Evaluation of the lactic acid consumption in yeast cultures by voltammetric means

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Abstract

The voltammetric study of the lactic acid reduction was performed in media suitable for yeast growth, using platinum microelectrodes. The decrease of the voltammetric peak current from square wave voltammetry, ΔI_p, in cultures of the yeasts *Candida utilis* and *Saccharomyces cerevisiae* was related to both the growth time and cellular biomass. Either the imposed variations of medium composition or those resulting from cellular growth did not significantly affect the ΔI_p values. For both yeast species, similar specific growth rates were estimated from the variation of I_p and absorbance at 640 nm.

Keywords: voltammetry, microelectrodes, lactic acid consumption, yeast growth, preservative determination

1. Introduction

Organic acids are widely spread in nature, occurring in many foodstuffs either as natural compounds, like in fresh fruits, or as a result of the microbial activity during the production of alcoholic beverages (wine and beer), fermented vegetables (sauerkraut and pickles) and fermented milks (yogurt and kefyr) [1]. In addition, organic acids are commonly added to food products as preservatives [2]. Therefore, the organoleptic characteristics as well as the microbiological stability of a wide variety of food products are greatly influenced by the presence of these compounds.

Yeasts are of capital importance in food industry as at least 20% of the ca. 700 of the yeast species currently recognized are associated with foods and beverages [3]. In addition, about one-half of the total listed yeast species contains strains that are able to use organic acids as carbon and energy source [4] and thus they can potentially be a serious threat in the food industry. Typically, yeasts become the dominant contaminants of foods and beverages when competition from other microorganisms, particularly bacteria and moulds, is restricted mainly by low pH and the presence of preservatives [5]. By consuming the organic acids yeasts may propitiate the conditions for the attack by other spoilage microorganisms. At present, control of microbial spoilage is becoming an increasing challenge for the food industry because the emergence of new products and the consumer demand of mild preservation systems to obtain better tasting and healthy foods [6]. In this context, the development of new analytical approaches for the in-situ determination of organic acids is of great importance.

Among other methods, voltammetry can be used for the determination of organic acids [7,8]. The electrochemical techniques present several advantages toward chromatographic and enzymatic assays, related to the time and the cost of the analysis.

In this work, the quantification of lactic acid consumption in cell cultures is performed by square wave voltammetry. *Candida utilis* and *Saccharomyces cerevisiae* were selected because both yeast species are able to use lactic acid as the carbon and energy source [4]. The effect of the medium composition on the voltammetric current of the lactic acid reduction was analysed varying the concentration of the phosphorous source (potassium dihydrogenphosphate) as well as the nature of the nitrogen source (urea or ammonium sulphate). Both variables are expected to influence the medium acid-base equilibria as potassium dihydrogenphosphate is a medium buffer and the nitrogen assimilation can bring up significant extracelular pH variations [9].

2. Experimental

2.1. Microorganisms and growth conditions

The yeasts *Candida utilis* IGC 3039 (CBS 890) and *Saccharomyces cerevisiae* IGC 4072 were maintained on a medium containing glucose (2 %, *w*/*v*), peptone (1 %, *w*/*v*), yeast extract (0.5 %, *w*/*v*) and agar (2 %, *w*/*v*). Four mineral media containing lactic acid (0.500 %, *w*/*w*) and supplemented with vitamins were used for growth in liquid medium [10]. The mineral media were composed by $MgSO_4.7H_2O$ (0.050 %, *w*/*v*), $CaCl_2.2H_2O$ (0.013 %, *w*/*v*) and: i) $(NH_4)_2SO_4$, (0.50 %, *w*/*v*) and KH_2PO_4 (0.50 %, *w*/*v*) or ii) $(NH_4)_2SO_4$, (0.50 %, *w*/*v*) and KH_2PO_4 (0.050 %, *w*/*v*) or iii) urea (0.23 %, *w*/*v*) and KH_2PO_4 (0.50 %, *w*/*v*) or iv) urea (0.23 %, *w*/*v*) and KH_2PO_4 (0.050 %, *w*/*v*). The components of the culture medium were sterilized at 120 ºC for 20 minutes, with exception of urea and vitamins, which were sterilized by filtration (Filtropur S 0.2 µm, Sarstedt).

All cultivations were carried out at initial pH 4.10 and 26 ºC, with mechanical shaking (120 r.p.m.) in 500 mL Erlenmeyer flasks in a void volume of half of the total flask volume. Growth was monitored through turbidity measurements at 640 nm, A_{640nm} (Spectronic 21, Bausch & Lomb). The number of yeast cells was determined through direct counting in a Neubauer chamber.

2.2. Sample preparation for voltammetry

Voltammetric assays were performed in 5 mL samples obtained from cell growing cultures and in solutions of different concentration of lactic acid prepared by the dilution of each culture medium (pH 4.10±0.02) with the respective mineral medium at the same pH.

2.3. Voltammetric measurements

Voltammetric measurements were performed using a potentiostat (Autolab type PGSTAT30) controlled by the GPES 4.9 software. The parameters of square wave voltammetry were f=8 Hz, ΔE=5 mV and E_{SW}=25 mV.

The voltammetric experiments were carried out at room temperature, in a two-electrode arrangement placed in a common two-neck flask in a Faraday cage. The reference / counter electrode was a saturated calomel electrode (SCE). All potentials are quoted vs. SCE. The platinum microdisk of 26.7 µm diameter used as working electrode was made of cross section of a metal wire (Goodfellows) sealed into soft glass. The electrode was polished by successive steps, first using fine abrasive papers and then with alumina (0.3 µm size, Tonerde, ATM) on a wet tissue (Mambo, Ziesmer). Prior to each experiment the electrode surface was polished with 0.05 µm alumina, rinsed with water, polished with a wet polishing cloth, rinsed again and dried. Electrode diameter was calibrated by measuring the limiting current of steady state voltammograms of a ferricyanide solution of known concentration.

All current intensity data correspond to the average

of at least five determinations.

The detection limit of the analytical measurement of lactic acid in the culture media without cells was determined through the standard deviation of the intercept (s_a) of the calibration straight-line as 3 s_a [11].

3. Results and Discussion

3.1. Characterization of the voltammetric response of lactic acid in media suitable for yeast growth

The voltammetric study of the lactic acid electrochemical reduction was performed in different media. The medium composition was exclusively selected on the basis of the yeast growth requirements. Besides the lactic acid used as the carbon and energy source, sources of nitrogen, phosphorous and sulphur as well as minerals and vitamins were also included in the media.

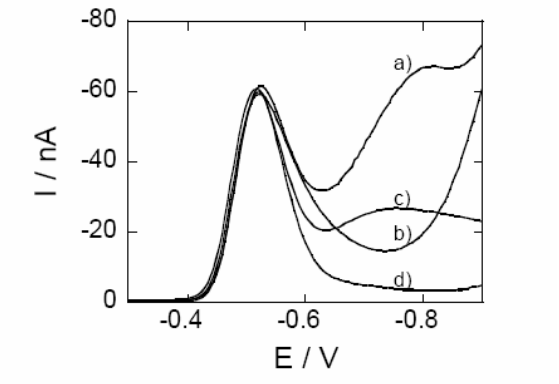

Fig. 1- Square wave voltammograms recorded with a 26.7 μm diameter platinum microelectrode in 0.500 % of lactic acid media containing: a) ammonium sulphate and 0.50 % of KH_2PO_4; b) ammonium sulphate and 0.050 % of KH_2PO_4; c) urea and 0.50 % of KH_2PO_4 or d) urea and 0.050 % of KH_2PO_4.

The square wave voltammograms acquired in the different media at pH 4.10 are presented in Figure 1. All the voltammograms displayed a peak at approximately -0.57 V, characteristic of the reduction of lactic acid [7,8]. Curve a) obtained in the presence of ammonium sulphate and 0.50 % of KH_2PO_4 displayed another important reduction process at -0.75 V. The magnitude of this peak decreased when either the concentration of KH_2PO_4 was decreased or the ammonium sulphate was substituted by urea. Although the lactic acid peak definition has been affected by the presence of this second reduction process, its height and position did not change. Regardless of the medium composition, the peak current, I_p, was found to be proportional to the lactic acid concentration up to 60 mM at pH 4.10±0.02 (I_p (nA)=(0.83±0.02) c_{HL} (mM), where c_{HL} is the analytical concentration of lactic acid; n=16 and r=0.996). The detection limit of the analytical measurement was 2.6 mM.

In the former analysis the variation of the concentration of lactic acid was performed settling constant the concentration of the other medium constituents. However, in the evaluation of the lactic acid consumption by microorganisms it must be considered that changes on the concentration of other components of the medium may occur, due to either consumption of nutrients or production of metabolites. Therefore, the voltammetric monitoring of the lactic acid was conducted in yeast cultures where the cellular activity is considerable.

3.2. Monitoring of the lactic acid consumption by the yeasts Candida utilis *and* Saccharomyces cerevisiae

The monitoring of lactic acid was performed during the growth of the yeast *C. utilis* and *S. cerevisiae*. As the lactic acid was the only compound in the medium that could be used by the cells as the carbon and energy source, it is expected that the decrease of its concentration should be related to the increase of the cellular biomass.

Figure 2 presents a set of voltammograms acquired in a culture of *C. utilis* with lactic acid, ammonium sulphate and 0.050 % of KH_2PO_4. The voltammogram recorded at the earliest time (t=0)

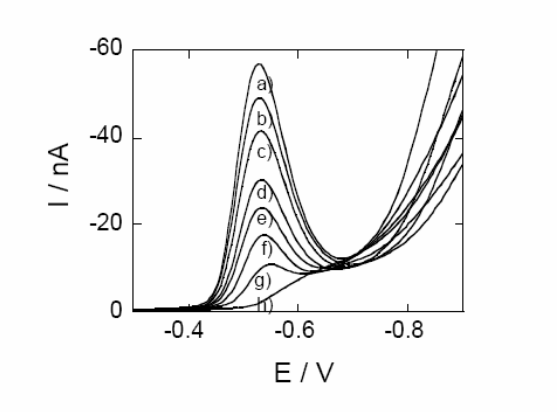

Fig. 2- Square wave voltammograms from a culture of the yeast *C. utilis* grown in lactic acid (0.500 %) containing medium with ammonium sulphate and 0.050 % of KH_2PO_4. Time of growth (and A_{640nm}): a) 0 (0.086); b) 1.25 (0.125); c) 2.83 (0.222); d) 5.58 (0.377); e) 7.17 (0.456); f) 8.42 (0.538); g) 9.67 (0.602) or h) 10.92 h (0.721).

presented the highest peak current (curve a)). Thereafter, the peak current gradually decreased and vanished after about 11 hours. Figure 3 (A) presents the variation of the cellular biomass evaluated by the light scattering at 640 nm (A_{640nm}) along time. The increase of A_{640nm} was tracked by the decrease of $|I_p|$ (Figure 3 (B)). Since I_p is a measure of the lactic acid present in the medium, its variation defined as $\Delta I_p = I_p - I_{p_0}$ (where I_{p_0} is the peak current of lactic acid in the culture medium before cell inoculation) is associated with the lactic acid consumption by the cells (Figure 3 (C)).

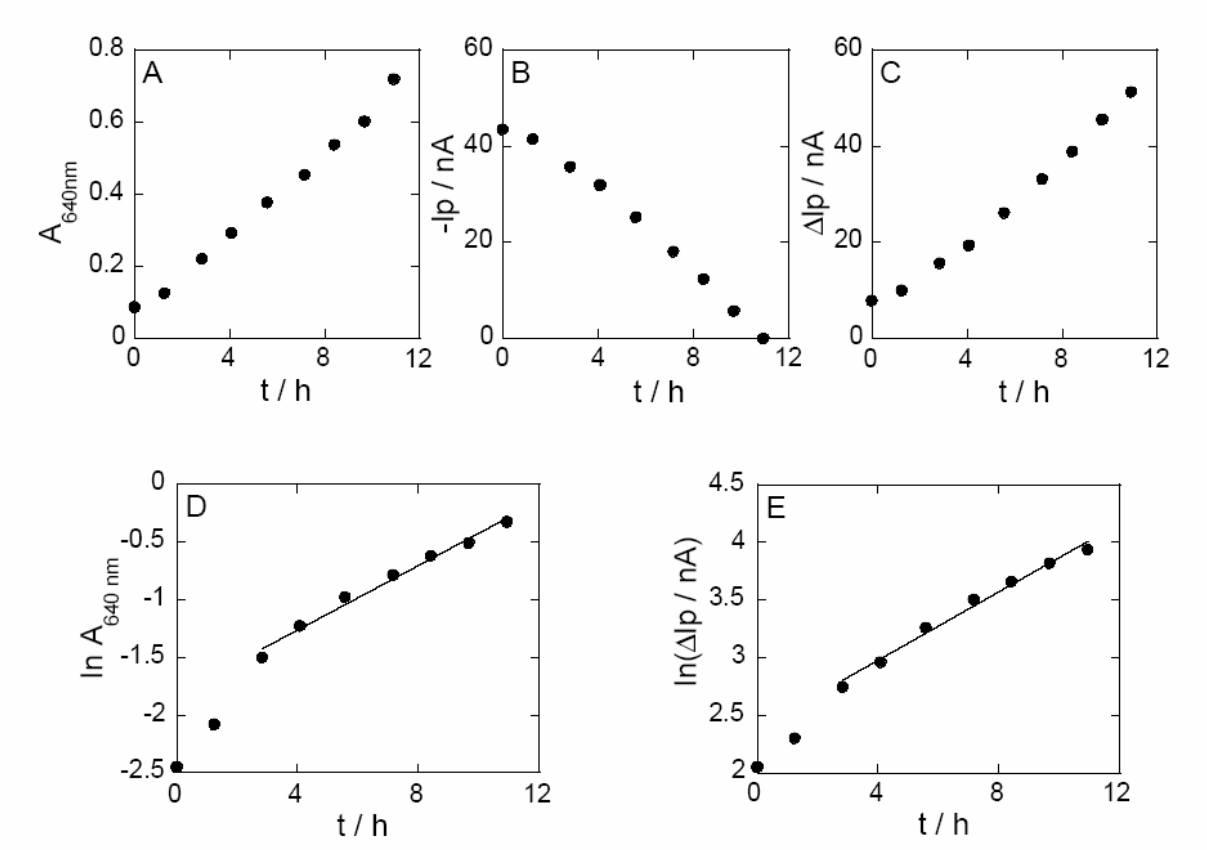

Fig. 3- Representation of turbidity data, A_{640nm}, (A), peak current, I_p, (B) and variation of peak current, ΔI_p, (C) vs. time; ($\Delta I_p = I_p - I_{p_0}$, where I_{p_0} is the peak current of lactic acid in the culture medium before cell inoculation). Semi-logarithmic plots of A_{640nm} (D) and ΔI_p (E) vs. time obtained from a growing culture of *C. utilis*. Lines were obtained from linear regression, using data from the exponential growth phase. The experimental conditions were the same as in Figure 2.

Table 1- Specific growth rates (μ_{max}) of *Candida utilis* and *Saccharomyces cerevisiae* grown in different culture media, estimated from turbidimetry and voltammetry.

Yeast	Nitrogen source	$[KH_2PO_4]$ / %	μ_{max} / h^{-1}	
			Turbidimetry (A_{640nm})	Voltammetry
C. utilis	NH_4^+	0.050	0.16 ± 0.02	0.21 ± 0.04
		0.50	0.15 ± 0.02	0.18 ± 0.05
	Urea	0.050	0.27 ± 0.03	0.22 ± 0.04
		0.50	0.24 ± 0.01	0.17 ± 0.06
S. cerevisiae	NH_4^+	0.050	0.053 ± 0.005	0.06 ± 0.01
		0.50	0.055 ± 0.006	0.06 ± 0.02
	Urea	0.050	0.055 ± 0.002	0.055 ± 0.008
		0.50	0.049 ± 0.001	0.049 ± 0.004

During the exponential growth, the variation of the cell number, or any related variable x, with time (t) is described by $x = x_0\ e^{\mu_{max} t}$, where x_0 is the value of the variable at t=0 and μ_{max} is the specific growth rate [12]. Therefore, the logarithm of the x values should be a linear function of time with the slope being μ_{max}. The A_{640nm} data obtained from the culture of *C. utilis* displayed this trend for t>3 hours, corresponding to the exponential growth phase (Figure 3 (D)). The $\ln\Delta I_p$ values also followed a linear variation with time in the same period (Figure 3 (E)). Furthermore, the slopes of the straight lines were identical within the experimental uncertainty, as shown in Table 1.

The specific growth rates of *C. utilis* in the different media characterized in Figure 1, evaluated by means of turbidimetry and voltammetry, are reported in Table 1. Under all conditions, a good agreement was found between the μ_{max} values.

Figure 4 (A) shows the plot of ΔI_p vs. A_{640nm} using data from cultures of the yeast *C. utilis* grown in different media. The existence of a unique linear relationship indicates that the correlation between both variables did not depend on the medium composition. This univocal relation also demonstrates that neither the selected variations of the medium composition nor those resulting from the cellular growth did impose any considerable influence on the peak current. Otherwise, it would be unlikely that the ΔI_p values would be influenced in the same manner regardless of the nitrogen source, the concentration of KH_2PO_4 and the nature of the metabolites produced by the cells in the different media.

Under all the tested conditions, cellular growth resulted in a pH increase. Figure 4 (B) shows the plot of $\Delta 10^{-pH}$ vs. A_{640nm}, where $\Delta 10^{-pH} = 10^{-pH} - 10^{-pH_0}$ and pH_0 is the pH of the culture medium before cell inoculation. For a fixed A_{640nm}, different values of $\Delta 10^{-pH}$ were obtained depending on the medium composition. The differences between the $\Delta 10^{-pH}$ values do not seem to be significant regarding its effect on the acid dissociation extent because identical ΔI_p values were obtained in spite of the non-electroactive nature of the acid dissociated form. This could be due to the small magnitude of the differences between $\Delta 10^{-pH}$ values (from 0.02 to 0.04 mM), or else they did not reflect an actual difference in the H^+ concentrations. The pH measurement could have been affected by the variation of either the ionic strength or the liquid junction potentials that are likely to change during cell growth, as a result the variation of the medium composition. In addition,

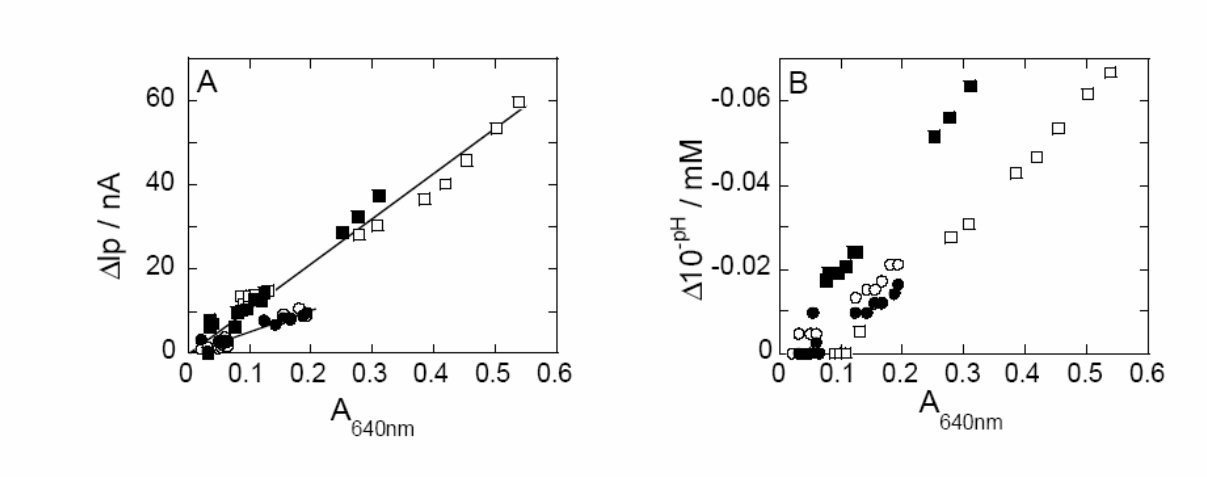

Fig. 4- Representation of ΔI_p, (A) and $\Delta 10^{-pH}$ (B) vs. A_{640nm}, where $\Delta 10^{-pH}=10^{-pH} - 10^{-pH_0}$ and pH_0 is the pH measured in the culture medium before cell inoculation. Data were obtained from cultures of *C. utilis* grown in lactic acid medium containing: (○) ammonium sulphate and 0.050 % of KH_2PO_4; (●) ammonium sulphate and 0.50 % of KH_2PO_4; (□) urea and 0.050 % of KH_2PO_4 or (■) urea and 0.50 % of KH_2PO_4.

the glass electrode response could have been biased by the presence of the cells, which form a colloidal suspension. Growth experiments were also performed with the yeast *Saccharomyces cerevisiae* in lactic acid medium with urea or ammonium sulphate and different concentrations of KH_2PO_4 (0.050 or 0.50 %). The A_{640nm} values of the cultures increased along time while those of $|I_p|$ decreased, as in the *C. utilis* assays. The specific growth rates of *S. cerevisiae* estimated by means of turbidimetry and voltammetry were identical (Table 1).

The correlations between ΔI_p and $\Delta 10^{-pH}$ vs. A_{640nm} obtained from cultures of the yeast *S. cerevisiae* grown in different media are presented in Figure 5 (A) and (B), respectively. The plot of ΔI_p vs. A_{640nm} displays two different trends, which are described by two linear relations (Figure 5 (A)). Each set of results consists of two series of data obtained in cultures where the same nitrogen source (urea or ammonium sulphate) was used but with different concentrations of KH_2PO_4. The corresponding pH data are not coupled in the same manner (Figure 5 (B)), as the $\Delta 10^{-pH}$ values from the culture with urea and 0.050 % of KH_2PO_4 (open squares) are more alike to those from the cultures with ammonium sulphate (circles) than to those from the culture with urea and 0.50 % of KH_2PO_4 (solid squares). Therefore, the two trends defined by ΔI_p vs. A_{640nm} do not seem to be originated from the effect of the dissociation equilibria on the I_p data. On the other hand, the relation between the turbidity measurements and the number of cells (N_{cells}) was not affected by the nature of the nitrogen source, as a unique correlation between both variables was found ($N_{cells}=3.1\times10^7$ A_{640nm} (cells mL^{-1})). In this way, it can be concluded that the two correlations between I_p and A_{640nm} (Figure 5 (A)) result from different cellular activity. Moreover, the slopes of ΔI_p vs. A_{640nm} can be regarded as a measure of the efficiency of the carbon source utilization by the yeast *S. cerevisiae*.

4. Conclusions

Voltammetry was used as a mean to evaluate the consumption of lactic acid in yeast cultures of *C. utilis* and *S. cerevisiae* using lactic acid as the carbon and energy source. Under all tested media a linear correlation between I_p and the lactic acid concentration was found. The variation rates of ΔI_p

and A_{640nm} in the yeast cultures at the exponential growth phase were similar. Furthermore, in all yeast cultures linear correlations were found between the values of ΔI_p and A_{640nm} during the whole growth experiment. Therefore, the ΔI_p values can be regarded as a measure of the lactic acid consumption.

Further work is now underway to validate and extend this method to a wider set of experimental conditions. Studies to be performed comprise assays in yeast cultures in which mixtures of acids, or acids and sugars are supplied as carbon and energy sources.

5. References

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