m^{-3} gallic acid equivalent, GAE)

PAN	polyacrylonitrile
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
R	average bead radius (m)
r	enzymatic transformation rate ($\text{mol m}^{-3} \text{s}^{-1}$)
r^2	coefficient of determination (dimensionless)
r_A	ammonium formation rate ($\text{mol m}^{-3} \text{min}^{-1}$)
r_{A0}	ammonia formation rate in the immobilising solution ($\text{mmol m}^{-3} \text{min}^{-1}$)
r_{AB}	ammonia formation rate for immobilised enzyme ($\text{mmol m}^{-3} \text{min}^{-1}$)
r_{Af}	ammonia formation rate in the filtrate solution ($\text{mmol m}^{-3} \text{min}^{-1}$)
$r_{\max}$	maximum rate of an enzymatic transformation ($\text{mol m}^{-3} \text{s}^{-1}$)
Re	Reynolds number ($[\rho_L n D^2/\mu_L]$)
r_S	urea degradation rate ($\text{mol h}^{-1} \text{m}^{-3}$; $\text{mol m}^{-3} \text{min}^{-1}$)
r_{Si}	urea degradation rate for immobilized enzyme referred to the unit volume of biocatalyst ($\text{mol m}^{-3} \text{h}^{-1}$)
S	substrate concentration (mol m^{-3})
Sc	Schmidt number $[=\mu_L/(\rho_L D_S)]$
Sh	Sherwood number $(=d_p k_L/D_S)$
s_j^2	generic variance
S_L	urea concentration in the liquid bulk (mol m^{-3})
S_{L0}	initial concentration of urea (mol m^{-3})
S_R	urea concentration at the biocatalyst surface (mol m^{-3})
SRM	standard reaction mixture
T	reaction temperature ($^{\circ}\text{C}$)
TA	concentration of tartaric acid (kg m^{-3})
t	reaction time (h; min)
t_I	immobilisation time (h)
t_S	storage time (day)
V_0	volume of the immobilising solutions (cm^3)
V_{E0}	immobilising solution volume (cm^3)
V_f	volume of the filtrate and washing solutions (cm^3)

V_L	liquid volume (m^3)
V_S	support soaking volume (cm^3)
v_{max}	maximum value urea degradation rate ($mol\ h^{-1}\ m^{-3}$)
v_S	overall biocatalyst volume the per unit volume of liquid phase ($= c_{Bw}/\rho_B$, dimensionless)
x_{Bw}	water fraction of wet biocatalyst ($g\ g^{-1}$)
X_i	generic principal axis of Eq.s.(3.15) and (5.3), as defined by Eq.s (3.16)- (3.19) and (5.4)-(5.7)
x_i	generic coded independent variables, as defined by Eq.s (3.14) and (5.8)
y_E	ethanol volumetric fraction (% v/v)
$Y_{E/B}$	enzyme loading (g bound enzyme/g dry support)
$Y_{GA/CHI}$	ratio between GA and chitosan bead masses ($g\ g^{-1}$)
$Y_{P/B}$	protein loading as defined by Eq. (4.5) (g bound protein g^{-1} wet support)
$Y_{P/S}$	protein added per unit mass of wet support (mg BSAE g^{-1} wet support)

Greek Symbols

Φ	Thiele modulus for pseudo-first order kinetics, as defined in Table 3.7 (dimensionless)
Ω	effectiveness factor for a spherical biocatalyst in the presence of the external film transport resistance, as defined in Table 3.7 (dimensionless)
δ_j	degrees of freedom
η_L	liquid viscosity (Pa s)
η	effectiveness factor for a spherical biocatalyst in the absence of the external film transport resistance, as defined in Table 3.7 (dimensionless)
μ_L	liquid viscosity (Pa s)
ν_L	liquid kinematic viscosity ($=\mu_L/\rho_L$, $m^2\ s^{-1}$)
π_A	specific enzyme activity ($=r_A/E_0$, $U\ mg^{-1}$)
θ	biocatalyst porosity (dimensionless)
ρ_B	biocatalyst density ($kg\ wet\ support\ m^{-3}$; $kg\ m^{-3}$)
ρ_L	liquid density ($kg\ m^{-3}$)

τ	biocatalyst tortuosity (dimensionless)
ζ_A	enzyme activity coupling yield, as defined by Eq. (4.12) (dimensionless)
ζ_E	enzyme coupling yield, as defined by Eq. (4.11) (dimensionless)

Subscripts

m	referred to model wines
---	-------------------------

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 (Schlatter *et al.*, 1990; Zimmerli *et al.*, 1991), EC levels in food products are to be greatly reduced. In particular, to minimise EC levels in wine the Food and Drug Administration of the USA 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 (Butzke *et al.*, 1997).

The feasibility of acid urease application for the removal of urea from several type wines has been extensively demonstrated (Bertrand, 2003; Fujinawa *et al.*, 1990; Fujinawa *et al.*, 1992 ; Kodama *et al.*, 1996; Ough *et al.*, 1988).

However, the effectiveness of this treatment differed depending on the type of wine, content of some inhibiting factors [i.e., in order of importance, fluoride, malate, ethanol, and phenolic compounds (Famuyiwa *et al.*, 1991; Kodama, 1996; Trioli *et al.*, 1989)], and usage conditions.

In previous experimentation (Fidaleo *et al.*, 2006) the effects and interactions of the concentrations of malic (M) and lactic (L) acids, and potassium metabisulphite (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 2nd-order polynomial reduced to its canonical form so as to identify the only statistically significant principal axes.

The kinetics of urea hydrolysis was described by a modified Michaelis-Menten reaction rate expression, incorporating pH dependent kinetics, substrate inhibition, and noncompetitive product inhibition by NH_4^+ (Moynihan *et al.*, 1989). In model and real wines for urea concentrations (S_L) less than 1 mol m^{-3} , acid urease kinetics was safely regarded of the pseudo-first order with respect to S_L (Esti *et al.*, 2007; Fidaleo *et al.*, 2006). Moreover, in model wine solutions the pseudo-first order kinetic rate constant was found to be largely greater (Fidaleo *et al.*, 2006) than that pertaining to some real wines, probably because of the competitive inhibition exerted by phenolic compounds (Esti *et al.*, 2007; Kodama, 1996; Trioli *et al.*, 1989).

Immobilisation 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 batch-wise hydrolysis), improved stability or resistance to shear or inhibitory compound inactivation. Moreover, in accordance with a recent extensive review about the immobilisation techniques and support materials of ureases (Krajewska, 2009b), the choice of the support material has to be strictly correlated with each specific application. In the food, pharmaceutical, medical and agricultural sectors, the support material has to be not only inexpensive, highly affine to proteins, mechanically stable, regenerable and easily preparable in different geometrical configurations, but what is more non-toxic, biocompatible and, thanks to the growing public health and environmental awareness, biodegradable.

Acid urease has been so far immobilized on various matrices, such as polyacrylonitrile (PAN) and chitosan derivatives (Matsumoto, 1993; Zhou *et al.*, 2008), nylon beads, sepharose and silica gels, or gelatine film coated on cellulose acetate membranes (Selvamurugan *et al.*, 2007). Continuous urea removal from sake by immobilised acid urease on PAN has been applied by many companies in Japan since 1988 (Matsumoto, 1993).

Among the well known commercial carriers currently used for enzyme immobilization, it is worth citing Eupergit[®] C 250L (Katchalski-Kazir *et al.*, 2000) and chitosan-derivative beads (their trade name being *Chitopearl*).

The former is an epoxy-(oxirane) activated macroporous support, that has been used to immobilize covalently a wide variety of different enzymes for industrial applications, except acid urease, because of its ability to stabilize protein conformation by multi-point attachment (Boller *et al.*, 2002; Katchalski-Kazir *et al.*, 2000; Mateo *et al.*, 2000).

On the contrary, the Chitopearls have an average particle diameter of 0.1 to 3.0 mm, a very narrow pore size distribution with pore diameters of 0.1-0.2 μm , this resulting in a good substrate diffusion, and a specific surface area of 20 to 200 $\text{m}^2 \text{g}^{-1}$ of wet carrier. Moreover, these beads are strong enough to overcome the compression stress exerted by a column bed, sterilizable, and safe for medical applications. To improve their acid resistance, as well as pH and thermal stability, the basic material [i.e., chitosan, that is a polysaccharide mainly made up of 2-amino-2-deoxy-*D*-glucose units, which are joined by β -(1,4)-linkages] is cross-linked with primary, secondary or quaternary amine groups

connected to the carbohydrate backbone via a six-carbon linear alkyl spacer. This spacer acts as a chain linking the enzyme with the carbohydrate backbone, thus allowing the enzyme itself to protrude into the solution and act to some extent as a free one (Chen *et al.*, 1999). These chitosan-derivative beads have been applied to immobilise not only a few ureases from *Lactobacillus fermentum* (Matsumoto, 1993), jack beans (Chen *et al.*, 1999), soybean (Kumar *et al.*, 2009), or pigeonpea (Kayastha *et al.*, 2001), but also several other enzymes, such as *L*-arabinose isomerase (Lim *et al.*, 2008), chitosanolytic enzyme from *Enterobacter* sp. G-1 (Yamasaki *et al.*, 1992), cycloisomaltooligosaccharide glucanotransferase (Kawamoto *et al.*, 2001), ferulic acid esterase (Matsuo *et al.*, 2008), β -galactosidase (Shin *et al.*, 1998), glucoamylase (Dhar *et al.*, 1993), *L*-lactate oxidase (Hasebe *et al.*, 1991), levan fructotransferase (Lim *et al.*, 2001), maltotetraose-forming amylase and pullulanase (Kimura *et al.*, 1990), rhamnose isomerase (Bhuiyan *et al.*, 1998; Morimoto *et al.*, 2006), proteases (Kise *et al.*, 1991), and D-tagatose 3-epimerase (Itoh *et al.*, 1995; Takeshita *et al.*, 2000).

Enzyme immobilisation on Eupergit® supports is generally carried out in accordance with the conventional method, that involves the direct enzyme binding on polymers via oxirane groups (Knezevic *et al.*, 2006); whereas that on chitosan-derivative beads is performed by using two different immobilisation procedures, that is *adsorption cross-linkage* and *covalent binding* (Matsumoto, 1993). In particular, the former gave rise to immobilised enzymes with specific activities of 148-170 IU g⁻¹ of wet carrier, whereas the latter yielded biocatalysts with lower specific activities (60-90 IU g⁻¹). Nevertheless, the covalent binding method is generally regarded as the method of choice (Chen *et al.*, 1999; Kayastha *et al.*, 2001).

Another controversial aspect is the titre of the support-activating reagent solution. Matsumoto (1993), as well as Kayastha and Srivastava (2001), activated the chitosan beads using a buffer containing 3% (w/v) glutaraldehyde (GA); whereas Chen and Chiu (1999) treated 0.4 g of chitosan beads with 20 ml of an aqueous solution at 25% (w/v) GA, this involving quite a great GA-to-chitosan bead ratio ($Y_{GA/CHI}$) of 12.5 g g⁻¹.

The main aims of this PhD thesis were the following:

- 1) immobilise acid urease onto Eupergit® C 250L using the conventional method (Knezevic *et al.*, 2006) and assess the specific activity (A_{Bi}) and protein loading

($Y_{P/B}$) per unit mass of wet or dry support, as well as the activity (ζ_A) and enzyme (ζ_E) coupling yields.

- 2) immobilise acid urease onto chitosan-derivative beads of different sizes (Chitopearl BCW-3001, BCW-3003, BCW-3010 and HP-3020) using the above two immobilisation procedures; establish the best immobilisation procedure and optimal $Y_{GA/CHI}$ value on Chitopearls BCW-3001; and finally assess the effect of particle size on the specific immobilised enzyme activity and protein loading when using the optimal conditions previously identified.
- 3) 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 (Fidaleo *et al.*, 2006), 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 250L or glutaraldehyde-cross-linked chitosan derivative beads.
- 4) compare the catalytic performance of acid urease immobilised on three 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.

Chapter 1

Ethyl carbamate

1.1 Introduction

Ethyl carbamate (EC, urethane) is a naturally occurring component of all fermented foods and beverages, such as yogurt, beer, wine and many alcoholic beverages produced from fruits or other vegetable matrices. Moreover, EC was found in cheese, tea and soy sauce (Ough, 1976). Owing to the carcinogenicity of EC when administered in high doses in animal tests, the wine industry is interested to reduce EC levels in their products (Butzke *et al.*, 1997).

It was demonstrated that EC led to liver and lung tumours in mice tests (Schmähl *et al.*, 1977).

Keeping the food products, also the wine, into hygienic/healthy parameters safety ranges is very important to products marketing

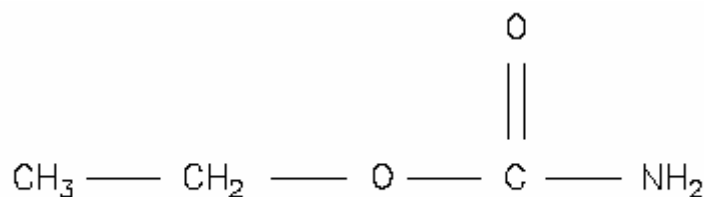
1.2 Ethyl Carbamate: properties and uses

Number: CAS 51-79-6

Molecular formula: $C_3H_7NO_2$

Molecular Weight: 89,09

Synonyms: Urethane, Ethyl Carbamate, Ethyl Urethane, Carbamic Acid, Ethyl Estere.



Urethane may occur as a colorless, odorless columnar crystals or a white, granular powder. It is slightly soluble in olive oil and soluble in water, ether, glycerol, and chloroform. When heated to decomposition, it emits toxic fumes of nitrogen oxides. In the United States urethane is available as a National Formulary (NF) grade fused solid or crystal, that contain 99.43% active ingredient.

In the past urethane has been produced commercially in the United States for over 30 years. It has been used as an antineoplastic agent and for other medicinal purposes. It

had been quite used in the treatment of multiple myeloma before it was found to be toxic and largely ineffective (Holland *et al.*, 1966).

Thus, by US FDA regulation urethane has been withdrawn from pharmaceutical use. The primary use of urethane has been as a chemical intermediate in preparation of amino resins. The process involves a reaction with formaldehyde to give hydroxymethyl derivatives, that are used as crosslinking agents in permanent press textile treatments designed to impart wash-and-wear properties to fabrics. Urethane is also utilized as a solubilizer and co-solvent in the manufacture of pesticides, fumigants, and cosmetics, as an intermediate for pharmaceuticals, and in biochemical research (Virginia Commonwealth University, Office of Environmental Health and Safety, 2006). Urethane was formerly used as an active ingredient in drugs prescribed for the treatment of neoplastic diseases, a sclerosing solution for varicose veins, a hypnotic, and a topical bactericide. In addition, two anticonvulsant drugs, trimethadione and paramethadione, can be contaminated with urethane. The allowable limit was set at 1 ppm. These anticonvulsant drugs may be used only to treat epilepsy that is refractory to other available drugs. It is also used in veterinary medicine as an anesthetic (Virginia Commonwealth University, Office of Environmental Health and Safety, 2006).

1.3 Potential ethyl carbamate carcinogenicity

Urethane is reasonably anticipated to be a human carcinogen, based on sufficient evidence of carcinogenicity in experimental animals. When administered in the drinking water, urethane induced lung adenomas, lymphomas (mainly lymphosarcomas), liver angiomas and hemangiomas, papillomas, sebaceous carcinomas of the skin, harderian gland tumors, pulmonary adenomas, squamous cell tumors, mammary carcinomas, and malignant mesenchymal tumors of the fat pad in mice of both sexes; malignant lymphomas, hemangiomas or hemangiosarcomas of the liver, spleen, or uterus, hepatomas, adrenal cortex adenomas, and fibrosarcomas of the mesentery or uterus in female rats; and papillomas, squamous cell carcinomas of the forestomach, malignant lymphomas, mammary tumors, hepatomas, hemangiomas or hemangiosarcomas of the liver, melanotic skin tumors, pulmonary adenomatosis, and adenomatous polyps of the cecum in hamsters of both sexes (Virginia Commonwealth University, Office of Environmental Health and Safety, 2006).

When administered by gavage in water followed by topical applications of croton oil, urethane induced an increase in skin papillomas and lung adenomas in mice of both sexes. When administered by gavage in dioctyl ester of sodium sulfosuccinic acid, urethane induced lymphocytic leukemias, stomach papillomas, hepatomas, and pulmonary adenomas in male mice. When applied topically followed by applications of croton oil solution in acetone or when administered in conjunction with dimethylbenz[a]anthracene, urethane produced skin tumors in mice of both sexes. When administered by inhalation in aerosol sprays (pressure and ultrasonic), urethane induced adenomas and solid squamous tumors in mice of both sexes. When injected subcutaneously postpartum in a gelatin suspension, urethane induced both thymic and malignant lymphomas and pulmonary adenomas in newborn mice of both sexes. When injected subcutaneously, urethane induced leukemia in mice in both sexes. A continuation of these studies also revealed the influence of age as a factor in the susceptibility of the liver to urethane carcinogenesis; hepatomas occurred rapidly among all groups treated neonatally with urethane. When injected subcutaneously, urethane induced melanotic lesions of the iris in newborn rats of both sexes, and adrenal cortical tumors, β -cell tumors of the pancreatic islet cells, forestomach papillomas, lung and intestinal tumors, dermal melanocytomas, and thyroid tumors in newborn hamsters of both sexes. When injected intraperitoneally, urethane induced adenomas in female mice; increased incidences of mammary tumors, Zymbal gland carcinomas, angiomas and sarcomas at various sites, malignant lymphomas, kidney tumors, and epidermal cysts in rats of both sexes; increased incidences of leukemia in male and female newborn mice; malignant lymphomas, lung adenomas, harderian gland tumors, and stromal and epithelial ovarian tumors in rats of both sexes; neoplasms, embryonal kidney tumors, harderian gland adenomas, and Anitschkow cell sarcomas of the heart in both newborn and adult rats of both sexes; and melanotic tumors in male and female newborn hamsters. When injected intraperitoneally in distilled water, urethane induced an increased incidence of hepatomas in partially hepatectomized and nonhepatectomized male mice. When injected intraperitoneally in sodium chloride followed by a second injection of butylated hydroxytoluene (BHT) in corn oil, urethane induced increased incidences of alveolar and papillary tumors of the lung in neonatal mice of both sexes (Beer *et al.*, 1985). When injected intraperitoneally or intravenously to female mice

before parturition, urethane induced an increased incidence of lung tumors in both sexes of offspring. Subcutaneous injections of urethane to pregnant female mice resulted in enhanced development of hepatomas, ovarian tumors, and pulmonary adenomas in the mothers. When injected intraperitoneally to pregnant female rats, urethane induced hepatomas and sarcomas of the heart in the offspring. When injected subcutaneously, urethane induced pulmonary adenomas in suckling offspring of mice of both sexes. Urethane also enhanced the leukemogenic effect of X irradiation in mice. X-irradiation combined with administration of urethane led to the induction of mammary carcinomas in mice. No adequate human studies of the relationship between exposure to urethane and human cancer have been reported (Virginia Commonwealth University, Office of Environmental Health and Safety, 2006).

In Table 1.1 are summarised the main studies on the carcinogenicity activity of CE, while Table 1.2 shows the tumour incidence on rats and mice tested with different CE doses (Schmähl *et al.* 1977).

Table 1.1 *Studies on ethyl carbamate carcinogenicity (Schmähl et al., 1977).*

Authors	Species	Exposition characteristics	Results	Notes
Otto and Plötz (1966)	mouse/3 strains BLH NMRI C57 BL	inhalation, aerosol concentrations: 0.5, 10, 15, 20% Xpo: 20-60 min/day days/week, 3.5 - 14.5 weeks	at 20% an increase in lung tumours (adenomas and solid tumours of a squamous type) was observed	duration of the exposure was less than one-fourth the standard lifespan
Toth <i>et al.</i> (1961)	mouse, Swiss albino 100/sex/ group	p.o. drinking water concentration: 0, 0.4% Xpo: two 10-day treatment periods Xpe: 42 weeks	treatment group: lymphomas (mainly lymphosarcomas) 15/100 male, 28/100 female; papillomas 3/100 male, 2/100 female. control group: lymphomas 4/100 male, 16/100 female	duration of the exposure was less than one-fourth the standard lifespan
Della Porta <i>et al.</i> (1963)	mouse, CTM albino 100/sex/ group	p.o. drinking water concentration: 0, 0.4% Xpo: varying between 5 and 35 days Xpe: 60-80 weeks	two 10-day treatments: lung adenomas 80-84%; lymphosarcomas 33% male, 27% female. control group: lung adenomas 2-7%; lymphosarcomas 4.5% male, 5% female	duration of the exposure was less than one-fourth the standard lifespan

Tannenbaum & Maltoni (1962)	mouse, C3H, 36 female	p.o. drinking water concentration: 0, 0.1% Xpo: 13 weeks Xpe: 76 weeks	treatment group: pulmonary adenomas 15/32; leukaemia 8/36. control group: pulmonary adenomas 2/36	duration of the exposure was less than one-fourth the standard lifespan.
Tannenbaum & Maltoni (1962)	mouse, DBA 54-62/sex/ group	p.o. drinking water concentration: 0, 0.1% Xpo: 31 weeks Xpe: 45 weeks	treatment group: pulmonary adenomatosis 20/54 male, 20/52 female; pulmonary adenomas 10/54 male, 11/52 female; squamous-cell tumours 11/54 male, 6/52 female. control group: pulmonary adenomatosis 1/59 male, 2/56 female	animals were 53-week old at the start of the treatment
Por76 Schm77 Schl90	mouse, NMRI	p.o. drinking water dose levels Xpo = Xpeb: 0, 0.1, 0.5, 2.5, 12.5 mg/kg bw/day	the number of animals (both sexes combined) with a malignant tumour were 6/74 (control), 11/65 (0.1 mg), 17/69 (0.5 mg), 20/59 (2.5 mg) and 32/65 (12.5 mg). difference statistically significant at 0.5 mg/kg bw/day and higher	$I = 3.5 \times 10^{-1}$ per mg/kg bw/day (TBA males + females)
Por76 Schm77 Schl90	rat, Sprague - Dawley	p.o. drinking water dose levels: 0, 0.1, 0.5, 2.5, 12.5 mg/kg bw/day Xpo = Xpec	the number of females with a malignant tumour were 2/36 (control), 1/37 (0.1 mg), 2/34 (0.5 mg), 6/39 (2.5 mg) and 12/38 (12.5 mg)	$I = 8 \times 10^{-2}$ per mg/kg bw/day (females TBA). for details see Table 2
Adenis et al. (1968)	rat, Sprague Dawley, 50 female	p.o. drinking water concentration: 0.1% Xpo = Xpe = lifespan	33/40 developed tumours (7 malignant lymphomas, 11 haemangiomas or haemangiosarcomas of the liver, spleen or uterus, 7 hepatomas, 10 adrenal cortex adenomas and 4 fibrosarcomas of the mesentery uterus)	there was no control group
Toth <i>et al.</i> (1961), Toth & Boreisha 1969	hamster, Syrian golden	p.o. drinking water concentration: 0, 0.2, 0.4% Xpo: 20 weeks 0.2% followed by 22 weeks 0.4% total 42 weeks. Xpe: 80 weeks	tumours were found in 22/27 male and 21/25 female survivors, compared with 9/54 in male and 3/47 female controls. tumours included melanotic tumours of the skin, papillomas and squamous-cell carcinomas of the forestomach,	

			malignant lymphomas, mammary tumours, hepatomas, haemangiomas, haemangiosarcomas, pulmonary adenomatosis and adenomatous polyps of the caecum. very few of these tumours occurred in 13 tumour-bearing controls	
Pietra & Shubik (1960)	hamster, Syrian golden	p.o. drinking water concentration: 0.2% Xpo = Xpe = lifespan	melanotic tumours of the skin in 8/20 animals, compared to 1/63 in controls	
Ina91	B6C3F1 mice 50 males/ group	p.o. drinking water, concentrations: 0, 0.6, 3, 6, 60, 600 ppm (0, 0.095, 0.58, 1.0, 10, 100 mg/kg bw/day) Xpo = Xpe = 70 weeks	exposure-related tumours were found in liver and lungs. the lung tumour incidence amounted to 9/49, 4/49, 7/48, 8/50, 34/50 (P<0.01), and 42/44 (p<0.01) in the successive exposure groups	1 per mg/kg bw based on lung tumour incidence in the 100 mg group amounted to 1.2×10^{-1} (lifespan exposure conditions)

Table 1.2 Tumors incidence on rats and mice treated with ethyl carbamate in drinking water (Schmähl et al., 1977).

	males				females		
dose of ethyl carbamate (µg/kg body weight/day)	no. of animals evaluated	no. with malignant (benign) Tumours	site of carcinoma or type of other malignant tumour		no. of animals evaluated	no. with malignant (benign) tumours	site of carcinoma or type of other malignant tumour
				<i>Rats</i>			
0	38	0(1)	-		36	2(0)	ovaries (1), fibrosarcoma (1)
100	33	1(0)	lymphoma/leukaemia (1)		37	1(3)	lymphoma/leukaemia
500	31	2(0)	fibrosarcoma (2)		34	2(2)	mammary gland (1), sarcoma (1)
2500	31	1(1)	lymphoma/leukaemia (1)		39	6(4)	mammary gland (2), mixed (4)
12500	36	3(0)	mixed (3)		38	12(8)	mammary gland (9), mixed (3)
				<i>Mice</i>			
0	4	4(0)	sarcoma (3), lymphoma/leukaemia (1)		38	2(2)	lymphoma/leukaemia (2)

100	6	6(2)	lymphoma/leukaemia (3), mixed (3)	33	5(9)	lymphosarcoma (2), mixed (3)
500	3	3(7)	mixed (3)	36	14(5)	mammary gland (2), lung (2), lymphosarcoma (4), lymphoma/leukaemia (4), mixed (3)
2500	10	10(10)	lung (4), blood vessels (2), mixed (4)	31	10(11)	Mammary gland (2), lymphoma/leukaemia (4), lymphosarcoma (3), mixed (3)
12500	14	14(15)	lung (5), lymphoma/leukaemia (3), blood vessels (3), mixed (3)	33	18(15)	mammary gland (6), lung (10), blood vessels (4), mixed (3)

1.4 Ethyl carbamate origin in wines

Ethyl carbamate is spontaneously produced by the reaction between urea and ethanol, as shown in Fig. 1.1.

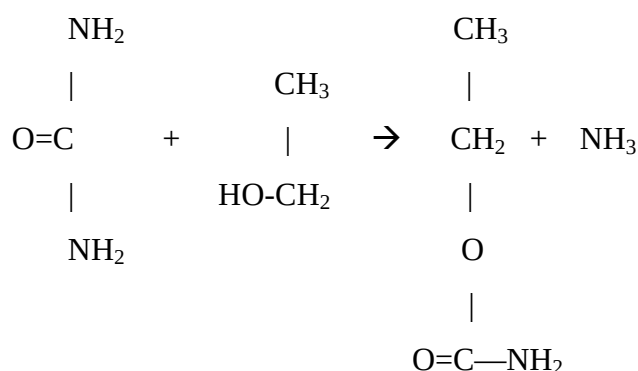


Figure 1.1 Ethyl carbamate formation

This reaction can be of hexogen origin. For example, in some oenological practices adopted in some countries (United States and Canada), urea is used as in grape and musts to favour yeast biomass fermentation and thus to increase ethanol fermentation rate. This kind of practice is no more use for the above toxicological problems, but this is not sufficient to avoid ethyl carbamate presence in wines (Sponholz *et al.*, 1991).

Tables 1.3 and 1.4 show urea contents in some commercial wines.

Table 1.3 Urea contents in (mpp) in some commercial wines.

Kinds of wines	White		Red			Rosé	
Origin	J	USA	UE	J	USA	J	USA
	2	2-2	1 >	1	1	2	1
	1 >	3-4	1 >		2		3
		1 > -64	2		17		
		2-3	1>				
		2-3					

Moreover, Table 1.3 shows the relationship between urea content and geographic area, and Table 1.4 shows the distribution of urea in 67 types of wine of 35 different producers in the States of Washington and California (U.S.A.).

Table 1.4 *Urea contents distribution in 67 types of wines (Fujinawa et al. 1990)*

Urea content						
(g m⁻³)	<1	1-2	2-3	3-4	4-5	>5
59 Table wines	28	9	6	7	4	5
8 Dessert wines	5	1	0	1	0	1

1.5 Ethyl carbamate precursors

In wine, the most important ethyl carbamate precursor is urea, that is produced during arginine metabolism. Arginine is produced by yeast nitrogen metabolism in wine (*Saccharomyces cerevisiae*; Monteiro *et al.*, 1989). Other precursors less important than urea are citrullin, carbammil-phosphate and arginin (Ough *et al.*, 1988). Citrullin and arginine are considered CE direct precursors, because they react directly with ethanol without chemically conversion by yeast enzymes in compound as urea (Tegmo-Larsson and Henik-Kling., 1990), as illustrated in Fig.1.2.

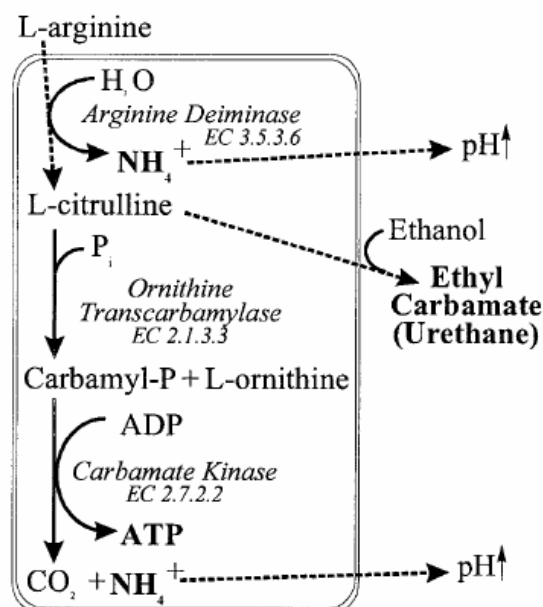


Figure 1.2 Ethyl carbamate formation from arginine and citrulline (De Orduna et al., 2001).

To better understand the possible actions that can be taken to minimize EC level in wine, it is necessary to review the basics of the major formation pathway and kinetics. Arginine, usually one of the most abundant yeast available amino acids in grape juice, is taken up by wine yeast as a nutrient and may be metabolized, yielding urea if present in excess amounts. If the urea cannot be further metabolized and accumulates above a critical concentration, yeast strains release it from their cells into the wine during or at the end of fermentation. Urea can spontaneously reacts with the alcohol in wine to form EC. The chemical reaction between urea and ethanol is exponentially accelerated at elevated temperatures. To a lesser extent citrulline, an amino acid which is not incorporated into yeast protein, and is formed during arginine biosynthesis, as reported in Fig. 1.2, can serve as an EC precursor. Lactic acid bacteria can also be a source of citrulline under winemaking conditions. However, the key reaction for EC formation in wine is between urea and ethanol (Butzke *et al.*, 1997).

Table 1.5 reports the arginine and citrulline contents in some unfermented musts.

Table 1.5 *Potential CE, arginine and citrulline contents in unfermented musts (Tegmo-Larsson and Henik-Kling. 1990).*

Grape variety	CE (mg m⁻³)	arginine (mg m⁻³)	citrulline (mg m⁻³)
Aurore	0.023	66,600	1,600
Catawaba	0.054	90,700	6,600
Chardonnay	0.019	45,500	1,100
Concord	0.043	34,900	<1000
Niagara	0.024	49,800	<1000
Cayuga	0.029	19,800	1,100
Elvira	0.029	34,100	1,000
Ravat	0.042	136,100	1,100
Riesling	0.036	15,700	2,400
Seyval	0.020	26,100	1,100
Vidal	0.022	88,600	2,100
Baco Noir	0.039	113,100	2,200
Cabernet Sauvignon	0.052	47,100	2,100
Chancellor	0.050	84,500	1,000
Pinot Noir	0.038	52,000	1,100

1.6 Ethyl carbamate maximum allowed dosage

Ough (1976) determined ethyl carbamate concentration in several food products. In particular, CE concentration in several wines and beers was found to be lower than 5 µg m⁻³. The lowest CE concentration was assessed in an American beer (60 ng m⁻³), instead the highest one was found in a Japanese sake (0.192 mg m⁻³). The CE concentration was about 90 ng m⁻³ in white bread and 2.4 µg m⁻³ in a sourdough. If bread was treated with N-hydrochloridric acid and warmed, CE concentration increased about 40 time.

1.7 Ethyl Carbammate maximum allowed levels in alcoholic beverages

Tables 1.6, 1.7 and 1.8 list the EC levels assayed in some alcoholic beverages.

Table 1.6 Ethyl carbamate levels (mg m^{-3}) assayed on some commercial wines (BATF 1986-1987) (<http://www.fst.vt.edu/extension/enology/contenttextethylcar>).

Products	Range	Media
Wines produced in U.S.A.	mg m^{-3}	mg m^{-3}
Table Wine	0 -0.102	0.001
Porto	0.007 -0.254	0.093
Sherry	0.018 – 0.209	0.082
Imported Wine	mg m^{-3}	mg m^{-3}
Table Wine	0 – 0.08	0.012
Porto/Madeira	0.017 – 0.108	0.055
Sherry	0.023 – 0.082	0.062

(ATF- Bureau of Alcohol, Tobacco and Firearms, U.S. Department of Justice)

Table 1.7 EC levels (mg m^{-3}) (<http://www.cfsan.fda.gov/~frf/fc0293ur.html>)

Products	ethyl carbamate levels (mg m^{-3})		
	1987	1991	
	Average	National	Imported
Brandy (grape)	0.04	0.01	0.045
Brandy (fruits)	1.2	0.005	0.255
Bourbon (retail)	0.15	0.07	0.055
Rum	0.02	0.002	0.005
Liqueurs	0.1	0.01	0.025
Sherry	0.13	0.01	0.04
Porto	0.06	0.023	0.026
Wine	0.013	0.01	0.015
Sakè	0.3	0.055	0.06

(FDA-ATF; Food and Drug Administration- Bureau of Alcohol, Tobacco and Firearms U.S. Department of Justice)

Despite there are not ethyl carbamate legal limits, yet, for wine (excepted for Canada, 0.03 mg m^{-3}), some International and National Organisms defined the recommended CE levels in some alcoholic beverages.

Table 1.8 Maximum allowed EC levels for the U.S. Food and Drug Administration
(http://www.le.camcom.it/aziende_speciali/multilab.asp)

Products	Levels for EC (mg m ⁻³)
Table Wine	0.03
Quality wines	0.1
Distilled beverages	0.15
Sake	0.4
Liqueurs	0.4

The U.S. Food and Drug Administration informed all Countries exporting wines in USA that the EC levels in all products had be in the range of 0-0.015 mg m⁻³ for base wines and in the range of 0-0.06 mg m⁻³ for alcoholic wines (Kodama, 1996), as stabilised by the American Wine industry (<http://www.fst.vt.edu/extension/enology/contenttextethylcar>).

1.8 Ethyl carbamate preventative action

Because ethyl carbamate has shown potential carcinogenic activity, United States Food and Drug Administration compiled a series of recommendations drawn from scientific research so as to help all winegrape growers, winemakers, and other industry members to minimize EC levels in wine. These recommendations are advisory only, and not intended to restrict the freedom and diversity of winemaking styles (Butzke *et al.*, 1997).

Therefore the following actions were recommended to minimise EC formation in wine.

1.8.1. Viticulture

1.8.1.1 Vineyard fertilization

Nitrogen (N) fertilization in the vineyard has direct influence on the nitrogen contents of the grape berry and the resulting must. Excessive fertilization with urea, ammonia and other N-fertilizers is considered partly responsible for the generally higher EC levels found in wines from traditional wine producing countries (Butzke *et al.*, 1997).

The OIV Expert Committee on Vine Physiology suggested to sample grapevine leaves to determine the nutritional status and provide sufficient, but not excessive, nutrients for vine growth.

There appears to be a growing opinion among viticulturists that nitrate-N in petioles at bloom has little if any relationship to growth, fruit set or general N status of the vine or N status of the berries and resulting juice (Butzke *et al.*, 1997).

In general, grapevines have a very low nitrogen requirement relative to most other crops, as reported in Table 1.9. For example, 10,000 kg of grapes remove only about 11.3 kg of nitrogen from the vineyard.

Growing grapes in soils, previously used for vegetables and thus heavily fertilized, can result in excessive nitrogen contents in grape juice and EC levels in wine.

The concentration of nitrogenous components, such as arginine in grape juice and urea in wine, proportionally increase with increasing nitrogen fertilization in the vineyard. If arginine concentrations in juice exceed 1 kg m^{-3} , the vineyard must be considered over-fertilized.

Table 1.9 *The mainly treatments adopted in vineyards to determine levels of nitrogen.*

Operation	Leaf Blade	Petiole
Timing	- Berry set - Véraison	- Véraison
Minimum number of vines ¹	50-100	100
Position of the leaf on the shoot	Leaf blade opposite the first basal cluster	Petioles opposite the cluster
Position of the shoot on the cordon or cane	Fruitful shoot in the middle	Fruitful shoot in the middle
Minimum number of leaves	50-100 leaf blades	100 petioles
Treatment of the leaves	Wash quickly in distilled water ² and then dry	Wash quickly in distilled water and then dry

¹ In relation to the size of the vineyard.

² Only for the determination of copper, zinc and manganese.

In a nitrogen deficient soil, an application of about $0.011 \text{ kg N m}^{-2}$ would achieve a level of about 150 g of yeast available nitrogen per m^{-3} of juice. This is considered sufficient for completing the fermentation. However, application of such high fertilizer

rates can lead to excessive canopy growth and delayed fruit maturation. Potential nitrogen deficiencies in juices in regard to yeast nutrition should not be addressed through vineyard fertilization (Butzke *et al.*, 1997).

1.8.1.2 Cover Crops

Growers need to be aware that they may be adding a significant amount of nitrogen to their vineyards when they disk under winter legumes used as cover crops in the vineyard. The legumes include vetches (*Vicia ssp.*), clover (*Trifolium ssp.*), and pea (*Pisum ssp.*). Use of these cover crops can increase vine nitrogen to excessively high levels. For example, clover tissue contains about 2.5% nitrogen, while vetch tissue about 4%. Growing vetch can accumulate up to 8.4 g of nitrogen per m², compared to an average of 2.8-5.6 g per m² applied through commercial fertilizer additions. If legumes are used as cover crops, soil and vine nitrogen status should be monitored to avoid excessive arginine levels in grape juices. If additional nitrogen fertilization of the soil is to be avoided, plowing-under of winter legumes should not be practiced (Butzke *et al.*, 1997).

1.8.1.3 Cultivars and Rootstocks

Different grape cultivars exhibit variations in nitrogen uptake, with some varieties being generally lower in level of arginine than others. However, low nitrogen status of cultivars is largely related to own-root characteristics and will change with the use of different rootstocks. Total bloom petiole nitrogen can vary by more than 40% on average, while nitrate-nitrogen may even vary 10-12 fold depending on the rootstock-scion combination used. Rootstocks can therefore have a profound impact on vineyard nitrogen status and fertilizer management. Local viticulture farm advisors can provide information on nitrogen uptake by different rootstocks (Butzke *et al.*, 1997).

Growers should be aware of vine and grape nitrogen status and modify vineyard procedures if juice arginine concentrations are significantly greater than 1 mg m⁻³.

1.8.2. Juice Nutrient: status and additions

Winemakers should determine the nitrogen content to evaluate correctly the nutritional status of an individual grape juice to sustain the yeast metabolic activity. Nitrogen status

of grapes varies widely with vineyard site, soil, irrigation and fertilization practices, vintage weather, scion and rootstock, and grape maturity. The two major sources for nitrogen in must are ammonia and amino acids with the exception of proline. Proline cannot be metabolised by yeast in anaerobiosis.

Both available nitrogen sources can be analyzed in a winery lab or at contract laboratories. Ammonia assays include enzymatic/photometric tests or use of an ammonia probe. Yeast available amino acids may be measured rapidly by a colorimetric OPA/NAC test (Dukes *et al.*, 1996), which requires a spectrophotometer. Analysis by HPLC determines all nitrogen sources simultaneously, but requires highly skilled personnel and yields delayed results under winemaking conditions. It is necessary to obtain analytical results within a few hours after yeast inoculation in order to make decisions about nutrient additions to deficient musts (Butzke *et al.*, 1997).

It would also be desirable to determine critical arginine levels through a simple analytical procedure which can be applied in a winery lab. There is currently no rapid analysis for arginine in juice available to wineries today. Development of a procedure has been proposed by University of California (Davis CU, USA; Butzke *et al.*, 1997).

To avoid sluggish or stuck fermentations, it is permitted by Bureau of Alcohol, Tobacco and Firearms (BATF) to add diammonium phosphate (DAP) up to 960 g m^{-3} to a nitrogen deficient must, which is equivalent to ca. 200 g of nitrogen m^{-3} . However, excessive levels of nitrogen may contribute to urea formation and excretion by yeast. Although some nutrients are required to accomplish an optional malolactic fermentation, high nutrient levels at the end of fermentation can contribute to wine microbial instability.

Yeast food preparations may add an unidentified level of yeast available nitrogen to a juice. It is recommended that winemakers request the supplier to specify the different nitrogen sources (Butzke *et al.*, 1997).

The use of urea as a fermentation supplement is prohibited. BATF has found that the use of urea is not considered acceptable in good commercial practice among wine producers and has rescinded the listing of urea as an authorized treatment.

1.8.3. Yeast Strains

Wine yeast strains differ in their ability to catabolise rapidly urea during fermentation. When excess urea accumulates in the cell cytoplasm, it is released into its environment,

that is the must. High urea producing yeasts are those that have a high capacity to degrade arginine to urea, but a low urea metabolizing ability. Low urea metabolizing ability may result from low activity of urea amidolyase, inhibition of amidolyase activity by the presence of high levels of ammonia, deficiencies of cofactors required by amidolyase, or apparently low activity due to hyperactive arginase. Genetic, as well as environmental factors, influence the amount of urea released by the cells. Some commercial yeast strains, such as Lallemend 71B[®], Red Star SC1120[®] and Premier Cuvée PdM[®] have been described as producing relatively low levels of urea. Yeast companies will be able to recommend their lowest urea excreting strains for each specific winemaking application, and it is suggested that they be consulted (Butzke *et al.*, 1997).

Spontaneous fermentations with undefined yeast strains will necessitate monitoring of arginine in juices and urea and EC levels in every fermentation. It is not clear what impact natural fermentations will have on EC levels, as this has not been thoroughly investigated. However, it is anticipated that indigenous yeast strains will display a similar variability in urea catabolism as observed in commercial strains.

1.8.4. Lactic acid bacteria

Some wine lactic acid bacteria are capable of forming small amounts of citrulline, a precursor of ethyl carbamate, from arginine, and excreting this precursor into the wine. Routine nitrogen supplement of juices with unknown nutritional status can increase the potential available nitrogen for bacteria after primary fermentation. Additionally, even strains unable to degrade arginine may produce small increases in ethyl carbamate, suggesting that nitrogenous precursors other than those derived from arginine may be involved.

Research results indicate the need for caution in the selection of starter cultures for conducting malolactic fermentation in wine, since citrulline formation from arginine degradation could result in high levels of ethyl carbamate, even at normal temperatures, during prolonged storage. In addition, spontaneous malolactic fermentation by undefined strains should be avoided, as this may lead to formation of ethyl carbamate precursors.

If malolactic fermentation is desired, winemakers should either use a commercial strain that does not produce high levels of citrulline or monitor post-fermentation citrulline content in juice (Butzke *et al.*, 1997).

1.8.5. Ureases application

Since urea is the major precursor for EC in wine, enzymatic hydrolysis of urea to ammonia and CO₂ appears to be a suitable way to eliminate formation from this source. Preparations of urease enzyme are commercially available and permitted by BATF for the treatment of wine (Butzke *et al.*, 1997).

However, urease activity is severely limited under normal wine conditions, specifically with respect to low pH and ethanol. Urease is especially inhibited by high concentrations of malic acid, and fluoride residues (from cryolite® application in the vineyard) in excess of 1 g m⁻³. Any combination of these factors can make it practically impossible to reach the desired low urea levels in reasonable time, even at a very high enzyme dosage. A complete elimination of EC is not possible.

If wine is high in residual urea, winemakers may be able to use a urease treatment to reduce urea levels. However, the effectiveness of the urease addition must be evaluated for each wine to confirm the enzyme is active (Butzke *et al.*, 1997).

1.8.6. *Sur Lie* aging

It is a common winemaking style to age wine *sur lie* (on yeast lees) after primary fermentation in order to impact the wine organoleptic properties. Aging on the lees allows nitrogenous compounds, amino acids and protein to be dissolved, into wine: a rapid excretion from the intracellular pool of the yeast cells during the first weeks of storage, and a slow increase during further storage due to autolysis of the yeast.

However, it has been reported that, in wines made from grapes having a low amino acid concentration, after extended lees contact, no increase in ethyl carbamate concentration was found, and that no additional ethyl carbamate precursors were released from the yeast during extended lees contact. Therefore, under the above conditions, the practice of extended yeast lees contact appears not to raise ethyl carbamate potentials.

No data are available to document an influence of *sur lie* aging on urea concentrations of wine made from grapes with excessive concentrations of yeast assimilable nitrogen. Similarly, no data are available regarding the production of sparkling wine and the evolution of levels of urea and other EC precursors during yeast autolysis, e.g., during long-term aging of *tirage*-bottled wines (Butzke *et al.*, 1997). *Sur lie* aging has not been shown to dramatically impact ethyl carbamate levels, but this has not been thoroughly tested (Butzke *et al.*, 1997).

1.8.7. Distillation and fortification

Although urea is not volatile and EC itself possesses a poor volatility, EC may still be found in wine distillates. It can be formed post-distillation via the reaction of a volatile precursor, isocyanate, and ethanol both at ambient and elevated temperatures.

Producers of fruit wine distillates have to be aware of another precursor for ethyl carbamate, in the form of cyanides. Stone-fruits, especially such as cherries, apricots or plums, contain sugar-bound cyanides in their seeds, which can be released during fermentation. Removal of stones prior to fermentation, and a secondary distillation are essential to avoid high concentrations of volatile EC precursors in this type of spirit.

Producers of fortified wines have to take the same considerations into account as table winemakers, since fortification itself may aggravate the problem of urea excretion by yeast. Urea is often formed during the early and middle stages of fermentation with subsequent yeast generations utilizing it during the later stages. Maximum excretion occurs frequently, but with exceptions at about 12 to 16°Brix. Arresting fermentation at this stage will lead to high urea concentrations in the fortified wine. It is recommended to perform a fortification test in the winery lab and to analyze both the fermenting wine and the resulting dessert wine for urea.

In addition, the fortifying grape spirit or brandy can serve as the primary source for EC taint in fortified wines, and should be monitored for EC and potential EC.

Since isocyanate is formed by break-down of urea, the same recommendations for table wine production apply. No recommendation can be given at this point regarding the fractionation of distillates due to lack of data regarding the distillation behavior of volatile EC precursors (Butzke *et al.*, 1997).

1.8.8. Shipment and Storage

The chemical reaction between urea and ethanol increases exponentially with temperature. It is therefore essential that a wine containing elevated levels of urea is not exposed to temperatures above 38°C during storage or shipment.

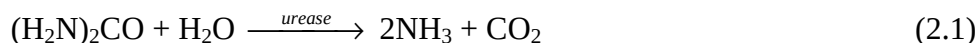
Since long-term exposure of wine to heat is also detrimental to its sensory properties and visual stability, wineries should educate and encourage the shipper, distributor, wholesaler, and retailer to minimize heat exposure by use of appropriate insulated containers, shipping schedules and storage facilities.

Chapter 2

Ureases

2.1 Ureases: function and brief story

Ureases (urea amidohydrolases) are a group of enzymes widespread in nature among plants, bacteria, fungi, algae and invertebrates that, although with different protein structures, exercise a single catalytic function, that is hydrolysis of urea, its final products being ammonia and carbon dioxide (Holm *et al.*, 1997; Krajewska, 2009):



Urea was first discovered in human urine by Hillaire M. Rouelle in 1773, later became the first organic compound synthesized from inorganic materials (Wöhler, 1828). That ammonia in urine derives from hydrolysis of urea was first recognized in 1798 by Fourcroy and Vauquelin, but the first ureolytic microorganism, *Micrococcus ureae*, was isolated from urine much later in 1864 by van Tieghem. By contrast, the first ureolytic enzyme was obtained in 1874 by Musculus from putrid urine, this being named urease by Miquel in 1890.

The discovery of urease in soybean *Glycine max* by Takeuchi in 1909 assured a plentiful source of the enzyme for increasingly intensified investigations (Fearon *et al.*, 1997; Krajewska, 2009).

In 1926 James B. Sumner crystallized urease from jack bean (*Canavalia ensiformis*) showing thus the first time ever that enzymes are proteins and can be crystallized (Krajewska, 2009; Sumner *et al.*, 1926). Dixon *et al.* (1975), discovered the essential presence of nickel ions in the active site (Krajewska, 2009). Since its discovery, urease has been a subject of extensive research that included its occurrence and roles in nature, the mechanisms by which it functions, the specificity of action and reaction to foreign compounds. More recently, the research have included amino acid sequencing, crystal structures of native ureases, their mutants and complexes with inhibitors, and the genetic organization. The knowledge of the molecular basis of the catalytic mechanism of urease is crucial for controlling the processes occurring with the participation of the enzyme.

2.2 Enzyme a real efficient biological catalyst

The enzymes are proteins able to catalyse many transformations related to cellular metabolism. These very efficient biocatalysts, often characterised by high selectivity and specificity, allow metabolic reactions to take place in a faster way also in environmental conditions (i.e. temperature) not so favourable from a catalytic point of view, but suitable to a living organism. In fact, they are able to increase the reaction rate also of 10^6 - 10^{15} times.

Enzymes can be conjugated proteins and sometimes contain a metal. When the metal is extracted from its enzymatic structure, the catalytic activity is immediately lost. In fact, this atom represents a fundamental component of the active site and its ability to interact simultaneously with many chemical substances makes it able to promote the formation of enzyme-metal-substrate complex.

Sometimes, metals can act as a sort of bridge between the protein fraction of an enzyme with a non-protein part.

According to Henry Michaelis, Menten's approach, the enzymatic catalysis would realise according to the following reaction mechanism:



The controlling step is related to the transformation of the enzyme-substrate complex (ES) to give the product (P) and to restore the free enzyme, its rate of decomposition (r) is given by:

$$r = k_2 [\text{ES}] \quad (2.3)$$

As it is practically impossible to collect experimental data regarding the concentration of the activated complex ([ES]) during an enzymatic transformation, and this concentration can be evaluated utilising a mathematical expression which is a function of kinetic constants or concentrations of components easily detectable in the reaction medium.

To individuate this mathematical relation the following hypothesis were assumed:

a) all the concentrations of the species involved in any transformation have an exponential coefficient equal to one (first order);

b) the concentration of the activated complex reaches the stationary state so that the difference occurring between the rates which induce its accumulation minus those related to its consumption, will be always equal to zero:

On these basis the following relation can be written:

$$d[ES]/dt = r_1 - r_{-1} - r_2 = k_1 [E] [S] - k_{-1} [ES] - k_2 [ES] = 0 \quad (2.4)$$

where k_{-1} , and k_2 are first-order kinetic rate constant and k_1 is second order kinetic rate constant, as shown in Eq.(2.2).

By establishing the enzyme mass balance:

$$[E]_{tot} = [E] + [ES] \quad (2.5)$$

it is possible to express the concentration of the free enzyme $[E]$ as the difference occurring between total amount of enzyme present in the reaction medium $[E]_{tot}$ minus that bond to substrate to give the activated complex $[ES]$:

$$[E] = [E]_{tot} - [ES] \quad (2.6)$$

So combining suitably Eq.(2.6) with Eq.(2.5), it is possible to express the concentration of the activated complex as a function of: the concentration of the substrate a specie largely present and easily detectable in the reaction medium; the total amount of enzyme present in the reaction medium; kinetic constants involved in the transformations reported in the scheme 3.1.

$$[ES] = \frac{k_1 [E]_{tot} [S]}{k_1 [S] + (k_{-1} + k_2)} = \frac{[E]_{tot} [S]}{[S] + \frac{k_{-1} + k_2}{k_1}} = \frac{[E]_{tot} [S]}{([S] + K_M)} \quad (2.7)$$

In fact the linear combination of more constant (k_1 , k_{-1} and k_2) yields a new constant K_M (Michaelis constant). So Eq.(2.3) becomes:

$$r = k_2 [ES] = \frac{k_2 [E]_{tot} [S]}{K_M + [S]} \quad \text{Henry Michaelis, Menten's equation} \quad (2.8)$$

When the substrate concentration increases also that of the activated complex (ES) increases to reach an asymptotic maximum value when all the enzyme is bound to substrate. In these y, also the total rate related to the product accumulation reaches its maximum value (r_{max}) as shown by the following expression:

$$\lim_{[S] \rightarrow \infty} r = \lim_{[S] \rightarrow \infty} \frac{k_2 [E]_{tot} [S]}{K_M + [S]} = k_2 [E]_{tot} = r_{max} \quad (2.9)$$

2.3 The enzymatic inhibitors

Sometimes, in the reaction medium may dissolve some compounds able to interact with an enzymatic specie involved in the catalytic cycle.

If the presence of some particular compounds needs to enzymatic conversion to take place and an increase of their concentration positively affects the reaction rate, they are *promoters* or *activators*. On the contrary, if an increase of their concentration induces a reduction of the enzymatic activity, they act as *inhibitors*.

This inhibitory activity can be exerted by two different mechanisms:

a) competitive inhibition. In this case the inhibitor (I) competes directly with substrate to bind the free enzyme (E) to give an enzyme-inhibitor stable complex (EI), which determines a decrease of the amount of the available enzyme (Fig. 2.1a).

b) not competitive inhibition. When the inhibitor interacts with the enzyme-substrate complex (ES) to give a stable and no more active form (ESI). Also in this case a lose of the enzymatic activity is obtained (Fig.2.1b).

As a consequence of their specific activity, they are able to induce an alteration of the mathematical form of Henry Michaelis, Menten's equation.

In the first case (*competitive inhibition*), the overall enzymatic mass balance would be so corrected:

$$[E]_{\text{tot}} = [E] + [ES] + [EI] \quad (2.10)$$

Assuming that inhibitor interacts with free enzyme to produce a stable and not reactive complex [EI] by a transformation which immediately achieves the equilibrium conditions:



The concentration assumed by this complex could be so evaluated:

$$[EI] = \frac{[E][I]}{K_I} \quad (2.12)$$

where K_I is the constant which rules the equilibrium occurring between free enzyme and the inhibitor.

On this basis Eq. (2.10) would become:

$$[E]_{\text{tot}} = [E] + [ES] + [EI] = \frac{[ES] KM}{[S]} + [ES] + \frac{[ES] KM [I]}{K_I [S]} = [ES] \left\{ \frac{KM}{[S]} + 1 + \frac{KM [I]}{K_I [S]} \right\}$$

From which:

$$[ES] = \frac{K_I E_{\text{tot}} [S]}{K_M (I + K_I) + K_I [S]} = \frac{[S][E]_{\text{tot}}}{(K_M(1 + \frac{I}{K_I}) + [S])} \quad (2.13)$$

$$\text{If } \alpha = 1 + \frac{[I]}{K_I} \quad (2.14)$$

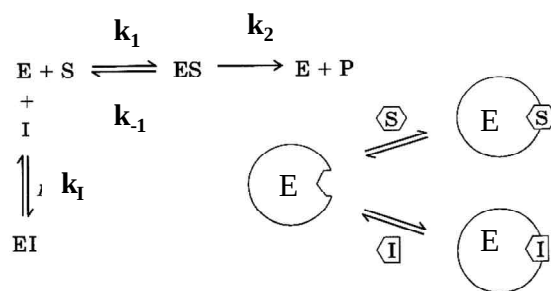
then

$$[ES] = \frac{[E]_{\text{tot}} [S]}{\alpha K_M + [S]} \quad (2.15)$$

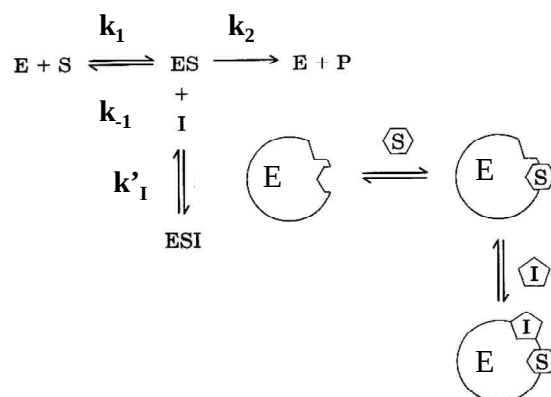
and the Michaelis-Menten equation in presence of a competitive inhibitor would become:

$$r = k_2[ES] = \frac{k_2 [E]_{\text{tot}} [S]}{\alpha K_M + [S]} = \frac{r_{\text{max}} [S]}{\alpha K_M + [S]} \quad (2.16)$$

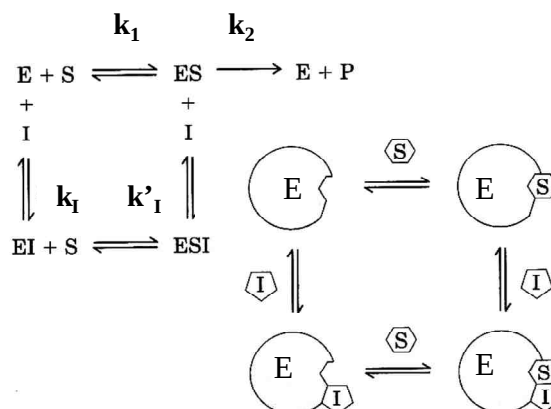
The term (αK_M) represent the so called “apparent” Michelis-Menten constant in the presence of a competitive inhibitor. It can be experimentally determined by varying $[I]$ in the reaction medium.



a) Competitive inhibition



b) Non-competitive inhibition



c) Mixed inhibition

Figure 2.1 Action mechanisms of competitive, non competitive and mixed inhibitors

The effect of a competitive inhibitor can be easily detected by using the well known graphical representations of the Michaelis–Menten equation in the plane ($1/[S]$; $1/r$), such as Lineweaver–Burk and Eadie–Hofstee plots. For example, in the Lineweaver–

Burk plot shown in Fig. 2.2 all the competitive inhibition lines intersect on the y-axis, illustrating that such inhibitors are not able to affect $r_{\max}$.

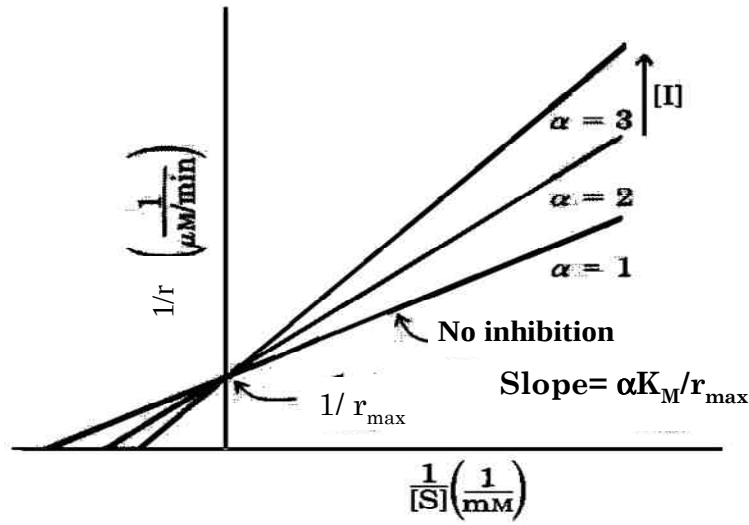


Figure 2.2 Lineweaver–Burk plot in the presence of a competitive inhibitor. The arrow shows the effect of increasing inhibitor concentrations $[I]$.

Other types of reversible inhibition are the non-competitive and mixed ones, this last usually observed in enzymatic reaction involving a single substrate.

In particular, a non-competitive inhibitor (Fig. 3.1b) binds the enzyme in a site different from that used to link the substrate and available only on the ES complex.

In this case the overall enzyme mass balance would be so written:

$$[E]_{\text{tot}} = [E] + [ES] + [ESI] \quad (2.17)$$

By assuming, even in this case, that the complex $[ESI]$ undergoes no other transformation and the inhibition reaction immediately achieves the equilibrium state:



it is possible to calculate the ESI concentration as:

$$[ESI] = \frac{[ES][I]}{K'_I} \quad (2.19)$$

where K'_I is the equilibrium constant.

By continuing to assume that $[ES]$ concentration is stationary, thus allowing to express $[E]$ as a function of $[ES]$ and substituting such equation together with Eq. (2.19) into Eq. (2.17), the following can be obtained:

$$[E]_{\text{tot}} = K_M \frac{[ES]}{[S]} + [ES] + [ES] \frac{[I]}{K_I} = [ES] \left\{ 1 + \frac{K_M}{[S]} + \frac{[I]}{K_I} \right\} = [ES] \left\{ \alpha' + \frac{K_M}{[S]} \right\} \quad (2.20)$$

with

$$\alpha' = 1 + \frac{[I]}{K_I} \quad (2.21)$$

By replacing Eq. (2.20) into Eq. (2.3), it is possible to determine the Michaelis-Menten equation in presence of a non-competitive inhibitor:

$$r = k_2 [ES] = \frac{k_2 [E]_{\text{tot}} [S]}{K_M + \alpha' [S]} = \frac{r_{\text{max}} [S]}{K_M + \alpha' [S]} \quad (2.22)$$

The effect of a non-competitive inhibitor can be easily detected by using the Lineweaver–Burk plot shown in Fig. 2.3, all the competitive inhibition lines having the same slope (K_M/r_{max}) and intercept (α'/r_{max}) increasing when the non-competitive inhibitor concentration.

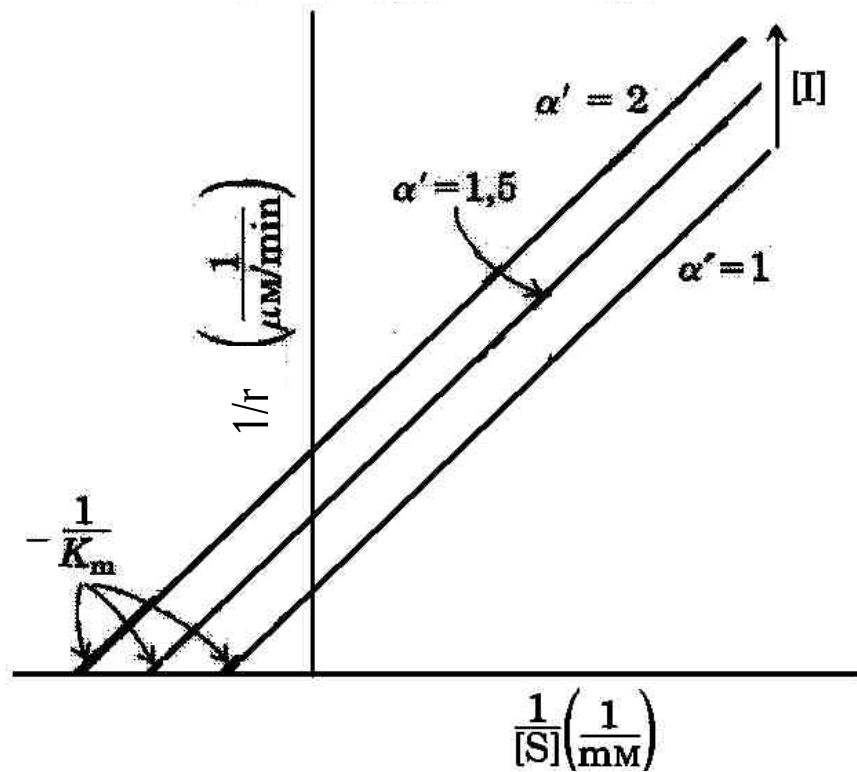


Figure 2.3 Lineweaver–Burk plot in the presence of a non-competitive inhibitor. The arrow shows the effect of increasing inhibitor concentrations $[I]$.

As shown in Fig. 2.1c, a mixed inhibitor is capable to bind either the free enzyme E or the enzyme-substrate complex (ES). In this case, the overall enzyme mass balance can be written as:

$$[E]_{\text{tot}} = [E] + [ES] + [EI] + [ESI] \quad (2.23)$$

By assuming that [EI] and [ESI] complexes undergo no other transformations and both inhibition reaction instantaneously achieve their equilibrium states and ES concentration is stationary, the concentrations of ES, EI, and ESI can be expressed by Eq.s (2.12) and (2.19), respectively. Once these equations were introduced into Eq. (2.23), it is possible to derive the following:

$$[E]_{\text{tot}} = [E] + \frac{[E][S]}{K_M} + \frac{[E][I]}{K_I} + \frac{[E][S]}{K_M} \frac{[I]}{K'_I} = [E] \left(1 + \frac{[I]}{K_I}\right) + \frac{[E][S]}{K_M} \left(1 + \frac{[I]}{K'_I}\right) = \alpha [E] + \alpha' \frac{[E][S]}{K_M} \quad (2.24)$$

By substituting the free enzyme concentration [E], as derived from Eq. (2.24), into Eq. (2.15), it is possible to obtain the enzyme-substrate complex concentration [ES]:

$$[ES] = \frac{[E]_{\text{tot}} [S]}{\alpha K_M + \alpha' [S]} \quad (2.25)$$

and finally, by replacing Eq. (2.25) into Eq. (2.3), to determine the Michaelis-Menten equation in presence of a mixed inhibitor:

$$r = k_2 [ES] = \frac{r_{\text{max}} [S]}{\alpha K_M + \alpha' [S]} \quad (2.26)$$

where α and α' are defined by Eq. (2.14) and (2.21), respectively.

The effect of a mixed inhibitor can be easily detected by using the Lineweaver–Burk plot, where the intercept (α'/r_{max}) and slope ($\alpha K_M/r_{\text{max}}$) vary with the inhibitor concentration [I].

2.4 Ureases Sources

As the key function of ureases is to provide living organisms with ammonia which represents the nitrogen form necessary for their growth, they are enzymes widely diffuse in nature (Krajewska, 2009; Wang *et al.*, 2008).

2.4.1 Ureases in plants

Being able to promote the degradation of urea permeated into plant cells, urease is an essential enzyme in plant (Kojima *et al.*, 2006; Krajewska, 2009; Wang *et al.*, 2008). Although not entirely yet well known, higher plants are characterised by passive or active urea transport systems, which allow them to optimize N-nutrition depending on the nitrogen form available from external environment or internally synthesized. From external environment, plants assimilate urea through roots as urea, but essentially as ammonia generated from urea hydrolysis, and this is possible due to the presence of ureases in soils, a fact exploited in urea fertilization practices.

Importantly, high inputs of urea fertilizers applied may constitute a serious hazard both to plants and the environment. To enhance fertilization practices, urea is also applied through the foliage. Foliar-applied urea, however, can be toxic in high concentrations (Wang *et al.*, 2008). Further knowledge on the mechanisms of urea-related plant nutrition is therefore needed for the development of balanced strategies of urea-fertilization for sustainable agricultural crop production.

Urea derives from arginine and possibly from degradation of purines and ureides (Polacco *et al.*, 1994). The nitrogen present in urea is unavailable to the plant unless hydrolyzed by urease. The product of urease activity, ammonia, is incorporated into organic compounds mainly by glutamine synthetase, as shown in Fig.2.4, (Sirko *et al.*, 2000). Arginase and urease may control the utilization of seed protein reserves during germination (Polacco *et al.*, 1993).

Metabolized rapidly, urea practically does not accumulate. However, constantly generated may serve as an N-source. It has also been hypothesized that due to the generation of ammonia, urease fulfils a defense function against plant pathogens (Polacco *et al.*, 1993). In the same context, recently, ureases insecticidal (Follmer *et al.*, 2004) and antifungal properties (Becker-Ritt *et al.*, 2007; Menegassi *et al.*, 2008) has

been recently provided. This corroborating their function in plant defense system. Present virtually in all plants, urea is especially abundant in leguminous seeds, those of soybean (*Glycine max*) containing 0.012% urease/dry mass and those of jack bean (*Canavalia ensiformis*) 0.07–0.14%, the latter thus being one of the commonest sources of the enzyme (Sumner *et al.*, 1926; Weber *et al.*, 2008).

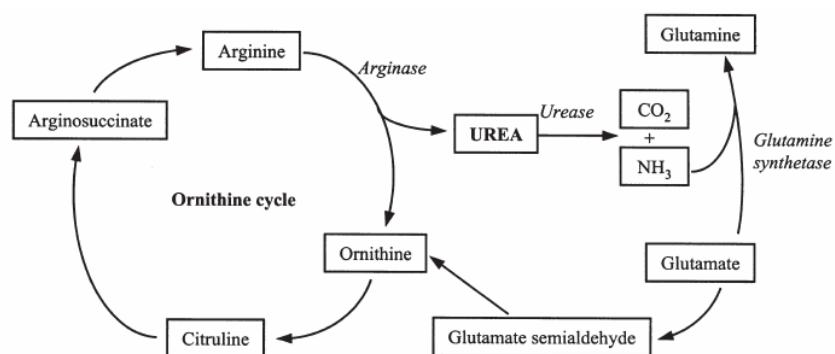


Figure 2.4 Urea metabolism in plants (Sirko *et al.*, 2000)

2.4.2 Ureolytic bacteria pathogenic to humans and animals

Urea is the major metabolic nitrogenous waste product of most terrestrial animals, it is produced in the liver, carried in the bloodstream to the kidneys, and excreted in urine (Burne *et al.*, 2000; Collins *et al.*, 1993).

Additionally, some 20–25% of all urea produced is estimated to remain in the intestinal tract, this renders urea readily available to ureolytic bacteria making the urinary and intestinal tracts the most common sites of ureolytic bacteria infections in humans (Burne *et al.*, 2000; Collins *et al.*, 1993).

Bacterial urease is directly associated with the formation of infection stones and contributes to the pathogenesis of pyelonephritis, ammonia encephalopathy, hepatic coma, urinary catheter encrustation, and peptic ulceration (Mobley *et al.*, 1989). The pathogenic effects arise from urea hydrolysis, that is an increase in pH (up to ca. 9.2) and the toxicity of released ammonia and its derivatives (Burne *et al.*, 2000). This is the reason of the infection stones: the normally soluble polyvalent ions, present in stones, become supersaturated as the pH increases from 6.5 to 9.0. This alkalinisation results in the stone crystallization (Mobley *et al.*, 1989).

One of the most important ureolytic pathogenic bacteria infecting the intestinal tract is *Helicobacter pylori* (Burne *et al.*, 2000; Collins *et al.*, 1993; Dunn *et al.*, 1998; McGee

et al., 2000). *H. pylori* is acid-sensitive and replicates in a pH range of 6.9 to 8.0 (Goodwin *et al.*, 1986; Mobley *et al.*, 1989), yet it survives in the highly acidic environment of the human stomach and it produces high levels of urease activity and consequently of ammonia which protects the bacterium from stomach acid (Brady *et al.*, 1986; Mobley *et al.*, 1989).

2.4.3 Acid ureases

Acid ureases are a distinct subgroup among ureases. Their salient characteristic is that unlike typical neutral ureases, with the optimal activity at pH close to neutral they have the optimal pHs in the range 2–4.5, as shown in Table 2.1.

Acid ureases can be produced by bacteria strains as lactic acid bacteria isolated from milk and dairy products, from sourdoughs, soil (*Arthrobacter mobilis*: Miyagawa *et al.* 1999) or produced in the intestinal tracts (*Lactobacillus*, *Streptococcus*, *Escherichia*, *Morganella* and *Bifidobacterium*) (Kakimoto *et al.*, 1990; Takebe *et al.*, 1988; Yamazaki *et al.*, 1990). To date it is unclear why bacteria growing in the alimentary tract, the pH of which is neutral, produce acid ureases.

These bacteria can be also obtained, from culture collections, belonging to different species of the genera *Enterococcus* (*E. faecalis* strains, *E. faecium*, *E. pseudoavium*), *Lactobacillus* (*L. alimentarius*, *L. brevis*, *L. casei*, *L. coryniformis*, *L. curvatus*, *L. farciminis*, *L. fermentum*, *L. helveticus*, *L. paracasei*, *L. paraplantarum*, *L. pentosus*, *L. plantarum*, *L. rhamnosus*, *L. sakei*, *L. zeae*), *Lactococcus* (*Lc. lactis*), *Leuconostoc* (*Le. mesenteroides*), *Pediococcus* (*P. acidilactici* and *P. pentosaceus*), *Streptococcus* (*S. macedonicus*, *S. thermophilus*, *S. salivarius*), *Weissella* (*W. cibaria*, *W. confusa*, *W. viridescens*; Zotta *et al.*, 2008).

Like neutral ureases, acid ureases are nickel-containing enzymes with related K_M values, but their activities are mostly lower, as reported in Table 2.1. Also, like neutral ureases they are inhibited by acetohydroxamic acid, Hg^{2+} , Cu^{2+} , Ag^+ ions and by p-chloromercuribenzoic acid (Kakimoto *et al.*, 1990; Yamazaki *et al.*, 1990; Fidaleo *et al.*, 2006).

Table 2.1 Kinetic parameters of selected ureases (Krajewska, 2009).

Ureases	K_M^a (mM)	Activity ^a (μ mol urea/min mg)	pH _{opt} ^a	pI
Plants				
<i>Canavalia ensiformis</i> (jack bean)	2.9–3.6	2700–3500	7.0–7.5	5.0–5.1
<i>Glycine max</i> (soybean)	0.2–0.6	650–800	7	–
<i>Cajanus cajan</i> (pigeon pea)	3.0	3120	7.3	–
<i>Gossypium hirsutum</i> (cotton seeds)	0.12–0.15	14.5	8.0	–
Fungi				
<i>Aspergillus nidulans</i>	1.33	670	8.5	–
<i>Aspergillus niger</i>	3.0	1341	8.0	–
<i>Coccidioides immitis</i>	4.1	1750	8.0	5.5
<i>Schizosaccharomyces pombe</i>	1.03	700–800	8.2	–
Bacteria				
<i>Aerobacter aerogenes</i>	2.8	690	7.5	–
<i>Arthobacter oxydans</i>	12.5	219	7.6	4.3–4.7
<i>Bacillus pasteurii</i>	17.3	2500	8.0	4.6
<i>Brevibacterium ammoniagenes</i>	32	3570	7	4.1
<i>Brucella suis</i>	5.60	540	7.0	5
<i>Helicobacter pylori</i>	0.2–0.8	1700	8.0–8.2	5.9; 5.93; 5.99
<i>Klebsiella aerogenes</i>	2.8	2500	7.75	–
<i>Proteus mirabilis</i>	13	2000	7.5	5.2–5.9
<i>Providencia rettgeri</i>	10.5	30.6	7.5	5.1–5.2
<i>Providencia stuartii</i>	9.3	7100	–	5.4
<i>Selenomonas ruminantium</i>	2.2	1100	8.0	–
<i>Staphylococcus leei</i>	1.66	730	–	–
<i>Staphylococcus saprophyticus</i>	9.5	1979	6.0; 7.0	4.7
<i>Staphylococcus xylosus</i>	–	1573	7.2	4–5
<i>Ureaplasma urealyticum</i>	2.5	180,000	6.9–7.5	5.0–5.2; 4.60
Acid ureases				
<i>Streptococcus mitior</i>	2.0	230	4.5	4.6
<i>Lactobacillus fermentum</i>	2.7	220	2	4.8
<i>Lactobacillus reuteri</i>	2.8	290	2	4.7
<i>Arthrobacter mobilis</i>	3.0	2370	4.2	6.8
Algae				
<i>Nitzschia obtusa</i>	4.5	–	–	–
Invertebrates				
Land snail <i>Otala lactea</i>	0.11	0.79	8.4–9.0	–

Although urease production is widespread among prokaryotes (Mobley *et al.*, 1995), with the exception of *S. thermophilus* (Mora *et al.*, 2002; Mora *et al.*, 2004) and *S. salivarius* (Chen *et al.*, 1996; Chen *et al.*, 1996; 1998, 2000) only a few species of lactic acid bacteria have been tested for urease activity and found positive (Kakimoto *et al.*, 1989, 1990a, b; Yamazaki *et al.*, 1990). Acid urease activity has been found in *L. fermentum* (Kakimoto *et al.*, 1990a), *Lactobacillus reuteri* (Kakimoto *et al.*, 1989), and *Streptococcus mitior* (Yamazaki *et al.*, 1990). Urease activity was always intracellular, as for the large majority of urease producing prokaryotes (Mobley *et al.*, 1995; Zotta *et al.*, 2008).

Acid ureases, those from *Lactobacillus* sp. in particular, are now commercially available in soluble and insoluble forms. A commercial acid urease preparations dried, ethanol-permeabilized obtained from *L. fermentum* cells, are currently used for the degradation of urea in sake and wine, their pH values being very low (e.g. pH of sake, 4.4; pH of wine, 3.2; Fidaleo *et al.*, 2006; Kodama, 1996), in order to prevent the accumulation of the potentially carcinogenic ethyl carbamate. To be used for this purpose, urease should be active at the low pH and low urea concentrations and in the presence of inhibitors found in alcoholic beverages (Esti *et al.*, 2007; Fidaleo *et al.*, 2006; Kodama, 1996). An

example of acid urease preparation principally used to remove urea from Japanese sake and wines is Nagapsin®. It is used by dissolving 0.003-0.01 kg of enzymatic preparation in 1-2 kg of sake, its activity is optimal in the pH range 3-4. The enzyme is tolerant to alcohol and is able to hydrolyse effectively the urea to ammonia and CO₂. The effect of pH and temperature is shown in Fig.2.5. An additional requirement may be a low heat tolerance to allow its inactivation by pasteurization (Miyagawa *et al.*, 1999) and the ability to retain sufficient activity at the temperatures used to store wines and alcoholic beverages (Zotta *et al.*, 2008).

Acid urease from *Lactobacillus fermentum* has a molecular weight of 220,000 Dalton, and consists of three kinds of subunits, designated α , β and γ , with molecular weights of 67,000, 16,800 and 8,600, respectively in a ($\alpha_1\beta_2\gamma_1$)₂ structure. The isoelectric point of the enzyme was 4.8. The nickel content was found to be 1.9 atoms of nickel per $\alpha_1\beta_2\gamma_1$ unit. The amino acid profile was different from those of known bacterial neutral ureases. The enzyme was most active at pH 2 and around 65°C. It was stable between pH 3 and 9, and below 50°C. The Michaelis-Menten's constant (K_M) for urea was 2.7 mM at pH 2. The amino terminal amino acid sequences of the subunits α , β and γ were Ser-Phe-Asp-Met-, Met-Val-Pro-Gly- and Met-Arg-Leu-Thr-, respectively (Kakimoto *et al.*, 1990).

Recently, some studies on *S. thermophilus* urease have shown a relatively high residual activity of the enzyme at low pH. Moreover Zotta *et al.*, (2008) showed for the first time that *S. thermophilus* urease exhibited a does have significant residual activity at low pH (3–4) and in the presence of ethanol, this result corroborating its use in wine and other alcoholic beverages.

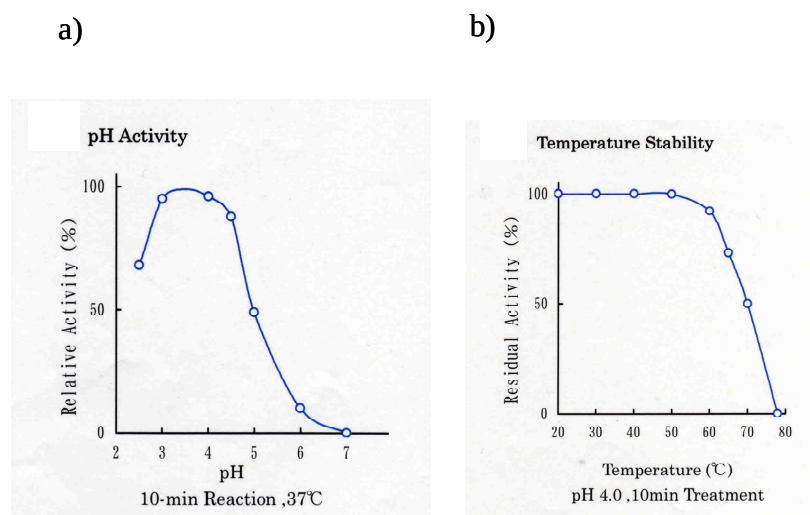


Figure 2.5 Effect on the pH (a) and on temperature (b) on the relative activity of Nagapsin preparation

2.5 Proteic structures of ureases

Plant and fungal ureases are homo-oligomeric proteins of ca. 90 kDa subunits, made up of identical subunits, as shown in Fig.2.6, unlike plant and fungal, bacterial ones are composed of three distinct subunits called UreA, UreB and UreC (Moblely *et al.*, 1995), one large (α , 60–76 kDa) and two small (β , 8–21 kDa and γ , 6–14 kDa), commonly forming $(\alpha\beta\gamma)_3$ trimers as shown in Fig.2.6, resulting in the enzyme molar masses between 190 and 300 kDa (Krajewska, 2009).

By referring to the structures of microbial ureases, it were, both the secondary and tertiary structures, extensively simila despite some differences in the number of chains. The sequence of urease from jack bean (*Canavalia ensiformis*) is closely related to the sequence of all bacterial ureases, this suggesting a common evolutionary origin. *Bacillus pasteurii* and most other bacterial ureases are collinear with the single chain of fungal and plant ureases.

plant urease from jack bean <i>Canavalia ensiformis</i> [84]	N—	90.77 kDa	—C	840 aa
plant urease from soybean <i>Glycine max</i> [82]		93.5 kDa		
plant urease from pigeonpea <i>Cajanus cajan</i> [83]		90 kDa		
plant urease from cotton seeds <i>Gossypium hirsutum</i> [36]		98.3 kDa		
fungus urease from [101] <i>Schizosaccharomyces pombe</i>		91.2 kDa		835 aa
fungus urease from <i>Coccidioides immitis</i> [102]		91.5 kDa		839 aa
bacterial urease from <i>Klebsiella aerogenes</i> [103]	γ 11.1 kDa	β 11.7 kDa	α 60.3 kDa	101/106/567 aa
bacterial urease from <i>Proteus mirabilis</i> [104]	γ 11.0 kDa	β 12.2 kDa	α 61.0 kDa	100/109/567 aa
bacterial urease from <i>Bacillus pasteurii</i> [105]	γ 11.1 kDa	β 14.0 kDa	α 61.4 kDa	101/126/570 aa
bacterial urease from [106] <i>Ureaplasma urealyticum</i>	γ 11.2 kDa	β 13.6 kDa	α 66.6 kDa	102/121/614 aa
bacterial urease from <i>Proteus vulgaris</i> [107]	γ 11.0 kDa	β 12.1 kDa	α 61.0 kDa	100/108/567 aa
bacterial urease from <i>Staphylococcus xylosus</i> [108]	γ 11.0 kDa	β 15.4 kDa	α 61.0 kDa	98/138/571 aa
bacterial urease from <i>Staphylococcus leei</i> [92]	γ 12 kDa	β 21 kDa	α 65 kDa	
bacterial urease from [91] <i>Staphylococcus saprophyticus</i>	γ 13.9 kDa	β 20.4 kDa	α 72.4 kDa	
bacterial urease from <i>Providencia stuartii</i> [109]	γ 9 kDa	β 10 kDa	α 73 kDa	
bacterial urease from <i>Helicobacter pylori</i> [99]		β 26.5 kDa	α 61.0 kDa	238/569 aa

Figure 2.6 Structural subunits of ureases (Krajewska, 2009).

By comparing of the amino acid sequence of the major urease subunits from *Proteus mirabilis*, *P. vulgaris* and *H. pylori*, it was found that they are identical to the sequence of urease from jack bean in over 50% of amino acid residues inspite of the very distant phylogenetic relation between plants and bacteria (Follmer, 2008; Mobley *et al.*, 1989). Moreover, on electron-microscopic images of negatively-stained jack bean urease suggested that the enzyme was composed of two cyclic trimers (Fishbein *et al.*, 1977). Analogous electron-microscopic images of the *H. pylori* enzyme (Austin *et al.*, 1991; Austin *et al.*, 1992; Hawtin *et al.*, 1990) similarly suggested a threefold rotational symmetry, whereas those of ureases from *S. saprophyticus* and *S. xylosus* showed symmetric particles divided in two by a dark-staining line (Schafer *et al.*, 1994). It was postulated that all known ureases are variants of the same enzyme and are likely to

possess the same general three-dimensional structure and catalytic residues at the active site (Mobley *et al.*, 1995).

Several ureases display a highly conserved amino acid sequence in the region of their catalytic site with respect to both critical residues and adjacent amino acids as shown in Fig.2.7 (Follmer, 2008). This conservation of amino acid sequence in the region of catalytic site suggested a similar kinetic mechanism of the several known ureases. Moreover, for many inhibitors the inhibition kinetics of bacterial and plants enzyme were generally similar (Mobley *et al.*, 1995).

The protein is a trimer ($\alpha\beta\gamma$)₃ of trimers, possessing three active sites per enzyme. The amino and carboxy termini for each of the *K. aerogenes* urease subunits are accessible; thus, additional residues, as are found in urease sequences from other microorganisms, can be accommodated without disturbing the structure. In particular, there is adequate room for inclusion of a connecting linker between the UreA and UreB subunits, as is present in the *H. pylori* and jack bean enzymes, as well as between the UreB and UreC subunits, as exists in the jack bean enzyme (Mobley *et al.*, 1995).

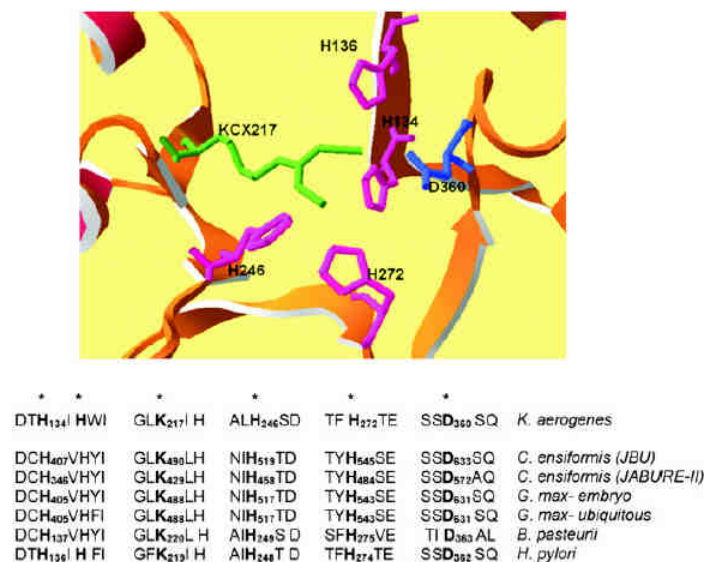


Fig. 2.7 Catalytic site of *Klebsiella aerogenes* urease (Follmer, 2008).

Regarding the structure of urease active sites, all purified ureases analyzed for metal content possessed nickel ions. More specifically, the great part of acid ureases analysed

by atomic absorption spectroscopic measurement, showed the presence of two nickel ions per active site (Mobley *et al.*, 1995).

X-ray absorption spectroscopic analysis of the bacterial urease revealed an edge spectrum that is most consistent with five-coordinate geometry, while extended X-ray absorption fine-structure analysis suggests the presence of two or three histidyl imidazole groups per nickel ion, as shown in Fig.2.8 (Wang *et al.*, 1994). Whereas the spectra indicated that metal ions in native enzyme possess all N or O ligands and Ni-S, as well as Ni-Ni scattering interactions, the Ni-Ni distance was estimated to be 0.326 nm by this technique, as shown in Fig.2.8.

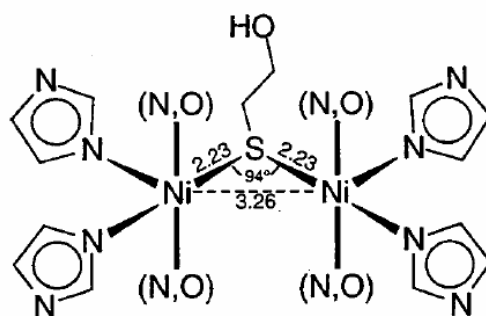


Figure 2.8 Model of the urease bi-Nickel center based on biophysical and spectroscopic analyses (Mobley *et al.*, 1995).

2.6 Urease catalyst properties

2.6.1 Proposed reaction mechanisms for urease-catalyzed urea hydrolysis

The currently accepted view of the mechanism of urease is based on the different roles played by the two Nickel ions: one of them binding and activating urea, the other binding and activating the nucleophile water molecule (Dixon *et al.*, 1980; Jabri *et al.*, 1995; Karplus *et al.*, 1997; Zerner, 1991). In particular, in view of the crystal structure of urease from *Klebsiella aerogenes* and of its mutants, it was proposed that urea binds to the five-coordinated Ni(1) while the hydroxide ion, bound to the hexa-coordinate Ni(2), acts as nucleophile (Jabri *et al.*, 1995; Karplus *et al.*, 1997).

Alternatively, Benini *et al.*, (1999), proposed that urea enters in the active site cavity and binds the more electrophilic five coordinated Ni(1) with the carbonyl oxygen, approaching the six-coordinated Ni(2) with one of its amino groups and eventually bridging the two metal ions, as shown in Fig.2.9a,b. The protonation state of His^{α222} Ne

stabilizes the binding of the urea carbonyl O atom to Ni(1). Furthermore, the conformational change from the open to the closed position of the flexible flap induces a rearrangement of Ala^{α366} carbonyl group such that its oxygen atom points towards Ni(2) Fig.2.9b. The carbonyl groups of Ala^{α170} and Ala^{α366} are therefore now positioned to act as hydrogen-bond acceptors towards the urea NH₂⁻ group, thereby assisting its binding to Ni(2). Urea is a poor chelating ligand because of the low Lewis base character of its NH₂⁻ groups; the hydrogen bonds with the nearby carbonyl oxygens of Ala^{α170} and Ala^{α366} would, however, enhance the basicity of the amino group and facilitate its binding to Ni(2). In conclusion, the orientation of the substrate is induced by the asymmetric structural features of the active-site residues, positioned to act as hydrogen bond donors in the vicinity of Ni(1) and as hydrogen-bond acceptors in the vicinity of Ni(2). The bidentate ligation of urea to the two very electrophilic nickel ions, together with the hydrogen-bonding network described, strongly activates the inert urea molecule towards nucleophilic attack, by polarizing the C–O and the C–NH₂ bonds. This binding mode of the substrate brings the carbon atom of urea in close proximity to the nickel-bridging ligand to be the nucleophile in the form of a hydroxide anion, as shown in Fig. 2.9b,c (Benini *et al.*, 1999).

2.6.2 Kinetic properties of ureases and enzyme substrates

Ureases typically exhibit simple Michaelis–Menten behaviour, though at high concentrations substrate and product inhibitions are seen. Kinetic characteristics of ureases from different sources, including their Michaelis constants K_M , activities, optimum pH and isoelectric points pI , are shown in Table 2.1.

Unlike K_M , the activity of ureases is strongly dependent on pH, the enzymes being active in a pH range of ca. 4.5–10.5 with the optimum activity at pH 7–8.

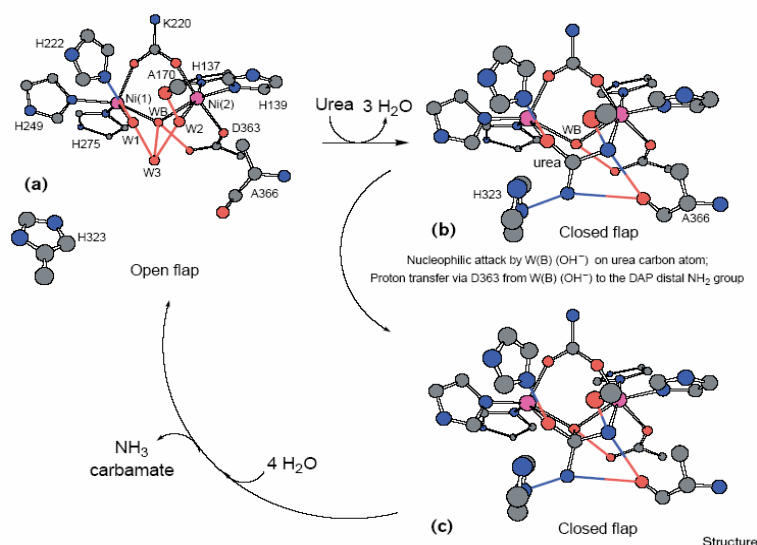


Figure 2.9 mechanisms of urease-catalyzed hydrolysis of urea proposed by Benini *et al.*, 1999.

Frequently interpreted as bell shaped, thereby indicating the involvement of two active-site functional groups in the catalysis of $pK_{as} \sim 6.5$ and ~ 9 (Krajewska *et al.*, 1999; Todd *et al.*, 1987), the activity–pH profiles of ureases were shown to possess shapes with two activity maxima, one dominant at pH 7–8 and the other one at an acidic pH (Evans *et al.*, 1991; Krajewska *et al.*, 2005; Mulrooney *et al.*, 2001; Taylor *et al.*, 1988). These shapes imply the involvement [$(pK_{as}: 3.0, 6.25$ and 9.0 (Dixon *et al.*, 1980) or $5.3, 6.6$ and 9.1 for jack bean urease (Lopreore *et al.*, 1998)] of either three or four functional groups ($pK_{as}: 4.5, 6.3, 6.9$ and 9.1 for *Klebsiella aerogenes* urease; Mulrooney *et al.*, 2001).

The identity of the functional groups detected by the pH-variation studies has not been elucidated yet. Although originally believed to be absolutely urea-specific, today a number of urease substrates are known which, however, are hydrolyzed at a much lower rate than urea, as shown in Table 2.2. Among the substrates two distinct groups are seen, namely urea analogues and phosphoric acid amides and esters. The reaction with compounds other than urea is complex, which is because most of them are both enzyme substrates and inhibitors (Krajewska, 2009).

Table 2.2 Substrates for ureases (Andrews *et al.*, 1984; Krajewska., 2009).

Substrates	Formula	K_{cat} (s^{-1})	K_M (M)
Urea	$H_2N-CO-NH_2$	5870	0.0029
Semicarbazide	$H_2N-CO-NHNH_2$	30	0.06
Formamide	$H_2N-CO-H$	92	1.06
Acetamide	$H_2N-CO-CH_3$	.55	0.75
N-methylurea	$H_2N-CO-NHC_2H_5$	.075	0.22

2.7 Urease inhibitors

Ureases are inhibited by a number of compounds. Substrate urea, product ammonium ions, and substrate analogues are weak inhibitors of urease (Krajewska, 2009).

Several urea analogues have been examined as urease inhibitors, including alkylated ureas, various thioureas, hydroxyurea, and numerous hydroxamic acids. The effects of alkylated ureas on urease activity have been examined in cell extracts from *Corynebacterium renale* (Lister, 1956), *K. aerogenes* (Kamel *et al.*, 1973), *Proteus vulgaris* (Pianotti *et al.*, 1966), and several other enteric bacteria (Rosentein *et al.*, 1981).

No significant urease inhibition was observed for any of the cell extracts by urea analogues such as methylurea, ethylurea, and phenylurea. Phenylurea was shown to be a poor competitive inhibitor with a K_i of 94 mM for purified *Brevibacterium ammoniagenes* urease (Nakano *et al.*, 1984). Mixed inhibition by methylurea was observed in the case of *K. aerogenes* urease.

Despite the close structural similarity between urea and thiourea, the latter compound is not a substrate for urease. By studying either whole cells or cell extracts, urease activity was found to be inhibited by 100 mM thiourea in *Corynebacterium renale* (Lister, 1956) and 0.2 mM in *Proteus vulgaris*, whereas substituted thioureas were generally much poorer inhibitors of urease (Pianotti *et al.*, 1966). Although 0.65 mM thiourea was reported to inhibit cell extracts of *K. aerogenes* (Kamel *et al.*, 1973), no significant inhibition was observed for a purified preparation of *K. aerogenes* urease at levels exceeding 25 mM thiourea. A similar lack of inhibition was noted with isolated

Brevibacterium ammoniagenes urease (Nakano *et al.*, 1984) and with crude preparations of urease from a mixed ruminal population (Mahadevan *et al.*, 1976).

Hydroxyurea is both an inhibitor and a substrate of microbial ureases (Mahadevan *et al.*, 1977, Rosentein *et al.*, 1984). Addition of this compound to a sample of urease results in rapid competitive inhibition, followed by a slow recovery of activity as hydroxyurea is hydrolyzed.

As revealed by pH-dependent kinetic study, thiols inhibit urease competitively in their thiolate anion form $R-S^-$ (Todd *et al.*, 1989). The crystal structure of urease with β -mercaptoethanol, a model compound of this group, demonstrated that β -mercaptoethanol binds to urease by displacing all four water/hydroxide molecules in the active site (Blakeley *et al.*, 1983; Dixon *et al.*, 1980; Todd *et al.*, 1989).

A similar inhibition shown by thiols, is observed for the binding of acetohydroxamic acid with urease. This compound was shown in 1962 to be potent inhibitor of jack bean urease (Kobashi *et al.*, 1962). The best-studied hydroxamate is acetohydroxamic acid, which has been shown to inhibit ureases from *Clostridium sordelli*, *E. coli*, *Morganella morganii*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, *Staphylococcus aureus* (Rosentein *et al.*, 1984), and many other microorganisms, as well as ureases from soil (Pugh *et al.*, 1969). Hydroxamic acids are good metal chelators; thus, the mechanism of inhibition has been generally assumed to involve binding to the active site nickel ion. Spectroscopic evidence obtained for jack bean urease is consistent with this assumption (Andrews *et al.*, 1984). By measuring rates of urease inactivation under varied conditions, and by measuring reactivation rates after removal of free inhibitor, acetohydroxamic acid was found to be a reversible, competitive inhibitor of this enzyme (Mobley *et al.*, 1989).

Amides and esters of phosphoric acid are classified as the strongest inhibitors. The kinetic analysis of their inhibition implied that irrespective of the compound, the inhibition is always brought about by the same diamidophosphate (DAP), a product of their hydrolysis (Andrews *et al.*, 1986; Faraci *et al.*, 1995; McCarty *et al.*, 1990). Phosphoroamide compounds possess a tetrahedral geometry that may mimic an intermediate state in enzymatic catalysis, thus acting as transition state analogue. For jack bean urease there is spectroscopic evidence that phosphoramidate binds to the active site nickel (Andrews *et al.*, 1984).

This contention was verified by the crystal structure of phenylphosphorodiamidate (PPD)-inhibited urease, in which the presence of DAP in the active site was demonstrated (Benini *et al.*, 1999).

An interesting group of compounds within this class of inhibitors are derivatives of thiophosphoric acid, chiefly amides, which were shown to effectively be only precursors that become inhibitors upon their conversion into oxygen analogues (Christianson *et al.*, 1990; Creason *et al.*, 1990). Due to their efficacy, a variety of derivatives of both phosphoric and thiophosphoric acids have been intensively studied for retarding urease hydrolysis in soils (Christianson *et al.*, 1990; Font *et al.*, 2008; Qui-Xiang *et al.*, 1994; Watson *et al.*, 2008) and against ureolytic bacteria infections (Blanchard *et al.*, 1988; Kenny, 1983; Mobley *et al.*, 1988; Pope *et al.*, 1998).

Phosphate buffer, very common in the kinetic studies of urease, and long known to be inhibitory at neutral pHs (Howell *et al.*, 1934), had its inhibitory strength shown to be pH-dependent. This strength decreases with an increase in pH and stops at pH 7.0–7.5 (Krajewska *et al.*, 1999; Todd *et al.*, 1989). The inhibitory action of the buffer was ascribed to H_2PO_4^- ion (Dixon *et al.*, 1980; Krajewska *et al.*, 1999; Todd *et al.*, 1989), a point verified by the crystal structure of urease–phosphate complex at pH 6.3 (Benini *et al.*, 2001).

Boric and boronic acids are rapidly binding urease inhibitors, comparatively weak. For boric acid, the maximum inhibitory activity was observed at pH between 6 and 9, suggestive of its action in the molecular form $\text{B}(\text{OH})_3$ (Breitenbach *et al.*, 1988; Krajewska *et al.*, 2005).

In contrast to the above inhibitors, the data on fluoride inhibition are less consistent. Namely, in a comprehensive study of *Klebsiella aerogenes* urease (Todd *et al.*, 2000), fluoride was found to be an uncompetitive slow-binding inhibitor, however, for jack bean urease, by virtue of F^- binding to an active-site nickel ion, this inhibition was defined as competitive, while its time-dependent character also suggested that it was uncompetitive (Dixon *et al.*, 1980), and interpreted as competitive slow-binding (Krajewska *et al.*, 2001). Heavy metal ions inhibit both plant (Krajewska, 1991; Preininger *et al.*, 1996; Shaw, 1954; Shaw *et al.*, 1961; Zaborska *et al.*, 2004) and bacterial ureases (Kenny, 1983; Nakano *et al.*, 1984) at the following approximate order of effectiveness: $\text{Hg}^{2+} \approx \text{Ag}^+ > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} > \text{Fe}^{3+} > \text{Pb}^{2+} > \text{Mn}^{2+}$

(Krajewska, 1991; Zaborska *et al.*, 2004), with Hg^{2+} , Ag^+ and Cu^{2+} ions nearly always listed as the strongest inhibitors (Kuswandi, 2003; Toren *et al.*, 1968). Classified on the basis of the initial reaction rates measurements as noncompetitive (Toren *et al.*, 1968; Zaborska *et al.*, 2004). This inhibition has been habitually ascribed to the reaction of the ions with the thiol groups of the enzyme, resulting in the formation of mercaptides (Hellerman *et al.*, 1943; Krajewska, 1991; Shaw, 1954; Shaw *et al.*, 1961;). However, evidence was also provided that Cu^{2+} and quite likely Ag^+ ions, in addition to SH groups, coordinate to nitrogen- (histidine) and possibly oxygen- (aspartic and glutamic acids) containing functional groups in urease (Follmer *et al.*, 2005; Krajewska *et al.*, 2008).

In practice, this inhibition is important for two reasons. One is that in view of heavy metal ion pollution, appropriate levels of urease activity in agricultural soils may be endangered. The other one is that this inhibition may be exploited in constructing urease inhibition-based sensing systems for *in situ* and real time determination of trace levels of the ions, *e.g.* in environmental monitoring, food control and biomedical analysis (Krawczynski *et al.*, 2000; Kuswandi, 2003; Preininger *et al.*, 1996). Likewise, the involvement of urease thiol groups was found in the inhibition of the enzyme by bismuth compounds (Zhang *et al.*, 2006). The data on this inhibition are of medical importance, because bismuth compounds are widely used as bactericidal agents in the treatment of peptic ulcers and *Helicobacter pylori* infections (Asato *et al.*, 1997; Murafuji *et al.*, 2006; Zhang *et al.*, 2006).

Most recently, a group of novel inhibitors, *P*-methyl phosphinic and thiophosphinic acids, were designed, synthesized and studied (Vassiliou *et al.*, 2008). They proved to be competitive inhibitors, simple and slow binding, respectively, with K_i constants varying between $1.7 \cdot 10^{-4}$ and 0.34 mM for *Bacillus pasteurii* urease. *P*-methyl thiophosphinic acids appeared to be stronger inhibitors than their oxygen analogues. A variety of other compounds were tested for their inhibitory potential towards ureases. Among those are ketones (α,β)-unsaturated (Tanaka *et al.*, 2003), α -hydroxyketones (Tanaka *et al.*, 2004) and cyclic β -triketones; Tarun *et al.*, 2004), Schiff base metal (Cu, Ni, Co, Cd, Mn) complexes, and notable for medicinal usage, compounds of natural origin, garlic- and herbs-derived (Juszkiewicz *et al.*, 2004; Khan *et al.*, 2006; Li *et al.*, 2007; Lodhi *et al.*, 2006; Shi *et al.*, 2007). Furthermore, there are reports on impact of

pesticides on ureases, which is unquestionably of prime significance, given the importance of adequate control of soil urease activity in the economic use of ureabased fertilizers (Cervelli *et al.*, 1975; Cervelli *et al.*, 1977; Lethbridge *et al.*, 1976; Lethbridge *et al.*, 1981).

Chapter 3

Enzymatic reaction engineering

3.1 Free urease kinetic model

Depending on their sources, ureases present a wide range of specific activities, these depending strongly on the buffer, pH, temperature and ionic strength (Qin *et al.*, 2002). The kinetics of urea hydrolysis was described by means of a modified Michaelis-Menten reaction rate expression, which incorporates pH dependent kinetics, substrate inhibition, and non-competitive product inhibition by NH_4^+ (Moynhan *et al.*, 1989):

$$r_s = \frac{r_{\max} S}{\left(K_M + S + \frac{S^2}{K_S} \right) \left(1 + \frac{A}{K_A} \right)} \quad (3.1)$$

where r_s is the urea degradation rate, $r_{\max}$ the maximum specific reaction rate, K_M the Michaelis-Menten constant, K_S and K_A are the substrate and product inhibition constants, while S and A are the concentrations of urea and ammonium ions.

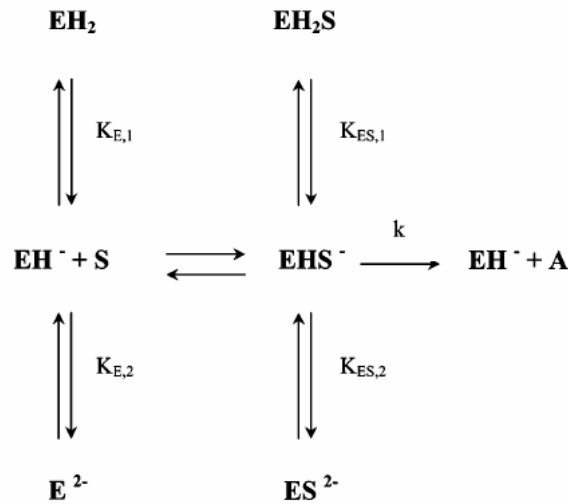


Figure 3.1 Schematic diagram of the mechanism assumed to describe the effect of pH on the enzymatic urea hydrolysis, where E , S and A represent the enzyme, substrate and product species, being the enzyme distributed among three differently protonated forms (Trypton *et al.*, 1979).

To explain the pH dependence of $r_{\max}$ and K_M , Trypton *et al.* (1979) proposed the reaction scheme shown in Fig. 3.1, thus yielding the following:

$$r_{\max} = \frac{k E_0}{1 + \frac{[\text{H}^+]}{K_{ES,1}} + \frac{K_{ES,2}}{[\text{H}^+]}} \quad (3.2)$$

$$K_M = K_{M0} \frac{1 + \frac{[H^+]}{K_{E,1}} + \frac{K_{E,2}}{[H^+]}}{1 + \frac{[H^+]}{K_{ES,1}} + \frac{K_{ES,2}}{[H^+]}} \quad (3.3)$$

where k is the kinetic rate constant of the dissociation of the monoprotonated enzyme-substrate (EHS^-) complex to NH_3 , E_0 and $[H^+]$ are the concentrations of total enzyme and hydrogen ions, $K_{E,1}$ and $K_{E,2}$ the molecular dissociation constants for the free protonated enzyme (EH^+), and $K_{ES,1}$ and $K_{ES,2}$ those for the monoprotonated enzyme-substrate complex.

The value of the substrate inhibition constant (K_S) was reported to vary from 3.2 to 6.4 $kmol\ m^{-3}$ (Qin *et al.*, 2002), thus showing that substrate inhibition can be neglected for S smaller than 0.5 $kmol\ m^{-3}$. The product inhibition was found to be even weaker and decreasing with pH (Qin *et al.*, 2002).

Alternatively, the value of the Michaelis-Menten constant (K_M) was reported as practically independent of pH. In fact, for the urease from jack bean K_M was found to be about 4 $mol\ m^{-3}$ over the pH range of 4-9 (Fidaleo *et al.*, 2003), while for acid urease from *L. fermentum* K_M ranged from 1.2 to 1.7 $mol\ m^{-3}$ at pH values of 4.0 and 2.0, respectively (Matsumoto, 1993). The maximum value of urea degradation rate (r_{max}) was reported to exhibit the classic bell-shaped pH dependence (Moynhian *et al.*, 1989). For the urease from jack bean, r_{max} was estimated as about 1.5 $mol\ m^{-3}\ min^{-1}$ at pH 7.0 and $E_0=100\ g\ m^{-3}$ (Fidaleo *et al.*, 2003).

By referring to the stoichiometric reaction of urea hydrolysis by urease (2.1), the unsteady-state urea (S) and ammonium (A) mass balances in the well mixed liquid phase can be written as follows, considering the:

$$\frac{dS}{dt} = -r_s \quad (3.4)$$

$$r_A = \frac{dA}{dt} = -2 \frac{dS}{dt} = 2 r_s \quad (3.5)$$

to be integrated with the initial conditions:

$$S = S_0; \quad A = A_0; \quad \text{per } t = 0 \quad (3.6)$$

where r_A is the ammonium formation rate.

By determining the net increase in the ammonium concentration after a reaction interval of 10 min, Fidaleo *et al.* (2006) estimated the initial ammonium formation rate (r_{A0}), this being the double of the initial urea degradation rate (r_{S0}).

Table 3.1 *Experimental conditions (pH; temperature, T; initial enzyme, E_0 , and urea, S_0 , concentrations) used to estimate the specific enzyme activity in different buffer solutions and overall number of experiments (m_i), as extracted from Fidaleo *et al.* (2006).*

Trial series	m_i	Reaction medium	pH	T (°C)	E_0 (g m ⁻³)	S_0 (mol m ⁻³)
A	6	Acetate buffer 0.1 kmol m ⁻³	4	37	21.0 - 63.0	83.33
B	16	Acetate buffer 0.1 kmol m ⁻³ , ethanol 12.5 % (v/v)	4	37	38.7	0.833-83.33
C	18	Acetate buffer 0.1 kmol m ⁻³ , ethanol 12.5 % (v/v)	4	20	77.5	0.833-83.33
D	14	Citrate buffer 0.1 kmol m ⁻³ , ethanol 12.5 % (v/v)	3	20	82.8	0.833-83.33

In accordance with the data listed in Table 3.1 (trial series A), r_{A0} was found to increase almost proportionally to E_0 with E_0 increasing from 21.0 to 63.0 g m⁻³, thus yielding an almost constant specific enzyme activity $\pi_A (=r_{A0}/E_0)$ of 3.3 ± 0.1 U mg⁻¹ ($r^2 = 0.95$) in agreement with the manufacturer's claim (Fidaleo *et al.*, 2006).

As a result of the trial series B-C (Table 3.1), π_A exhibited a typical saturation pattern as the urea concentration was increased from 0.83 to 83.3 mol m⁻³ (Fig. 3.2). These data were non-linearly fitted by using the well known Michaelis-Menten kinetic model:

$$\pi_A = \frac{r_A}{E_0} = \frac{k' S}{K'_M + S} \quad (3.7)$$

where k' is the ammonia formation kinetic rate constant, and K'_M the saturation constant, their values being estimated by using a non-linear fitting method.

With reference to Fig. 3.1, by assuming that the enzyme underwent the same dissociation process whatever it was free or bound to the substrate (that is $K_{E,1} \approx K_{ES,1}$, $K_{E,2} \approx K_{ES,2}$), and the inhibitory effect of substrate and ammonia was negligible (that is $K_S \approx K_A \rightarrow \infty$), the combined use of Eq.s (3.5), (3.2), (3.3) and (3.7) gave rise to the following equivalences:

$$k' \approx \frac{2k}{1 + \frac{[H^+]}{K_{ES,1}} + \frac{K_{ES,2}}{[H^+]}} \quad (3.8)$$

$$K'_M \approx K_M \quad (3.9)$$

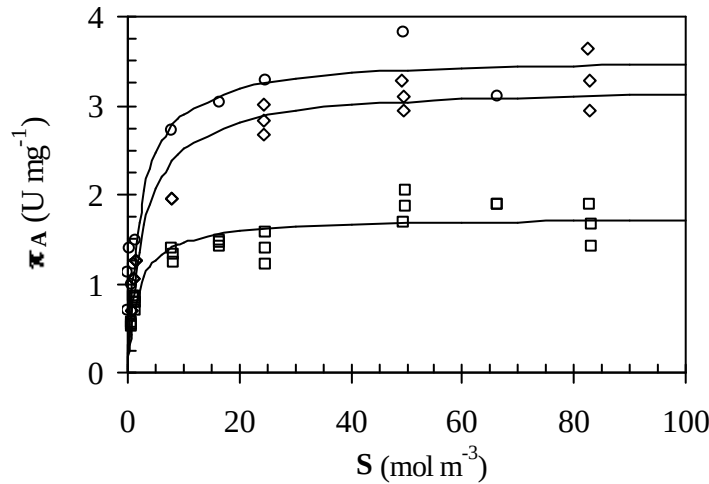


Figure 3.2 Effect of urea concentration (S) on the specific urease activity (π_A) in the buffers B ($\circ$), C ($\square$) or D ($\diamond$) listed in Table 3.1. The continuous lines were calculated using Eq. (3.32) together with the estimated parameters listed in Table 3.2.

Table 3.2 Mean values and standard deviations of the kinetic parameters of Eq. (3.32) as a function of pH and temperature (T), as extracted from Fidaleo et al. (2006).

Trial series	k' ($U^a \text{ mg}^{-1}$)	K'_M (mol m^{-3})	pH (-)	T ($^{\circ}\text{C}$)
B	3.5 ± 0.3	2.2 ± 1.0	4	37
C	1.7 ± 0.2	1.8 ± 1.0	4	20
D	3.2 ± 0.4	2.8 ± 1.6	3	20

^a1 U = 1 μmol of ammonia formed min^{-1} from urea at 37 $^{\circ}\text{C}$.

The effects of pH and temperature on K'_M (Table 3.2) were found to be practically negligible at least over the experimental ranges tested, whereas k' appeared to be dependent on pH and temperature in agreement with Eq. (3.8). In fact, at pH 4 its value was greater at 37 $^{\circ}\text{C}$ than at 20 $^{\circ}\text{C}$, but at 20 $^{\circ}\text{C}$ it almost doubled as pH was reduced to 3, thus corroborating the potential use of acid urease from *L. fermentum* to remove effectively urea in wines (Fidaleo et al., 2006).

Finally, from Fig. 3.2 it was also guessed that for $S \ll K_M'$ the specific enzyme activity (π_A) tended to vary linearly with the substrate concentration, its kinetic model reducing to the pseudo-first order one:

$$\pi_A \cong k'/K_M' S = k_I S \quad (3.10)$$

where k_I is the pseudo-first order kinetic rate constant.

Despite the initial urea concentration ($S_0 \approx 1 \text{ mol m}^{-3}$) used in Fidaleo *et al.* (2006)'s experimental design was not by far less than, but about one third of K_M' (Table 3.2), use of Eq. (3.7) allowed k_I to be estimated as:

$$k_I \cong \frac{\pi_A}{S_0} \quad (3.11)$$

In this way, by replacing Eq. (3.11) into Eq. (3.5) and integrating both Eq.s (3.4) and (3.5) with the initial conditions (3.6), it was possible to determine the time dependence of A and S as:

$$S = S_0 - \frac{1}{2} (A - A_0) = S_0 \exp\left(-\frac{1}{2} k_I E_0 t\right) \quad (3.12)$$

$$A = A_0 + 2 S_0 [1 - \exp\left(-\frac{1}{2} k_I E_0 t\right)] \quad (3.13)$$

where k_I is to be expressed in $\text{m}^3 \text{g}^{-1} \text{min}^{-1}$, E_0 in g m^{-3} , and A and S in mol m^{-3} (Fidaleo *et al.*, 2006).

To assess the main effects and interactions of the most inhibitory wine components, that is malic (M) and lactic (L) acids, potassium metabisulphite (K), and ethanol (E), as well as pH, on the acid urease activity, Fidaleo *et al.* (2006) carried out a half-fractionate 2^5 factorial experiment, where the four-factor interaction (M-L-K-E) was confounded with pH, that subsequently supplemented with extra-points according to a central composite design (Fidaleo *et al.*, 2006).

Table 3.3 show the experimental conditions in natural and coded levels using the following dimensionless equations:

$$\begin{aligned}
x_1 &= (M - 2.5)/1.25 \\
x_2 &= (L - 1.750)/0.875 \\
x_3 &= (K - 0.20)/0.05 \\
x_4 &= (E - 13.0)/1.5 \\
x_5 &= (\text{pH} - 3.50)/0.25.
\end{aligned} \tag{3.14}$$

Table 3.3 Natural and coded levels of the factors of the composite experimental design used by Fidaleo et al. (2006).

Factor	Factor level					Unit
	$-\alpha$	-1	0	+1	$+\alpha$	
Malic acid (M)	0	1.25	2.50	3.75	5.00	kg m ⁻³
L-lactic acid (L)	0	0.88	1.75	2.63	3.50	kg m ⁻³
K ₂ S ₂ O ₅ (K)	0.10	0.15	0.20	0.25	0.30	kg m ⁻³
Ethanol (E)	10.0	11.5	13.0	14.5	16.0	% (v/v)
pH	3.00	3.25	3.50	3.75	4.00	-

Statistical analysis of data collected by Fidaleo et al. (2006) allowed the significance of the main effects and interactions to be assessed as follows:

- i) factors L and K had no effect on π_A at the 90% confidence level;
- ii) factors M, E, and pH, as well as interaction K-E, were highly significant at the 99% confidence level;
- iii) all other binary interactions were statistically insignificant.

Moreover, the effects of M and pH on π_A were practically equal but opposite, that is π_A tended to decrease or increased as M or pH was increased. The effect of E on π_{A0} was negative, but about a half of that of M.

To determine the 2nd-order effects, the factorial design was supplemented with extra-points $\pm\alpha$ (Table 3.3) according to a central composite design, thus allowing the specific enzyme activity π_A to be reconstructed with a mean percentage error of 9.8% by means of the following 2nd-order canonical regression:

$$\pi_A = 0.029 + 0.0088 X_1^2 + 0.0104 X_2^2 + 0.0125 X_3^2 + 0.0034 X_4^2 \tag{3.15}$$

with

$$\begin{aligned}
X_1 = & 0.742 (x_1 - 1.045) - 0.028 (x_2 - 0.517) + 0.442 (x_3 - 0.747) \\
& - 0.413 (x_4 - 0.677) - 0.290 (x_5 + 0.964)
\end{aligned} \tag{3.16}$$

$$\begin{aligned}
X_2 = & -0.452 (x_1 - 1.045) - 0.332 (x_2 - 0.517) + 0.264 (x_3 - 0.747) \\
& + 0.042 (x_4 - 0.677) - 0.783 (x_5 + 0.964)
\end{aligned} \tag{3.17}$$

$$X_3 = 0.474 (x_1 - 1.045) - 0.012 (x_2 - 0.517) - 0.498 (x_3 - 0.747) + 0.603 (x_4 - 0.677) - 0.404 (x_5 + 0.964) \quad (3.18)$$

$$X_4 = 0.120 (x_1 - 1.045) - 0.931 (x_2 - 0.517) - 0.209 (x_3 - 0.747) - 0.119 (x_4 - 0.677) + 0.248 (x_5 + 0.964) \quad (3.19)$$

where X_i and x_i are the generic principal axis and coded input variable, respectively.

Thus, in the five-dimensional space ($X_1, X_2, X_3, X_4, \pi_A$), the response surface associated to π_A was an elliptic hyper-paraboloid. Fig. 3.3 shows the response surface (a) and contour (b) plots of the specific urease activity π_A as a function of malic acid concentration (M) and pH when setting the other independent variables to their zero level.

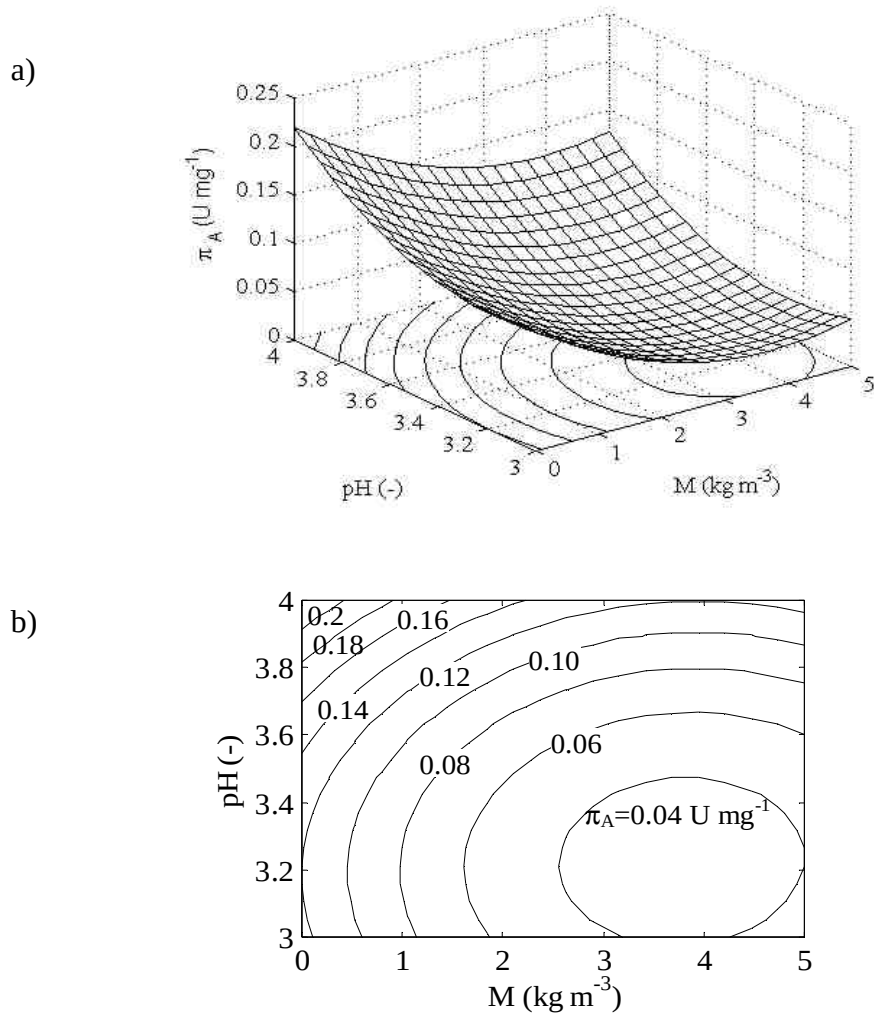


Figure 3.3 Response surface (A) and contour (B) plots of the specific urease activity (π_A) as calculated by using Eq. (3.40), as function of malic acid concentration (M) and pH at constant values for the other independent variables ($L = 1.75 \text{ kg m}^{-3}$, $K = 0.20 \text{ kg m}^{-3}$, $E = 13.0 \text{ \% v/v}$), as extracted from Fidaleo et al. (2006).

It can be noted that as the reaction medium was commuted from an acetate buffer at pH 4 to any of the other model wine solutions tested by Fidaleo *et al.* (2006), the specific acid urease activity reduced from the value claimed by the manufacturer (3.3 U mg^{-1}) to quite smaller values reduced by a factor in the range of 20 and 90 depending on the combined inhibitory action of the wine components accounted for.

To check for the accuracy of π_A estimation, a couple of full hydrolysis trials were carried out till urea exhaustion by setting the independent variables at the levels associated with the maximum and minimum π_A values observed throughout the half-fractionate 2^5 factorial experiment (Fidaleo *et al.*, 2006).

In the circumstances, the combined use of Eq.s (3.15) and (3.11) allowed the corresponding k_I values for these trials V_1 and V_2 to be estimated as 1.34×10^{-4} and $0.39 \times 10^{-4} \text{ m}^3 \text{ g}^{-1} \text{ min}^{-1}$, respectively. Then, it was possible to estimate the time course of ammonia formation by using Eq. (3.13).

Fig. 3.4 shows a satisfactory agreement between the experimental and calculated A values in both validation tests, thus confirming the capability of the pseudo-first order empirical model developed here to reconstruct the evolution of urea hydrolysis in the operating conditions tested for as long as 24 h, even if the pseudo-first order kinetic rate constant k_I was derived from short tests of 10 min each.

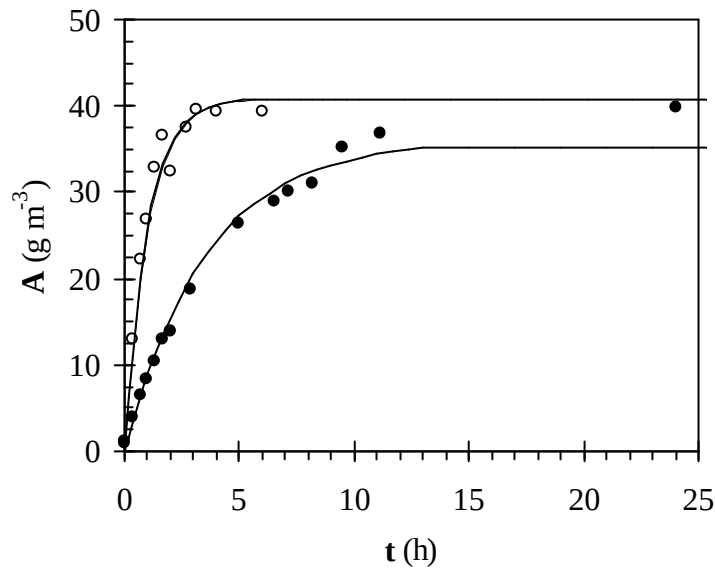


Figure 3.4 Comparison between the experimental (●, ○) and calculated (continuous line) ammonium concentrations (A) as a function of time (t) for the model wine solutions V_1 (○) and V_2 (●) described in the text.

3.2 Free acid urease kinetics in real wine

Despite the feasibility of acid urease application for the removal of urea from several types of wines has been extensively demonstrated (Bertrand, 2003; Fujinawa *et al.*, 1990; Fujinawa *et al.*, 1992; Kodama *et al.*, 1996; Ough *et al.*, 1988), the efficiency of such a treatment was found to vary with the type of wine, content of inhibiting factors, and usage conditions. More specifically, the most effective inhibitors of urease present in wines were found to be, in order of importance, fluoride, malate, ethanol, and phenolic compounds (Famuyiwa *et al.*, 1991; Kodama, 1996; Trioli *et al.*, 1989).

The above pseudo-first order kinetic model (3.10) was applied to describe urea removal from some Italian wines manufactured in Apulia region of Italy in the 2003 (Esti *et al.*, 2007), by using the same operative conditions previously selected to study the enzyme activity in the above model wine solutions: $S_0=1 \text{ mol m}^{-3}$, $E_0=238 \text{ g m}^{-3}$ (Fidaleo *et al.*, 2006).

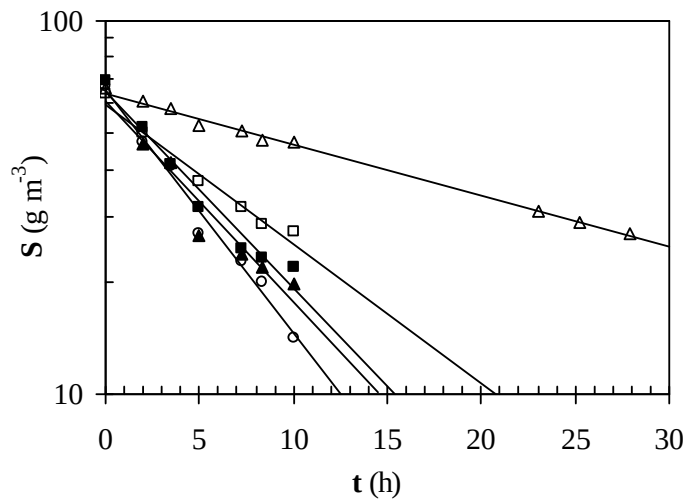


Figure 3.5 Time course of urea degradation for the 5 Italian wines used by Esti *et al.* (2007): $\circ$: A; $\blacksquare$: B; $\triangle$: C; $\square$: D, $\blacktriangle$: E, that were enriched with $1 \text{ mol of urea m}^{-3}$ and treated with 238 g m^{-3} of acid urease. The continuous lines represent the mean squares regression lines.

The semi-logarithmic plots of the current urea concentration (S) against time (t) for the 5 Italian wines assayed were quite linear (Fig. 3.5). Therefore, the pseudo-first order kinetic model was regarded as appropriate to describe the evolution of urea hydrolytic process not only in model wine solutions (Fidaleo *et al.*, 2006), but also in real wines (Esti *et al.*, 2007).

As reported before, in wines are present many urease inhibitors. Among the others, it is worth citing the phenolic compounds.

By assuming that such compounds behave as competitive inhibitors of acid urease by linking to the enzyme active site to form a stable enzyme-inhibitor complex (EP), it was possible to express the effective kinetic rate constant (k_{le}) as follows:

$$k_{le} = \frac{k_i}{1 + \frac{P}{K_p}} \quad (3.20)$$

where K_p is the equilibrium constant of the reaction between the enzyme (E) and inhibitor (P) (Esti *et al.*, 2007).

By fitting the ratio (k_{le}/k_i) as a function of P via a non-linear estimation method, Esti *et al.* (2007) minimized to 14.5% the mean percentage error among the experimental and calculated ratios by setting K_p to 21.1 ± 0.5 g GAE m^{-3} (see continuous line in Fig. 3.6).

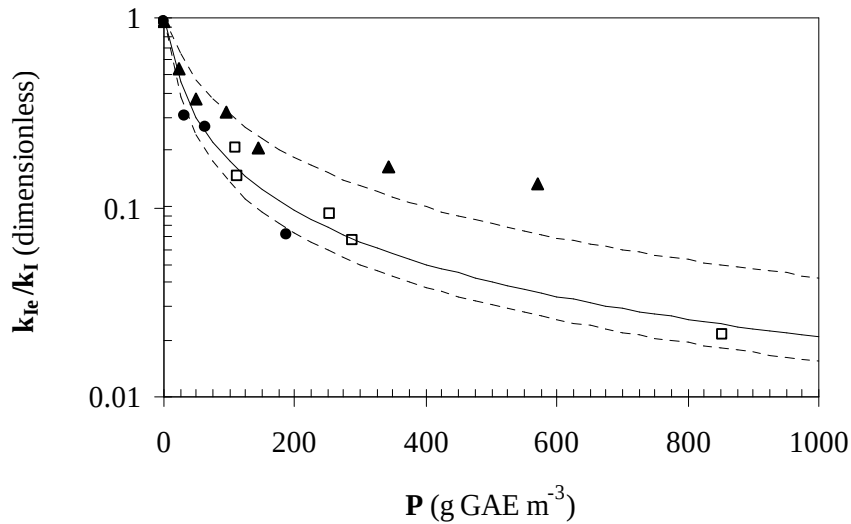


Figure 3.6 Effect of phenolic compounds on the ratio between the experimental pseudo-1st order kinetic rate constant (k_{le}), pertaining either to the real wines ($\square$) assayed by Esti *et al.* (2007) or to the model wines enriched with grape skin ($\blacktriangle$) or seed ($\bullet$) tannins, and calculated constant (k_i) as referred to model wines devoid of any phenolic compound, but with the same composition and pH.

To verify whether the observed correlation between k_{le} and P was due to the inhibitory effect of the phenolic compounds, the model wine solution corresponding to the composite design performed by Fidaleo *et al.* (2006) was enriched with two different tannin sources extracted from grape skins or seeds in the ranges of 0 to 1.2 kg m^{-3} and 0 to 0.3 kg m^{-3} , respectively.

By resorting to Eq. (3.20), it was possible to determine the inhibition constant for both tannin sources as:

$$K_p = 44.7 \text{ g GAE m}^{-3} \quad \text{for grape skin extract}$$

$$K_p = 15.7 \text{ g GAE m}^{-3} \quad \text{for grape seed extract}$$

The broken lines in Fig. 3.6 show a quite satisfactory agreement between the experimental and calculated kinetic constant rate ratios in both tests, the corresponding mean percentage errors being about 23.8 and 12.3%, respectively (Esti *et al.*, 2007).

This result clearly confirmed the greater inhibitory effect of phenolic compounds extracted from grape seeds than those extracted from grape skins. Moreover, they agreed with previous findings by Trioli and Ough (1989), that observed a greater reduction in the activity of 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 to 880 g GAE m⁻³. However, Esti *et al.* (2007) were unable to explain why the phenolic content of the real Italian wines assayed tended to inhibit the acid urease activity in just a little milder way ($K_p \approx 21 \text{ g GAE m}^{-3}$) than grape seed tannins ($K_p \approx 16 \text{ g GAE m}^{-3}$).

3.3 The enzyme immobilization

3.3.1 Definition

According to a definition by Katchalski-Katzir *et al.*, (2000) at the 1st Enzyme Engineering Conference in 1971, immobilized enzymes denote “enzymes physically confined or localized in a certain defined region of space retaining their catalytic activities, so as to be repeatedly and continuously used”.

Essentially, an immobilized enzymes is a complex system, consisting of two essential components:

1. the non-catalytic structural component (carrier), which is designed to aid separation and reuse of the catalyst and facilitates control of the process;
2. the catalytic functional component (enzyme), which is designed to convert the substrates of interest into the desired products (Cao *et al.*, 2003). The non-catalytic parameters are related to the physical and chemical nature of the non-catalytic part, especially the geometric parameters, such as shape, size and

length. Whereas the catalytic parameters are linked to the catalytic functions, such as activity, selectivity and stability.

Practically, the catalytic functions are designed to achieve the desired activity, selectivity, substrate specificity, productivity and space-time yield. The aim is to achieve fewer side reactions, high toleration of structural variation of the substrates, high productivity and space-time yield and high durability of the catalyst. The selection criteria for non-catalytic functions, especially the geometric parameters, are largely dependent on the design of reactor configurations (batch, stirred tank, column and plug-flow), the type of reaction medium (aqueous, organic solvent or two-phase system), the reaction system (slurry, liquid-to-liquid, liquid-to-solid or solid-to-solid), and the process conditions (pH, temperature and pressure). Parameters are chosen in order to achieve easy separation of the immobilised enzymes from the reaction mixture, flexibility of reactor design and broad applicability in various reaction media and reaction systems. The selected parameters should also facilitate down-stream processing and, in particular, control of the process.

Apart from these functional requirements, like many other industrial biocatalysts (e.g. free enzymes or whole cells), an ideal industrial immobilised enzyme has to meet some other criteria as well, such as being recyclable, broadly applicable, cost effective and safe for use (Cao *et al.*, 2003).

Many details about the changes of molecular catalytic properties associated with the chemical modification of proteins and effects of the microenvironment other than due to electrostatic force are still unknown. On the other hand, measurements with particles of simple, defined structure have established the applicability of diffusion laws to immobilised enzymes, and the implications have been successfully used to design carriers for applications requiring high reaction rates per units catalyst volume. However, the mathematical equations relating to the fundamentals physical parameters and external variables to the properties of interest become very complex unless the structure of the carrier matrix and the distribution of the catalyst within the matrix are simple or unless diffusion limitations are negligible (WIPIG, 1983).

There are several reasons to use immobilised enzymes. In addition to the convenient handling of enzyme preparations, the two main targeted benefits are easy separation of enzyme from the product and the reuse of enzyme from the product simplifies enzyme

applications and permits reliable and efficient reaction technology. Enzyme reuse is an essential prerequisite for establishing an economically viable enzyme-catalysed process. The properties of immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material. The interaction between the two provides an immobilised enzyme with specific chemical, biochemical, mechanical and kinetic properties (Tischer *et al.*, 1999).

As far as manufacturing costs are concerned, the yield of immobilised enzyme activity is determined by the immobilisation method in relation to the amount of soluble enzyme used. Under process conditions, the resulting activity can be further reduced by mass-transfer effects. The immobilised yield of enzyme activity does not only depend on losses caused by the binding procedure, but it can be further reduced as a result of the diminished availability of enzyme molecules within pores or by slowly diffusing substrates molecules. Such limitations lead to lowered efficiency. However, improved stability under working conditions (optimum values of pH, temperature ecc.) can compensate for such drawbacks, resulting in an overall benefit (Tischer *et al.*, 1999).

The main industrial application of immobilised enzymes are reported in the Table 3.4.

3.3.2 The main enzymatic immobilization methods

Diverse enzyme immobilization techniques have been developed (Cao, 2005; Chaplin *et al.*, 1990; Sheldon., 2007). Traditionally can be classified as chemical and physical ones, though very frequently their combinations or various follow-up treatments are applied (Cao, 2005). Among the chemical techniques it is possible to distinguish: (i) covalent attachment to solid supports, and (ii) crosslinking with multifunctional, low molecular reagents, also sometimes performed with the addition of neutral compounds (co-crosslinking).

By contrast, the physical techniques include: (i) adsorption on solid supports, (ii) gel entrapment, (iii) microencapsulation with solid or liquid membranes, (iv) containment in membrane reactors, (v) formation of Langmuir–Blodgett films, and (vi) layer-by-layer assembling.

The choice of materials to be used in these techniques is practically unlimited, and include organic and inorganic, natural and synthetic materials, that may be configured as micro or nano-beads, membranes, fibers, hollow-fibers, micro- capsules, sponges to

best suite a chosen biotransformation in a chosen bioreactor. On the whole, to immobilize an enzyme is not a difficult task, but to obtain an enzyme with desired properties is a complex challenge. This is because, there are no universal protocols how to immobilize enzymes and how to exactly predict their resulting properties (Krajewska, 2009b).

Enzyme cross-linking was investigated thoroughly during the pioneer phase of enzyme immobilisation. However, because of the low mechanical and hydrodynamic stability of the proteinaceous materials obtained by this method, interest turned to other methods (Tischer *et al.*, 1999).

Enzyme immobilization on a solid carrier is carried out by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier. If the carrier is a porous matrix, the enzyme is allowed to penetrate inside the pores of the carrier to remain trapped inside of them (Kourkoutas *et al.*, 2004).

Immobilisation by inclusion involves the enzyme being retained within a membrane device, such as a hollow-fibre, polymeric network or microcapsule. In simple inclusion, there is no need for derivatization of the enzyme or contact with another surface and so none of the detrimental effects of binding forces have to be taken into account. However, there is no additional stabilization as a result of protein carrier interactions. Incorporation into a polymeric network is most efficient when combined with additional binding methods by, for example, the attachment of vinyl groups to the enzyme surface and covalent integration into a growing polymer chain. Such chemically-modified enzymes can be incorporated into biocatalytic plastics and are active in aqueous and inorganic media (Tischer *et al.*, 1999). This immobilisation method is largely used when values of mass-transfer are low, this is the mainly disadvantage presented by using this method.

Table 3.4 *Industrial applications of immobilised enzymes.*

Enzyme	Substrate	Product
Glucose isomerase	Glucose	Fructose (high-fructose corn syrup)
β -Galactosidase	Lactose	Glucose and galactose (lactose-free milk and whey)
Lipasi	Triglycerides	Cocoa butter substitutes
Nitrile hydratase	Acrylonitrile 3-Cyanopyridine Adiponitrile	Acrylamide Nicotinamide 5-Cyanovaleramide
Aminoacylase	D,L-Aminoacides	L-aminoacides (methionine, alanine, phenylalanine, tryptophan, valine)
Raffinase	Raffinose	Galactose, sucrose (raffinose free-solutions)
Invertase	Sucrose	Glucose/fructose (invert sugar)
Aspartate ammonia-lyase	Ammonia and Fumaric acid	L-aspartic acid (used for production of synthetic sweetener aspartame)
Glucoamylase	Starch	D-Glucose
Papain	Proteins	Removal of “chill haze” in beers
Hydantoinase	D,L-Amino acid hydantoins	D,L-Aminoacides
Penicillin amidase	Penicillins G and V	6-Aminopenicillanic acid (precursor of semi-synthetic penicillins)
β -Tyrosinase	Pyrocatechol	L-DOPA

Using apposite cross-linkers, it can be possible to bind two or more molecules of enzyme forming protein assemblies, crystals very similar to protein assemblies, but multiple non-covalent forces present in crystals, form and stabilize them and make them more rigid than cross-linked amorphous protein assemblies. Additional chemical bonding via cross-linkers is also required to stabilize the crystals against dissolution in aqueous environment (Tischer *et al.*, 1999).

Immobilization by non-covalent adsorption was found to be very useful in non-aqueous systems, in which desorption can be neglected owing to the low solubility of enzymes in

such solvents. This method is widely used for reactions of lipases in water-immiscible solvents. The use of adsorbed enzymes and proteins is widespread in commercial applications because of its simplicity. Immobilisation is also achieved by the simple dispersion of dried water-soluble enzyme preparations in water-immiscible organic solvent. In particular, lipases can be successfully immobilised by this means. In organic solvents, they catalyse otherwise hydrolytic reactions in the reverse direction, if the water content is carefully controlled (Tischer *et al.*, 1999).

3.4 Immobilised ureases and their applications

The most typical examples of such applications, where immobilized ureases are preferably used in place of free enzymes, are presented below.

The removal of urea from aqueous solutions is a problem faced in numerous areas, examples being urea-producing industry, agriculture and natural environment, food production and medicine. In the natural environment urea comes from many sources, fertilized crop-planted soils, fertilizer wastewater effluents from households, but primarily from urine excretion by animals. Medically immobilised ureases are used to treat kidney diseases or to detoxify blood. Other instances include the removal of urea from industrial wastewaters, where the product ammonia can be recovered by air-stripping or by ion-exchange, as well as the removal from fertilizer wastewater effluents (Latkar *et al.*, 1994).

Moreover, ureases is crucial as indicators of liver and kidney function, the blood urea test is being used as a marker for quantify and monitoring haemodialysis treatment. By contrast, in food analyse, urea is routinely quantified in cow milk and alcoholic beverages (Francis *et al.*, 2002). In the former, as the prime component of non-protein nitrogen in milk, the level of urea (typically 3–6 mM) is utilized as an indicator of protein feeding efficiency.

In addition to these applications, ureases are also immobilized for other purposes. For instance certain urease-entrapped gels are studied as smart materials having enzyme reaction-regulated properties. Owing to the controlled hydrolysis of urea, the gels are capable of converting biochemical energy into mechanical work through swelling and shrinking. Ureases are also immobilized on selected soil materials in order to gain insights into behaviour and properties of soil urease. In the same agricultural context

adsorption of urease on selected materials is tested as a possible means of reducing the activity of soil urease. Also, various multi-enzyme immobilizations are performed mainly for analytical purposes (Krajewska, 2009b).

3.5 The carriers used in this PhD work

The properties of immobilized enzymes are governed by the properties of both the enzyme and the support material (Kennedy *et al.*, 1983; Tischer *et al.*, 1999). Their interaction gives rise to specific physico-chemical and kinetic properties that may be decisive for the practical application, of immobilised enzymes. Thus, a support judiciously chosen can significantly enhance the operating performance of the immobilized system. There is no universal support for all enzymes and their applications, a number of desirable characteristics should be common to any material considered for immobilizing enzymes. These include: high affinity to proteins, availability of reactive functional groups for direct reactions with enzymes and for chemical modifications, hydrophilicity, mechanical stability and rigidity, regenerability, and ease of preparation in different geometrical configurations that provide the system with permeability and surface area suitable for a chosen biotransformation. For food, pharmaceutical, medical and agricultural applications, nontoxicity and biocompatibility of the materials are also required (Krajewska, 2004).

3.5.1 Eupergit[®] C

Eupergit[®] C was identified as a carrier suitable for the covalent immobilization of several enzymes for industrial applications. It was developed between 1974 and 1980 by Rohm, Darmstadt, Germany (Kraemer *et al.*, 1975; Kraemer *et al.*, 1985).

Eupergit[®] C consists of microporous beads with a diameter of 100–250 µm, made by copolymerization of *N,N*-methylene-bis-(meth-acrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide, as shown in Fig.3.7. The procedure was based on a novel bead-polymerization method, developed by Roehm in 1970 (Pennewiss *et al.*, 1970), in which two immiscible organic phases were employed. Because of its structure, Eupergit[®] C is stable, both chemically and mechanically, over a pH range of 0-14, and does not swell or shrink even upon drastic pH changes in this range. Its mechanical stability is outstanding, as it does not show significant attrition after 650 cycles in

stirred tank reactors (STR) with substrate volumes larger than 1000 l (Katchalski-Kazir *et al.*, 1999).

No change in distribution of its particle size was observed when Eupergit[®] C was stirred under such conditions. Eupergit[®] C is highly “reactor-compatible” since almost any common type of reactor, stirred-tank or fixed-bed can be used, so long as it is equipped with a bottom sieve to withhold the carrier particles. Eupergit[®] C binds proteins via its oxirane groups which react at neutral and alkaline pH with the amino groups of the protein molecules, (e.g. enzymes) to form covalent bonds which are long-term stable within a pH range of pH 1 to 12, Fig.3.7. Eupergit[®] C can also bind enzyme molecules via their sulfhydryl groups and via their carboxyl groups in the acidic, neutral and alkaline pH range. Thus, Eupergit[®] C can covalently bind enzyme molecules between pH 1 and 12, *i.e.* it can bind a given enzyme at the pH range where the enzyme is stable and does not lose its activity. Due to the high density of oxirane groups on the surface of the beads (600 $\mu\text{mol/g}$ dry Eupergit[®] C), enzymes are immobilized at various sites of their structure. This phenomenon is called “multi-point-attachment,” which is considered a major factor for the high operational stability of enzymes bound to Eupergit[®] C. The procedure of immobilization of enzymes on Eupergit[®] C is simple. The enzyme is dissolved in a buffer, mixed with Eupergit[®] C and allowed to stand at 20–25°C for 24 to 100 h. The immobilized enzyme is then washed with water on buffer to be used in its subsequent application.

The binding capacity of an enzyme is about 100 mg protein/g Eupergit[®] C (dry weight). Eupergit[®] C is commercially available worldwide, and quantities as large as several tons per year have been employed by individual companies for the production of biocatalysts (Katchalski-Kazir *et al.*, 1999).

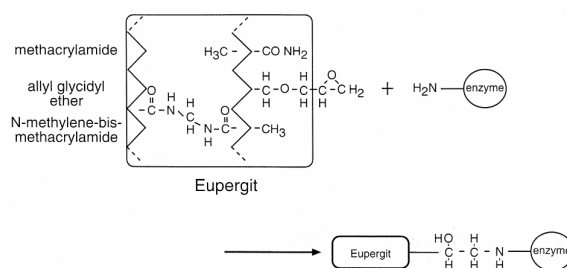


Figure 3.7 Structure of Eupergit[®] C and covalent immobilisation of an enzyme (Katchalski-Kazir *et al.*, 1999).

After enzyme immobilization on Eupergit[®], the bulk of oxirane groups are still present and can be easily modified to achieve the best “surroundings” for the enzyme (Data Sheet/Info EP-4/E). Eupergit[®] has > 600 µmol oxirane groups/g dry beads. When ~ 100 mg enzyme are immobilized on 1 g of beads, less than 1% of these groups are involved in covalent attachment to the enzyme molecule. The residual 99% of the oxirane groups slowly hydrolyze into diol groups. However, in some cases, it is advantageous to modify these groups, which can be done easily with thio-compounds, e.g. with 2-mercaptoethanol or Cleland’s reagent (dithiothreitol) (Katchalski-Kazir *et al.*, 1999; Kraemer *et al.*, 1981).

Eupergit[®] C is commercial available in three different typologies with different particles dimensions and different average diameter of micropores:

- Eupergit[®] C, the average micropore diameter is about 150 µm;
- Eupergit[®] C 250 L, the average micropore diameter is about 200 µm;
- Eupergit[®] C 1 Z, the average micropore diameter is about 1 µm.

Eupergit[®] C e C 250 L are used, also, in fixed bead and stirred batch reactors, Eupergit[®] C 1 Z is adopted in membrane reactors.

The main enzymes immobilised onto Eupergit[®] and their industrial applications are listed in Table 3.5.

3.5.2 Chitosan

Chitin and chitosan are natural polyaminosaccharides (Hudson *et al.*, 1998; Khor, 2002; Peter, 1995), chitin being one of the world’s most plentiful, renewable organic resources. It is the major constituent of the shells of crustaceans, exoskeletons of insects and cell walls of fungi, where it provides strength and stability. Chitin is estimated to be synthesized and degraded in the biosphere in the vast amount of at least 10 Gt each year. Chemically, chitin is composed of $\beta(1 \rightarrow 4)$ linked 2-acetamido-2-deoxy- β -D-glucose units (or *N*-acetyl-D-glucosamine) (Peter, 1995), forming a linear long-chain polymer, as shown in Fig.3.8. It is insoluble in most solvents. Chitosan is a collective name given to a group of polymers obtained by *N*-deacetylation of chitin, as shown in Fig.3.8, to a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine.

Table 3.5 *main enzymes immobilised onto Eupergit® and their industrial applications (Knezevic et al., 2006).*

enzyme	application	support	Immobilized activity (%)
Alcohol dehydrogenase	Reduce aliphatic ketones (5-chloropentan-2-one)	Eupergit C	15
		CL-Sepharose-4B	30
Pyranose oxidase	Oxidation of D-glucose to 2-keto-D-glucose	Eupergit C	50
Phenylalanine Dehydrogenase	Synthesis of the vasopeptidase inhibitor	Eupergit C	17
		Eupergit C 250 L	41
D-amino acid Oxidase	Synthesis of semisynthetic cephalosporin antibiotics	Eupergit C	28
Transketolase	Production of synthetic carbohydrates	Eupergit C	20
β-glucosidase	Wine making and juice processing Hydrophobic interaction chromatography	Eupergit C	40
β-Galactosidase	Hydrolysis of lactose	Eupergit C	84
Aldolase	Play an important role in a wide range of biological recognition process	Eupergit C	40

Chitin or chitosan can be chemically considered as analogues of cellulose, in which the hydroxyl at carbon-2 has been replaced by acetamido or amino groups, respectively. The difference between chitin and chitosan lies in the degree of deacetylation (Li *et al.*, 1992).

Chitosan was traditionally used in the East Asia for the treatment of abrasion and in America for the healing of machete gashes (Allan *et al.*, 1984).

This biopolymer was reportedly first discovered by Rouget in 1859 (Muzzarelli, 1977) when he boiled chitin in a concentrated potassium hydroxide solution. Fundamental

research on chitosan started about a century later. In 1934, two patents, one for producing chitosan from chitin and the other for making films and fibers from chitosan, were obtained by Rigby (Rigby, 1934a; Rigby, 1934b). In the same year, the first X-ray pattern of a well-oriented fiber made from chitosan was published by Clark and Smith (Clark *et al.*, 1936).

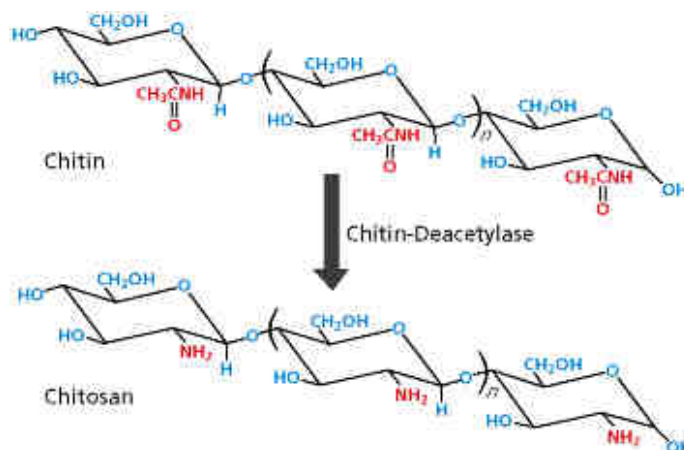


Figure 3.8 Structure of chitin and chitosan

The main driving force in the development of new applications for chitosan lies in the fact that polysaccharide is not only natural abundant, but it is also non-toxic and biodegradable. Unlike oil and coal, chitosan is a naturally regenerating resource (e.g. crab and shrimp shells) that can be further enhanced by artificial culturing.

One of the most useful properties of chitosan is for chelation. Chitosan can selectively bind desired materials, such as cholesterol, fats, metal ions, proteins and tumor cells. Chelation has been applied to the areas of food preparation, health care, water improvement and pharmaceuticals. Some properties that make chitosan very useful include inhibition of tumor cells (Sirca *et al.*, 1971), antifungal effects (Allan *et al.*, 1979), acceleration of wound healing (Balassa *et al.*, 1978; Malette *et al.*, 1986), stimulation of the immune system (Eida *et al.*, 1988; Suzuki *et al.*, 1984) and acceleration of plants germination (Yano *et al.*, 1988).

Moreover chitosan is a good cationic polymer for membrane formation. It was shown that membranes formed from this polymer could be exploited for water clarification, filtration, fruit coating, surgical dressing and controlled release (Li *et al.*, 1992).

The molecular weight of native chitin is usually larger than one million Da, while commercial chitosan products fall between 100,000 and 1,200,000 Da. Chitosan is insoluble in water, alkali, organic solvent, but soluble in most solutions of organic acids when the pH of the solution is less than 6 (Li *et al.*, 1992).

The industrial production and use of chitosan has been steadily increasing since 1970s. In Japan, for example, the production of chitosan increased 37% each year from 1978 to 1983. At the time, the major applications of chitosan were centred on sludge dewatering, food processing and metal-ion chelation. The present trend, in industrial applications, however, is toward producing high value products, such as cosmetic, drug carriers, feed additives, semipermeable membranes and pharmaceuticals (Li *et al.*, 1992). For example, chitosan is hemostatic and it is used as blood anticoagulants, in fact, chitosan bandages and sponges were prepared for surgical treatment and wound protection (Kibune *et al.*, 1988; Motosugi *et al.*, 1988).

Chitosan has many potential applications, also, in agriculture because the polymer is naturally occurring and biodegradable. One application that is widely employed is seed coating. It was found that chitosan coating of seeds have many beneficial effects, such as inhibition of fungal pathogens in the vicinity of the seeds and enhancement of plant resistant responses against diseases (Li *et al.*, 1992).

Owing to the high chelating and coagulating ability of chitosan, the polymer has been widely utilised in the food industry. In the case of beverages, this polymer was used to remove dyes from orange juices and to remove solids, β -carotene and acid substances from apple or carrot juices. In addition, chitosan was used to extend the preservation time of foods because of its antibiotic properties.

Chitosan is also used for colour stabilisation of fish-protein paste and increased firmness of cucumber pickles (Kuwahara *et al.*, 1988; Li *et al.*, 1992; Wakayama *et al.*, 1987).

As enzyme immobilization supports, chitin- and chitosan based materials are used in the form of powders, flakes and gels of different geometrical configurations. Chitin/chitosan powders and flakes are available as commercial products from Sigma-Aldrich and chitosan gel beads (Chitopearl) from Fuji Spinning Co. Ltd. (Tokyo, Japan). Otherwise, the chitinous supports are laboratory manufactured. Preparation of chitosan gels is promoted by the fact that chitosan dissolves readily in dilute solutions

of most organic acids, including formic, acetic, tartaric and citric acids, to form viscous solutions that precipitate upon an increase in pH and by formation of water-insoluble ionotropic complexes with anionic polyelectrolytes. In this way, chitosan gels in the form of beads, membranes, coatings, capsules, fibres, hollow-fibers and sponges, can be manufactured. Commonly, different follow-up treatments and modifications are applied to improve gel stability and durability. The methods of chitosan gel preparation described in the literature can be broadly divided into four groups: solvent evaporation method, neutralization method, crosslinking method and ionotropic gelation method (Dutta *et al.*, 2002; Felt *et al.*, 1998; Hudson *et al.*, 1998; Krajewska, 2004; Paul *et al.*, 2000).

3.5.2.1. Solvent evaporation method

The method is mainly used for the preparation of membranes and films. A solution of chitosan in organic acid is cast onto a plate or an electrode tip and allowed to dry, if possible at temperature of 65°C. Upon drying, the membrane/film is normally neutralized with a dilute NaOH solution and crosslinked to avoid disintegration in solutions of pH < 6.5.

Enzymes may be immobilized on such prepared membranes either on their surfaces by adsorption, frequently followed by cross-linking (reticulation), or covalent binding, commonly preceded by chemical activation of the surface, or included into chitosan solution to achieve inclusion (Krajewska, 2004).

3.5.2.2. Neutralization method

If an acidic chitosan solution is mixed with alkali, an increase in pH results in precipitation of solid chitosan. This method is exploited to produce chitosan precipitates, membranes, fibers, but foremost spherical beads of different sizes and porosities. These are obtained by adding a chitosan solution drop wise to a solution of NaOH, most frequently prepared in water-ethanol mixtures, where ethanol, being a non-solvent for chitosan, facilitates the solidification of chitosan beads. Following the preparation, the beads are commonly subjected to crosslinking. Enzyme immobilization, similar to the solvent evaporation method, is achieved by binding onto the gel surface

by adsorption, reticulation or covalent binding, or by inclusion if the enzyme is dissolved in the initial chitosan solution (Krajewska, 2004).

3.5.2.3. Cross-linking method

In this method an acidic chitosan solution is subjected to straightforward cross-linking by mixing with a cross-linking agent, which results in gelling.

Gels obtained in bulk solution are later crushed into particles. The enzyme may also be entrapped in the gel if mixed with chitosan prior to cross-linking. Glutaraldehyde is largely used as a cross-linking and surface activating agent. This is due to its reliability and ease of use, but more importantly, due to the availability of amino groups for the reaction with glutaraldehyde not only on enzymes, but also on chitosan (Krajewska, 2004).

3.5.2.4. Ionotropic gelation method (or coacervation)

By virtue of the attraction of oppositely-charged molecules, chitosan, owing to its cationic polyelectrolyte nature, spontaneously forms water-insoluble complexes with anionic polyelectrolytes (Dumitriu *et al.*, 1998; Kubota *et al.*, 1998; Singla *et al.*, 2001). The anionic polyelectrolytes used include alginate, carrageenan, xanthan, various polyphosphates and organic sulfates or enzymes themselves (Zhang *et al.*, 2003). The method is chiefly utilized for preparing of gel beads, these being obtained by adding an anionic polyelectrolyte solution dropwise into an acidic chitosan solution. Enzyme immobilization is achieved here by preparing an enzyme-containing anionic polyelectrolyte solution prior to gelation. The enzyme is immobilized by inclusion in the interior of the beads/capsules.

An overview of enzymes immobilized on chitin- and chitosan-based materials utilised in food industry are reported in Table 3.6. It implies that there is vivid interest in utilizing chitin-based materials, predominantly chitosan, as a promising enzyme immobilization support for a multiplicity of applications ranging from wine, sugar and fish industries (Krajewska, 2004).

Table 3.6 *most common enzymes immobilised onto chitosan based material used in food industry (Krajewska, 2004)*

Enzyme	Application	Support (preparation method)	Immobilization
Acid phosphatase	Hydrophobic interaction chromatography	Mercapto-chitin powder Chitosan beads Chitosan precipitate	I III, IV III
Alkaline phosphatase	Hydrophobic interaction chromatography Molecular cloning	Chitosan precipitate Chitosan beads	III III
Alcohol oxidase	Determination of ethanol	Chitosan beads	III
Aminoacylase	Production of phenylalanine	Chitosan-coated alginate beads	V ($\geq 100\%$)
α -Amylase	.Hydrolysis of starch for glucose syrup and for BOD analysis in waters .Hydrophobic interaction chromatograph	Chitin powder Chitosan beads Chitosan precipitate Chitosan microbeads	III (38%) I III V
β -Amylase	Production of high maltose syrup from starch	Chitosan beads	I
α -L-Arabinofuranosidase	Aromatization of musts, alcoholic beverages and fruit juices	Chitosan powder Glyceryl-chitosan powder Chitosan particles Chitosan beads Chitosan coated alginate beads	I, II (3,2%), III II V IV V
Catalase	Removal of H_2O_2 from food treatment of hyperoxaluria	Chitosan powder Chitosan film Chitosan membrane Chitosan-organosilane particles Chitosan beads	I, IV, II V III I V
Cellulase	Decrease in viscosity of fruit/vegetable juices; affinity chromatography	Chitin powder Chitosan beads Chitosan solution	IV (15%) I Protectitive additive
α -Galactosidase	Raffinose hydrolysis in beet molasses Blood group specificity fabry disease	Chitosan powder	IV (67%)
β -Galactosidase	Hydrolysis of lactose	Chitin powder	III
Glucoamylase	Hydrolysis of starch (ethanol productin)	Chitin powder Chitosan magnetic beads Chitosan powder Chitosan beads	III I I I, III
β -glucosidase	Wine making and juice processing Hydrophobic interaction chromatography	Chitosan powder Chitosan particles Chitosan flakes Chitosan solution Chitosan beads Chitosan precipitate Chitosan magnetic beads	III, II (29%) V III (60%) I (90%) III, IV III I

Glutamate dehydrogenase	Glutamate determination (food industry and medicine)	Chitosan membrane Succinyl-, glutaryl-, phtalyl-chitosan membranes	III IV
β -Glycosidase	Cellobiose hydrolysis for glucose production	Chitosan powder Chitosan precipitate	II II
Lysozyme	Affinity membrane chromatography Cheesemaking	Microporous chitin membrane Chitosan powder PHEMA-chitin membrane microporous chitin membrane	I I (10%) I
Pectin lyase	Reduction of fruit/vegetable juices' viscosity	Chitin powder	III (26%)
Urease	Artificial kidney Urea biosensor Treatment of fertilizer effluents Removal of urea from beverages and food	Chitosan-triphosphate beads Chitosan beads Chitosan membrane Chitosan-PVA capsules Chitosan-PGMA precipitate Chitosan-coated alginate beads Chitosan –organosilane particles	III (64%) III (100%) I, II, III (94%) V I (82%) V I

3.6 Immobilised acid urease kinetic model

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 (Fidaleo *et al.*, 2006). In these circumstances, the urea degradation rate referred to the unit volume of immobilized acid urease (r_{Si}) may be expressed using the following equation:

$$r_{Si} = k_{li} S \quad (3.21)$$

with

$$k_{li} = k'_{li} \rho_B Y_{E/B} \quad (3.22)$$

where k_{li} is the urea degradation pseudo-first order kinetic rate constant of the biocatalyst of concern (expressed in h^{-1}), $Y_{E/B}$ the *enzyme loading* expressed as kg enzyme per kg dry (ds) or wet (ws) support, ρ_B the biocatalyst density ($kg\ ds\ or\ ws\ m^{-3}$) and k'_{li} the specific pseudo-first order kinetic rate constant relative to immobilised enzyme [expressed in $m^3(kg\ enzyme)^{-1} h^{-1}$].

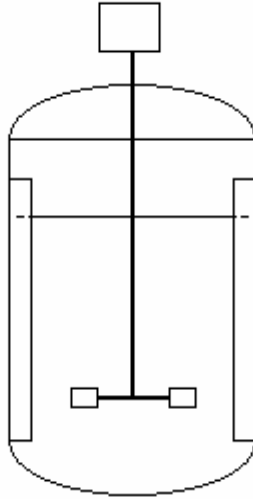


Figure 3.9 Schematic diagram of a stirred bioreactor.

When using a perfectly mixed bioreactor (Fig. 3.9), charged with a volume (V_L) of a buffer solution at an initial concentration of urea S_{L0} and inoculated with a prefixed concentration (c_{BW}) of wet 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 Fig. 3.10.

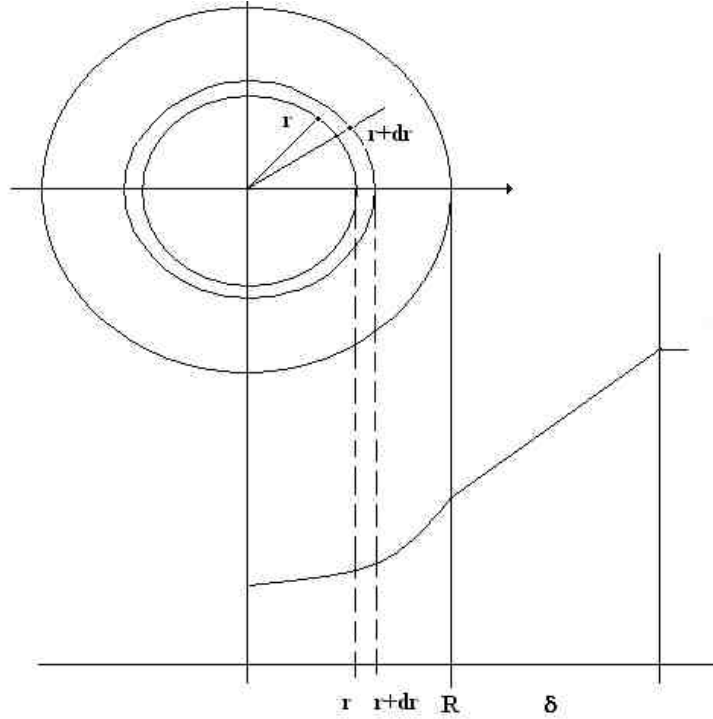


Figure 3.10 Substrate concentration profile within the spherical biocatalyst of average radius R and liquid film adhering to the external surface of the biocatalyst itself.

In the circumstances, the overall surface (a_s) and volume (v_s) for the biocatalyst per unit volume of liquid phase may be estimated as (Andrich *et al.*, 2009):

$$a_s = a_p c_B / \rho_B \quad (3.23)$$

$$v_s = c_B / \rho_B \quad (3.24)$$

The unsteady-state material balance for the reagent S may be written as follows:

$$S_L V_L \Big|_t = S_L V_L \Big|_{t+dt} + k_L a_s V_L (S_L - S_R) dt \quad (3.25)$$

with the boundary condition:

$$k_L a_s V_L (S_L - S_R) = \Omega (k_{ii} S_L) v_s V_L \quad (3.26)$$

with

$$\Omega = \frac{\eta}{1 + \frac{\eta \Phi}{3 Bi}} \quad (3.27)$$

$$\eta = \frac{3}{\Phi} \left(\frac{1}{\tanh(\Phi)} - \frac{1}{\Phi} \right) \quad (3.28)$$

$$\Phi = R \sqrt{\frac{k_{Li}}{D_{Se}}} \quad (3.29)$$

$$Bi = \frac{k_L R}{D_{Se}} \quad (3.30)$$

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, and S_R is the reagent concentration at the biocatalyst surface (Bailey and Ollis 1986). Eq.(3.25) can be converted into the following first-order different equation:

$$-\frac{dS_L}{dt} = k_L a_S (S_L - S_R) = \Omega v_S k_{Li} S_L \quad (3.31)$$

and integrated with the following initial conditions:

$$S_L = S_{L0} \text{ for } t = 0. \quad (3.32)$$

By introducing Eq.s (3.22) and (3.24) into the right-hand side of Eq. (3.32) and integrating by variable separation, the following can be obtained:

$$\ln\left(\frac{S_L}{S_{L0}}\right) = \int_{S_{L0}}^{S_L} \frac{dS_L}{S_L} = - \int_0^t \Omega c_B Y_{E/B} k'_{Li} dt \quad (3.33)$$

Finally, by referring to reaction (2.1) and Eq.(3.33), it would be possible to evaluate the instantaneous concentration of the ammonium ions in the liquid bulk:

$$A_L = A_{L0} + 2 (S_{L0} - S_L) \quad (3.34)$$

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

The basic relationships useful to estimate the effectiveness factor for a spherical biocatalyst in the presence (Ω) or absence (η) of the external film transport resistance are reassumed in the Table 3.7, extracted from Bailey and Ollis (1986).

Table 3.7 Basic relationships useful to estimate the effectiveness factors for a spherical biocatalyst in the presence (Ω) or absence (η) of the external film transport resistance and Thiele modulus (Φ) for pseudo-first order kinetics as functions of well known dimensionless numbers, as extracted from Bailey and Ollis (1986).

Parameter	Equation
Overall Effectiveness Factor	$\Omega = \frac{\eta}{1 + \frac{\eta \Phi}{3 \text{Bi}}} 2$
Biocatalyst Effectiveness Factor	$\eta = \frac{3}{\Phi} \left(\frac{1}{\tanh(\Phi)} - \frac{1}{\Phi} \right)$
Thiele modulus	$\Phi = R \sqrt{\frac{k_{\text{Li}}}{D_{\text{Se}}}}$
Biot number	$\text{Bi} = \frac{k_{\text{L}} R}{D_{\text{Se}}}$
Grashof number	$\text{Gr} = \frac{d_{\text{p}}^3 \rho_{\text{L}} \rho_{\text{L}} - \rho_{\text{B}} g}{\eta_{\text{L}}^2}$
Schmidt number	$\text{Sc} = \frac{\eta_{\text{L}}}{\rho_{\text{L}} D_{\text{S}}}$
Sherwood number	$\text{Sh} = \frac{d_{\text{p}} k_{\text{L}}}{D_{\text{S}}} = 2 + 0.31 (\text{Sc Gr})^{1/3}$

Chapter 4

Materials and Methods

4.1 Raw materials

4.1.1 Nagapsin preparation

Three lots #1 (ref. no. 2735159), #2 (ref. no. 3707118) and #3 (ref. no. 5870734) of the commercial preparation Nagapsin[®] were donated by Nagase Europa GmbH (Duesseldorf, Germany) and used here.

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. Their average specific activities were 766±7 IU g⁻¹, 420±27 IU g⁻¹ and 647±54 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).

4.1.2 Supports

During this PhD thesis, two kind of supports were used, that is Eupergit[®] C 250 L and Chitopearl. Their main characteristics are listed in Table 4.1.

4.1.2.1 Eupergit[®] C 250 L

Eupergit[®] C is an acrylic polymer with about a spherical form and an average particle size of 180 μm. It was kindly provided by Röhm GmbH (Darmstadt, Germany).

4.1.2.2. Chitopearl

Different chitosan-derivative beads were used, such as Chitopearl BCW-3001 (Wako cat. #301-02061), BCW-3003 (#308-02071), and BCW-3001 (#302-02091), all carrying primary amine groups and a six-carbon linear alkyl spacer, and Chitopearl HP-3020 (#300-04231), carrying primary, secondary and quaternary amine groups. They were obtained from Wako Chemicals GmbH (Neuss, Germany).

Table 4.1 *Main characteristics of the porous matrices used in this work.*

Parameter	Eupergit® C250L	BCW-	BCW-	BCW-	HP-3020	Unit
		3001	3003	3010		
Manufacturer	Röhm GmbH (Darmstadt, D)	Wako Chemicals GmbH (Neuss, D)				
Bead size	180	74-210	177-420	1190	840- 1,900-2,500	µm
Average bead diameter (d _p)	180	142	298.5	1,015	2,200	µm
Specific surface area(a _p)	66667	42254	20101	5911	2727	m ² /m ³
Water fraction (x _{BW})	3.4±0.2	84.6±0.4	91.0±0.6	86±1	80±1	% w/w

4.1.3 Real wine assayed

Two commercial Italian white wines, produced in the Umbria region of Italy by *Sauvignon Blanc* or *Grechetto* grapes (2008 vintage) in Monrubio winery (Monterubiaglio di Castel Viscardo, Terni, Italy), were used.

4.2 Solution preparations

The catalytic activity of the free enzymatic preparation (Nagapsin), as well enzymes immobilised onto Eupergit® C 250L or Chitopearls, was determined in a standard reaction mixture (SRM), a model wine solution as such or enriched with different concentrations of grape seed tannins, and two real wines.

The buffers used in this work were 0.1 kmol m⁻³ sodium-acetate buffer pH 4.0 (KAB4) and 0.05 kmol m⁻³ sodium-acetate buffer pH 5.0 (KAB5). They were prepared by diluting acetic acid in 800 cm³ of deionised water at the above concentration. After addition of 1 kmol m⁻³ NaOH to adjust the pH to 4.0 or 5.0, the volume of the mixture was set to 1 dm³ using deionised water.

The standard reaction mixture (SRM) consisted of KAB4, enriched with urea (83.33 mol m⁻³).

The 0.05 M potassium phosphate buffer pH 7 (KBP7) was prepared dissolving 6.81 g of KH₂PO₄ in 291 cm³ of 0.1 M NaOH and made up to 1 dm³.

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 used by Fidaleo *et al.* (2006). 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 metabisulphite (0.2 kg m^{-3}), and ethanol (13% v/v) in deionised water and then adjusting the resulting pH to 3.50. All reagents were of analytical grade.

An extract of grape seed (Grap'tanPC), manufactured by Ferco Oenologie (Saint Montan, France), was used as a source of phenolic compounds. Its moisture and total phenolics contents were found to be $2.3 \pm 0.3\%$ (w/w) and $0.62 \pm 0.01 \text{ g of GAE per g of extract}$, respectively.

4.3 Analytical Methods

4.3.1 Ammonium ion

The colorimetric kit Spectroquant® 14752 Ammonium (Merck, Whitehouse Station, N.J., U.S.A) was used. This kit uses the Berthelot reaction to determine the ammonium content in aqueous solutions in the range of $0.06\text{--}3.86 \text{ g}^{-3}$.

An alkaline reagent ($\text{NH}_4\text{-1B}$) ($600 \mu\text{l}$) is added to the sample to correct the pH to 12.5 and thus convert the ammonia ion into ammonia. By adding a powder colorant reagent ($\text{NH}_4\text{-2B}$), containing sodium hypochlorite (NaClO), ammonia reacts with sodium hypochlorite, thus forming monochloramine:



After 5 min another reagent ($\text{NH}_4\text{-3B}$) containing nitroprussiate and thymol is added to favour the formation of indophenol. In alkaline environment the latter can be detected in the visible spectrum with a peak of absorbance at 690 nm. This reaction is completed in 5 min and it is stable for 50 min.

Calibration curve

Several standard solutions were prepared by diluting a mother solution containing ammonium sulphate, $(\text{NH}_4)_2\text{SO}_4$, at about 1 kg m^{-3} . By plotting the optical density (OD) at 690 nm against the NH_4^+ concentration $[\text{NH}_4^+]$, an almost linear relationship is obtained. The calibration curve shown in Fig. 4.1 was used to assess the ammonium ion content of any sample under testing.

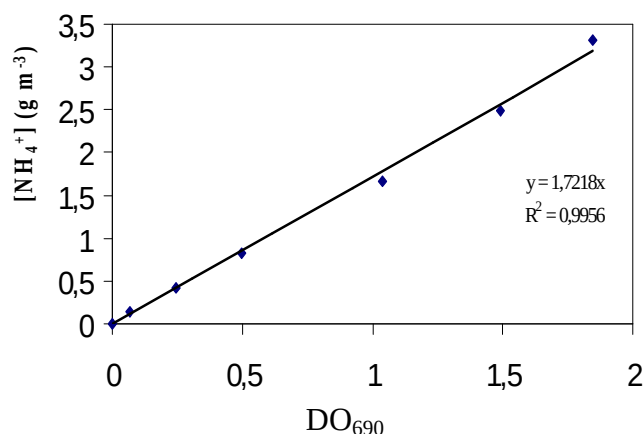


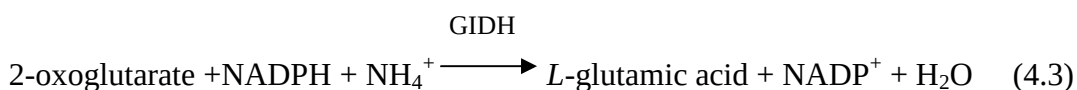
Figure 4.1 Ammonium ion calibration curve by using Spectroquant® 14752 Ammonium kit

4.3.2 Urea

Urea was determined using the enzymatic kit K-URAMR (Megazyme International Ireland Ltd., Wicklow, Ireland), that retorts to urease to hydrolyse urea to ammonia (NH₃) and carbon dioxide (CO₂):



In the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), ammonium ion (NH₄⁺) reacts with 2-oxoglutarate to form *L*-glutamic acid and NADP⁺:



For each mole of urea reacted, two moles of NADPH are thus consumed, this consumption being measured by a decrease in the absorbance at 340 nm.

The smallest detectable difference in DO for this assay is 0.005 absorbance units, this corresponding to 0.018 g m⁻³ of ammonia or 0.031 g m⁻³ for urea. The maximum detection limit is 0.071 g m⁻³ of ammonia and 0.1258 g m⁻³ for urea.

4.3.3 Raw protein

Total protein content was assessed according to the method of Lowry *et al.* 1951 using the Total Protein Micro Lowry Kit (Onishi & Barr modification, Sigma, St. Louis, MO).

The Lowry procedure is limited by poor stability of combined reagents, non-reproducibility of colour, especially at low protein concentrations owing to a non-linear chromogenic response with protein concentration (Lowry *et al.*, 1951). For all these reasons, Onishi and Barr modified the Biuret reagent, thereby simplifying it, while improving the stability of the combined reagent (Ohnishi *et al.*, 1978).

Lowry procedure is based on the reaction between two specific amino acids (*i.e.*, tryptophan and tyrosine) and Folin and Ciocalteu's phenol reagent.

This procedure consists of two steps. In the first one, the Biuret reagent, mainly containing 0.75 mM cupric sulphate and 94 mM sodium hydroxide, is added to the sample to adjust the sample pH in the alkaline region. In the second step, the addition of Folin and Ciocalteu's phenol reagent binds the above aminoacids present in the sample, thus turning the alkaline solution in blue. This colour change can be photometrically detected in the visible spectrum at wavelengths ranging from 550 to 750 nm, the maximum absorbance being observed at 700-750 nm.

Because the method is very sensitive, the sample is to be diluted to a final protein concentration in the range of 0.25-1 kg m⁻³. Protein concentration is determined via a calibration curve.

Calibration curve

A protein standard solution, containing 100 kg m⁻³ of bovine serum albumin (BSA) in 0.85% sodium chloride solution with 0.05% sodium azide as preservative, is provided with the Total Protein Micro Lowry kit. Such a standard solution is generally diluted with a solution obtained by dissolving 0.85 g of sodium chloride in 100 cm³ of KAB5 or KBP7, so as to vary the BSA content from 0 to 1000 g m⁻³ with a step of 250 g m⁻³.

Fig.4.2 shows the average calibration curve obtained in this work.

4.4 Procedures and operating conditions

4.4.1 Enzyme immobilisation on Eupergit[®] 250 L

Enzyme immobilisation on Eupergit[®] C 250 L was carried out in accordance with the conventional method, that involves the direct enzyme binding on polymers via oxirane groups (Knezevic *et al.*, 2006).

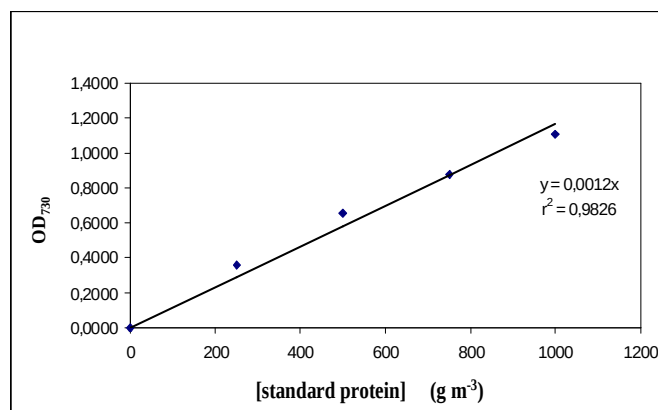


Figure 4.2 Total protein calibration curve to assess the total protein content by using the Micro Lowry kit.

In the first procedure, unmodified Eupergit C 250 L beads, 25 mg in each test, were charged in 10 cm³ rubber-capped flasks and soaked in 2 cm³ of KPB7 at 4°C for 24 h before immobilization. Then, 3 cm³ of an immobilisation solution containing different concentration of Nagapsin (4-12 kg m⁻³) were added. After gentle shaking (*i.e.*, 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 portion of the biocatalyst was 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. This solution was prepared by dissolving about 0.5 g of glycine in 0.1 kg of deionised water (Oliveira *et al.*, 2001). Alternatively, another portion was soaked in KPB7 enriched with 5 kg m⁻³ of BSA at room temperature for 24 h, as suggested by Torres-Bacete *et al.*, (2000).

Once had stabilised with glycine or BSA, the beads were recovered by centrifugation, washed with KPB7, and newly centrifuged before assessing their activity 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.

Another procedure was used to prepare adequate amounts of biocatalyst for kinetic studies. To this end, 0.6-1.2 g of dry beads and 30-35 cm³ of KPB7 at 20°C were charged into a 150 cm³ 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 centre 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 and pre-conditioned at 20°C , were added while continuing to mix the dispersion.

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 (Oliveira *et al.*, 2001), washed with KPB7, and stored at 4°C in the wet state.

4.4.2 Enzyme immobilisation on Chitopearls

The enzymatic preparation Nagapsin was immobilised onto Chitopearls BCW-3001, BCW-3003, BCW-3010, and HP-3020.

For Chitopearl BCW-3001, two different immobilisation methods, that is the absorption cross-linkage (method A) and covalent binding (method B), both described by Matsumoto (Matsumoto, 1993), were slightly modified and tested.

About 200 milligrams of any of the above wet supports were washed with distilled water (50 cm^3), collected by vacuum filtration using a glass filter (1.2- μm Whatman GF/C disc) and rinsed with 50 cm^3 of KAB5. According to the method A, the wet beads were charged in a 50- cm^3 rubber-capped tube and soaked in 14 cm^3 of KAB5 enriched with $\sim 24 \text{ kg m}^{-3}$ of the enzyme preparation. Each tube was horizontally positioned in a rotary shaker and kept rotating at 100 rev min^{-1} and 20°C for 48 h. After incubation, the beads were decanted, thus allowing most of the clear immobilising solution to be removed using a pipette; diluted with deionised water (10-15 cm^3); collected by vacuum filtration as reported above; and rinsed twice with 50 cm^3 of KAB5. The enzyme absorbed on such beads was immediately cross-linked with different volumes of the 2.5% (w/v) glutaraldehyde solution [obtained by diluting 1:10 the glutaraldehyde solution at 25% (w/v) with KAB5], so as to vary the GA-to-chitosan bead ratio ($Y_{\text{GA/CHI}}$) from 0 to 3 g g^{-1} . By shaking gently 3 times an hour, any dispersion was left stand at 20°C for 2 h. The GA-treated beads were thoroughly washed 3 times with deionised water (10 cm^3) to eliminate the excess of GA, and finally filtered as previously detailed.

According to the method B, the Chitopearls BCW-3001 were firstly activated with GA by suspension into different volumes of the 2.5% (w/v) GA solution in KAB5 ($Y_{GA/CHI}$ in the range of 0-3 g g⁻¹) at 20°C for 2 h. The GA-activated beads were thoroughly washed 3 times with deionised water (10 cm³), filtered as reported above, and transferred to 14 cm³ of KAB5 containing ~24 kg m⁻³ 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 recovered by using the same procedure of method A. For all the other tests, the biocatalysts were prepared according to method B, as reported below.

About 1 g of any Chitopearl was washed with distilled water (50 cm³), collected by vacuum filtration using a glass filter (1.2-µm Whatman GF/C disc) and rinsed with 50 cm³ of KAB5.

After that, the wet supports were charged in 150-cm³ rubber-capped flask and activated with GA by suspending them into 30 cm³ of the 2.5% (w/v) GA solution in KAB5 ($Y_{GA/CHI} = 0.625$ g g⁻¹) at 20°C for 2 h. The GA-activated beads were washed as reported before, and transferred to 70 cm³ of KAB5 containing ~24 kg m⁻³ 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 recovered by using the same aforementioned procedure.

4.4.3 Bound enzyme assessment

The protein concentration in all solutions was determined according to the method by Lowry *et al.*, (1951) using the Total Protein Kit (Sigma, Saint Louis, Missouri, USA).

The amount of bound protein (m_{pb}) was indirectly assessed by subtracting the amount of protein in the filtrate and washing solutions ($c_{pf} V_f$) from the amount of protein present in the immobilising solution ($m_{p0}=c_{p0} V_0$):

$$m_{pb} = (c_{p0} V_0) - (c_{pf} V_f) \quad (4.4)$$

where V_0 and V_f are the volumes of the immobilising solutions and filtrate and washing solutions, respectively; c_{p0} and c_{pf} the protein concentrations in the corresponding solutions.

The *protein loading* was defined as the amount of bound protein (m_{pb}) per g of wet support (m_{Bw}) and calculated as follows:

$$Y_{P/B} = m_{Pb}/m_{Bw} \quad \text{g bound protein/g wet support} \quad (4.5)$$

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

4.4.4 Enzyme activity assays

The acid urease activity in the immobilising solution (A_{E0}), filtrate (A_{Ef}), or immobilised enzyme (A_{BEI}) per unit mass of protein was estimated as follows.

For immobilising and filtrate solutions, the activity assay was assessed adding the following liquids in sequence: 5.65 cm³ of KAB4, 5 cm³ of the same buffer containing 11 kg m⁻³ of urea, and 0.35 cm³ of the sample to be tested, in a 25-cm³ beaker containing a 10-mm magnetic stirrer.

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 immobilising solution was estimated by dividing the ammonia formation rate (r_{A0}) by its corresponding enzymatic content (c_{p0}):

$$A_{E0} = r_{A0}/c_{p0} \quad \text{IU/g}^{-1} \text{ BSAE} \quad (4.6)$$

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

$$A_{Ef} = r_{Af}/c_{pf} \quad \text{IU g}^{-1} \text{ BSAE} \quad (4.7)$$

The immobilised enzyme activity was assessed by using about 50 mg of biocatalyst and adding the following liquids in sequence: 12 cm³ of KAB4 and 10 cm³ of the same buffer containing 11 kg m⁻³ of urea, the assay was tested in a 50-cm³ beaker containing a 10-mm magnetic stirrer.

The resulting reaction mixture was agitated at 400 rev min⁻¹ and incubated in a water bath at 20°C for 10 min.

Whatever the enzyme activity test carried out, a 200-μl sample was withdrawn from any beaker after 10 min, and quickly submitted to the Colorimetric kit Spectroquant[®] 14752 Ammonium.

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

$$A_{Bi} = r_{AB}/m_{Bd} \quad \text{IU g}^{-1} \text{ dry support} \quad (4.8)$$

$$A_{Bi} = r_{AB}/m_{Bw} \quad \text{IU g}^{-1} \text{ wet support} \quad (4.9)$$

$$A_{BEi} = A_{Bi}/Y_{P/B} \quad \text{IU g}^{-1} \text{ bound protein} \quad (4.10)$$

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

$$\zeta_E = m_{pb}/m_{p0} \quad (4.11)$$

$$\zeta_A = A_{BEi}/A_{E0} \quad (4.12)$$

4.4.5 Enzyme storage stability

All the biocatalysts prepared in this work were stored in the wet form, that is in KPB7 enriched with 2% (v/v) isopropanol and 0.5 kg m⁻³ ethyl parabene, at 4°C. Their storage stability was assessed by repeating the immobilised enzyme activity assay every two or three weeks for as long as 34 days for the Eupergit® C 250 L- based biocatalysts or 170 days for the chitosan-based ones.

4.4.6 Urea degradation kinetics by free or immobilised acid urease

To assess the time course of the hydrolytic process under study, 80 cm³ of the model wine solution, as such or enriched with Grap'tan® PC, or real wines were spiked with 60 g m⁻³ of urea and pre-conditioned at 20°C. Then, they were poured into a 100-cm³ rubber-capped flask, pre-charged with given amounts of free or immobilised acid urease. The flask was immersed in a water bath to keep the reaction temperature at 20±0.2°C, using a thermostat mod. F3 (Haake, Karlsruhe, Germany), and placed over a magnetic multistirrer mod. Multistirrer 15 (Velp Scientifica, Milan, Italy) to assure a constant stirring level of 100, 250 or 400 rev min⁻¹. Several samples (1 cm³) were withdrawn from any flask for time intervals of 7-150 h, and assayed for ammonium and urea using the K-URAMR kit (Megazyme International Ireland Ltd, Wicklow, Ireland). Any interference between the enzymatic kits and the phenolic compounds, present in real wines or grape-seed tannin enriched model wines, was limited by diluting 0.5 cm³ of any samples with an equal volume of an aqueous solution containing 1 kg of polyvinylpyrrolidone (PVP) m⁻³. 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 x g) for 3 min via a Centrifuge® 4204 (ALC Apparacchi per Laboratori Chimici, Milan, Italy). Such a procedure was newly

repeated, once 0.5 cm³ of the first supernatant had been collected. Finally, an aliquot (0.2 cm³) of the second supernatant was enzymatically 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 at zero tannin content (P=0) was also assessed by setting the initial concentration of Nagapsin to about 0.075, 0.10, 0.25, 0.3, 0.51, 0.76, 0.82 and 1.25 kg m⁻³, this being equivalent to an enzymatic protein content in the range of 3.8-47.2 g m⁻³ BSAE.

The kinetics of free acid urease in the real wines was also assessed by setting the initial concentration of Nagapsin[®] to about 1.2 kg m⁻³, this being equivalent to an enzymatic protein content of about 47.5 g m⁻³ BSAE.

On the contrary, the kinetics of immobilised biocatalysts in the basic model wine solution was assessed at three different levels, that is 3.8, 5.6 and 9.4 kg m⁻³ of wet carrier, for Eupergit based-biocatalysts. For chitosan based-biocatalysts, their concentration was varied in accordance with their corresponding protein loading, this being 1.25 or 3.13 kg m⁻³ of wet carrier so as to keep the immobilised enzymatic protein concentration (E_i) dispersed in the liquid phase about constant (47±3 g BSAE m⁻³) during any kinetic test.

Since the grape-seed tannins exerted a high inhibitory effect on free acid urease (Esti *et al.*, 2007), the sensitivity of acid urease immobilized on Eupergit[®] C250 L to such phenolic compounds was assessed by enriching the model wine solution with 5-1000 g m⁻³ of Grap'tan[®]PC, whereas the concentration of wet biocatalyst was constant at about 6.25 kg m⁻³.

To assess the inhibitory effect of tannins on the activity of acid urease immobilised onto Chitopearls, the above model wine solution was enriched with 302±2 or 602±3 g m⁻³ of Grap'tan[®]PC, whereas the biocatalysts concentration used coincided with that used in the trials aforementioned.

To estimate the kinetics of the Eupergit[®] C250 L-, BCW-3003- and BCW-3010-based biocatalysts in the real wines, the biocatalysts concentration was respectively set to 1.25, 3.13 or 3.53 kg m⁻³ of wet carrier, depending on their corresponding protein loading, to keep the immobilised enzymatic protein concentration (E_i) dispersed in the liquid phase about constant (47±3 g BSAE m⁻³).

4.5 Wine Analyses

The target wines were stored in 0.75 dm³ bottles at (10±1)°C in the dark before testing. The following analyses were carried out as reported below.

4.5.1 Alcohol content

It is determined by distilling any wine sample. Once the temperature of the wine sample had been regulated to 20°C, calcium carbonate and an antifoam agent were added. About 100 cm³ of the filtered sample were submitted to distillation so as to obtain about 75 cm³ of distillate. The latter was diluted to 100 cm³ by adding deionised water. Then, its density at 20°C was determined using a pycnometer. Its mass was compared to that of 100 cm³ of deionised water, so as to assess the alcohol titre (% w/w) and relative density of the distillate, as suggested by Amerine and Ough (1974).

4.5.2 pH

The pH of any wine sample was measured using a pHmeter.

4.5.3 Total acidity

It was determined by titrating 25 cm³ of wine, enriched with a few drops of bromothymol blue (SIGMA-ALDRICH, Saint Louis, Missouri, USA), with 0.1 N sodium hydroxide until to pH 7. Total acidity was expressed as kg m⁻³ of tartaric acid equivalent .

4.5.4 Volatile acidity

The volatile acidity was assessed by titrating the distillate of the wine sample under study with 0.1 N sodium hydroxide solution.

A sample of wine (10 cm³) was diluted to 250 cm³ by adding deionised water, then steam distilled up to recover 100 cm³ of distillate. The distillate was brought to boil for no more than 30 s and, after adding about 3 drops of 1% phenolphthalein solution (SIGMA-ALDRICH, Saint Louis, Missouri, USA), it was titrated to a faint pink endpoint using 0.1 N NaOH solution. The volatile acidity was expressed as kg m⁻³ acetic acid equivalent (Amerine & Ough, 1974).

4.5.5 Glycerol

Glycerol is a yeast metabolite and is generally determined via enzymatic method, consisting of three reactions.

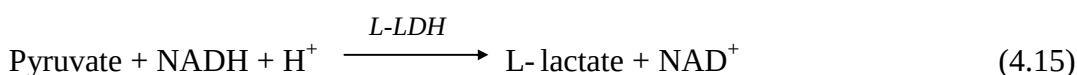
The first reaction is the phosphorylation of glycerol to glycerol-3-phosphate by adenosine5'-triphosphate (ATP), that is catalysed by glycerolkinase:



The adenosine-5'-diphosphate (ADP) is converted in presence of phosphoenolpyruvate (PEP) by pyruvatekinase into ATP and pyruvate:



In the presence of L-lactate dehydrogenase (L-LDH) and nicotinamide-adenine dinucleotide (NADH), pyruvate is reduced to L-lactate and NAD^+ :



The amount of NAD^+ is stoichiometrically related to the initial amount of glycerol. The decrease in the absorbance at 340 nm is proportional to NADH consumption.

4.5.6 Ash

Ash is the inorganic matter remaining after wine evaporation and incineration at 500-550 °C. During ashing, the cations are fixed as carbonates or other anhydrous mineral salts, while ammonium ion is lost.

A wine sample (25 cm^3) was transferred into a platinum dish, previously dried in an oven at 600°C for 10 min. The wine-sample is firstly evaporated in a water bath, and then in an oven at 100°C. After the dish had been weighed, it was moved into a muffle furnace at $525 \pm 25^\circ\text{C}$ for 5 min. After the dish had been removed from the furnace and cooled at room temperature, 5 cm^3 of distilled water were drop by drop added, this moisture being dried firstly in the oven and then in the muffle furnace for 15 min. After the dish had been cooled in a vacuum desiccator, the dish was reweighed (Amerine & Ough, 1974).

4.5.7 Overall SO₂

The total sulphur dioxide content of wines is generally determined by using the Ripper procedure. It is based upon the alkaline hydrolysis of acetaldehyde- α -hydroxy sulfonate and acidification.

A wine sample (20 cm³) and 25 cm³ of 1N NaOH were pipetted into a 250-cm³ beaker, mixed and then let stand for 10 min. Then, once sodium bicarbonate, 5 cm³ of a starch indicator solution and 10 cm³ of 1:3 sulphuric acid had been added, the mixture was rapidly titrated with 0.02 N iodine solution to a bluish end-point, this persisting no longer than 30 s (Amerine & Ough, 1974).

4.5.8 Total phenolics

Total phenolics content was assessed via the Folin-Ciocalteu method.

Any wine sample (1 cm³) and 60 cm³ of deionised water were pipetted into a 100- cm³ calibrated flask and mixed, before adding 5 cm³ of the Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, Missouri, USA). After no longer than 8 min, 15 cm³ of 20% sodium carbonate were added. After mixing, the mixture was diluted to 100 cm³ using distilled water. After the solution had been kept at room temperature for 30 min, its optical density (OD₇₀₀) was measured at 700 nm against a blank consisting of deionised water, 15 cm³ of 20% sodium carbonate solution and 5 cm³ of Folin-Ciocalteu reagent.

Calibration curve

The total phenolic content was expressed as kg m⁻³ of gallic acid equivalent (GAE). To this end, a series of standard solutions was prepared by dissolving prefixed amounts of gallic acid in aqueous solution containing 13% (v/v) ethanol, so as to vary the GAE concentration in the range 0-0.8 kg m⁻³. Each standard solution was assayed by using the Folin-Ciocalteu method, thus yielding an almost linear relationship between OD₇₀₀ and GAE content.

Fig.4.3 shows the average calibration curve used in this work.

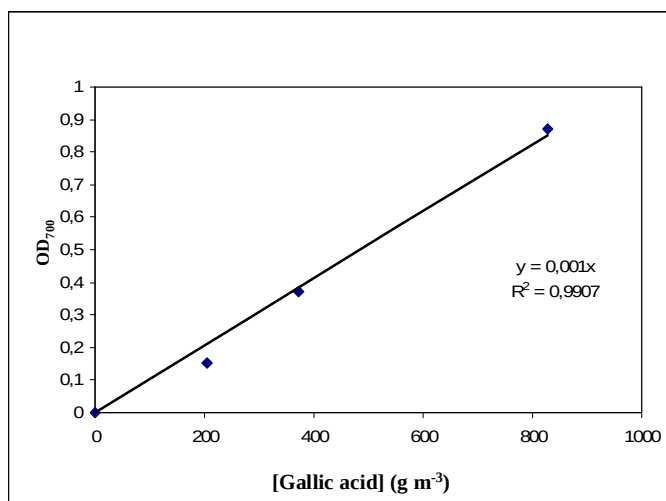


Figure 4.3 Calibration curve used to assess the total phenolic content by using the Folin-Ciocalteu method.

4.5.9 Total dried extract

A wine sample (50 cm³) was dried in a oven at 105°C until a constant mass had been reached.

This mass was used to express the total dried extract as kg m⁻³. By subtracting the residual sugars present in the wine sample, it was possible to estimate the total pure dried extract.

4.5.10 Organic acids and reducing sugars

The acidic wine composition was expressed by determining the concentrations of tartaric, *L*-malic, *L*-lactic, and citric acids and reducing sugars content by high-pressure liquid chromatography (HPLC-DAD) (Cane, 1990) 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 C₁₈, 250 x 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, (1990).

Chapter 5

Results and discussion

5.1 Immobilisation of acid urease

5.1.1 Enzyme immobilisation onto Eupergit® C 250 L

Two different lots #1 and #2 of Nagapsin were used. Table 5.1 shows the experimental conditions used to immobilise conventionally acid urease on Eupergit® and stabilise the biocatalyst with glycine, as well as the protein concentrations, expressed as Lowry protein, in the immobilising solution (c_{p0}) and overall filtrate (c_{pf}), protein bound on support (m_{pb}), protein loading ($Y_{p/B}$), immobilised 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 the lot#1 $Y_{p/B}$ (=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, i.e. lipase (39 mg g⁻¹; Knezevic *et al.*, 2006) or dextranucrase (23.1 mg g⁻¹; Gómez de Segura *et al.*, 2004). However, when using the lot#2, $Y_{p/B}$ declined to about 7 mg BSA equiv. g⁻¹ dry support. By trials and errors, it was possible to improve $Y_{p/B}$ to 22.5 mg g⁻¹ by increasing both the Nagapsin concentration in the immobilising solution and the immobilisation time (t_i) from 16 to 24 kg m⁻³ and from 24 to 48 h, respectively, as shown in Table 5.1. Under this circumstances, the enzyme (ζ_E) and activity (ζ_A) coupling yields did not differ, being approximately equal to 24 and 27%, respectively (see Table 5.1).

5.1.2 Enzyme immobilisation onto chitosan-based biocatalysts beads

To assess which of the two immobilisation methods A (absorption cross-linkage) and B (covalent binding), described in section 4.4.2, was the most appropriate for immobilising acid urease, the BCW-3001 chitosan-derivative beads, either after or before having been dispersed in the immobilising solution, were activated with glutaraldehyde (GA) by varying the GA-to-chitosan ratio ($Y_{GA/CHI}$) in the range of 0 to 3 g per g of wet support.

Fig. 5.1 shows the effect of $Y_{GA/CHI}$ on the specific activity (A_{Bi}) per unit mass of wet support (Chitopearl BCW-3001). Despite the absorption cross-linkage method (A) applied to immobilise acid urease on chitosan beads (BCW-3005 and BCW-3505) of 0.5 mm in diameter yielded biocatalysts with specific activities (A_{Bi} =150-170 IU g⁻¹ wet support) about double of that exhibited by the counterparts obtained with the covalent

method (B) , the main results shown in Fig. 5.1 disagreed with Matsumoto's finding (Matsumoto, 1993).

In this PhD work, when using the method A, A_{Bi} tended to decrease from about 300 to 230 IU g⁻¹ as $Y_{GA/CHI}$ was increased from 0 to 3 g g⁻¹, whereas when using the GA-cross-linking method (B) A_{Bi} reached a peak value (320±20 IU/g ws) at $Y_{GA/CHI}$ =0.625 g/g.

Such an optimal ratio is by far smaller than that (12.5 g g⁻¹) used by Chen *et al.*, (1999); however, it coincided with that pertaining to the covalent bounding of chloroperoxidase to aminopropyl-glass beads (Kadima *et al.*, 1990).

In all probability, the greater the aldehyde groups available on the beads, the greater the multiple-point attachments of enzyme molecules to the bead become, thus adversely influencing the enzyme activity. Even if recent studies seem to exclude mutagenic and cancerogenic effects of GA, such a finding suggested a safer GA level than that generally encountered under normal cross-linking operating conditions (Clair *et al.*, 2006; Zeiger *et al.*, 2005).

Hence, in agreement with other researchers (Chen *et al.*, 1999; Kayastha *et al.*, 2001; Kuma *et al.*, 2009), the covalent binding method (B) was regarded as more efficient and was used to immobilise acid urease onto all four the of chitosan spheres used here.

Table 5.1 *Experimental conditions and assessment of the enzyme and activity coupling yields of soluble acid urease as a result of direct binding to Eupergit® C 250 L beads followed by stabilisation with glycine (Andrich et al., 2009).*

Parameter	Symbol	Values Lot#1	Values 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
Immobilising solution volume	V_0	72	100	cm^3
Nagapsin concentr. in the immobilising solution	C_N	16.0	24.0	$kg\ m^{-3}$
Protein concentration in the immobilising solution	C_{P0}	881 ± 14	1058 ± 18	$g\ m^{-3}$ BSA equiv.
Initial protein mass	m_{P0}	63 ± 1	106 ± 2	mg BSA equiv.
Protein added per unit mass of support	$Y_{P/S}$	106	91	mg BSA equiv. g^{-1} support
Immobilising solution activity	A_{E0}	$13,918 \pm 119$	$9,632 \pm 957$	$IU\ g^{-1}$ BSA equiv.
Protein concentration in the filtrate	C_{Pf}	192 ± 9	319 ± 6	$g\ m^{-3}$ BSA equiv.
Filtrate volume	V_f	250	250	cm^3
Protein bound on support	m_{Pb}	15.5	26.1	mg BSA equiv.
Protein loading	$Y_{P/B}$	25.9	22.5	mg BSA equiv. g^{-1} support
Specific immobilised enzyme activity	A_{Bi}	99 ± 17	61 ± 3	$IU\ g^{-1}$ dry support
Immobilised enzyme specific activity	A_{BEi}	3816 ± 665	2713 ± 125	$IU\ g^{-1}$ BSA equiv.
Enzyme coupling yield	ζ_E	24.4	24.7	%
Enzyme activity coupling yield	ζ_A	27 ± 5	28 ± 1	%

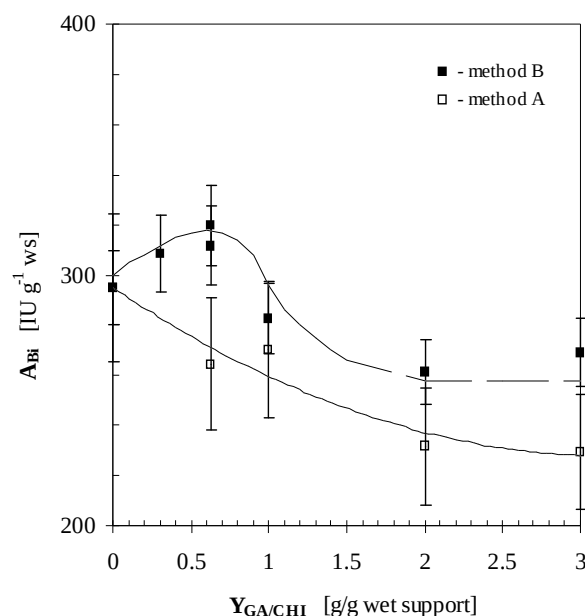


Figure 5.1 Effect of GA-to-chitosan ratio ($Y_{GA/CHI}$) on the specific activity (A_{Bi}) per unit mass of wet support (Chitopearl BCW-3001) depending of the immobilisation methods A and B described in Section 4.4.2.

5.1.3 Effect of bead size

By using the method B and setting $Y_{GA/CHI}$ at the above optimal value, acid urease was immobilised onto the four different chitosan-derivative beads, the main characteristics are shown in Table 4.1.

Table 5.2 shows the experimental conditions used, as well as the protein concentrations in the immobilising solution (C_{P0}) and overall filtrate (C_{Pf}), protein bound on support (m_{pb}), protein loading ($Y_{P/B}$), immobilised acid urease activity per unit mass of wet support (A_{Bi}) or bound protein (A_{BEi}), as well as enzyme (ζ_E) and activity (ζ_A) coupling yields.

It is worthy of note that the specific activity ($71\text{--}308 \text{ IU g}^{-1}$ wet support, ws) of the chitosan-based biocatalysts was by far greater than that ($19 \pm 3 \text{ IU g}^{-1}$ ws) of the biocatalysts obtained when using Eupergit® C 250 L (Andrich *et al.*, 2009), whereas the protein loading of the latter ($26 \text{ mg BSAE g}^{-1}$ ws) fell within the range of protein loading values ($17\text{--}40 \text{ mg BSA}_e \text{ g}^{-1}$ ws) detected for all the biocatalysts immobilised onto chitosan-derivatives beads.

Table 5.2 *Experimental conditions and specific activities per unit mass of wet support (ws) of acid urease immobilised on some GA-activated chitosan beads of different size, as compared to those pertaining to the enzyme immobilised on Eupergit® C 250 L.*

Parameter		Eupergit® C250L	Chitopearl BCW-3001	Chitopearl BCW-3003	Chitopearl BCW-3010	Chitopearl HP-3020	Unit
Mass of wet support	m_{Bw}	601.3	1200	1001.4	1200.4	1200.2	mg
Support soaking volume	V_S	35	0	0	0	0	cm ³
Immobilising solution volume	V_0	72	70	70	70	70	cm ³
Nagapsin® conc. in the immob. sol.	c_N	16	24	24	24	24	kg m ⁻³
Protein conc. in the immobilizing sol.	c_{P0}	881±14	886±17	895±17	885±4	893±7	g BSAE m ⁻³
Initial protein mass	m_{P0}	63±1	62±1	63±1	62.0±0.3	62.5±0.5	mg BSAE
Prot. added per unit mass of ws	$Y_{P/S}$	105.5	52	52	52	52	mg BSAE g ⁻¹ ws
Immobilising solution activity	A_0	13.9±0.1	17.3±0.1	15 ± 1	17.8 ± 0.1	19.8±0.4	IU mg ⁻¹ BSAE
Protein concentration in the filtrate	c_{Pf}	192±9	29.3±0.4	48 ± 1	48 ± 1	85 ± 3	g m ⁻³ BSAE
Filtrate volume	V_f	250	500	500	500	500	cm ³
Protein bound on support	m_{pb}	15.5	47.4	38.8	20.8	20.1	mg BSAE
Protein loading	$Y_{P/B}$	25.8	39.5	38.7	17.3	16.8	mg BSAE g ⁻¹ ws
Specific immobilised enz. Activity	A_{Bi}	19±3	308.2±0.1	260±3	151±16	71±1	IU g ⁻¹ ws
Immobilised enz. specific activity	A_{BEi}	3816±665	7805±3	6725±69	8729±907	4239±79	IU g ⁻¹ BSAE
Enzyme coupling yield	ζ_E	24.4	76.4	61.9	33.6	32.2	%
Enzyme activity coupling yield	ζ_A	27±5	45±0.1	43.9±0.5	49±5	21.4±0.4	%

In particular, as shown in Fig. 5.2, the specific activity (A_{Bw}) of the biocatalyst tended to decrease almost linearly with the logarithm of the bead average diameter (d_p). This result will help to optimise the geometry (diameter and height and particle size) of a prospective packed-bed bioreactor in order to maximise the urea conversion rate and simultaneously minimise the pressure drop across the packed column.

Moreover, the protein loading ($Y_{P/B}$) and enzyme coupling yield (ζ_E) were found to be of the order of 39 mg BSAE per g of wet support and 76% for the smaller chitosan derivative beads (BCW-3001 and BCW-3003), but reduced to about 17 mg BSAE g⁻¹ and 32 % for the larger ones (BCW-3010 and HP-3020). On the contrary, the activity coupling yield (ζ_A) was of about 45% for all the beads of the BCW series, but it dropped to 21% for the Chitopearl HP-3020. Approximately, the enzyme and activity coupling yields of the last biocatalysts ($d_p \sim 2,200 \mu\text{m}$) were quite similar to those (21.4 and 27%, respectively) of the Eupergit® C 250 L-based counterpart ($d_p \sim 180 \mu\text{m}$).

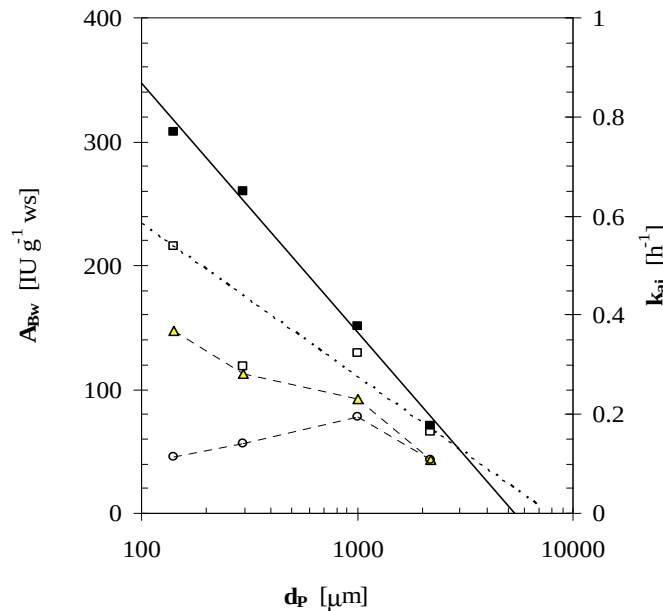


Figure 5.2 Effect of the average diameter (d_p) of the chitosan beads used to immobilise acid urease via the method B with $Y_{GA/CHI} = 0.625 \text{ g/g}$ on their specific activity (A_{Bi} ; $\blacksquare$) and apparent pseudo-first order kinetic rate constants (k_{ai}) at 20°C in the model wine solution as such ($\square$) or enriched of grape seed tannins ($\blacklozenge$, $P = 187 \pm 1 \text{ g GAE m}^{-3}$; $\circ$, $P = 374 \pm 2 \text{ g GAE m}^{-3}$).

5.2 Storage stability of immobilised acid urease

All biocatalysts were stored in the wet form at 4°C using a potassium phosphate buffer 0.05 M pH 7 (KBP7) enriched with 2% (v/v) isopropanol and 0.5 kg m⁻³ ethyl parabene, as suggested by the Eupergit[®] manufacturer.

5.2.1 Eupergit[®]-based biocatalysts

The storage stability of soluble acid urease immobilised in Eupergit[®] C 250 L, used as such or after stabilisation with glycine or BSA, was assessed for as long as 34 days, as reported in Fig. 5.3. Despite a fast partial inactivation in the first 10 days, the immobilised biocatalyst as such or stabilised with glycine tended to recover its initial activity, especially when using immobilising solutions containing as much as 8-12 kg m⁻³ of Nagapsin[®] (lot#1). Moreover, it was impossible to confirm the stabilising effectiveness of BSA observed in the covalent immobilisation of penicillin acylase from *Streptomyces lavendulae* on Eupergit C (Torres-Bacete *et al.* 2000).

When the Nagapsin[®] concentration in the immobilising solution was varied from 8 to 16 kg m⁻³, the immobilized biocatalyst stabilised 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 independently of the enzyme added per unit mass of support used, as shown in Fig. 5.4.

The initial abrupt inactivation might be a consequence of the heterogeneity of the immobilised biocatalyst with acid urease molecules differing in their orientation toward the bulk solvent (Gómez de Segura *et al.*, 2004).

5.2.2 Chitopearl-based biocatalysts

Fig. 5.5 shows the storage stability at 4°C of acid urease immobilised on GA-crosslinked chitosan beads, for as long as 170 days.

Similarly to the biocatalysts prepared by Matsumoto, (1993), that exhibited about a 6% loss in A_{BI} after preservation in 0.1 M acetate buffer (pH 5.5) at 5°C for 6 months, the biocatalysts obtained from Chitopearls of the BCW and HP series exhibited less than 5% loss in A_{BI} after preservation in the aforementioned buffer at 4°C for 150-170 days.

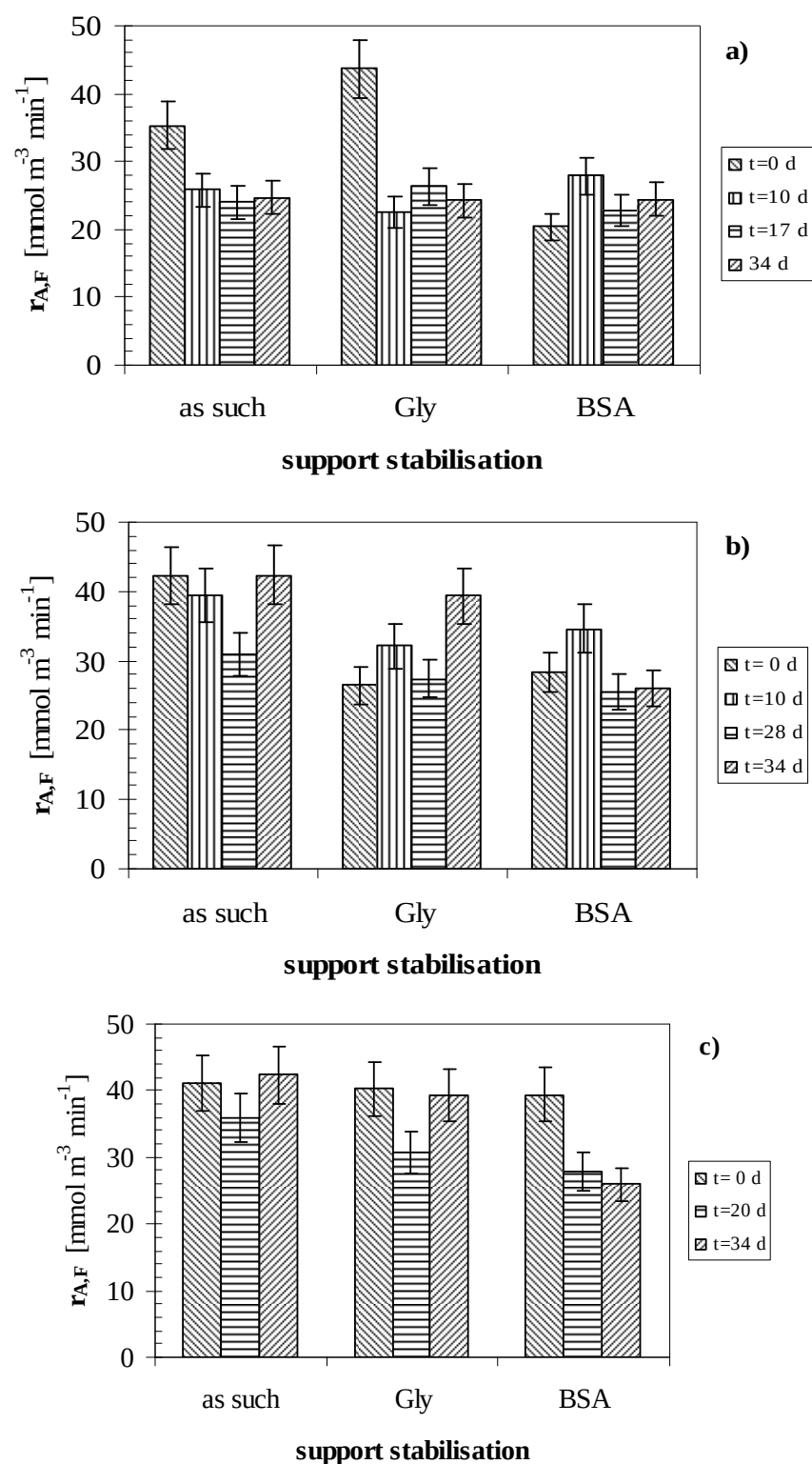


Figure 5.3 Storage stability at 4°C of soluble acid urease immobilised on 25-mg Eupergit® C 250 L using different immobilising solutions at 4 (a), 8 (b) and 12 (c) kg m⁻³ of Nagapsin® and used as such or after stabilisation with glycine (Gly) or bovine serum albumin (BSA): ammonia formation rate (r_{AB}) against time (t).

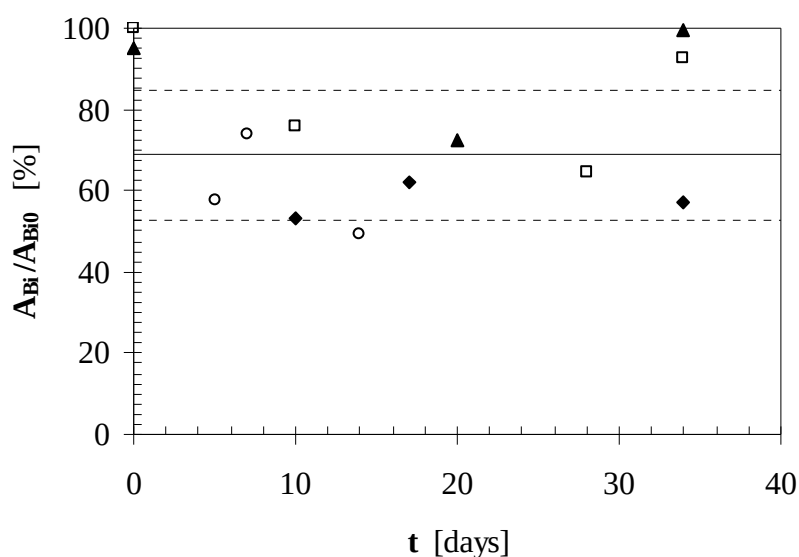


Figure 5.4 Effect of the concentration of Nagapsin® preparation (c_N : $\blacklozenge$, 4.0; $\square$, 8.0; $\blacktriangle$, 12.0; $\circ$, 16.0 kg m^{-3}) in the immobilising solution on the storage stability at 4°C of soluble acid urease immobilised on 25-mg Eupergit® C 250 L and stabilised with glycine: percentage specific biocatalyst activity (A_{Bi}/A_{Bi0}) versus storage time (t).

Only the specific activity of the biocatalysts prepared using the Chitopearl BCW-3001 tended to reduce by about 40% after 62-day storage. However, throughout the following 78 days its residual specific activity was found to be approximately constant ($181 \pm 16 \text{ IU g}^{-1}$). Nevertheless, it was greater than that of the biocatalysts obtained using Chitopearl BC-3010 ($133 \pm 18 \text{ IU g}^{-1}$) or HP-3020 ($58 \pm 4 \text{ IU g}^{-1}$) after 152 or 160 days.

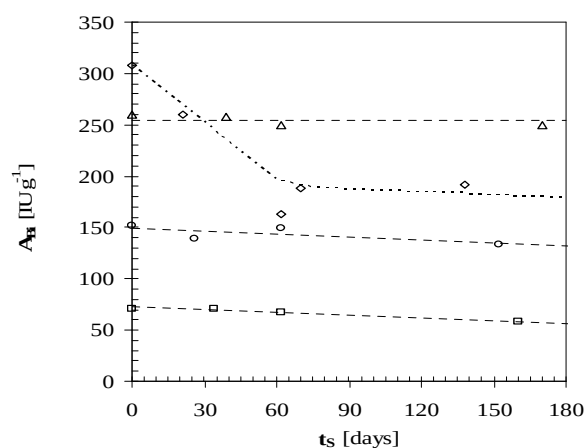


Figure 5.5 Storage stability of acid urease immobilised onto different GA-cross-linked chitosan beads (BCW-3001: $\diamond$; BCW-3003: $\square$; BCW-3010: $\circ$; HP-3020: $\triangle$); specific activity (A_{Bi}) against storage time (t_s).

5.3 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 to 820 g m⁻³, this being equivalent 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. 5.6.

It can be noted that the semilogarithmic plots of the dependent variable (S_L) against reaction time (t) were approximately linear, thus allowing the product (Ω c_B Y_{E/B} k'_{ii}) of Eq. (3.33) to be regarded as practically constant. Actually, in the case of free enzyme, the overall effectiveness factor (Ω) is intrinsically unitary, the product of c_B by Y_{E/B} coincides with the free enzyme concentration (E_f) dissolved in the liquid phase, hence, 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 enzyme concentration (E_f) (Fig. 5.6), 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:

$$k'_{if} = (8.5 \pm 0.3) \times 10^{-3} \text{ m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSA eq.} \quad (r^2=0.99) \quad (5.1)$$

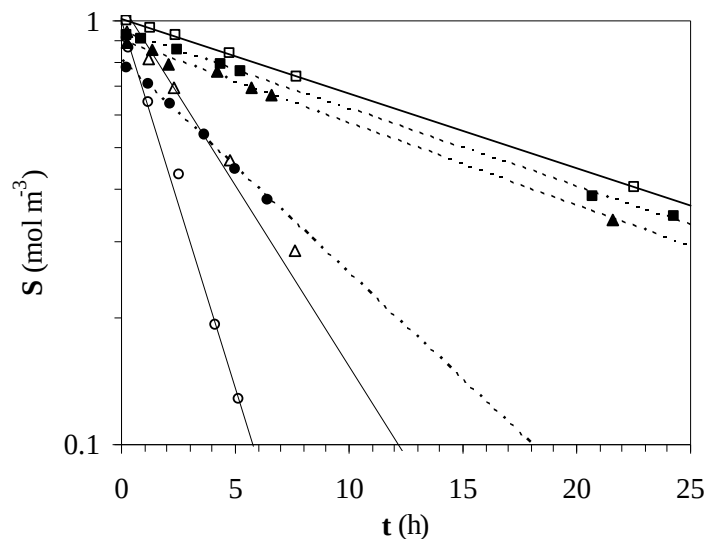


Figure 5.6 Time course of urea degradation at 20°C for a model wine solution when using different concentrations of free ($\square$, $c_N=75$; $\triangle$, $c_N=300$; $\circ$, $c_N=820 \text{ g m}^{-3}$ Nagapsin®) or immobilised onto Eupergit® ($\blacksquare$, $c_{Bw}= 3.8$; $\blacktriangle$, $c_{Bw}= 5.7$; $\bullet$, $c_{Bw}= 9.4 \text{ kg m}^{-3}$ wet carrier) enzyme.

5.4 Urea degradation rate by immobilised acid urease

5.4.1 Immobilised Acid Urease kinetics

The kinetics of immobilised acid urease in the basic model wine solution in a stirred bioreactor was measured using the Eupergit®-based biocatalysts, obtained using the second procedure described in section 4.4.1 and lot#1 of the commercial enzymatic preparation, at three different levels, that is 3.8, 5.6 and 9.4 kg m⁻³ of wet carrier.

The closed symbols in Fig. 5.6 show almost linear relationships between the natural logarithm of the current urea concentration (S_L) and time (t) for any c_{BW} level tested, thus confirming that even in this case the product ($\Omega c_{BW} Y_{E/B} k'_{li}$) of Eq. (3.42) may be approximately regarded as a constant.

Even, the kinetic analysis of acid urease immobilised onto Chitopearls in the model wine solution yielded, a series of approximately linear semilogarithmic plots of (S_L/S_{L0}) against t (Fig. 5.7), thus confirming the appropriateness of the mathematical modelling described in section 3.5.

In accordance with Eq. (3.33), the slopes of such diagrams express the apparent pseudo-first order kinetic rate constant ($k_a = \Omega c_{BW} Y_{P/B} k'_{li}$), which is to be proportional to the free (E_f) or immobilised (E_i) enzymatic protein concentration dissolved or dispersed in the liquid phase.

As shown in Fig. 5.2 (see squared open symbols), the apparent pseudo-first order kinetic rate constant of acid urease immobilised onto Chitopearls of different size [$k_{ai} = -0.13 \ln(d_p) + 1.2$] exhibited an almost linearly decreasing trend with $\ln(d_p)$. This being in agreement with the aforementioned trend of A_{Bi} -vs.- $\ln(d_p)$, as also shown in Fig. 5.2

When using Eupergit® C 250 L, the specific pseudo-first order kinetic rate constant relative to the free enzyme [$k'_{lf} = (8.5 \pm 0.3) \times 10^{-3} \text{ m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSAE}$] was estimated to be about 4 times greater than that relative to the immobilized counterpart [$k'_{li} = (2.4 \pm 0.2) \times 10^{-3} \text{ m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSAE}$], as shown by the broken lines and closed symbols in Fig. 5.8.

As the lot#3 of the enzyme preparation was used, the apparent pseudo-first order kinetic rate constants of free (k_{af}) or immobilised (k_{ai}) acid urease differed from those determined when using the lot#1 (see open symbols in Fig. 5.8).

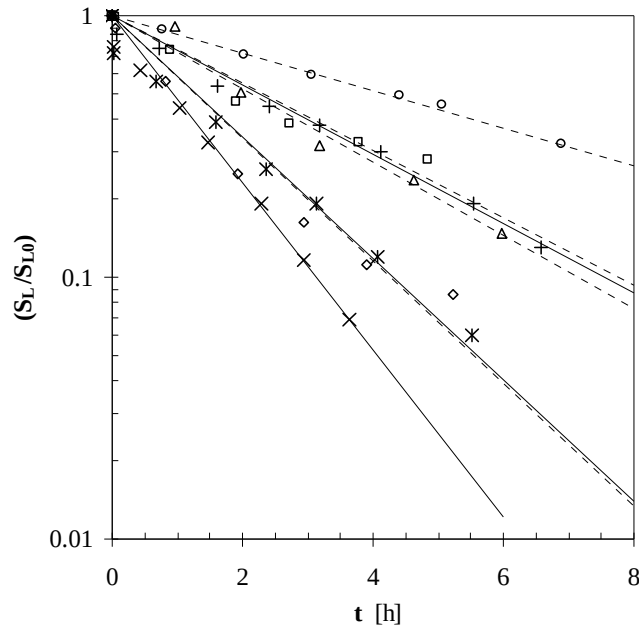


Figure 5.7 Time course of urea degradation at 20°C for a model wine solution when using different concentrations of free enzyme ($\times$, $c_N=1.25$; $*$ $c_N=0.76$; $+$, $c_N=0.51 \text{ kg m}^{-3}$ Nagapsin®) or immobilised enzyme onto GA-activated chitosan beads of different size (BCW-3001: $\diamond$, $c_{Bw}=1.25$; BCW-3003: $\square$, $c_{Bw}=1.26$; ; BCW-3010: $\triangle$, $c_{Bw}=3.13$; HP-3020: $\circ$, $c_{Bw}=3.13 \text{ kg m}^{-3}$ wet carrier): Semi-logarithmic plot of the ratio between the current and initial urea concentrations ($S_L/S_{L,0}$) against time (t).

In this case, by plotting k_{aj} against the corresponding free (E_f) or immobilised (E_i) enzymatic protein concentration dissolved or dispersed in the bulk medium, it was possible to derive a greater k'_{If} value [$(17 \pm 1) \times 10^{-3} \text{ m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSA}_e$, $r^2 = 0.984$] as shown by the continuous line in Fig.5.8.

Thus, the lot#3 of the purified acid urease preparation used to immobilise acid urease onto Chitopearl was found to be more active than lot#1, initially used to immobilise acid urease onto Eupergit®.

It was also observed that k'_{If} was 1.5-5.3 times greater than k'_{Ii} , thus confirming the greater performance of the GA-activated Chitopearl of the BCW series with respect to Chitopearl HP-3020 and Eupergit® C 250 L, their kinetic performance being quite similar.

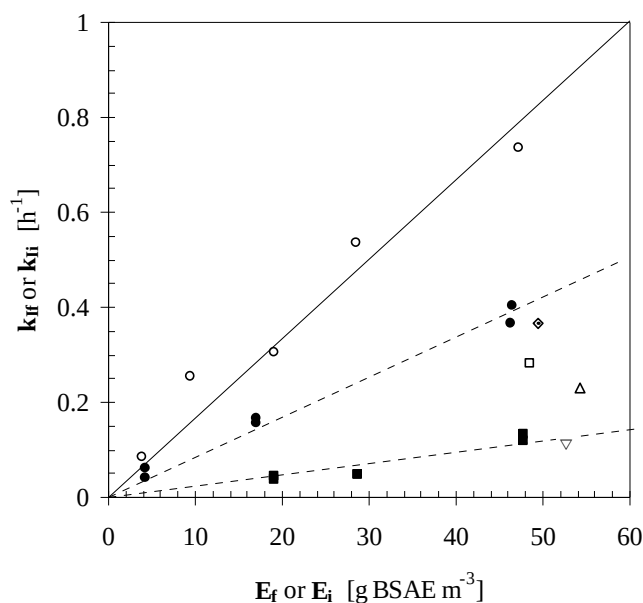


Figure 5.8 Effect of free (E_f : ●, lot#1; ○, lot#3) and Eupergit® C 250 L- (■) or chitosan- (◇, □, △, ▽) immobilised (E_i) enzymatic protein concentrations in the model wine solution on the corresponding apparent pseudo-first order kinetic rate constant (k_{af} or k_{ai}) of urea degradation at 20°C by free or immobilized acid urease.

5.4.2 Effectiveness factor estimation

To check for the contribution of the external and/or internal 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 (Baley & Ollis, 1986; Gómez de Segura *et al.*, 2004; Kosmerl *et al.*, 2000; Satterfield *et al.*, 1963; Spieß *et al.*, 1999; Tagatsuji *et al.*, 1998; Treybal, 1968) on the assumption that the density and viscosity at 20°C of the model wine solution coincided with those of a typical white wine with the same alcohol content (Kosmerl *et al.*, 2000). All estimates are listed in Table 5.3.

By referring to the Eupergit®-based biocatalysts, both the effectiveness factors (Ω) and (η) resulted to be practically unitary.

To check further the negligible contribution of the external film resistance to the urea reaction rate, two further tests using free or immobilised acid urease onto Eupergit® were performed at low (250 rev. min⁻¹) and high (400 rev. min⁻¹) stirring rates under

constant concentrations of free enzyme ($c_N=1.0 \text{ kg m}^{-3}$) or wet biocatalyst ($c_{Bw}=9.4 \text{ kg m}^{-3}$), respectively.

The estimated pseudo-first order kinetic rate constants for the free (k_{if}) and immobilized enzyme (k_{ii}), as obtained by the least squares method and characterized by a variance (s_j^2) and degrees of freedom (δ_j) 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 also 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 Fig. 5.9.

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

Even when using the Chitopearl-based biocatalysts, both the effectiveness factors (Ω) and (η) were found to be practically unitary, despite Ω slightly decreased from 0.9999 to 0.996 as d_p increased from 140 to 2,200 μm . Thus, with all the biocatalysts tested here the overall urea degradation rate was controlled by the reaction kinetics, being negligible the contribution of the external film and intraparticle mass-transfer resistances.

Despite the estimates shown in Table 5.3 were referred to biocatalysts with a unitary tortuosity factor (τ) (Spieß *et al.*, 1999), these estimates still hold even if τ was as great as 7, the typical τ values for industrial catalysts ranging from 1 to 7 (Satterfield *et al.*, 1963). In fact, the loss in both effectiveness factors ranged from as small as 0.03 to 2% for the range of bead size used.

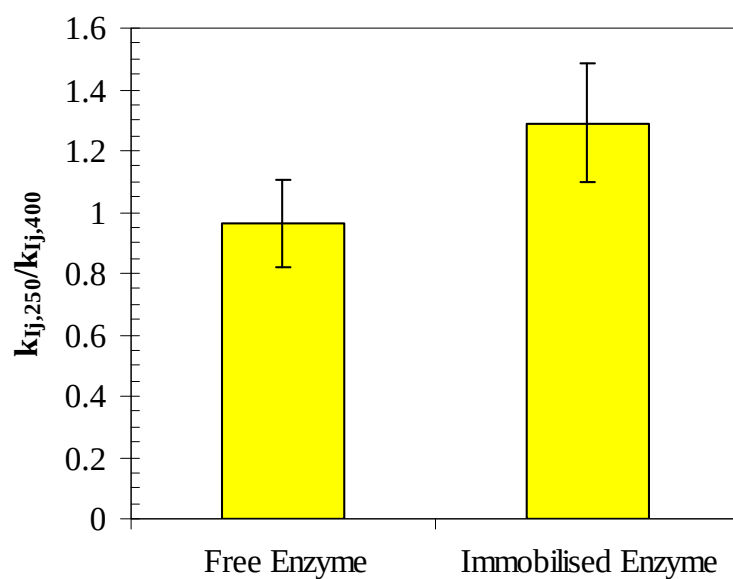


Figure 5.9 Enhancement in the pseudo-first order kinetic rate constant for the free (k_{if}) or immobilized (k_{ij}) enzyme onto Eupergit® by increasing the stirring level from 250 to 400 rev. min⁻¹ when using constant concentrations of free enzyme ($c_N=1.0$ kg m⁻³) or wet biocatalyst ($c_{Bw}=9.4$ kg m⁻³).

Table 5.3 Estimates of the effectiveness factors for acid urease immobilized onto both the supports used in this experimentation, different Chitopearl and Eupergit® C250L (Andrich *et al.*, 2009), in the presence (Ω) or absence (η) of the external film transport resistance by resorting to well known literature relationships (Baley & Ollis, 1986; Gómez de Segura *et al.*, 2004; Kosmerl *et al.*, 2000; Satterfield *et al.*, 1963; Spieß *et al.*, 1999;; Tagatsuji *et al.*, 1998; Treybal, 1968).

Parameter	Eupergit® C 250 L	Chitopearl BCW-3001	Chitopearl BCW-3003	Chitopearl BCW-3010	Chitopearl HP-3020	Unit
Average bead diameter (d_p)	180	142	298.5	1,015	2,200	μm
Bead porosity (θ)	0.6	0.83	0.83	0.83	0.83	-
Bead tortuosity factor (τ)	1.0	1.0	1.0	1.0	1.0	-
Particle density (ρ_B)	370	1136	1136	1136	1136	kg/m^3
Reaction temperature (T)	20	20	20	20	20	$^{\circ}\text{C}$
Model wine density (ρ_L)	998	998	998	998	998	kg/m^3
Model wine viscosity (μ_L)	0.00168	0.00168	0.00168	0.00168	0.00168	Pas
Urea diffusivity (D_S) (Treybal, 1968)	1.29×10^{-9}	1.29×10^{-9}	1.29×10^{-9}	1.29×10^{-9}	1.29×10^{-9}	$\text{m}^2 \text{s}^{-1}$
Urea effective diffusivity ($D_{Se} = \theta/\tau D_S$) (Satterfield <i>et al.</i> , 1963)	7.77×10^{-10}	1.07×10^{-9}	1.07×10^{-9}	1.07×10^{-9}	1.07×10^{-9}	$\text{m}^2 \text{s}^{-1}$
Wet biocatalyst concentration (c_{Bw})	10	1.25	1.26	3.13	3.13	kg/m^3
Biocatalyst water fraction (x_{Bw})	84 ± 3	79 ± 1	75 ± 5	79 ± 3	66 ± 2	% w/w
Protein loading ($Y_{p/B}$)	25.9	39.5	38.7	17.3	16.8	$\text{g BSAE kg}^{-1} \text{ws}$
Specific pseudo-1 st order kinetic rate constant (k_{ii}')	0.0024	0.0109	0.0061	0.006	0.0032	$\text{m}^3 \text{h}^{-1} \text{g}^{-1} \text{BSAE}$
Pseudo-first order kinetic rate constant (k_{ii})	0.099	0.539	0.296	0.323	0.166	h^{-1}
Schmidt number (Sc)	1300	1300	1300	1300	1300	-
Grashof number (Gr)	12.7	1.4	12.7	500	5089	-
Sherwood number (Sh) (Baley & Ollis, 1986)	9.9	5.8	9.9	28.8	60.2	-
Mass transfer coefficient (k_L)	7.11×10^{-5}	5.25×10^{-5}	4.29×10^{-5}	3.68×10^{-5}	3.54×10^{-5}	m s^{-1}
Biot number [$Bi = R k_L/D_e$]	8.2	3.5	6.0	17.4	36.2	-
Thiele modulus (θ)	0.017	0.027	0.041	0.147	0.228	-
Effectiveness factor (η)	0.99998	0.99995	0.9999	0.9986	0.9966	-
Global effectiveness factor (Ω)	0.99997	0.99989	0.9998	0.9981	0.9961	-

5.5 Effect of grape seed tannins on immobilised enzyme kinetics

Owing to the greater inhibitory effect of the tannins extracted from grape seeds observed by Esti *et al.*, (2007), the sensitivity of the activity of acid urease immobilized on Eupergit® C250 L to such phenolic compounds was assessed by enriching the model wine solution of concern with 5-500 g m⁻³ of the above tannin source. To this end, another batch of biocatalysts was prepared using the second procedure, described in section 4.4.1 and lot#2 of the purified enzyme preparation (Table 5.1). These biocatalysts were added to the reaction mixture so as to assure a constant wet biocatalyst concentration of about 6.25 kg m⁻³.

Fig. 5.10 shows a semi-logarithmic 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 concentration (P) 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 pseudo-first order kinetic rate constant (k_{fi}) for immobilised acid urease as a function of P .

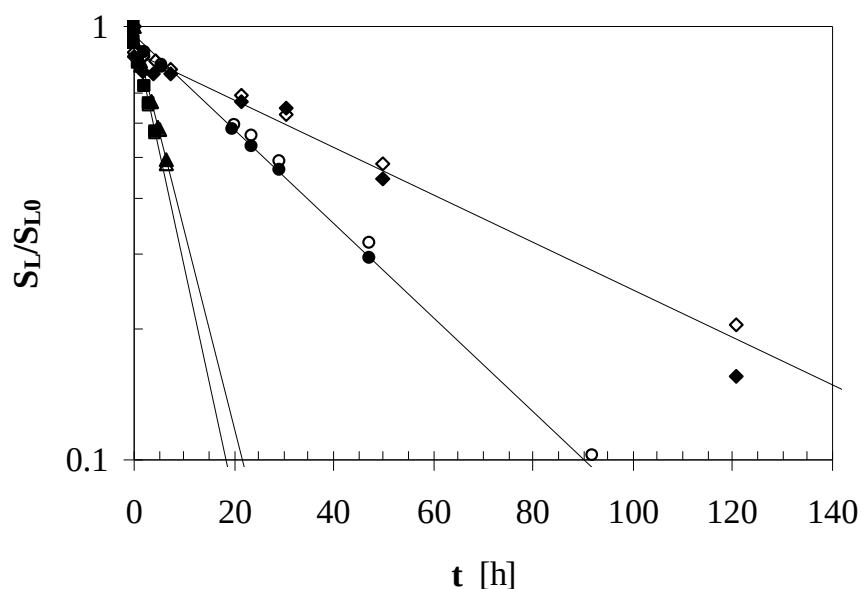


Figure 5.10 Semi-logarithmic plot of the ratio between the current and initial urea concentrations (S_L/S_{L0}) against time (t) for the model wine solution enriched with different amounts of grape seeds extract ($\square, \blacksquare - P=0$; $\triangle, \blacktriangle - P=5 \text{ g m}^{-3}$; $\circ, \bullet - P=50 \text{ g m}^{-3}$; $\diamond, \blacklozenge - P=500 \text{ g m}^{-3}$) using a constant wet biocatalyst concentration ($c_{Bw}=6.25 \text{ kg m}^{-3}$). The continuous lines represent the mean squares regression lines.

The inhibitory effect of grape seed tannins was also assessed on the activity of acid urease immobilised onto Chitopearls by enriching the model wine solution of concern with about 300 or 600 g m⁻³ of the above tannin source. The resulting almost linear $\ln(S_L/S_{L0})$ -vs.-t plots allowed the experimental pseudo-first order kinetic rate constant (k_{li}) for acid urease immobilised onto chitosan derivative beads to be determined (see open symbols in Fig. 5.2).

It was observed a general reduction in k_{ai} as P was increased from 0 to 370 g GAE m⁻³. In particular, at $P=187\pm1$ g GAE m⁻³ the apparent pseudo-1st order kinetic rate constants for immobilized acid urease (k_{li}) reduced to $75\pm14\%$ of that pertaining to the same biocatalyst in the model wine solution devoid of phenolics ($P=0$).

At $P=374\pm2$ g GAE m⁻³, such a ratio in k_{li} values reduced to $58\pm9\%$, with the exception of the biocatalysts of the smaller size (BCW-3001), its corresponding k_{ai} value being as small as 21% of that (0.54 ± 0.03 h⁻¹) observed in the model wine solution containing no phenolics.

This finding may be probably attributed to the higher adsorption capacity of BCW-3001 chitosan-derivative beads for grape seed tannins because of their greater specific surface area (Table 4.1). This probably resulted in a local increase in the phenolic content and thus in a greater inhibitory effect on its overall urea degradation kinetics. In fact, as recovered from the urea-exhausted wine model solutions by vacuum filtration, these biocatalysts resulted to be by far more tainted with the same dark brown colour of grape seed tannins than the other larger beads.

Fig. 5.11 compares the effect of phenolic substances on the performance of the chitosan-based biocatalysts prepared here to that of the Eupergit® C 250 L-based counterparts, as well as to that of free acid urease in the same model wine solution enriched with grape seed tannins and some real wines (Esti *et al.*, 2007).

It can be noted that the general reduction in the ratio ($k_{ai}/k_{ai,P=0}$) with increasing phenolic content (P) was by far attenuated for the biocatalysts obtained from Chitopearls BCW-3003, BCW-3010 and HP-3020 with respect to the Eupergit®-based catalysts (Andrich *et al.*, 2009).

These results confirmed the protective action of the chitosan matrix against the greatest inhibitory effect of grape seed tannins towards free enzymes..

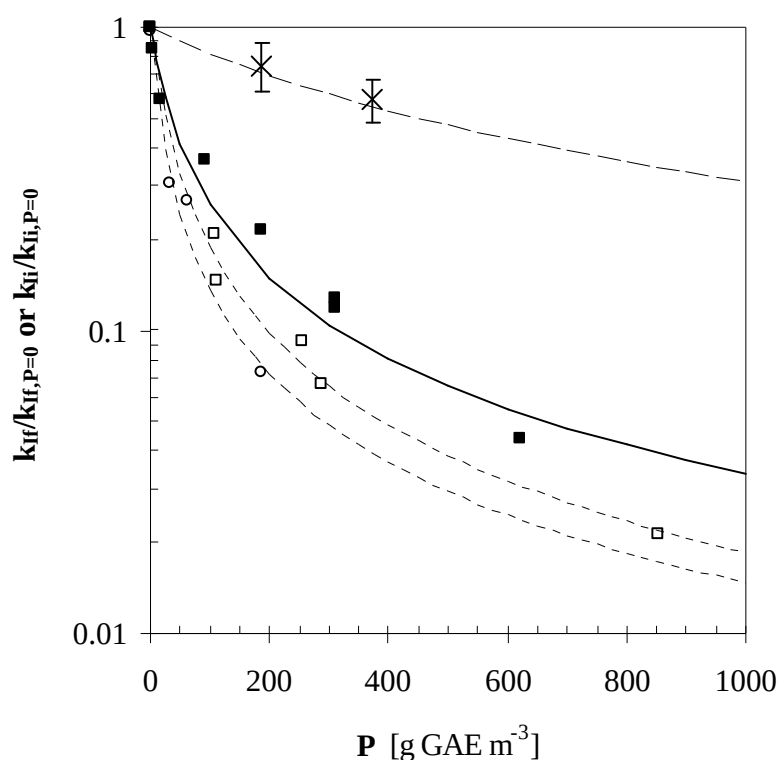


Figure 5.11 Effect of phenolic compound concentration (P) on the experimental pseudo-1st order kinetic rate constants ($k_{ij,p}$) for acid urease immobilized onto chitosan derivative- (*) or Eupergit® C 250 L- (■) beads in model wine solutions enriched with grape seed extract or for free acid urease in real wines (□) or model wines enriched with grape seed tannins (○) (Esti et al., 2007), 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.

5.6 Urea degradation in real white wines by immobilised acid urease

5.6.1 Wine characteristics

Two commercial Italian white wines, produced in the Umbria region of Italy by *Sauvignon Blanc* or *Grechetto* grapes (2008 vintage) in Monrubio winery (Monterubiaglio di Castel Viscardo, Terni, Italy) were used as target real wines to assess the performance of immobilised acid urease.

The main characteristics of these wines are shown in Table 5.4.

Table 5.4 Mean values and standard deviations of the main chemico-physical analyses for the commercial Italian white wines investigated in this PhD thesis.

Wine type	Sauvignon Blanc	Grechetto	Unit
Parameter	Value		
Alcohol content	12.7 ± 0.6	12.90 ± 0.6	% v/v
pH	3.48 ± 0.04	3.27 ± 0.04	-
Total Acidity	5.5 ± 0.3	5.5 ± 0.3	kg [*] m ⁻³
Volatile Acidity	0.18 ± 0.02	0.21 ± 0.02	kg [#] m ⁻³
Glycerol	5.7 ± 0.3	5.1 ± 0.3	kg m ⁻³
Reducing Sugars	0.66 ± 0.07	1.7 ± 0.2	kg m ⁻³
Total Extract	21.8 ± 1.7	20.1 ± 1.6	kg m ⁻³
Ash	2.7 ± 0.2	2.2 ± 0.2	g m ⁻³
Overall SO ₂	102 ± 8	95 ± 7	g m ⁻³
Urea	nd	nd	mmol m ⁻³
Ammonium	5.0 ± 0.4	11 ± 1	g m ⁻³
Total Phenolics	364 ± 24	450 ± 29	g GAE m ⁻³
Tartaric Acid	3.1 ± 0.2	3.6 ± 0.2	kg m ⁻³
L-Malic Acid	2.2 ± 0.2	1.5 ± 0.1	kg m ⁻³
L-Lactic Acid	0.04 ± 0.00	nd	kg m ⁻³
Citric Acid	0.22 ± 0.03	0.16 ± 0.02	kg m ⁻³
Density	992 ± 3	990 ± 3	kg m ⁻³

* as tartaric acid equivalent

as acetic acid equivalent

nd not detectable

5.6.2 Urea degradation in real wines by immobilised enzyme

To assess the urea degradation rate in *Sauvignon Blanc* and *Grechetto* wines, acid urease (lot#3) was newly immobilised onto Eupergit® and Chitopearl BCW-3003 and BCW-3010. Table 5.5 lists the experimental conditions used for all the support matrices used, as well as the protein concentrations in the immobilising solution (c_{p0}) and overall filtrate (c_{pf}), protein bound on support (m_{pb}), protein loading ($Y_{p/B}$), immobilised acid urease activity per unit mass of dry (A_{Bd}) or wet (A_{Bw}) support or protein bound (A_{BEi}), as well as enzyme (ζ_E) and activity (ζ_A) coupling yields.

It is worth noting that acid urease, as immobilized onto Chitopearl 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 to be quite near to those obtained previously (Table 5.2), 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 case ($\sim 10 \text{ IU g}^{-1}$ wet support, ws), was found to be slightly smaller than that (19 IU g^{-1} ws) pertaining to the same biocatalysts attained earlier (Table 5.2).

Moreover, the enzyme coupling yield (ζ_E) was of 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. Finally, the activity coupling yield (ζ_A) was of more or less 50% for the beads of the BCW series, but it dropped to as little as 5% for the 180- μm Eupergit® C 250 L particles.

Table 5.5 *Experimental conditions and specific activities per unit mass of dry (ds) or wet (ws) support of acid urease immobilised on Eupergit® C 250 L and some GA-activated chitosan beads of different size.*

Parameter		Eupergit® C 250L	Chitopearl BCW-3003	Chitopearl BCW-3010	Unit
Mass of wet support	m_{Bw}	1204.0	1200.7	1200.2	mg
Water fraction	x_{BW}	80 ± 1	75 ± 5	79 ± 3	% w/w
Immobilising solution volume	V_0	70	70	70	cm ³
Nagapsin® conc. in the immob. sol.	C_N	24	24	24	kg m ⁻³
Protein conc. in the immobilizing sol.	C_{p0}	978 ± 19	950 ± 24	913 ± 4	g BSAE m ⁻³
Initial protein mass	m_{p0}	68 ± 1	66 ± 2	63.9 ± 0.3	mg BSAE
Prot. Added per unit mass of ws	$Y_{p/S}$	57	55	53	mg BSAE g ⁻¹ ws
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}	106 ± 1	47 ± 1	93 ± 4	g m ⁻³ BSAE
Filtrate volume	V_f	500	500	500	cm ³
Protein bound on support	m_{pb}	15.3	43.2	17.3	mg BSAE
Protein loading	$Y_{p/B}$	12.75	36.0	14.4	mg BSAE g ⁻¹ ws
Specific immobilised enz. activity	A_{Bd}	51.3 ± 0.4	1059 ± 11	609 ± 20	IU g ⁻¹ ds
Specific immobilised enz. activity	A_{Bw}	10.3 ± 0.1	260 ± 3	127 ± 4	IU g ⁻¹ ws
Immobilised enz. specific activity	A_{BEi}	0.80 ± 0.01	7.24 ± 0.07	8.79 ± 0.29	IU mg ⁻¹ BSAE
Enzyme coupling yield	ζ_E	22.4	64.9	27.1	%
Enzyme activity coupling yield	ζ_A	4.8 ± 0.1	47.2 ± 0.5	56 ± 2	%

5.6.3 Effectiveness factor of immobilised acid urease in real wines

By using the same stirred bioreactor and operating conditions ($S_{L0}=1 \text{ mol m}^{-3}$, $E_i \sim 47 \text{ g BSAE m}^{-3}$) previously used to study urea degradation in model wine solutions, it was assessed the time course of urea degradation in two target Italian white wines of concern.

Depending on the different protein loadings of three biocatalysts used (BCW-3003, BCW-3010, or Eupergit® C 250 L), their concentration (c_{Bw}) in any wine sample under testing was respectively estimated as about 1.25, 3.13 or 3.53 kg m^{-3} of wet carrier, in order to keep the immobilised enzymatic protein concentration ($E_i=c_{Bw} Y_{P/B}$) dispersed in the liquid phase approximately constant and equal to $47 \pm 3 \text{ g BSAE m}^{-3}$.

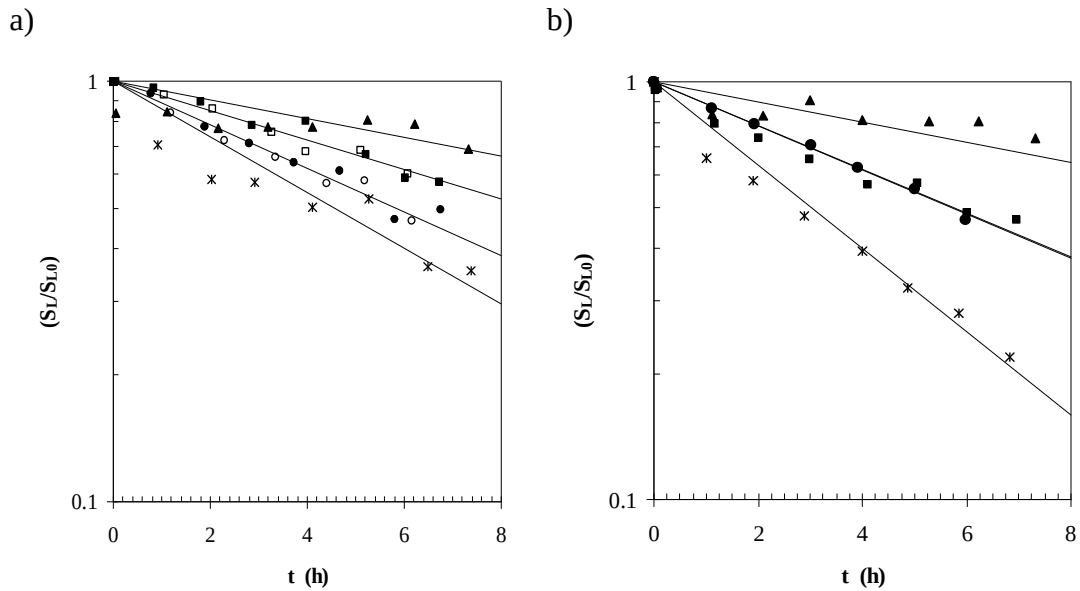


Figure 5.12 Time course of urea degradation at 20°C for Grechetto (a) and Sauvignon Blanc (b) wines when using free enzyme (*, $c_N=1.2 \text{ kg m}^{-3}$ Nagapsin®) or immobilised enzyme onto different matrices (BCW-3003: $\circ$, $\bullet$, $c_{Bw}=1.25$; BCW-3003: $\blacksquare$, $\square$, $c_{Bw}=3.13$; Eupergit® C 250 L: $\blacktriangle$, $c_{Bw}=3.53 \text{ kg m}^{-3}$ wet carrier): Semi-logarithmic plot of the ratio between the current and initial urea concentrations (S_L/S_{L0}) against time (t).

Fig.5.12 shows the semi-logarithmic 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 immobilised acid urease. In all cases, such plots were approximately linear.

By accounting for Eq.(3.33) 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 pseudo-first order kinetic rate constant (k_{ali}) for any wine sample and biocatalyst examined, as listed in Table 5.6.

The apparent pseudo-first order kinetic rate constant (k_{alf}) for urea degradation by free acid urease in *Grechetto*, *Sauvignan Blanc* and model wines at 20°C was also listed in Table 5.6.

To contribution for the contribution of the external and/or internal diffusion resistances to the overall substrate reaction for these biocatalysts was assessed as reported in Table 5.7.

For any of the three biocatalysts used, both the effectiveness factors (Ω) and (η) resulted to be practically unitary, as established for the biocatalysts used to remove urea in model wine solution. Thus, whatever the biocatalyst used here, in a model wine solution and in real wines, the overall urea degradation rate was controlled by the reaction kinetics, being negligible the contribution of the external film and intraparticle mass-transfer resistances.

Despite the estimates shown in Table 5.7 were referred to biocatalysts with a unitary tortuosity factor (τ) (Spieß *et al.*, 1999), these would still hold even if τ was as great as 7, the typical τ values for industrial catalysts ranging from 1 to 7 (Satterfield *et al.*, 1963). In fact, for $\tau=7$ the loss in both effectiveness factors varied from as small as 0.01 to 0.4% for the range of bead size used.

The specific pseudo-first order kinetic rate constant (k'_{alf}) for free acid urease from lot#3 was equal to $[(17\pm1)\times10^{-3} \text{ m}^3 \text{ h}^{-1} \text{ g}^{-1} \text{ BSAE}]$ ($r^2=0.984$).

In these final experiments, since E_f was equal to $48\pm1 \text{ g BSAE m}^{-3}$, the apparent kinetic rate constant (k_{ajf}) should have been equal to $(0.82\pm0.02) \text{ h}^{-1}$. It was instead estimated as equal to $(0.74\pm0.02 \text{ h}^{-1})$, thus differing by about 10% from the aforementioned value (Table 5.6).

Table 5.6 Mean values and standard deviations of the apparent urea degradation pseudo-first order kinetic rate constant (k_{alij}) in model, Grechetto, and Sauvignon Blanc wines at 20°C by free or immobilized enzyme at different concentrations of the commercial acid urease preparation (c_N) or wet biocatalysts (c_{Bw}) and their corresponding enzymatic protein dissolved (E_i) or dispersed (E_f) 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	kg m ⁻³
	E_i or E_f	49.5	19.0	48.6	54.3	g BSAE m ⁻³
	k_{alij}	0.74±0.02	0.18±0.01	0.43±0.02	0.45±0.01	
		($r^2=0.994$)	($r^2=0.986$)	($r^2=0.991$)	($r^2=0.998$)	h ⁻¹
Grechetto wine	c_{Bw} or c_N	1.20	3.53	1.26	3.13	kg m ⁻³
	E_i or E_f	47.5	45.0	45.4	45.2	g BSAE m ⁻³
	k_{alij}	0.23±0.01	0.06±0.01	0.122±0.002	0.120±0.006	
		($r^2=0.992$)	($r^2=0.800$)	($r^2=0.998$)	($r^2=0.983$)	h ⁻¹
Sauvignon Blanc wine	c_{Bw} or c_N	1.20	3.53	1.25	3.13	kg m ⁻³
	E_i or E_f	47.4	45.0	45.0	45.1	g BSAE m ⁻³
	k_{alij}	0.152±0.01	0.051±0.008	0.139±0.005	0.135±0.005	
		($r^2=0.961$)	($r^2=0.828$)	($r^2=0.994$)	($r^2=0.987$)	h ⁻¹

Table 5.7 Estimates of the effectiveness factors for acid urease immobilized on Chitopearl BCW-3003 and -3010 or Eupergit® C250L in the presence (Ω) or absence (η) of the external film transport resistance by resorting to well known literature relationships (Baley & Ollis, 1986; Gómez de Segura et al., 2004; Kosmerl et al., 2000; Satterfield et al., 1963; Spieß et al., 1999; Tagatsuji et al., 1998; Treybal, 1968).

Biocatalyst matrix	Eupergit® C 250L		Chitopearl BCW-3003		Chitopearl BCW-3010		Unit
	Wine sample	Grechetto Sauvignon Blanc	Grechetto Sauvignon Blanc	Grechetto Sauvignon Blanc	Grechetto Sauvignon Blanc	Grechetto Sauvignon Blanc	
Parameter							
Average bead radius (R)		90		149		508	μm
Bead porosity (θ)		0.6		0.83		0.83	-
Bead tortuosity factor (τ)		1.0		1.0		1.0	-
Particle density (ρ_B)		1136		1136		1136	kg ws/m^3
Reaction temperature (T)		20		20		20	$^{\circ}\text{C}$
Wine density (ρ_L)		990.5	991.5	990.5	991.5	990.5	kg/m^3
Wine viscosity (μ_L)		1.56	1.61	1.56	1.61	1.56	mPa s
Urea diffusivity (D_S) (Treybal, 1968)		1.29×10^{-9}		1.29×10^{-9}		1.29×10^{-9}	$\text{m}^2 \text{s}^{-1}$
Urea effective diffusivity ($D_{Se} = \theta/\tau D_S$) (Satterfield et al., 1963)		7.77×10^{-10}		1.07×10^{-9}		1.07×10^{-9}	$\text{m}^2 \text{s}^{-1}$
Wet biocatalyst concentration (c_{BW})		3.53	3.53	1.26	1.26	3.13	kg/m^3
Biocatalyst water fraction (x_{BW})		80 $\pm$ 1		75 $\pm$ 5		79 $\pm$ 3	% w/w
Protein loading ($Y_{P/B}$)		12.8		36.0		14.4	$\text{g BSAE kg}^{-1} \text{ws}$
Apparent pseudo-first order kinetic rate constant (k_{ji})		0.060	0.051	0.122	0.139	0.120	h^{-1}
Schmidt number (Sc)		1217	1255	1217	1255	1217	-
Grashof number (Gr)		3.4	3.2	15.5	14.4	607	-
Sherwood number (Sh) (Baley & Ollis, 1986)		7.0	6.9	10.2	10.1	30.0	-
Mass transfer coefficient (k_L)		5.01×10^{-5}	4.97×10^{-5}	4.44×10^{-5}	4.39×10^{-5}	3.83×10^{-5}	m s^{-1}
Biot number ($Bi = R k_L/D_{Se}$)		5.8	5.8	6.2	6.1	18.1	-
Thiele modulus (θ)		0.013	0.012	0.027	0.028	0.089	-
Effectiveness factor (η)		0.99999	0.99999	0.99995	0.99995	0.99947	-
Global effectiveness factor (Ω)		0.99998	0.99998	0.99992	0.99990	0.99932	-

By referring the experimental k_{aj} values to k_{alf} , it was possible to compare directly the performance of the three biocatalysts tested in the model and real wines, as shown in Fig. 5.13.

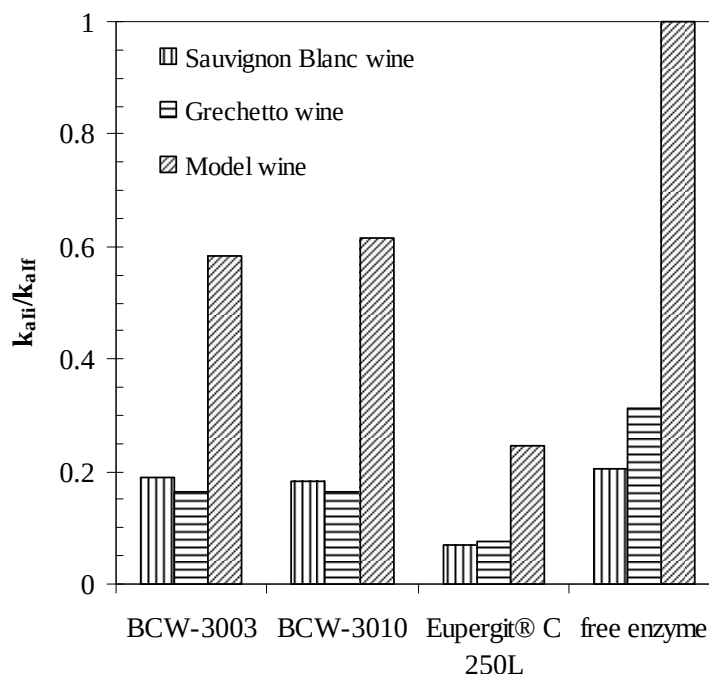


Figure 5.13 Experimental apparent pseudo-1st order kinetic rate constants (k_{aj}) for free acid urease or enzyme immobilized onto different matrices (BCW-3003, BCW-3010, Eupergit® C 250 L) in Grechetto, Sauvignon Blanc and model wines as referred to the value (k_{alf}) pertaining to free acid urease in the model wine solution.

When using free acid urease, the apparent pseudo-first order kinetic rate constant in *Grechetto* and *Sauvignon Blanc* wines reduced to 30 or 20% of that observed in the model wine solution owing to their different composition, and especially to their higher phenolic content (Table 5.4).

When using acid urease immobilised onto Chitopearls BCW-3003 and BCW-3010 or Eupergit® C 250 L, the apparent pseudo-first order kinetic rate constant in *Grechetto* and *Sauvignon Blanc* wines reduced to about 18 and 17% or 7% of that for free enzyme (k_{alf}) in the model wine solution, 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 behaviour 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 (Fidaleo *et al.*, 2006). In the circumstances, using an initial urea concentration of 1 mol m^{-3} throughout all the experimental design, the effects of the main inhibitory wine components [i.e., malic (M) and lactic (L) acids, potassium metabisulfite (K), and ethanol], as well as pH, on the pseudo-first order kinetic rate constant for free acid urease (k_{If}) in different model wine solutions were reconstructed by using the 2nd-order canonical regression developed by Fidaleo *et al.* (2006) – see Eq.s (3.15), and (3.16)-(3.19).

Then, to account for the inhibitory effect of the phenolic compounds (P) present in the real wines under testing, the effective pseudo-first order kinetic rate constant (k_{Ife}) was guessed by resorting to the competitive inhibition model set up by Esti *et al.* (2007) – see Eq. (3.20) – with the inhibition constant ($K_P=21.1\pm0.5 \text{ g GAE m}^{-3}$) referred to the Italian white and rosé wines studied by Esti *et al.* (2007).

As shown in Table 5.8, the different composition of the real wines under study exerted a limited effect on the effective pseudo-first order kinetic rate constants, this corroborating the overall results shown in Fig. 5.13.

Thus, use of Eq.s (3.15) and (3.16)-(3.19) may be recommended as a short-cut procedure to assess roughly the kinetic response of free acid urease in wines of different composition and figure out the likely effectiveness of the wine treatment by immobilised enzyme.

Table 5.8 Apparent pseudo-first-order kinetic rate constants for a killed cell acid urease preparation in model wine solutions having the same pH and characteristics of the real wines assayed in this work in the absence (k_{If}) or presence (k_{Ife}) of phenolic compounds (P), as estimated by using Eq.s (3.36) and (3.40)-(3.45).

Wine sample	Coded levels					Real Values		
	Malic acid x_1	Lactic acid x_2	$K_2S_2O_5$ x_3	Ethanol x_4	pH x_5	P g GAE m ⁻³	$k_{If} \times 10^5$ dm ³ mg ⁻¹ min ⁻¹	$k_{Ife} \times 10^6$ dm ³ mg ⁻¹ min ⁻¹
<i>Sauvignon</i>								
<i>Blanc</i>	-0.24	-1.95	-0.47	-0.23	-0.09	364	8.0	4.4
<i>Grechetto</i>	-0.80	-2.00	-0.70	-0.07	-0.92	450	9.4	4.2

Conclusions

The immobilisation of acid urease on Eupergit® C 250 L via the direct binding of the enzyme to the carrier oxirane groups (Knezevic *et al.*, 2006), followed by stabilisation with glycine (Oliveira *et al.*, 2001), gave rise to quite a stable biocatalyst, since its residual activity resulted to be as much as $69\pm 16\%$ of its initial value even after 34 days of storage at 4°C. This finding was independent of the enzyme added per unit mass of support used. However, the observed enzyme loading ($Y_{E/B}$) appeared to be extremely dependent on the stability of the original source of purified acid urease used. In fact, $Y_{E/B}$ reduced from about 27 to 11 mg of bound enzyme per g of dry carrier when the lot#1 was replaced with the lot#2 of the same purified acid urease preparation, probably because at the moment of their use to prepare the immobilising solutions the specific activity of such lots reduced from 766 ± 7 to 420 ± 27 IU per g of Nagapsin®, respectively.

The immobilisation of acid urease onto glutaraldehyde-activated chitosan-derivative beads gave rise to more stable biocatalysts, with since their residual activity resulted to be as much as 95% of their initial values even after 150-170 days of storage at 4°C. Only the specific activity of the biocatalysts prepared using Chitoparl BCW-3001 reduced by about 40% after 62-day storage and then was approximately constant for the subsequent 78 days.

The specific activity (A_{BI}) of the chitosan-based biocatalysts decreased from circa 308 to 70 IU g⁻¹ wet support as the bead average diameter (d_p) increased from 0.14 to 2.2 mm. Nevertheless, in all cases tested, these biocatalysts were by far more active than the biocatalysts obtained when using Eupergit® C 250 L (19 ± 3 IU g⁻¹ ws). On the contrary, the protein loading of the latter (26 mg BSAE g⁻¹ ws) fell within the range of protein loading values (17-40 mg BSAE g⁻¹ ws) detected for all the biocatalysts prepared in this experimentation.

The kinetics of urea degradation in a model wine solution by free purified acid urease was confirmed to be of the pseudo-first order with respect to urea concentration, in agreement with previous findings in real and model wines using killed cell preparations (Esti *et al.*, 2007; Fidaleo *et al.*, 2006; Kodama *et al.*, 1996; Trioli *et al.*, 1989). Such a mechanism was even corroborated when using purified acid urease immobilised on all the aforementioned matrices. More specifically, the pseudo-first order kinetic rate constant relative to the free enzyme (k_{If}) resulted to be 1.5-5.3 times greater than that

relative to the immobilised counterpart (k_{ii}) under constant concentration of the enzymatic protein dissolved (E_f) or dispersed (E_i) in the liquid bulk.

For all biocatalysts tested here, k_{if} accounted for the effect of the external resistance of urea transport from the bulk solution to the enzyme sites only, while k_{ii} incorporated the effects of simultaneous film and interparticle mass-transfer resistances (Treybal., 1968). However, by resorting to well known literature relationships (Baley & Ollis, 1986; Gómez de Segura *et al.*, 2004; Satterfield *et al.*, 1963; Spieß *et al.*, 1999; Treybal, 1968), the contribution of the external film and/or intraparticle diffusion resistances to the overall substrate reaction rate was found to negligible, the overall urea degradation rate being just controlled by the reaction kinetics.

Further testing allowed the activity of acid urease immobilized onto Eupergit® or Chitopearl® to be assessed in the presence of the high-inhibitory tannins extracted from grape seeds (Esti *et al.*, 2007) at levels ranging from 5 to 600 g GAE m⁻³. For all the immobilised biocatalysts tested here, a protective action against such compounds of enzyme immobilization towards free enzymes was assessed. In particular, the chitosan-based biocatalysts exerted a higher protective action not only about free acid urease, but also towards acid urease immobilised onto Eupergit® C 250 L.

The kinetics of urea degradation by immobilised ureases in two target Italian wines (that is *Grechetto* or *Sauvignon Blanc* wines) using a stirred bioreactor was of the pseudo-first order with respect to urea concentration, in agreement with previous findings in real and model wines using killed cell preparations (Bertrand, 2003; Esti *et al.*, 2007; Fidaleo *et al.*, 2006; Trioli *et al.*, 1989) and in model wines using immobilised biocatalysts (Andrich *et al.*, 2009). Moreover, urea removal rate was not limited by urea external and intraparticle mass transfer. Owing to the peculiar phenolic content of *Grechetto* or *Sauvignon Blanc* wine, the apparent pseudo-first order urea degradation kinetic rate constant for acid urease immobilised onto Eupergit® C 250 L-, BCW-3003- or BCW-3010-particles at E_i approximately constant reduced to about 7, 18 or 17% of that observed for free enzyme in the model wine solution, respectively.

By accounting for their intrinsic positive characteristics of being resistant to the compression stress exerted by a column bed (Matsumoto, 1993), sterilizable, non-toxic, biocompatible and biodegradable (Chen *et al.*, 1999), and for being more than twice less sensitive to the wine phenolic content than the Eupergit®-based biocatalysts, the

chitosan-based catalysts, in particular Chitopearls BCW-3003 ($d_p \sim 0.3$ mm) or BCW-3010 ($d_p \sim 1.0$ mm), seem to be potentially employable in the make up of packed-bed cartridges to remove continuously urea not only from sake (Matsumoto, 1993), but also from commercial wines.

Wine treatment by using the commercially available killed cell acid urease preparations generally involves too 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 (Esti *et al.*, 2007; Fidaleo *et al.*, 2006), the maximum dose allowable was equivalent to quite a low enzymatic protein concentration (11.0 ± 0.6 g BSAE m^{-3}) and yielded a low urea degradation rate.

To accelerate the process it would be necessary to resort to a dosage by far higher than the maximum allowable one (i.e., 75 g m^{-3}) for wine treatment (Bertrand, 2003). Thus, strictly speaking, the wine detoxification trials performed here with free acid urease (see * symbols in Fig. 5.12) could not be practically exploited, since the dissolved enzymatic protein concentration (E_f) was about four times greater than the maximum allowable one.

In contrast, the wine treatment tests using immobilized acid urease allowed the present regulatory to be circumvented because immobilized enzyme was insoluble in wine. In addition, recovery of the catalyst from the urea-exhausted wine by filtration may result in significant cost savings owing to multiple enzyme recycles in consecutive batch trials with no negative effect on the typical bouquet of the wine treated, this being altered at >50 g m^{-3} of the aforementioned killed cell preparation (Bertrand, 2003).

To increase the urea degradation rates achieved here, one needs to increase further the biocatalyst concentrations (c_{Bw}) in the agitated bioreactor or alternatively to resort to specifically designed packed-bed cartridges.

Further work is required to assess both the operational performance and stability of a laboratory-scale packed-bed bioreactor to detoxify real wines and economic feasibility.

References

Allan C.R., Hadwiger L.A. (1979), *Exp. Micol.*, **3(3)**:285-287.

Allan G.G., Altman L.C., Bensinger R.E., Ghosh D.K., Hirabayashi Y., Neogi A.N., Neogi S. (1984), "In chitin, Chitosan and related enzymes", *J.P. Zikakis, ed, Academic Press, Inc*, pp119-133.

Amerine M.A., Ough C.S. (1974), "Wine and must analysis", Wiley-Interscience: New York.

Andrews K. R., Blakeley R. L., Zerner B., (1984), "Urea and Urease", *Adv. Inorg. Biochem.*, **6**: 245-282.

Andrews R.K., Dexter A., Blakeley R.L., Zerner B. (1986), *J. Am. Chem. Soc.*, **108**:7124–7125.

Andrich L., Esti M., Moresi M. (2009), "Urea Degradation in Model Wine Solutions by Free or Immobilized Acid Urease in a Stirred Bioreactor", *J. Agr. Food Chem.*, **57**:3533–3542.

Asato E., Kamamuta K., Akamine Y., Fukami Y., Nukada R., Mikuriya M., Deguchi S., Yokota Y. (1997), *Bull. Chem. Soc. Jpn.*, **70**: 639–648.

Austin, J. W., Doig P., Stewart M., Trust T. J. (1991), "Macromolecular structure and aggregation states of *Helicobacter pylori* urease", *J. Bacteriol.*, **173**:5663–5667.

Austin, J. W., Doig P., Stewart M., Trust. T. J.(1992), "Structural comparison of urease and a GroEL analog from *Helicobacter pylori*", *J. Bacteriol.*, **174**:7470–7473.

Bailey JE, Ollis DF (1986) Biochemical Engineering Fundamentals. 2nd ed, McGraw-Hill Book Co: New York, pp. 204-227.

Balassa L.L., Prudden J.F. (1978), MIT Sea Grant Rep. MITSG, 78-7, *Proc. Int. Conf. Chitin/Chitosan*, 1st, PB 285 640, pp.296-305.

Becker-Ritt A.B, Martinelli A.H.S., Mitidieri S., Feder V., Wassermann G.E., Santi L., Vainstein M.H., Oliveira J.T.A., Fiuza L.M., Pasquali G., Carlini C.R., (2007), *Toxicon*, **50**:971–983.

Beer D. G., Malkinson A. M. (1985) “Genetic influence on type 2 or Clara cell origin of pulmonary adenomas in urethan-treated mice”, *J Natl Cancer Inst*, **75**(5): 963-9.

Benini S., Rypniewski W.R., Wilson K.S., Ciurli S., Mangani S. (2001), *J. Biol. Inorg. Chem.*, **6**:778–790.

Benini S., Rypniewski W.R., Wilson K.S., Miletto S., Ciurli S., Mangani S. (1999), *Structure*, **7**:205–216.

Bertrand A. (2003), “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, 171-192.

Bhuiyan S.H., Itami Y., Rokui Y., Katayama T., Izumori K. (1998), “D-allose production from D-psicose using immobilized L-rhamnose isomerase”, *J Ferment Bioeng*, **85**:539-541.

Blakeley R.L., Dixon N.E., Zerner B. (1983), *Biochim. Biophys. Acta*, **744**: 219–229.

Blanchard A., Razin S., Kenny G.E., Barile M.F. (1988), *J. Bacteriol.*, **170**:2692–2697.

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

Brady C.E., Hadfield T.L., Hyatt J.R., Utts S.J. (1986), “*Campylobacter pyloridis*: survival in a hostile environment”, *Am. J. Gastroenterol.*, **81**:855.

Breitenbach J.M., Hausinger R.P. (1988), *Biochem. J.*, **250**:917–920.

Burne R.A., Chen Y.-Y.M. (2000), *Microb. Infect.*, **2**:533–542.

Butzke C. E., Bisson L. F. (1997) “*Ethyl Carbamate Preventative Action Manual*” Department of Viticulture & Enology University of California, Davis, Center for Food Safety and Applied Nutrition U. S. Food and Drug Administration (<http://vm.cfsan.fda.gov/~frf/ecactit.html>).

Cane P. (1990), “Quality control of wine by HPLC: determination of organic acids”, *L’Enotecnico*, **1**:69-72.

Cao L, van Langen L, Sheldon RA (2003) “Immobilised enzymes: carrier bound or carrier free?”, *Current Opinion in Biotechnology* **14**: 387-394.

Cao L.Q. (2005), *Curr. Opin. Chem. Biol.*, **9**:217–226.

Cervelli S., Nannipieri P., Giovannini G., Perna A. (1975), *Pestic. Biochem. Physiol.*, **5**:221–225.

Cervelli S., Nannipieri P., Giovannini G., Perna A. (1977), *Soil Biol. Biochem.*, **9**:393–396.

Champluvier B., Marchal F., Rouxhet P.G. (1989), *Enzyme Microb. Technol.*, **11**(7):422-430.

Chaplin M.F., Bucke C. (1990), *Enzyme Technology*, Cambridge University Press.

Chen J.P., Chiu S.H.(1999), “Preparation and characterization of urease immobilized onto porous chitosan beads for urea hydrolysis”, *Bioprocess Eng*, **21**(4): 323-330.

Chen Y.-Y.M., Burne R.A. (1996), “Analysis of *Streptococcus salivarius* urease expression using continuous chemostat culture”, *FEMS Microbiol. Lett.*, **135**: 223–229.

Chen Y.-Y.M., Clancy K.A., Burne R.A. (1996), “*Streptococcus salivarius* urease: genetic and biochemical characterization and expression in a dental plaque *Streptococcus*”, *Infect. Immun.*, **64**:582–585.

Chen Y.-Y.M., Weaver C.A., Burne R.A. (2000), “Dual functions of *Streptococcus salivarius* urease”, *J. Bacteriol.*, **182**:4667–4669.

Chen Y.-Y.M., Weaver C.A., Mendelsohn D.R., Burne, R.A. (1998), “Transcriptional regulation of the *Streptococcus salivarius* 57.I urease operon”, *J. Bacteriol.*, **180**:5769–5775.

Christianson C.B., Byrnes B.H., Carmona G. (1990), *Fertil. Res.*, **26**:21–27.

Clair M.B.G.St., Bermudez E., Gross E.A., Butterworth B.E., Recio L., Carrano A.V. (2006), “Evaluation of the genotoxic potential of glutaraldehyde”, *Environmental and Molecular Mutagenesis*, **18**:113-119.

Clark G.L., Smith A.F. (1936), “X-ray diffraction studies of chitin, chitosano and derivatives”, *J. Phys. Chem.*, **40**:863-879.

Collins C.M., D’Orazio S.E.F. (1993), *Mol. Microbiol.*, **9**:907–913.

Creason G.L., Schmitt R.M., Douglas E.S., Hendrickson L.L. (1990), *Soil Biol. Biochem.*, **22**:209–211.

Data Sheet/Info EP-4/E, available from Roehm, Kirschenallee, D-64293 Darmstadt, Product Manager Eupergit® C, fax: q49-6151-183520. Matrix Modification of Eupergit® C and Eupergit® C 250 L after Protein Binding/ Removal of Excess of Oxirane Groups.

De Orduna M.R., Patchett M.L., Liu S-Q., Pilone G.J. (2001), “Growth and arginine metabolism of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus*

oeni at different pH values and arginine concentrations”, *appl. environm. microbiol.*, **67(4)**:1657-1662.

Dhar G.M., Mitsutomi M., Ohtakara A. (1993), “Immobilization of glucoamylase on chitosan beads and application to the conversion of starch to glucose”, *Saga Daigaku Nogakubu Iho*, **74**:59-68.

Dixon N.E., Blakeley R.L., Zerner B. (1980), *Can. J. Biochem.*, **58**: 481–488.

Dixon N.E., Gazzola C., Blakeley R.L., Zerner B.(1975), *J. Am. Chem. Soc.*, **97**:4131-4133.

Dixon N.E., Riddles P.W., Gazzola C., Blakeley R.L., Zerner B. (1980), *Can. J. Biochem.*, **58**:1335–1344.

Dukes B., Butzke C., (1996) “Concentration of α -Amino Compounds in Grape Juice can be Rapidly Determined Using an o-Phthalaldehyde/N-acetyl-L-cysteine Spectrophotometric Assay”, *47th Annual Meeting of the American Society for Enology & Viticulture*, Reno, NV.

Dumitriu S, Chornet E. (1998) “Inclusion and release of proteins from polysaccharide-based polyion complexes”, *Adv Drug Deliv Rev*, **31**:223–46.

Dunn B.E., Phadnis S.H.(1998), *Yale J. Biol. Med.*, **71**:63–73.

Dutta PK, Ravikumar MNV, Dutta J. (2002), “Chitin and chitosan for versatile applications”, *J Macromol Sci*, **C42**:307–54.

Eida T., Hidaka H. (1988), *Jpn. Fudo Saiensu*, **27(12)**:56-63.

Esti M., Fidaleo M., Moresi M., Tamborra P. (2007), *J. Agric. Food Chem.*, **55**:

Evans Jr. D.J., Evans D.G., Kirkpatrick S.S., Graham D. (1991), *Microb. Pathogen.*, **10**:15–26.

Famuyiwa O., Ough C. (1991), “Modification of acid urease activity by fluoride ions and malic acid in wines”, *Am. J. Enol. Viticult.*, **42**:79-80.

Faraci W.S., Yang B.V., O’Rourke D., Spencer R.W. (1995), *Bioorg. Med. Chem.*, **3**:605–610.

Fearon W.R., Sander C.(1997), *Proteins*, **28**:72-82.

Felt O, Buri P, Gurny R. (1998), “Chitosan: a unique polysaccharide for drug delivery”, *Drug Dev Ind Pharm*, **24**:979–93.

Fidaleo M., Esti M., Moresi M. (2006), *J. Agric. Food Chem.*, **54**:6226–6235.

Fidaleo M., Lavecchia R. (2003), “Kinetic study of enzymatic urea hydrolysis in the pH range 4-9”, *Chem. Biochem. Eng. Q.*, **17(4)**:311-318.

Fishbein W. N., Engler W. F., Griffin J. L., Scurzi W., Bahr G. F.(1977), “Electron microscopy of negatively stained jack bean urease at three levels of quaternary structure, and comparison with hydrodynamic studies”, *Eur. J. Biochem*, **73**:185–190.

Follmer C., (2008), *Phytochemistry*, **69**:18-28.

Follmer C., Carlini C.R. (2005), *Arch. Biochem. Biophys.*, **435**:15–20.

Follmer C., Wassermann G.E., Carlini C.R., (2004), *Plant Sci.* **167**:241–246.

Font M., Domínguez M.-J., Sanmartín C., Palop J.A., San-Francisco S., Urrutia O., Houdusse F., García-Mina J.M. (2008), *J. Agric. Food Chem.*, **56**:8451–8460.

Francis P.S., Lewis S.W., Lim K.F. (2002), *Trends Anal. Chem.*, **21**:389–400.

Fujinawa S., Burns G., De La Teja P. (1990), “Application of acid urease to reduction of urea in commercial wines”, *Am. J. Enol. Viticult.*, **41(4)**:350-354.

Fujinawa S., Kodama S., Todoroki H., Suzuki T. (1992), “Trace urea determination in red wine and its degradation rate by acid urease”, *Am. J. Enol. Viticult.*, **43(4)**:362-366.

Fujinawa S., Todoroki H., Ohashi N., Toda J., Terasaki M. (1990), “Application of an acid urease to wine: determination of trace urea in wine”, *J. Food Sci.*, **55 (4)**:1018-1038.

Gómez de Segura A., Alcalde M., Yates M., Rojas-Cervantes M.L., López-Cortés N., Ballesteros A., Plou F.J. (2004), “Immobilization of dextranucrose from *Leuconostoc mesenteroides* NRRL B-512F on Eupergit C supports”, *Biotechnol. Progr.*, **20**:1414-1420.

Goodwin C.S., Armstrong J.A., Marshall B.J. (1986), “*Campylobacter pyloridis*, gastritis, and peptic ulceration”, *J Clin Pathol.*, **39(4)**:353–365.

Hasebe K., Hikima S., Yoshida H. (1991), “Determination of L-lactate by differential-pulse polarography with covalently immobilized enzyme on Chitopearl”, *Fresenius J Anal Chem*, **339**:261-263.

Hawtin, P. R., Stacey A. R., Newell D. G. (1990), “Investigation of the structure and localization of the urease of *Helicobacter pylori* using monoclonal antibodies”, *J. Gen. Microbiol.*, **136**:1995–2000.

Hellerman L., Chinard F.P., Deitz V.R. (1943), *J. Biol. Chem.*, **147**:443–462.

Holland JR, Hosley H, Scharlau C, Carbone PP, Frei E 3rd, Brindley CO, Hall TC, Shnider BI, Gold GL, Lasagna L, Owens AH Jr, Miller (1966) "A controlled trial of

urethane treatment in multiple myeloma", *Blood*, **27(3)**: 328–42.
<http://bloodjournal.hematologylibrary.org/cgi/content/abstract/27/3/328>.

Holm L., Sander C.(1997), *Proteins*, **28**:72-82.

Howell S.F., Sumner J.B. (1934), *J. Biol. Chem.*, **65**:380–383.

Hudson SM, Smith C. (1998), "Polysaccharides: chitin and chitosan: chemistry and technology of their use as structural materials", In: Kaplan DL, editor. Biopolymers from renewable resources. Berlin: Springer; p. 96–118.

Itoh H., Sato T., Takeuchi T., Khan A.R., Izumori K. (1995), "Preparation of D-sorbose from D-tagatose by immobilized D-tagatose 3-epimerase", *J Ferment Bioeng*, **79**:184-185.

Jabri E., Carr M.B., Hausinger R.P., Karplus P.A. (1995), *Science*, **268**:998–1004.

Jabri, E., Lee M. H., Hausinger R. P., Karplus P. A. (1992), "Preliminary crystallographic studies of ureases from jack bean and from *Klebsiella aerogenes*", *J. Mol. Biol.*, **227**:934–937.

Juszkiewicz A., Zaborska A., Łapta's A., Olech Z. (2004), *Food Chem.* **85**:553–558.

Kadima T.A., Pickard M.A. (1990), "Immobilisation of chloroperoxidase on aminopropyl-glass", *Appl Environ Microbiol*, **56**:3473-3477.

Kakimoto S., Miyashita H., Sumino Y., Akiyama S. (1990a), "Properties of acid ureases from *Lactobacillus* and *Streptococcus* strains", *Agric. Biol. Chem.*, **54**: 381–386.

Kakimoto S., Okazaki K., Sakane T., Imai K., Sumino Y., Akiyama S., Nakao Y. (1989), *Agric. Biol. Chem.*, **53**:1111–1117.

Kakimoto S., Sumino Y., Kawahara K., Yamazaki E., Nakatsui I. (1990b), "Purification and characterization of acid urease from *Lactobacillus fermentum*", *Appl. Microbiol. Biotechnol.*, **32**:538–543.

Kamel N.M.Y., Ragaa R.H. (1973), "Constitutive synthesis of urease in *Aerobacter aerogenes* PRL-R3. The effect of different nitrogenous compounds on its inhibition and formation", *Acta Med. Biol. Ger.*, **30**:457-466.

Karplus P.A., Pearson M.A., Hausinger R.P. (1997), *Acc. Chem. Res.*, **30**:330–337.

Katchalski-Kazir E, Kraemer DM (2000) "Eupergit C, a carrier for immobilization of enzymes of industrial potential", *Journal of Molecular Catalysis B: Enzymatic* **10**: 157-176.

Kawamoto H., Oguma T., Sekine H. (2001), "Kobayashi M. Immobilization of cycloisomaltooligosaccharide glucanotransferase for the production of cycloisomaltooligosaccharides from dextran", *Enzyme Microb Technol*, **28**:515-521.

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

Kennedy J.F., Cabral J.M.S. (1983), "Immobilized enzymes", In: Scouten WH, editor. Solid phase biochemistry. Analytical and synthetic aspects. New York: John Wiley & Sons; p.253–391.

Kenny G.E. (1983), *Yale J. Biol. Med.*, **56**:717–722.

Khan W.N., Lodhi M.A., Ali I., Haq A.-U., Malik A., Bilal S., Gul R., Choudhary M.I. (2006), *J. Enzyme Inhib. Med. Chem.*, **21**:17–19.

Khor E. (2002), “Chitin: a biomaterial in waiting”, *Curr Opin Solid State Mater Sci*, **6**:313–7.

Kibune K., Yamaguchi Y., Motosugi K. (1988), Jpn. Kokai Tokkyo Koho JP 63,209,661 [88,209,661].

Kimura T., Ogata M., Kobayashi H., Yoshida M., Oishi K., Nakakuki T. (1990), “Continuous production of maltotetraose using a dual immobilized enzyme system and maltotetraose-forming amylase and pullulanase”, *Biotechnol Bioeng*, **36**:790-796.

Kise H., Hayakawa A. (1991), “Immobilization of proteases to porous chitosan beads and their catalysis for ester and peptide synthesis in organic solvents”, *Enzyme Microb Technol*, **13**:584-588.

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

Kobashi K., Hase J.-I., Uehara K. (1962), “Specific inhibition of urease by hydroxamic acids”, *Biochim. Biophys. Acta*, **62**:380-383.

Kodama S, Yotsuzuka F. (1996), “Acid urease: reduction of ethyl carbamate formation in sherry under simulated baking conditions”, *J. Food Sci.*, **61(2)**:304-307.

Kodama S. (1996), “Optimal conditions for effective use of acid urease in wine”, *J. Food Sci.*, **61(3)**:548-552.

Kodama S., (1996) “Optimal Conditions For Effective Use Of Acid Urease in Wine”, *Journal Of Food Science*, **61 (3)**:548-552.

Kojima S., Bohner A., von Wirén N. (2006), *J. Membr. Biol.*, **212**:83–91.

Košmerl T., Abramovič H., Klofutar C. (2000), "The rheological properties of Slovenian wines", *J Food Eng*, **46**:165-171.

Kourkoutas Y, Bekatorou A, Banat IM, Marchant R, Koutinas AA (2004) "Immobilization technologies and support materials suitable in alcohol beverages production:a review", *Food Microbiology* **21**:377-397.

Kraemer D.M., Lehmann K., Pennewiss M., Plainer H. (1975), "Photo-beads and oxirane-beads as solid supports for catalysis and biospecific adsorption, in: H. Peeters Ed. , *Protides of Biological Fluids*", *23rd Colloquium, Pergamon, Oxford*, pp. 501–511.

Kraemer D.M., Pennewiss H., Plainer H., Schnee R., Schleier W. (1985), Roehm, United States Patent, 4511694.

Kraemer D.M., Sauber K. (1981), Roehm. European Patent 0000486.

Krajewska (2009a), "Ureases I. Functional, catalytic and kinetic properties: a review", *Journal of Molecular Catalysis B: Enzymatic*, **59**:9-21.

Krajewska (2009b), "Ureases II.Properties and their customizing by enzyme immobilisations: a review", *Journal of Molecular Catalysis B: Enzymatic*, **59**:22-40.

Krajewska B. (1991), *J. Chem. Technol. Biotechnol.*, **52**:157–162.

Krajewska B. (2004), "Application of chitin- and chitosan- based materials for enzyme immobilisations: a review", *Enzyme Microbial. Technol.*, **35**:126-139.

Krajewska B. (2008), *J. Enzyme Inhib. Med. Chem.*, **23**:535–542.

Krajewska B., Ciurli S. (2005), *Plant Physiol. Biochem.*, **43** 651–658.

- Krajewska B., Zaborska W. (1999), *J. Mol. Catal. B: Enzym.*, **6**:75–81.
- Krajewska B., Zaborska W., Leszko M. (2001), *J. Mol. Catal. B: Enzym.*, **14**:101–109.
- Krawczyński T. vel Krawczyk T., Moszczyńska M., Trojanowicz M. (2000), *Biosens. Bioelectron.*, **15**:681–691.
- Kubota N, Kikuchi Y. (1998), “Macromolecular complexes of chitosano”, In: Dumitriu S, editor. *Polysaccharides*. New York: Dekker; p. 595–628.
- Kumar S., Dwevedi A., Kayastha A.M. (2009), “Immobilization of soybean (*Glycine max*) urease on alginate and chitosan beads showing improved stability: Analytical applications”, *J Molecular Catalysis B: Enzymatic*, **58**:138-145.
- Kuswandi B. (2003), *Anal. Bioanal. Chem.*, **376**:1104–1110.
- Kuwahara Y., Otsuka N., Manabe M. (1988), *Nippon Shokuhin Kogyo Gakkaishi*, **35(11)**:776-780.
- Latkar M., Chakrabarti T. (1994), *Water Environ. Res.*, **66** :12–15.
- Lethbridge G., Bull A.T., Burns R.G. (1976), *Soil Biol. Biochem.*, **8**:99–102.
- Lethbridge G., Bull A.T., Burns R.G. (1981), *Pestic. Sci.*, **12**:147–155.
- Li Q., Kikuchi K., Yamamoto Y., Yagi N. (1988), “Application and properties of chitosan”, *J. Bioact. Comp. Polym.*, **7**:371-397.
- Li Y.-G., Shi D.-H., Zhu H.-L., Yan H., Ng S.W. (2007), *Inorg. Chim. Acta*, **360**: 2881–2889.

- Lim B-C, Kim H-J, Oh D-K. (2008), “Tagatose production with pH control in a stirred tank reactor containing immobilized L-arabinose from *Thermotoga neapolitana*”, *Appl Biochem Biotechnol*, **149**: 245-253.
- Lim S., Lee K-Y, Lee Y-B, Song K-B. (2001), “Immobilization of levan fructotransferase for the production of di-fructose anhydride from levan”, *Biotechnol Lett*, **23**:1335-1339.
- Lister A. J. (1956), “The kinetics of urease activity in *Corynebacterium renale*”, *J. Gen. Microbiol.*, **14**:478-484.
- Lodhi M.A., Hussain J., Abbasi M.A., Jassbi A.R., Choudhary M.I., Ahmad V.U. (2006), *J. Enzyme Inhib. Med. Chem.*, **21**:531–535.
- Lopreore C., Byers L.D. (1998), *Arch. Biochem. Biophys.*, **349**:299–303.
- Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. (1951), “Protein measurement with the Folin phenol reagent”, *J Biol Chem*, **193**:265-275.
- Mahadevan S., Sauer F., Erfle J.D. (1976), “Studies on bovine rumen bacterial urease” *J. Anim. Sci.*, **42**:745-753.
- Mahadevan, S., Sauer F. D., Erfle J. D. (1977), “Purification and properties of urease from bovine rumen”, *Biochem. J.*, **163**:495-501.
- Malette W.G., Quigley H.J., Adiches E.D. (1986), In *Chitin in Nature and Technology*, R. Muzzarelli, C. Jeniaux and G.W. GooDay, eds, New York; Plenum Press, pp.435-442.
- Mateo C., Abian O., Fernandez-Lafuente R. (2000), “Increase in the conformational stability of enzymes immobilized on epoxy-activated supports by favouring additional multipoint attachment”, *Enzyme Microb. Technol.*, **26**:509-515

Matsumoto K. (1993) "Removal of urea from alcoholic beverages by immobilised acid urease", *Bioprocess Tech.*, **16**:255-273.

Matsuo T., Kobayashi T., Kimura Y., Tsuchiyama M., Oh T., Sakamoto T., Adachi S. (2008), "Synthesis of glyceryl ferulate by immobilized ferulic acid esterase", *Biotechnol Lett*, **30(12)**: 2151-2156.

McCarty G.W., Bremner J.M., Lee S.J. (1990), *Plant Soil*, **127**:269–283.

McGee D.J., Mobley H.L.T. (2000), *Curr. Opin. Gastroenterol.* **16**:24–31.

Menegassi A, Wassermann G.E., D. Olivera-Severo, Becker-Ritt A.B., Martinelli A.H.S., Feder V, Carlini C.R., (2008), *J. Agric. Food Chem.* 56:4399–4405.

Miyagawa K., Sumida M., Nakao M., Harada M., Yamamoto H., Kusumi T., Mobley H.L.T., Hausinger R.P. (1989), "Microbial ureases: significance, regulation and molecular characterization", *Microbiol. Rev.*, **53(1)**:85-108.

Mobley H.L.T., Hausinger R.P. (1989), "Microbial ureases: significance, regulation, and molecular characterization", *Microbiol. Rev.*, **53(1)**:85-108.

Mobley H.L.T., Island M.D., Hausinger R.P. (1995), "Molecular biology of microbial ureases", *Microbiol. Rev.*, **59(3)**:451-480.

Monteiro F., Trousdale K., Bisson L.(1989), "Ethyl Carbamate Formation in Wine: Use of Radioactively Labeled Precursors to Demonstrate the Involvement of Urea.", *Am. J. Enol. Vitic.*, **40 (1)**: 1-8.

Mora D., Fortina M.G., Parini C., Ricci G., Gatti M., Giraffa G., Manachini P.L. (2002), "Genetic diversity and technological properties of *Streptococcus thermophilus* strains isolated from dairy products", *J. Appl. Microbiol.*, **93**:278–287.

Mora D., Maguin E., Masiero M., Parini C., Ricci G., Manachini P.L., Daffonchio D. (2004), "Characterization of the urease genes cluster of *Streptococcus thermophilus*", *J. Appl. Microbiol.*, **96**:209–219.

Mora D., Monnet C., Parini C., Guglielmetti S., Mariani A., Pintus P., Molinari F., Daffonchio D., Manichini P.L. (2005), "Urease biogenesis in *Streptococcus thermophilus*", *Res. Microbiol.*, **156**:897–903.

Morimoto K., Park C-S, Ozaki M., Takeshita K., Shimonishi T., Granstroem T.B., Takata G., Tokuda M., Izumori K. (2006), "Large scale production of D-allose from D-psicose using continuous bioreactor and separation system", *Enzyme Microb Technol.*, **38**:855-859.

Motosugi K., Yamaguchi Y., Kibune K. (1988), Jpn. Kokai Tokkyo Koho JP 63,90,507 [88,90,507].

Moynihan H. J., Lee C.K., Clark W., Wang N.-H.L. (1989), "Urea hydrolysis by immobilized urease in a fixed-bed reactor: analysis and kinetic parameter estimation", *Biotechnol. Bioeng.*, **34**:951-963.

Mulrooney S., Zakharin T., Schaller R.A., Hausinger R.P. (2001), *Arch. Biochem. Biophys.*, **394**:280–282.

Murafuji T., Azuma T., Miyoshi Y., Ishibashi M., Rahman A.F.M.M, Migita K., Sugihara Y., Mikata Y. (2006), *Bioorg. Med. Chem. Lett.*, **16**:1510–1513.

Muzzarelli R.A.A. (1977), "Chitin", Toronto:Pergamon of Canada Ltd.

Nakano H., Takenishi S., Watanabe Y. (1984), *Agric. Biol. Chem.*, **48**:1495–1502.

National Toxicology Program (2005), NIEHS, National Institutes of Health, "Eleventh Report on Carcinogens, Urethane", accessed May 13,2006 at: <http://ntp.niehs.nih.gov/roc/eleventh/profiles/s184uret.pdf>

Ngo T.T. (1980) "Minireview: Bioanalytical applications of immobilized enzymes", *Int. J. Biochem.* **11**: 459-465.

Nishimura K., Nishimura S., Nishi N., Tokura S., Azuma I. (1986), In *Chitin in Nature and Technology*, R. Muzzarelli, C. Jeniaux and G.W. GooDay, eds, New York; Plenum Press, pp.477-483.

Ohnishi S.T., Barr J.K. (1978), "A simplified method of quantitating proteins using the biuret and phenol reagents", *Anal. Biochem.*, **86**:193.

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

Ough C. S., Trioli G. (1988), "Urea removal from wine by an acid urease", *Am. J. Enol. Viticult.*, **39(4)**:303-7.

Ough, C.S. (1976), "Ethyl carbamate in fermented beverages and foods. I. Naturally occurring ethyl carbamate.", *J. Agr. Food Chem.*, **24**: 323-327.

Paul W, Sharma CP. (2000), "Chitosan, a drug carrier for the 21st century: a review", *STP Pharmaceut Sci*, **10**:5–22.

Pennewiss M., Knoell H., Masanek H. (1970), Roehm, German Patent DE2009218C3.

Peter M. (1995), "Applications and environmental aspects of chitin and chitosano", *J Macromol Sci Pure Appl Chem*, **A32**:629- 40.

Pianotti, R. S., Mohan R. R., Schwartz B. S. (1966), "Proteus vulgaris urease: in vitro inhibition by urea analogues", *Proc. Soc. Exp. Biol. Med.*, **122**:506-508.

Polacco J.C., Holland M.A. (1993), *Int. Rev. Cytol.*, **145**:65-103.

Polacco, J.C., Holland M.A. (1994) “Genetic control of plant ureases; in Genetic Engineering”, (Setlow, J.K., ed.),**16**, pp. 33-48, Plenum Press, New York.

Pope A.J., Toseland C.D.N., Rushant B., Richardson S., McVey M., Hills J. (1998), *Digest. Dis. Sci.*, **43**:109–119.

Preininger C., Wolfbeis O.S. (1996), *Biosens. Bioelectron.*, **11**:981–990.

Pugh K.B., Waid J.S. (1969), “The influence of hydroxamates on ammonia loss from an acid loamy sand treated with urea”, *Soil Biol. Biochem.*, **1**:195-206.

Qin Y, Cabral J. M. S. (2002), “Properties and application of urease”, *Biocatal. Biotransor*, **20(1)**:1-14.

Qui-Xiang L., Freney J.R., Keerthisinghe D.G., Peoples M.B. (1994), *Soil Biol. Biochem.*, **26**:1059–1065.

Rigby G.W. (1934b), “Process for the preparation of films and filaments and products thereof”, U.S. Patent, 2,040, 880.

Rigby G.W. (1934a), “Substantially undergraded deacetylated chitin and process for producing the same”, U.S. Patent 2, 040, 879.

Rosenstein I. J., Hamilton-Miller J. M., Brumfitt W. (1981), “Role of urease in the formation of infection stones: comparison of ureases from different sources”, *Infect. Immun.*, **32**:32-37.

Rosenstein I.J.M., Hamilton-Miller J.M.T. (1984), *CRC Crit. Rev. Microbiol.*, **11**:1–12.

Sabbaj J., Sutter V. L., Finegold. S. M. (1970), “Urease and deaminase activities of fecal bacteria in hepatic coma”, *Antimicrob. Agents Chemother.* 1969, p. 181-185.

Samtoy B., DeBeukelaer M.M. (1980), “Ammonia encephalopathy secondary to urinary tract infection with *Proteus mirabilis*”, *Pediatrics*, **65**:294-297.

Sanford P.A., Steinnes A. (1991), “Biomedical applications of high-purity chitosan”, In: Shalaby SW, McCormick CL, Butler GB (Eds) *Water-soluble polymers: Synthesis, solution properties and applications*. ACS Symp. Series 467. Washington DC: American Chemical Society, pp. 430-445.

Satterfield C.N., Sherwood T.K. (1963), “The role of diffusion in catalysis”, Addison-Wesley Publ Co, Reading (Mass, USA), p. 47.

Schäfer U.K., Kaltwasser H. (1994), *Arch. Microbiol.*, **161**:393–399.

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

Schmahl D., Port R., Wardendorf J. (1977), “A dose-response study of urethane carcinogenesis in rats and mice”. *Int J Cancer*. **19**: 77-80.

Selvamurugan C., Lavanya A., Sivasankar B. (2007), “A comparative study on immobilisation of urease on different matrices”, *Journal of Scientific & Industrial Research*, **66**:655-659.

Shaw W.H.R. (1954), *J. Am. Chem. Soc.*, **76**:2160–2163.

Shaw W.H.R., Raval D.N. (1961), *J. Am. Chem. Soc.*, **83**:3184–3187.

Sheldon R.A. (2007), *Adv. Synth. Catal.*, **349**:1289–1307.

Sherlock S. (1981), "Diseases of the liver and biliary system", Blackwell Scientific Publications. Oxford, p.91-106.

Shi D.-H., You Z.-L., Xu C., Zhang Q., Zhu H.-L. (2007), *Inorg. Chem. Commun.*, **10**:404–406.

Shin H-J, Park J-M, Yang J-W. (1998), "Continuous production of galactooligosaccharides from lactose by *Bullera singularis* β -galactosidase immobilized in chitosan beads", *Process Biochem*, **33**: 787-792.

Singla AK, Chawla M. (2001), "Chitosan: some pharmaceutical and biological aspects—an update", *Pharm Pharmacol*, **53**:1047–67.

Sirca A.E., Woodman R.J. (1971), *J. Nat. Cancer Inst.*, **47(2)**:377-388.

Sirko A., Brodzik R. (2000), "Plant ureases: Roles and regulation", *Acta Biochim. Pol.*, **47(4)**:1189-1195.

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

Sponholz W.R., Kürbel H.,Dittrich H. (1991), "Beitrage zur Bildung von Ethylcarbamat in Wein ", *Wein-Wissenschaft.*, **46(1)**:11-17.

Sumner J.B (1926), *J.Biol. Chem.*, **69**:435-441.

Sumner J.B., (1926), *J. Biol. Chem.* **70**:97–98.

Suzuki K., Ogawa Y., Hashimoto K., Suzuki S., Suzuki M. (1984), *Microbiol. Immunol.*, **28**:903-912.

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

Takebe S., Kobashi K. (1988), *Chem. Pharm. Bull.*, **36**:693–699.

Takeshita K., Suga A., Takada G., Izumori K. (2000), "Mass production of D-psicose from D-fructose by a continuous bioreactor system using immobilized D-tagatose 3-epimerase", *JBB (Journal of Bioscience and Bioengineering)*, **90**: 453-455.

Tanaka T., Kawase M., Tani S. (2003), *Life Sci.*, **73**:2985–2990.

Tanaka T., Kawase M., Tani S. (2004), *Bioorg. Med. Chem.*, **12**:501–505.

Tarun E.I., Rubinov D.B., Metelitz D.I. (2004), *Appl. Biochem. Microbiol.*, **40**: 398–406.

Taylor M., Goodwinn C.S., Karim Q.N. (1988), *FEMS Microbiol. Lett.*, **55**: 259–262.

Tegmo-larsson I., Henick-Kling T., (1990) "The Effect Of Fermentation And Extended Lee Contact On Ethyl Carbamate Formation In New-York Wine.", *Am. J. Enol. Vitic.*, **41(4)**:269-272.

Tischer W., Wedekind F. (1999), "Immobilized enzymes: methods and applications", *Top. Curr. Chem.*, **200**:95–126.

Tischer W., Wedekind F. (1999), "Immobilized enzymes: methods and applications", *Top Curr Chem*, **200**:95–126.

Todd M.J., Hausinger R.P. (1987), *J. Biol. Chem.*, **262**:5963–5967.

Todd M.J., Hausinger R.P. (1989), *J. Biol. Chem.*, **264**:15835–15842.

Todd M.J., Hausinger R.P. (2000), *Biochemistry*, **39**:5389–5396.

Toren Jr. E.C., Burger F.J. (1968), *Mikrochim. Acta*, 1049–1058.

Torres-Bacete J., Arroyo M., Torres-Guzmán R., de la Mata I., Castellón M.P., Acebal C. (2000), “Covalent immobilization of penicillin acylase from *Streptomyces lavendulae*”, *Biotechnology Letters*, **22**: 1011-1014.

Treybal R.E. Mass Transfer Operations. 2nd Edn, McGraw-Hill Book Co, New York, 1968.

Trioli G., Ough C. S. (1989), “Causes for inhibition of an acid urease from *Lactobacillus fermentus*”, *Am. J. Enol. Viticult*, **40(4)**:245-52.

Tripton K.F., Dixon H.B.F. (1979), “Effects of pH on enzymes”, In *Methods in Enzymology*, Academic Press: New York, **63**:183-234.

Vassiliou S., Grabowiecka A., Kosikowska P., Yiotakis A., Kafarski P., Berlicki Ł. (2008), *J. Med. Chem.*, **51**: 5736–5744

Virginia Commonwealth University (2006), The Chemical/Biological Safety Section (CBSS) of the Office of Environmental Health and Safety, “Working with Urethane” ; <http://www.vcu.edu/oehs/chemical/biosafe/urethane.pdf>.

Wakayama Y., Sekino Y. (1987), “Stabilization of coloring agents and coloured products with chitosan”, *Jpn. Kokai Tokkyo Koho JP 62,297,365* [877,297,365].

Wang W.H., Kohler B., Q-Cao F., Liu H., (2008), *Plant. Sci.*, **175**:467-477.

Wang, S., Lee M. H., Hausinger R. P., Clark P. A., Wilcox D. E., Scott R. A. (1994), “Structure of the dinuclear active site of urease. X-ray absorption spectroscopic study of native and 2-mercaptoethanol-inhibited bacterial and plant enzymes”, *Inorg. Chem.*, **33**:1589–1593.

Watson C.J., Akhonzada N.A., Hamilton J.T.G., Matthews D.I. (2008), *Soil Use Manage.*, **24**:246–253.

Weast R.C. (1982/83), *CRC Handbook of Chemistry and Physics*, 63rd Edn., CRC Press Inc., Boca Raton, Florida.

Weber M., Jones M.J., Ulrich J. (2008), *Food Bioprod. Process*, **86**:43–52.

Wei X, Zhang M, Gorski W. (2003), “Coupling the lactate oxidase to electrodes by ionotropic gelation of biopolymer”, *Anal Chem*, **75**:2060–4.

WPIG (1983) “Guidelines for characterization of immobilized biocatalysts”, *Enzyme Microbiol Technol*, **5**: 304-307.

Yamasaki Y., Fukumoto I., Kumagai N., Ohta Y., Nakagawa T., Kawamukai M., Matsuda H. (1992), “Continuous chitosan hydrolyzate production by immobilized chitosanolytic enzyme from *Enterobacter* sp. G-1. BBB”, *Biosc. Biotechnol. Biochem.*, **56(10)**:1546-51.

Yamazaki E., Kurasawa T., Kakimoto S., Sumino Y., Nakatsui I. (1990), *Agric. Biol. Chem.*, **54**:2433–2435.

Yano S., Tsugita T. (1988), “Chitosan containing seeds coatings for yield enhancement”, Jpn. Kokai Tokkyo Koho JP 63,139,102 [88,139,102].

Yoshizawa K., Amachi T., Nakayama T. (1999), *J. Biotechnol.*, **68**:227–236.

Zaborska W., Krajewska B., Olech Z. (2004), *J. Enzyme Inhib. Med. Chem.*, **19**:65–69.

Zeiger E., Gollapudi B., Spencer P. (2005), “Genetic toxicity and carcinogenicity studies of glutaraldehyde - a review”, *Mutation Research/Reviews in Mutation Research*, **589**:136-151.

Zerner B. (1991), “Recent advances in the chemistry of an old enzyme, urease”, *Bioorg. Chem.*, **19**:116-131.

Zhang L., Mulrooney S.B., Leung A.F.K., Zeng Y., Ko B.B.C., Hausinger R.P., Sun H. (2006), *Biometals*, **19**:503–511.

Zhou J., Chen S., Wang J. (2008), “Research on orientedly immobilised urease via *Concanavalin A*”, *Chin. J. Biotech.*, **24**:617-621.

Zimmerli B., Schlatter J. (1991), “Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment”, *Mutat. Res.*, **259**: 325–350.

Zotta T., Ricciardi A., Rossano R., Parente E. (2008), “Urease production by *Streptococcus thermophilus*”, *Food Microbiol.*, **25**:113-1.

