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Assessment of Urea Degradation Rate in Model Wine Solutions by Acid Urease from *Lactobacillus fermentum*

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The specific activity (π_A) of a whole cell acid urease preparation was assessed in model wine solutions at different levels of malic (*M*) and lactic acids, metabisulfite, ethanol, and pH by performing a central composite design. *M* and then pH were found to be the most controlling variables, their effects being practically coincident but of opposite sign. For urea concentrations up to ≈1 mol m^{-3} the ammonium formation rate was assumed of the pseudo-first-order with respect to urea, this being confirmed by two independent validation tests performed at 20 °C for as long as 24 h. In the case of real wines the effective pseudo-first-order kinetic rate constants were found to be smaller than those pertaining to the model solutions having the same wine composition and pH by a factor varying from 10 to 1000, this affecting significantly the specific urease treatment costs per liter of wine treated.

KEYWORDS: Acid urease; composite design; enzyme processing costs; ethanol; lactic and malic acids; model wine solutions; pH; metabisulfite; pseudo-first-order kinetic constant rate; specific activity; urea degradation kinetics

INTRODUCTION

Ethyl carbamate (urethane, EC) is a naturally occurring component in all fermented foods and beverages, being spontaneously produced by the reaction between urea and ethanol (*1*). Because EC has shown a potential carcinogenic activity when administered in high doses in animal tests (*2*, *3*), there is a great deal of interest in reducing EC levels in food products.

Canadian regulation limits the maximum EC concentration in wines to 30 mg m^{-3}, that in fortified wines (sherries and ports) to 100 mg m^{-3}, that in distilled spirits to 150 mg m^{-3}, and that in fruit brandies and liqueurs to 400 mg m^{-3} (*4*−*6*); in all other countries a safe level in wines of 15 mg m^{-3} has been so far recommended (*7*, *8*).

Arginine and citrulline (*1*) and urea natural concentrations in grape musts, as well as the biological activity of yeasts and lactic acid bacteria (LAB), affect EC content in wines. Arginine, usually one of the most abundant yeast-available amino acids in grape juice, is converted into urea by wine yeast (*9*). To a lesser extent citrulline, an amino acid that is formed during arginine deamination by LAB, can serve as an EC precursor (*10*). However, the key reaction for EC formation in wine is that between urea and ethanol, no correlation being found between the amount of potential EC and the concentrations of the above amino acids (*6*). Moreover, the rate of such a chemical reaction varies exponentially as the temperature is increased (*11*).

Besides the numerous preventive actions to reduce EC levels (i.e., control of fertilization techniques, adjustment of nutrient contents in grape musts, use of suitable yeast and lactic acid bacteria strains, and control of storage conditions) issued by the U.S. Food and Drug Administration (*7*), the hydrolysis of urea to ammonia and carbon dioxide by a highly specific enzyme, such as acid urease (EC 3.5.1.5), seems to be a suitable way to avoid EC formation from such a precursor:

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

This enzyme is mainly found in the jack bean (*Canavalia ensiformis*) (*12*). Unfortunately, its maximum activity is at pH 6.5−7.5, whereas the pH of alcoholic beverages, such as sake and wine, is on the acid side, that is, at about pH 4.4 and 3.2, respectively. Urease with an optimum pH on such a pH range was first found in *Lactobacillus* sp. from the rat gastrointestinal tract (*13*, *14*). Urease from *Lactobacillus fermentum* was partially purified, characterized, and named *acid urease* by Takebe and Kobashi (*15*). Although acid ureases were found in several *Lactobacillus*, *Streptococcus*, *Escherichia*, *Staphylococcus*, *Morganella*, *Bifidobacterium*, *Arthrobacter*, and *Zoogloea* species (*16*, *17*), the commercial grade acid ureases are currently obtained from *L. fermentum* (Takemate-AU, Nagapshin, Desterate, and Enzeco acid urease, supplied by Takeda Chemical Industries Ltd., Osaka, Japan; Nagase Biochemicals Ltd., Osaka, Japan; Toyo Jozo Co., Tokyo, Japan; and Enzyme Development Corp., New York, respectively) or *Arthrobacter mobilis* (U Enzyme supplied by Suntory Ltd., Osaka, Japan), and their use in sake and enology has been allowed in Japan by the National Tax Administration Agency since 1987 (*16*) and in the European Union (EU) by EU Regulation 1622/00 - All.XI.

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Table 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)

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

There are a great number of papers demonstrating the feasibility of acid urease application for the removal of urea from several types of wines (*11*, *18*−*22*). The efficiency of urease treatment varied 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 (*23*−*25*).

The greater content of fluoride in California wines than in European and Japanese wines was attributed to the extensive use of cryolite (AlF_6Na) as an insecticide in California vineyards (*24*). This fact was further confirmed by more recent trials performed by Seiichi and Kiyoshi (*26*), the rate of urea degradation by acid urease in European wines being 6−9-fold higher than that in American white and rose wines, but almost similar for red wines.

As the overall content of phenolic compounds was increased from 0 to 880 g m^{-3} of gallic acid equivalent by integrating the model wine samples with catechin or grape seed tannins, the relative activity of the killed cell preparation (Takemate-AU) tended to reduce from unity to about 0.87 or 0.59, respectively, whereas that of the purified preparation (Nagapshin) reduced to 0.55 or 0.14, respectively (*23*). In this way, it was shown that the grape seed extract had a stronger inhibitory effect than catechin, this being less effective in the killed cell preparation than in the purified one.

Among the organic acids present in wines, the acid urease from *L. fermentum* was inhibited in the following decreasing order by L-malic, L-lactic, D-lactic, pyruvic, α-ketoglutaric, and acetic acids, whereas tartaric or succinic acid exerted no inhibitory effect. Moreover, the relative enzyme activity tended to reduce linearly as the logarithm of the concentration of free malic acid increased, the latter being dependent on the initial L-malate content and pH (*23*).

Similarly, when the model wine samples were enriched with potassium metabisulfite ($K_2S_2O_5$) up to 200 g m^{-3}, the enzyme activity was found to be more sensitive to the free sulfur dioxide than the bound one, this reducing a great deal as the pH was lowered from 4.0 to 3.0 (*23*). Finally, the urease activity of the killed cell preparation tended to reduce almost linearly from unity to ≈68% as the ethanol concentration was increased from 0 to 15% (v/v) (*23*).

Although urea removal from sake (*16*) or California sherries (*11*) by acid urease has been industrialized in Japan and the United States, respectively, the enzyme process has a few drawbacks. Because the enzyme preparation specific costs are of the order of 1 euro/g, the enzymatic processing costs per each liter of product treated may be uneconomical, especially if the process time is required to be <7 days. To avoid kidney stones and other diseases, urease can be quite easily inactivated by resorting to pasteurization (*16*) and clarification in the case of sake production or removed via bentonite adsorption and filtration in the case of table wine production (*25*). This clearly shows that the enzyme is applicable to the elimination of urea in fermented beverages with minimal modification of the conventional processes. Moreover, by immobilizing acid urease on polyacrylonitrile (PAN) fibers (140 units/g of wet weight carrier), it was possible to operate the process in the continuous manner over 150 days under practically constant temperature (11−16 °C) and space velocity (20−50 vol vol^{-1} h^{-1}), thus reducing the urea content of sake from 12−37 to <3 g m^{-3} (*16*).

Despite this method having been industrialized and used for urea removal from sake by many companies in Japan, its extension to wines is restricted yet. The quite numerous experimental trials currently available refer to a limited number of wine types (*11*, *18*−*21*, *23*−*27*). However, their main results do not appear to be usable for a safe extrapolation of the urease activity in any wine of given characteristics as a function of the enzyme concentration. This information is necessary to estimate the enzyme specific costs per unit liter of wine treated, as well as to estimate the potential benefits of the immobilized urease process against the free one.

The main aim of this work was to set up a short-cut method to predict the urea removal rate in model wine solutions. To this end, the kinetics of urea degradation by a microbial acid urease in different buffers at two temperature levels (20 and 37 °C) was preliminary assessed. Then, a composite design experiment was replicated twice to determine the main effects and interactions of the most inhibitory wine components (i.e., malic and lactic acids, potassium metabisulfite, and ethanol), as well as pH, on the acid urease activity. Finally, the combined inhibitory action of these factors on urease activity as predicted from the empirical modeling was compared with the effective activity observed either in two model wine solutions at long treatment times or in a few real wines, as extracted from literature (*23*, *25*) and used to estimate roughly the processing costs for such a detoxification process.

MATERIALS AND METHODS

The commercial preparation Enzeco Acid Urease (Enzyme Development Corp., New York) from *L. fermentum* was used. It consisted of a partially soluble powder to be stored at 4 °C. Its claimed specific activity was 3.3−4.0 units mg^{-1}, where 1 unit corresponds to the amount of powder that liberates 1 μmol min^{-1} of ammonia from urea at 37 °C, once it is dissolved in a 0.1 kmol m^{-3} sodium acetate buffer (pH 4) enriched with urea (83.33 mol m^{-3}) and ethanol (12.5% v/v). Once the powder was suspended in deionized water just before use, its specific activity was checked using different buffer solutions by varying temperature, pH, and urea and urease concentrations (**Table 1**). All other chemicals were of reagent grade.

To investigate the effect of malic (M) and lactic (L) acids, potassium metabisulfite (K), ethanol (E) and pH on the enzyme activity, a half replicate 2^5 factorial experiment was used by confounding the four-factor interaction ($M-L-K-E$) with pH. All model wine solutions also contained constant concentrations of urea (1 mol m^{-3}) and tartaric acid (TA = 5 kg m^{-3}). Extra points were also added according to a central composite design (*28*).

Table 2 shows the experimental conditions in natural and coded levels using the following dimensionless equations:

Table 2. Natural and Coded Levels of the Factors of the Composite Experimental Design

	factor level					
factor	$-\alpha$	−1	0	+1	$+\alpha$	unit
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}
$K_2S_2O_5$ (*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	

$$x_1 = (M - 2.5)/1.25$$

$$x_2 = (L - 1.750)/0.875$$

$$x_3 = (K - 0.20)/0.05 \quad (2)$$

$$x_4 = (E - 13.0)/1.5$$

$$x_5 = (\mathrm{pH} - 3.50)/0.25$$

Initial reaction rates were calculated by the slopes of the concentration versus time plots. Data points were taken over a time period of 10 min. After the pH was adjusted by adding NaOH at 1 kmol m^{-3}, a buffer or a model wine solution was poured into 10 cm^3 rubber-capped flasks. These were immersed in a water bath placed over a magnetic multistirrer (model Multistirrer 15, Velp Scientifica, Milan, Italy) to maintain the reaction temperature constant within ±0.2 °C by means of a thermostat model F3 (Haake, Karlsruhe, Germany). After no less than 10 min, the hydrolytic reaction was started by adding 1 cm^3 of the enzyme dispersion so as to vary the initial enzyme concentration (E_0) in the range shown in **Table 1**. After 10 min, the reaction was stopped by adding NaOH at 1 kmol m^{-3} to a final pH of 12.5. Samples withdrawn from any flask were centrifuged at 14000 rpm (13000*g*) for 6 min in a microcentrifuge (ALC microcentrifugette 4204; ALC, Milan, Italy). The supernatants were collected and diluted for ammonium and/or urea analysis by using two reagent kits, that is, Spectroquant Ammonium (Merck KGaA, Darmstadt, Germany) and Boehringer Mannheim GmbH (Mannheim, Germany), respectively. Two validation tests, V_1 and V_2, were then performed using a 100 cm^3 magnetically stirred glass-jacketed vessel under the following experimental conditions:

V_1: $x_1 = x_2 = x_3 = x_4 = -1$, $x_5 = +1$, $S_0 = 1.132$ mol m^{-3}, $E_0 = 244$ g m^{-3}, TA = 5 kg m^{-3}

V_2: $x_2 = x_5 = -1$, $x_1 = x_3 = x_4 = +1$, $S_0 = 0.999$ mol m^{-3}, $E_0 = 240$ g m^{-3}, TA = 5 kg m^{-3}

RESULTS AND DISCUSSION

Kinetic Analysis. Ureases from different sources present a wide range of specific activities, these depending strongly on the buffer, pH, temperature, and ionic strength (*29*). 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 noncompetitive product inhibition by NH_4^+ (*30*)

$$r_S = \frac{\upsilon_{max} S}{\left(K_M + S + \frac{S^2}{K_S}\right)\left(1 + \frac{A}{K_A}\right)} \quad (3)$$

where r_S is the urea degradation rate, υ_{max} is the maximum specific reaction rate, K_M is the Michaelis−Menten constant, and K_S and K_A are the substrate and product inhibition constants, whereas S and A are the concentrations of urea and ammonium ions.

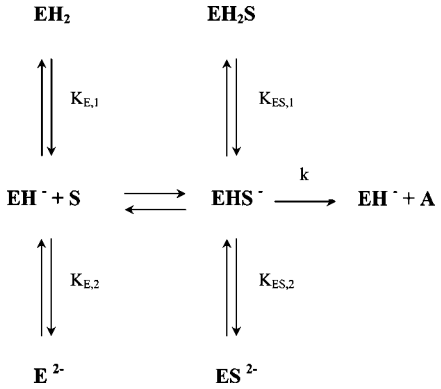

Figure 1. Schematic diagram of the mechanism assumed to describe the effect of pH on the enzymatic urea hydrolysis, where E, S, and A represent the enzyme, substrate, and product species, respectively, the enzyme being distributed among three differently protonated forms, as suggested by Tripton and Dixon (*31*).

The reaction scheme shown in **Figure 1** was proposed by Tripton and Dixon (*31*) to explain the pH dependence of υ_{max} and K_M

$$\upsilon_{max} = \frac{kE_0}{1 + \frac{[H^+]}{K_{ES,1}} + \frac{K_{ES,2}}{[H^+]}} \quad (4)$$

$$K_M = K_{M0}\frac{1 + \frac{[H^+]}{K_{E,1}} + \frac{K_{E,2}}{[H^+]}}{1 + \frac{[H^+]}{K_{ES,1}} + \frac{K_{ES,2}}{[H^+]}} \quad (5)$$

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, respectively, $K_{E,1}$ and $K_{E,2}$ are the molecular dissociation constants for the free protonated enzyme (EH^-), and $K_{ES,1}$ and $K_{ES,2}$ are 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} (*29*), thus showing that substrate inhibition can be neglected for $S < 0.5$ kmol m^{-3}. The product inhibition was found to be even weaker and decreasing with pH (*29*), thus allowing such a phenomenon to be disregarded in the case under study.

Several studies showed that K_M values of acid ureases were not significantly affected by pH. In fact, K_M for the urease from jack bean was found to be of ≈4 mol m^{-3} over a pH range of 4−9 (*32*), whereas that from *L. fermentum* ranged from 1.2 to 1.7 mol m^{-3} at the optimum pH value of 4.0 or 2.0, respectively (*16*). On the contrary, υ_{max} was reported to exhibit the classic bell-shaped pH dependence (*30*). For the urease from jack bean, a maximum value of ≈1.5 mol m^{-3} min^{-1} was detected at pH 7.0 and $E_0 = 100$ g m^{-3} (*32*), this being equivalent to 15 units mg^{-1}. On the contrary, no υ_{max} data are available for the urease from *L. fermentum*.

By referring to eq 1, the un-steady-state urea (S) and ammonium (A) mass balances in the well-mixed liquid phase can be written as

$$\frac{dS}{dt} = -r_S \quad (6)$$

$$r_A = \frac{dA}{dt} = -2\frac{dS}{dt} = 2r_S \quad (7)$$

to be integrated with the initial conditions

$$S = S_0 \text{ and } A = A_0 \text{ for } t = 0 \quad (8)$$

where r_A is the ammonium formation rate.

By determining the net increase in the ammonium concentration after a reaction interval of 10 min, it was possible to estimate its initial value (r_{A0}), this being the double of the initial urea degradation rate (r_{S0}).

By performing trial series A (**Table 1**), where E_0 was increased from 21.0 to 63.0 g m^{-3}, r_{A0} was found to increase almost proportionally to E_0 (**Figure 2**), thus yielding an almost constant specific enzyme activity $\pi_A (= r_{A0}/E_0)$ of 3.3 ± 0.1 units mg^{-1} ($r^2 = 0.95$) in agreement with the manufacturer's claim. Such activity was also in line with that (≈4 units mg^{-1}) pertaining to the killed cell preparation (Takemate-AU) used by Trioli and Ough (*23*).

As a result of the trial series B−C (**Table 1**), π_A exhibited a typical saturation pattern as the urea concentration was increased from 0.83 to 83.3 mol m^{-3} (**Figure 3**). All trials were nonlinearly fitted by using the well-known Michaelis−Menten kinetic model

$$\pi_A = \frac{r_A}{E_0} = \frac{k'S}{K'_M + S} \quad (9)$$

where k' is the ammonia formation kinetic rate constant and K'_M the saturation constant, their values being estimated by using a nonlinear fitting method (**Table 4**).

With reference to **Figure 1**, by assuming that the enzyme underwent the same dissociation process whether 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 that the inhibitory effect of substrate and ammonia was negligible (that is, $K_S \approx K_A \rightarrow \infty$), the combined use of eqs 3, 4, 5, and 9 gave rise to the following equivalences:

$$k' \approx \frac{2k}{1 + \frac{[H^+]}{K_{ES,1}} + \frac{K_{ES,2}}{[H^+]}} \quad (10)$$

$$K'_M \approx K_{M0} \quad (11)$$

The estimated K'_M values listed in **Table 4**, obtained by a nonlinear fitting method and characterized by a variance (s_i^2) and degrees of freedom (δ) equal to the overall number of trials (m_i) minus 2, were compared with the classic inequality of the hypothesis test for means by resorting to the two-sided Student *t*-test for the confidence level of 0.05. In this way, it was possible to assess that the pH and temperature effects on K'_M were practically negligible at least over the experimental ranges tested. On the contrary, k' appeared to be dependent on pH and temperature, in agreement with eq 10. In fact, at pH 4 its value was greater at 37 °C than at 20 °C, but at 20 °C it almost doubled as the pH was reduced to 3, thus corroborating the potential use of this enzyme to remove effectively urea in wines.

Finally, from **Figure 3** it can be 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

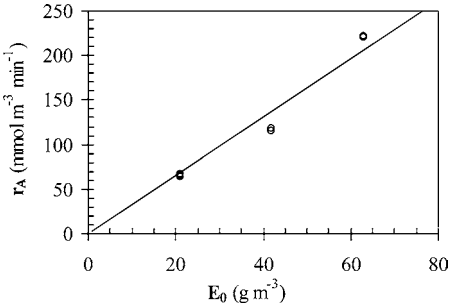

Figure 2. Effect of initial enzyme concentration (E_0) on ammonium formation rate (r_A) at pH 4, 37 °C, and urea concentration (S) of 5 kg m^{-3}. The continuous line represents the mean squares regression line, the coefficient of determination of which is 0.95.

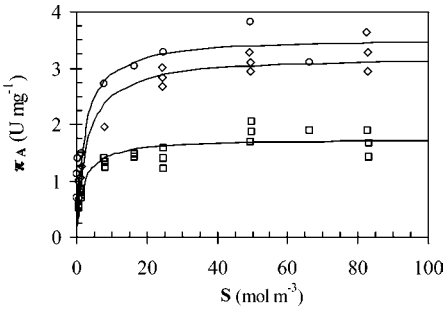

Figure 3. Effect of urea concentration (S) on the specific urease activity (π_A) in buffer B (○), C (□), or D (◇) listed in **Table 1**. The continuous lines were calculated using eq 9 together with the estimated parameters listed in **Table 4**.

the pseudo-first-order one

$$\pi_A \cong k'/K'_M S = k_I S \quad (12)$$

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

Although the initial urea concentration ($S_0 \approx 1$ mol m^{-3}) used in the following experimental design was not by far less than, but about one-third of, K'_M (**Table 4**), use of eq 12 allowed k_I to be estimated as

$$k_I \cong \frac{\pi_A}{S_0} \quad (13)$$

In this way, by replacing eq 12 into eq 7 and integrating both eqs 6 and 7 with the initial conditions (eq 8), it was possible to determine the time dependence of A and S as

$$S = S_0 - {}^1/_2(A - A_0) = S_0 \exp\left(-\frac{1}{2}k_I E_0 t\right) \quad (14)$$

$$A = A_0 + 2S_0\left[1 - \exp\left(-\frac{1}{2}k_I E_0 t\right)\right] \quad (15)$$

where k_I is to be expressed in m^3 g^{-1} min^{-1}, E_0 in g m^{-3} , and A and S in mol m^{-3}.

Statistical Analysis of Results. To maximize the specific urease activity (π_A) in model wine solutions, the effect of the concentrations of malic (M) and lactic (L) acids and potassium metabisulfite (K), ethanol volumetric fraction (E), and pH on the enzyme activity was previously studied by carrying out a replicate half-fractionate 2^5 factorial experiment, where the four-factor interaction ($M-L-K-$E) was confounded with pH (see **Table 3**, trials 1−16).

The pure error of this factorial design was estimated by combining the variances (s^2) of each duplicate experiment, thus

Table 3. Replicate Composite Design Used in This Work Together with the Number of Trials (m) and Mean and Standard Deviation (SD) Values of the Initial Specific Urease Activity (π_{A0}) at $T = 20$ °C Using Model Wine Solutions Containing Constant Initial Concentrations of Urea (1 mol m^{-3}), Tartaric Acid (TA = 5 kg m^{-3}), and Enzyme (E_0 = 240 g m^{-3})

		factor level					π_{A0} (units/mg)	
trial no.	m	M	L	K	E	pH	mean	SD
1	2	–1	–1	–1	–1	+1	0.142	0.010
2	2	+1	–1	–1	–1	–1	0.052	0.002
3	2	–1	+1	–1	–1	–1	0.083	0.017
4	2	+1	+1	–1	–1	+1	0.105	0.009
5	2	–1	–1	+1	–1	–1	0.124	0.011
6	2	+1	–1	+1	–1	+1	0.106	0.020
7	2	–1	+1	+1	–1	+1	0.164	0.013
8	2	+1	+1	+1	–1	–1	0.060	0.008
9	2	–1	–1	–1	+1	–1	0.099	0.024
10	2	+1	–1	–1	+1	+1	0.086	0.005
11	2	–1	+1	–1	+1	+1	0.131	0.005
12	2	+1	+1	–1	+1	–1	0.055	0.004
13	2	–1	–1	+1	+1	+1	0.114	0.012
14	2	+1	–1	+1	+1	–1	0.042	0.001
15	2	–1	+1	+1	+1	–1	0.071	0.001
16	2	+1	+1	+1	+1	+1	0.069	0.016
17	2	–α	0	0	0	0	0.126	0.010
18	2	+α	0	0	0	0	0.048	0.002
19	2	0	–α	0	0	0	0.069	0.001
20	2	0	+α	0	0	0	0.059	0.003
21	2	0	0	–α	0	0	0.081	0.011
22	2	0	0	+α	0	0	0.065	0.011
23	2	0	0	0	–α	0	0.083	0.001
24	2	0	0	0	+α	0	0.067	0.016
25	2	0	0	0	0	–α	0.041	0.004
26	2	0	0	0	0	+α	0.131	0.025
27	10	0	0	0	0	0	0.067	0.010

Table 4. Mean Values and Standard Deviations of the Kinetic Parameters of Equation 9 as a Function of pH and Temperature (T)

trial series	K' (unit[a] mg^{-1})	K'_M (mol m^{-3})	pH	T (°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

[a] 1 unit = 1 μmol of ammonia formed per minute from urea at 37 °C.

obtaining its mean sum of squares (MS_{PE}) as equal to 1.4×10^{-4} with 16 degrees of freedom. Graphical analysis of residuals revealed no severe violations of basic analysis of variance (ANOVA) assumptions. Thus, because for 1 and 16 degrees of freedom the 1, 5, or 10% value of F is 8.531, 4.494, or 3.048, respectively, an F test allowed the significance of the main effects and interactions to be assessed as follows (**Table 5**): (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 increase, respectively, as M or pH was increased. The effect of E on π_{A0} was negative, but about half of that of M (**Table 5**).

To determine the second-order effects, the factorial design was supplemented with extra points (**Table 2**) according to a central composite design (*28*). The point at the center of the design was randomly tested 10 times, whereas the 10 extra points in pairs along the five coordinate axes at distance α = ±2 (to ensure rotatability and uniform precision; *28*) from the central point were duplicated. In this case, a new estimate of

Table 5. Analysis of Variance for the Replicate Half-Fractionate 2^5 Factorial Design (Trials 1–16 Shown in **Table 3**)

term	alias	DF	effect	mean squares (MS)	MS/MS_{PE}[a]
M	(L–K–E–pH)	1	–0.0441	7.8×10^{-3}	54.60**
L	(M–K–E–pH)	1	–0.0034	4.7×10^{-5}	0.33
K	(M–L–E–pH)	1	–0.0006	1.2×10^{-6}	0.01
E	(M–L–K–pH)	1	–0.0211	1.8×10^{-3}	12.47**
pH	(M–L–K–E)	1	+0.0413	6.8×10^{-3}	47.91**
M–L	(K–E–pH)	1	+0.0040	6.4×10^{-5}	0.45
M–K	(L–E–pH)	1	–0.0050	9.9×10^{-5}	0.69
M–E	(L–K–pH)	1	+0.0033	4.2×10^{-5}	0.30
M–pH	(L–K–E)	1	–0.0022	1.9×10^{-5}	0.13
L–K	(M–E–pH)	1	–0.0021	1.7×10^{-5}	0.12
L–E	(M–K–pH)	1	$+2.63 \times 10^{-5}$	2.8×10^{-9}	0.00
L–pH	(M–K–E)	1	+0.0083	2.8×10^{-4}	1.94
K–E	(M–L–pH)	1	–0.0184	1.3×10^{-3}	9.47**
K–pH	(M–L–E)	1	–0.0023	2.1×10^{-5}	0.15
E–pH	(M–L–K)	1	–0.0083	2.8×10^{-4}	1.94
pure error		16		1.44×10^{-4}	

[a] Two asterisks (**) indicate high significance (probability p < 1%).

Table 6. Estimates of the Regression Coefficients of Equation 16 Together with Their Corresponding Standard Deviations (s), Estimated Student Values (t), and Probability Levels (p)

coefficient	value	s	t	p
a_0	0.054	0.012	4.476	0.004
a_1	-0.021	0.003	–7.619	0.000
a_2	–0.002	0.003	–0.668	0.529
a_3	–0.001	0.003	–0.509	0.629
a_4	–0.008	0.003	–2.944	0.026
a_5	0.021	0.003	7.548	0.000
a_{11}	0.0098	0.003	2.865	0.029
a_{22}	0.004	0.003	1.214	0.271
a_{33}	0.006	0.003	1.848	0.114
a_{44}	0.007	0.003	1.974	0.096
a_{55}	0.009	0.003	2.770	0.032
a_{12}	0.002	0.003	0.530	0.615
a_{13}	–0.003	0.003	–0.778	0.466
a_{14}	0.001	0.003	0.421	0.688
a_{15}	–0.001	0.003	–0.263	0.801
a_{23}	–0.001	0.003	–0.25	0.811
a_{24}	0.000	0.003	0.057	0.957
a_{25}	0.004	0.003	1.158	0.291
a_{34}	–0.009	0.003	–2.625	0.039
a_{35}	–0.001	0.003	–0.390	0.710
a_{45}	–0.004	0.003	–1.267	0.252

the pure error was obtained by combining the variances (s^2) of all replicated experiments (that is, $MS_{PE} = 1.27 \times 10^{-4}$ with 35 degrees of freedom).

Table 3 shows the experimental results that were correlated by the second-order polynomial

$$\pi_A = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} a_{ij} x_i x_j \quad (16)$$

where n is the number of factors and a_i, a_{ii}, and a_{ij} are the regression coefficients. These were evaluated by means of the method of least-squares and are reported in **Table 6** together with their standard errors, t statistics, and associated probabilities.

Equation 16 was found to be adequate to fit all experimental data with a coefficient of determination (r^2) of 0.96. Residuals, as plotted against the fitted values or the time at which experiments were carried out, and on a probability plot, showed no apparent departures from basic ANOVA assumptions (i.e.,

Table 7. Parameters (m_{ij}) of the Principal Axes (X_i) Together with Their Corresponding Eigenvalues (A_i), Coded (x_{iS}) and Natural Coordinates of the Stationary Point S, and Related Specific Urease Activity (π_{AS})

	m_{ij}							
factor	X_1	X_2	X_3	X_4	X_5	x_iS	factor level	unit
x_1	0.7417	–0.4522	0.4741	0.1196	0.0797	1.0451	3.81	kg m^{-3}
x_2	–0.0283	–0.3316	–0.0120	–0.9309	–0.1502	0.5174	2.20	kg m^{-3}
x_3	0.4416	0.2642	–0.4981	–0.2086	0.6660	0.7466	0.24	kg m^{-3}
x_4	–0.4127	0.04232	0.6032	–0.1185	0.6708	0.6772	14.01	% v/v
x_5	–0.2895	–0.7835	–0.4040	0.2482	0.2784	–0.9636	3.26	
A_i	0.0088	0.0104	0.0125	0.0034	0.0015	π_{AS}	0.029	U mg^{-1}

Table 8. Data Dispersion Analysis for the Canonical Equation (17) by Accounting for One or More than One Principal Axis (X_i)

source of variation		sum of squares	df	mean squares (MS)	MS/MS_{PE}	$F_{0.05}$[a]
model						
12345	$\pi_{AS} = 0$	2.37×10^{-02}	7	3.38×10^{-3}	26.61	2.29**
1234	A5 = 0	1.37×10^{-03}	10	1.37×10^{-4}	1.08	2.11
123	A4 = A5 = 0	3.78×10^{-03}	14	2.70×10^{-4}	2.13	1.99*
124	A3 = A5 = 1	3.28×10^{-02}	14	2.34×10^{-3}	18.43	1.99**
12	A3 = A4 = A5 = 0	4.10×10^{-02}	18	2.28×10^{-3}	17.95	1.91**
1	A2 = A3 = A4 = A5 = 0	6.24×10^{-02}	22	2.84×10^{-3}	22.35	1.85**
12345	$\pi_{AS} \neq 0$	1.11×10^{-03}	6	1.85×10^{-4}	1.46	2.37
pure error			35	1.27×10^{-4}		

[a] Two asterisks (**) indicate high significance (probability $p < 1\%$). One asterisk (*) indicates significance (probability $1 < p < 5\%$).

errors have constant variances and are normally distributed and independent of one another, respectively). Moreover, as the mean sum of squares for pure error (MS_{PE}) was compared to the mean sum of squares for the residual error ($MS_E = 1.88 \times 10^{-4}$ with 6 degrees of freedom) of the above polynomial, their ratio ($MS_E/MS_{PE} \approx 1.48$) was less than the corresponding critical value (2.37) of the F test at the 95% confidence level. Finally, by referring to the last column in **Table 6**, it was possible to note that the constant a_0, all of the linear coefficients (a_i), except for those pertaining to L and K, the quadratic ones a_{11} and a_{55} corresponding to M^2 and pH^2, respectively, and just the coefficient a_{ij} related to the binary interaction $K-E$ were significant at the 0.05 probability level.

A mere inspection of the second-order polynomial coefficients of eq 16 (**Table 6**) does not allow the shape of the fitted response surface to be easily understood. Moreover, with five independent variables, interpretation of contour plots is not straightforward. A formal analysis, called canonical analysis, is useful to facilitate this interpretation (*28*). It consists of transforming the model into a new coordinate system with the origin at the stationary point (S) and then rotating the axes of this system until they are parallel to the principal axes of the fitted response surface. When related to this new system of axes, the various forms of the second-order equations are greatly simplified and their geometrical nature becomes obvious. Furthermore, canonical analysis sometimes makes it possible to discover the *fundamental variables* that are driving the system, that is, functions of two or more experimental design variables (or *natural variables*, i.e., quantities that can be conveniently measured separately) in terms of which the behavior of the system could be described more economically (*33*). This is the case of occurrence of ridge systems.

To this end, eq 16 was reduced to its canonical form

$$\pi_A = \pi_{AS} + \sum_{i=1}^{n} A_i X_i^2 \quad (17)$$

with

$$X_i = \sum_{j=1}^{n} m_{ij}(x_j - x_{jS}) \quad (18)$$

where π_{AS} is the specific urease activity at the center point S, X_i the generic principal axis of eq 16, A_i the generic eigenvalue of the matrix of regression coefficients (a_{ij}), and m_{ij} the generic component of the associated eigenvector X_i (*28*). In particular, the center of the system represented by eq 16 was found by differentiating the right-hand side of eq 16 with respect to each variable x_i and equating to zero. All of these parameters are listed in **Table 7**.

From **Table 7** it was possible to ensure that the coordinates (x_{jS}) of the center of the system fell within the range of input variables initially set up.

Table 8 shows the analysis of variance of the experimental data (**Table 3**) by accounting for one or more than one principal axis (X_i) in eq 17 (*34*). The statistical significance of these models was analyzed by means of an F test, thus yielding the following: (a) factor X_5 had no effect on π_A at the 0.05 confidence level; (b) factor X_4 was insignificant at the 0.01 confidence level, but significant at the 0.05 confidence level; and (c) factors X_1, X_2, and X_3 were significant at the 0.01 confidence level.

Thus, the first four factors, each one being highly biased by M, pH, E, or L, respectively, were regarded as those associated with the most relevant eigenvalues A_i of the system under study and were used to reconstruct the specific urease activity as follows:

$$\pi_A = 0.029 + 0.0088X_1^2 + 0.0104X_2^2 + 0.0125X_3^2 + 0.0034X_4^2 \quad (19)$$

Such a canonical equation allowed the response surface (π_A) to be characterized from a geometric point of view as a preliminary step to identify the best strategy to maximize π_A with respect to x_i. Thus, in the five-dimensional space (X_1, X_2, X_3, X_4, π_A), the response surface associated with π_A was an elliptic hyper-paraboloid, its vertex (π_{AS}) coinciding with the center point S, the co-ordinates (x_{iS}) of which are listed in **Table 7**.

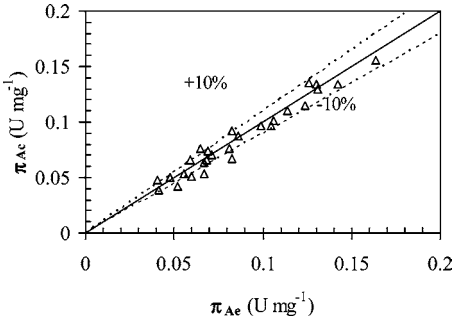

Figure 4. Comparison between the observed specific urease activity (π_A) in different wine model solutions and those calculated by using eq 19.

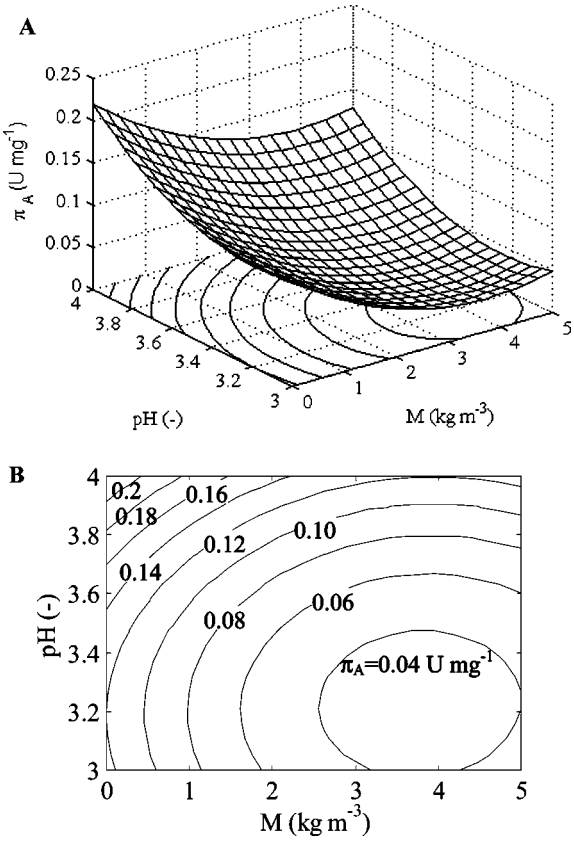

Figure 5. Response surface (**A**) and contour (**B**) plots of the specific urease activity (π_A) as calculated by using eq 19, as a function of malic acid concentration (*M*) and pH at constant values for the other independent variables (L = 1.75 kg m^{-3}, K = 0.20 kg m^{-3}, E = 13.0% v/v).

Figure 4 compares the calculated and experimental values of π_A. The mean percentage error is 9.8%, and ≈70% of all experimental data fall within a ±10% deviation band.

Figure 5 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 the other independent variables are set to their zero level.

It can be noted that as the reaction medium was commuted from an acetate buffer at pH 4 to any of the model wine solutions tested here, the specific acid urease activity reduced from the value claimed by the manufacturer (3.3 units mg^{-1}) to quite smaller values reduced by a factor in the range of 20 and 90 (**Table 3**) depending on the combined inhibitory action of the wine components accounted for. Obviously, this may be counterbalanced by increasing the enzyme concentration by the same factor, which results in a much more expensive detoxification procedure.

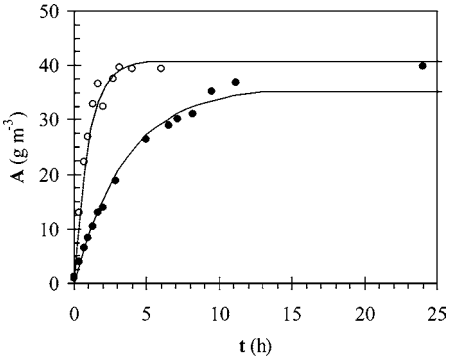

Figure 6. Comparison between experimental (●, ○) and calculated (continuous line) ammonium concentrations (*A*) as a function of time (*t*) for model wine solutions V_1 (○) and V_2 (●) described in the text.

Validation Tests Using Model Wine Solutions. To check for the accuracy of π_A estimation, a couple of full hydrolysis trials were carried out until urea exhaustion by setting the independent variables at the levels associated with the maximum (trial 1) and minimum (trial 14) π_A values observed throughout the half-fractionate 2^5 factorial experiment (**Table 3**).

In these circumstances, the combined use of eqs 19 and 13 allowed the corresponding k_I values for trials V_1 and V_2 to be estimated as 1.34 × 10^{-4} and 0.39 × 10^{-4} m^3 g^{-1} min^{-1}, respectively. Then, it was possible to estimate the time course of ammonia formation by using eq 15.

Figure 6 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.

Validation Tests Using Real Wines. To further check the short-cut method developed here, the time course of the urea degradation tests performed on a few real wines by Kodama (*25*) and Trioli and Ough (*23*) was characterized in terms of the half-life time ($t_{1/2}$), that is, the time needed to reduce the initial urea concentration by a factor of 2.

In agreement with previous work (*11*, *21*, *25*), this hydrolytic process was assumed to follow a pseudo-first-order kinetics. Thus, use of $t_{1/2}$ in conjunction with eq 14 allowed the experimental k_{Ie} value to be easily estimated.

All of these data are shown in **Table 9** together with the main wine components, as well as its pH, and the initial urea and urease concentrations used. It can be noted that it was impossible to determine all of the wine components affecting acid urease activity from the original papers. Thus, the missing concentrations were assumed on the basis of winery experience, as reported within parentheses in **Table 9**.

In the case of real wines the effective pseudo-first-order kinetic rate constants (k_{Ie}) were found to be smaller than those (k_{Ic}) pertaining to the model wine solutions having the same wine composition and pH by a factor (ζ) varying from as low as 10 to as great as 1000.

Because in the composite design used the specific urease activity (π_A) was found to vary from 0.05 to 0.15 units mg^{-1} (**Table 3**), the corresponding pseudo-first-order kinetic rate constants consequently varied by a maximum factor of 3. Thus, the great variation in ζ listed in **Table 9** seemed to be more likely related to the wine content in polyphenolic compounds or fluoride than to its content in malate, lactate, or metabisulfite.

Although the technical information available about the application of acid urease in wines appears to be so extremely

Table 9. Main Characteristics of a Few Varieties of Wine Submitted to Urea Degradation Tests by Kodoma (*25*) and Trioli and Ough (*23*): Wine Components [*M*, *L*, $K_2S_2O_5$, *E*, Equivalent Gallic Acid (*P*), and Fluoride (*F*) Concentrations] and pH; Initial Concentrations of Urea (S_0) and Acid Urease (E_0); Specific Urease Activity (τ_{Ac}) and Pseudo-First-Order Kinetic Rate Constant (k_{lc}) As Estimated via Equations 19 and 13, Respectively; Experimental Half-life Time ($t_{1/2}$) and (k_{le}) As Calculated by Using Equation 14

wine variety	M[a] (kg m^{-3})	L[a] (kg m^{-3})	E (% v/v)	pH	$K_2S_2O_5$[a] (kg m^{-3})	P (g m^{-3})	F (g m^{-3})	τ_{Ac} (units mg^{-1})	S_0 (mol m^{-3})	E_0 (g m^{-3})	$k_{lc}\times10^4$ (m^3 g^{-1} min^{-1})	$t_{1/2}$ (h)	$k_{le}\times10^6$ (m^3 g^{-1} min^{-1})	k_{le}/k_{lc}
French Colombard	(0)	(2.5)	10.2	3.68	(0.1)	252	na[b]	0.179	2.17	1000	1.79	6.4	3.62	0.020
Sauvignon Blanc	(0)	(2.5)	12.0	3.76	(0.1)	275	na	0.186	2.17	1000	1.86	200.0	0.12	0.001
French Colombard	(1.0)	(2.0)	12.5	3.31	(0.1)	278	na	0.088	2.17	1000	0.88	50.0	0.46	0.005
Chardonnay	(1.8)	(1.0)	14.1	3.22	(0.1)	280	na	0.096	2.17	1000	0.96	230.0	0.10	0.001
Symphony	(2.5	(0.5)	13.6	3.02	(0.1)	249	na	0.093	2.17	1000	0.93	6.4	3.62	0.039
Cabernet Sauvignon	(0)	(2.5)	13.6	3.62	(0.1)	1440	na	0.167	2.17	1000	1.67	6.4	3.62	0.022
Cabernet Sauvignon	(1.0)	(2.0)	12.8	3.32	(0.1)	1474	na	0.091	2.17	1000	0.91	3.2	7.13	0.078
Petite Sirah	(0.8	(1.8)	14.8	3.43	(0.1)	2835	na	0.133	2.17	1000	1.33	12.8	1.81	0.014
Carignane	(0)	(2.5)	13.2	3.87	(0.1)	1084	na	0.213	2.17	1000	2.13	50.0	0.46	0.002
zinfandel	(0)	(2.5)	12.0	3.52	(0.1)	1043	na	0.143	2.17	1000	1.43	160.6	0.14	0.001
red	0.39	(1.75)	12.0	3.50	(0.1)	na	0.5	0.126	0.08	100	1.26	47.2	4.89	0.039
white	1.96	(1.75)	11.0	3.40	(0.1)	na	0.5	0.067	0.08	100	0.67	35.6	6.49	0.097
port	0.65	(1.75)	19.0	3.60	(0.1)	na	1.0	0.270	0.08	100	2.70	67.4	3.43	0.013

[a] Data within parentheses were assumed on the basis of winery experience, being not available in the original papers (*23*, *25*). [b] Not available.

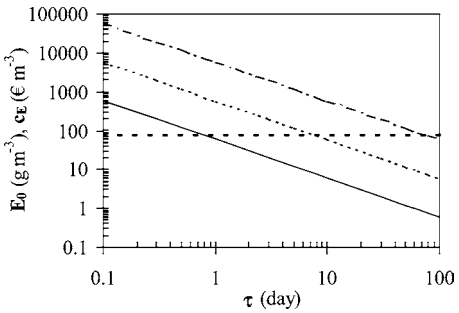

Figure 7. Effect of initial enzyme concentration (E_0) on the process time (τ) needed to reduce the initial urea concentration (S_0) from 10 to 1 g m^{-3} in a model wine solution corresponding to the center of the factorial design used in this work (—) and two simulated real wines with the same composition and pH and an overall content of inhibitory components characterized by a safe reduction factor (ζ) equal to 10 (- - -) or 100 (- - — - -). All curves were calculated by using eq 14. The horizontal broken line refers to the maximum allowable concentration of enzyme (75 g m^{-3}) in wine treatment (*18*).

dispersed to prevent anyone from reaching any reliable conclusion, the contribution of the other components, such as fluoride and phenolic compounds, generally regarded as inhibitory ones, is to be clearly determined to improve greatly the prediction capability of this empiric modeling. Conversely, it should be assessed whether the very low activity of acid urease in red wines (see wines with $P > 1000$ g m^{-3} in **Table 9**) is the outcome of the deactivating action exerted by phenolic compounds and/or unknown inhibitors naturally present in the wines tested.

Preliminary Cost Estimates. A rough estimate of the enzyme preparation costs for the hydrolytic treatment tested was carried out with reference to the model wine solution associated with the central point of the experimental design (trial 27 in **Table 3**) under the assumption of reducing the initial urea content from 10 to 1 g m^{-3} (EU Regulation 1622/00). In the circumstances, k_{lc} was found to be equal to 5.34×10^{-5} m^3 g^{-1} min^{-1}.

As the processing time was increased from 0.1 to 100 days, the dose of acid urease (E_0) needed to degrade the initial urea concentration by a factor of 10 exhibited an exponentially decreasing trend from 599 to 0.6 g m^{-3} (**Figure 7**).

When the overall inhibitory effect of all wine components was taken into account by reducing the above pseudo-first-order kinetic rate constant by a safe factor (ζ) of 10 or 100, E_0 had to be increased by the same factor (**Figure 7**).

Thus, because a 30-day hydrolytic treatment prior to final wine processing might be easily performed at the winery scale, E_0 had to be increased from about 20 to 200 g m^{-3}, respectively. In the case of $\zeta = 100$, the enzyme concentration required was by far greater than the maximum allowable one (75 g m^{-3}) in wine treatment (*18*). In these circumstances, use of such a maximum E_0 level would prolong the enzymatic processing up to 80 days (**Figure 7**).

Because the current market price of acid urease is of the order of 1 euro g^{-1}, the 30- or 80-day-long detoxification process under study would cost about 200 or 750 euros per hectoliter of wine treated depending on its aptitude to cause less ($\zeta = 10$) or more severe ($\zeta = 100$) inhibition of the enzyme, respectively.

In conclusion, these cost data are highly likely to explain why such an enzymatic process has not yet received systematic attention by the wine industry, owing to both its high specific processing costs and its insufficient reliability. Further testing of such an enzyme, once immobilized onto fibers or bead resins, is thus suggested to ameliorate its performance in real wines.

NOTATION

A	concentration of ammonium ions (mol m^{-3})
A_i	generic eigenvalue of the matrix of regression coefficients (a_{ij})
a_i	generic linear regression coefficient in eq 16
a_{ii}	generic quadratic regression coefficient in eq 16
a_{ij}	generic interaction regression coefficient in eq 16
c_E	specific enzymatic processing cost per liter of wine treated (euros dm^{-3})
E	ethanol volumetric fraction (% v/v)
E_0	initial enzyme concentration (g m^{-3})
[H$^+$]	concentration of hydrogen ions (mol m^{-3})
F	fluoride concentration (g m^{-3})
K	concentration of potassium metabisulfite (kg m^{-3})
K_A	product inhibition constant (mol m^{-3})
K_{Ei}	molecular dissociation constant for the free protonated enzyme (EH$^-$) (mol m^{-3})
$K_{ES,i}$	molecular dissociation constant for the monoprotonated enzyme−substrate complex (mol m^{-3})
K_M	Michaelis−Menten constant (mol m^{-3})
K'_M	saturation constant for urea (mol m^{-3})

K_{M0} pH-independent Michaelis−Menten constant (mol m^{-3})
K_S substrate inhibition constant (mol^2 m^{-6})
k kinetic rate constant of the dissociation of the monoprotonated enzyme−substrate (EHS^-) complex to NH_3 (m^3 g^{-1} min^{-1})
k' ammonia formation kinetic rate constant (m^3 g^{-1} min^{-1})
k_I pseudo-first-order kinetic rate constant for ammonia (m^3 g^{-1} min^{-1})
L concentration of lactic acid (kg m^{-3})
M concentration of malic acid (kg m^{-3})
MS_E mean sum of squares for residuals of the generic regression equation
MS_{PE} mean sum of squares for pure error of the factorial/composite experiment
m_i generic overall number of trials
m_{ij} generic component of the eigenvector X_i
n overall number of factors,
P gallic acid equivalent concentration (g m^{-3})
p probability level
r^2 coefficient of determination (dimensionless)
r_A ammonium formation rate (mol m^{-3} min^{-1})
r_S urea degradation rate (mol m^{-3} min^{-1})
S concentration of urea (mol m^{-3})
s_i^2 generic sample variance
t reaction time (min)
$t_{1/2}$ half-life time (h)
TA concentration of tartaric acid (kg m^{-3})
v_{max} maximum urea reaction rate (mol m^{-3} min^{-1})
X_i generic principal axis of eq 16
x_i generic coded independent variables, as defined by eq 2

Greek Symbols

δ degrees of freedom
π_A specific enzyme activity ($=r_A/E_0$, units mg^{-1})
π_{AS} specific urease activity at the center point S (units mg^{-1})
ζ safe reduction factor (dimensionless)
τ processing time needed to reduce urea concentration by a factor 10 (day)

Subscripts

i, j referred to a generic independent variable
0 referred to the initial condition
e referred to experimental
c referred to calculated
S referred to the stationary point

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