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New separation methodologies for the distinction of the growth phases of *Saccharomyces cerevisiae* cell cycle

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ABSTRACT

In the present work two separation techniques, namely the gravitational field-flow fractionation (GrFFF) and the reversed-flow gas chromatography (RFGC), are proposed for the distinction of the growth phases of *Saccharomyces cerevisiae* (AXAZ-1) yeast cycle at different temperatures (30 °C, 25 °C, 20 °C, and 15 °C) and pH (2.0, 3.0, 4.0 and 5.0) values. During the fermentation processes, differences observed in the peak profiles, obtained by GrFFF, can be related with the unlike cell growth. The distinction of the phases of AXAZ-1 cell cycle with the GrFFF, was also confirmed with the RFGC technique, which presented similar fermentation time periods for the alcoholic fermentation phases. Simultaneously, the reaction rate constant for each phase of the fermentation process and the activation energies were determined with the aid of the RFGC technique. Finally, the application of both the GrFFF and the RFGC techniques, in combination with high-performance liquid chromatography, allowed us to find the ideal experimental conditions (temperature and pH) for the alcoholic fermentation by AXAZ-1. The results indicate that *S. cerevisiae* cells performed better at 30 °C, whereas at lower temperatures decreases in the fermentation rate and in the number of viable cells were observed. Moreover, the pH of the medium (pH 5.0) resulted in higher fermentation rates and ethanol productivities.

1. Introduction

The employment of analytical techniques has been rapidly increased in terms of separation and characterization of macromolecules and micro-sized particles. One of the most widely used separation techniques is field-flow fractionation (FFF) which has proved over more than three decades the ability to characterize supramolecular species in a size range spanning many orders of magnitude [1]. FFF has a broad potential in many fields related to biotechnology. It has been successfully applied to the characterization and separation of colloids with biological interest such as yeasts [2,3], starch [4,5,6,7], proteins [8], of pharmaceutical interest emulsions and liposomes [9], as well as the fractionation of DNA and viruses and the separation of bacterial and blood cells [10]. Moreover, FFF has a vast number of applications on industrial colloids and particles [11], synthetic polymers and environmental materials [12].

FFF is classified as a one-phase chromatographic technique in which an externally adjusted force field is applied to the suspended particles under motion in a channel. The particles are pushed to one of the channel walls, so that they occupy a thin layer close to the accumulation wall. The thickness of the layer is an explicit function of the applied field, particle size and density of both particles and carrier solution. It is an elution technique and as in classical chromatography, sample components elute at *retention volumes* which are related, and often rigorously predictable, to various physico-chemical properties of the retained species. Measurements of retention volumes under selected experimental conditions are inextricably linked with the number and the weight average particle diameter or particle mass. FFF technique is ideally suited to the analytical scale separation and characterization of particles [13,14].

Different kind of fields has been applied to FFF. They can be a sedimentation field or a thermal gradient, an electrical field or a secondary flow. The type of field defines the FFF sub techniques which, therefore, show wide applicability. Gravitational field-flow fractionation is the simplest and cheapest among FFF techniques. It is a sub-set of sedimentation field-flow fractionation (SdFFF) suitable for the separation and characterization of various micrometer-size particles of different origin [15,16]. It employs the Earth's gravitational field applied perpendicularly to a very thin, empty channel. GrFFF has revealed appealing in the field of biological applications.

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It has been applied to living samples such as parasites [17] and blood cells [18] because of its simplicity, low-cost and reduced risk of sample degradation.

In industrial fermentation processes, such as baking, wine making, brewing, as well as potable and fuel grade alcohol production, yeasts are added as promoters. In the past few years the FFF technique has been applied for the study of yeasts. More specifically, GrFFF has been applied for the characterization of yeast cells [19,20] as well as the study of the viability and activity of *Saccharomyces cerevisiae* strains during wine fermentation [21,22]. As it has been demonstrated [21], differences in the elution curves and peak profiles during fermentation can be related to the different cell growth stage.

Reversed-flow gas chromatography (RFGC) is a sub technique of inverse gas chromatography and it was introduced in 1980 by Katsanos and Georgiadou [23] (in the University of Patras). RFGC involves the change of the carrier gas flow direction at various time intervals. It has a vast number of applications in a wide scientific field such as the determination of: diffusion and mass transfer coefficients [24], adsorption equilibrium constants [25], Lenard–Jones parameters [26], kinetic study of the alcoholic fermentation on industrial scale [27], and activity coefficients [28,29].

In the present study, GrFFF was proposed as a low-cost and simple method, for the distinction of the phases of *S. cerevisiae* growth cell cycle in different environmental conditions (pH, temperature). Moreover, reversed-flow gas chromatography (RFGC) and high-performance liquid chromatography (HPLC) were used in order to confirm the reliability of the GrFFF method.

2. Materials and methods

2.1. Yeast strain

The yeast strain used in the present work was isolated from an agricultural area in Greece [30] and was maintained by the stuff of the Food Technology Laboratory of the Chemistry Department at the University of Patras. *S. cerevisiae* AXAZ-1 strain is an alcohol resistant and pshychrotolerant yeast strain.

2.2. Growth media

AXAZ-1 was suspended in glucose culture medium of the following composition: glucose 40 g/L, KH_2PO_4 10 g/L$(NH_4)_2SO_4$ 10 g/L, $MgSO_4{\cdot}7H_2O$ 50 g/L, and yeast extract 40 g/L, in triply distilled water. All the mediums were sterilized at 150 °C for 15 min, at pressure 1.5 atm. Yeast cells were centrifuged at 5000 rpm for 10 min. Must was provided from a local wine industry, Achaia Clauss S.A. It was of the roditis variety and had total acidity 6–7 g tartaric acid/L.

2.3. Must fermentation

Fermentations were performed in 500 mL bottles containing 400 mL of must (of glucose concentration 247 g/L or 12°Be') and were inoculated with 7 g wet weight AXAZ-1 cells. The fermentations were incubated in duplicate at different values of temperatures (30 °C, 25 °C, 20 °C, and 15 °C) and pH (2.0, 3.0, 4.0 and 5.0). The different pH values (except for pH 5.0 which was the pH value of the medium) were adjusted using H_2SO_4 solutions. The end of the alcoholic fermentations was considered when no residual sugar was left (<2 g/L).

2.4. Biomass determination

For the viable cells determinations, 1 g of them was mixed with 9 mL of sterilized ringer solution and successive dilutions were carried out. The cells were countered on agar with 40 g/L of glucose monohydrate, 10 g/L of KH_2PO_4, 10 g/L of $(NH_4)_2SO_4$, 50 g/L of $MgSO_4{\cdot}7H_2O$ and 40 g/L of yeast extract. The incubation took place at 30 °C for 72 h.

2.5. Analytical methods

2.5.1. Gravitational field-flow fractionation (GrFFF)

The GrFFF system used in this work, which has been described in detail elsewhere [31] has the following dimensions: length l = 48.3 cm, breadth a = 2.0 cm and thickness w = 0.021 cm. The channel void volume V_0 measured by the elution of the non-retained sodium benzoate peak was found to be 2.025 cm^3. A Gilson Minipuls 2 peristaltic pump was used to pump the carrier solution and the sample to the channel, whereas a Gilson Model 112 UV/VIS detector, operated at 254 nm, and a Linseis Model L6522 recorder were also used for sampling analysis. Polystyrene particles of different diameters (nominal 2.013 ± 0.025 μm, 4.991 ± 0.035 μm, 9.975 μm ± 0.061, 15.02 ± 0.08 μm and 20.00 ± 0.10 μm) from Duke Scientific Corporation, dispersed in triply distilled water, were used for the optimization of the elution conditions. The carrier solution was triply distilled water containing 0.5% (v/v) FL-70 (Fisher Scientific Co.), a low-foaming, low-alcalinity, phosphate-, chromate- and silicate-free mixture of anionic and cationic surfactant and 0.02% (w/v) sodium azide (Fluka AG) as bacteriocide.

An aliquot of 25 μL was drawn from the fermentation mixture and was injected into the channel by a microsyringe. Following injection, the longitudinal flow (50 mL h^{-1}) was stopped for 10 min to allow sample relaxation.

2.5.2. Reversed-flow gas chromatography (RFGC)

A gas chromatograph (Shimadzu-8A) equipped with a flame ionization detector (F.I.D.) was used for the separation and quantitation of ethanol (Fig. 1). The system, which has been described elsewhere [27], contained in its oven the sampling column consisting of two sections of lengths $l = l'$ = 100 cm and the diffusion column with length L_1 = 43 cm. At the lower end of diffusion column a glass vessel (L_2 = 5 cm) containing the fermentation mixture was connected. The two columns comprise the sampling cell, which was connected to the carrier gas inlet and the detector in such a way that the carrier gas flow through the sampling column (no carrier gas flows through the diffusion column) can be reversed in direction at any time desired. This can be done by using a four-port valve to connect the ends D_1 and D_2 of the sampling column to the carrier gas supply and the detector, as shown schematically in Fig. 1.

An analytical column (2 m × 1/4 in. × 2 mm glass) packed with 5% Carbowax-20 M and 80/120 mesh Carbopack BAW, which was kept at 115 °C, was placed before the detector in order to separate

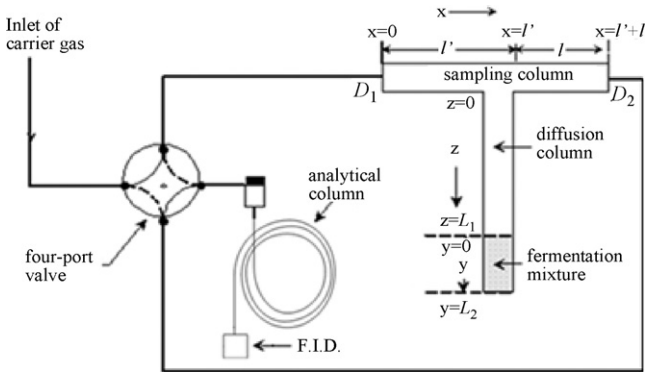

Fig. 1. Schematic representation of the columns and gas connections of the RFGC system used for the determination of ethanol concentration during the fermentation process.

ethanol from alcoholic fermentation's by-products. The temperature of the detector was set at 150 °C whereas the temperature of the chromatographic cell was 75 °C. The experiments were conducted under constant flow rate (20 mL min^{-1}) using helium as carrier gas.

By means of the four-port valve shown in Fig. 1, the direction of the carrier gas flow can be reversed for a short time interval. When the normal direction of the gas flow is restored again, fairly narrow symmetrical chromatographic peaks can be observed from the detector signal, which are called sample peaks.

An aliquot of 0.5 mL of the fermentation mixture was added in the glass vessel (Fig. 1), at constant temperature and pressure. By the time a concentration–time curve appears, the chromatographic procedure begins by reversing the carrier gas flow direction, through the Shimadzu valve, for 6 s. This period of time is shorter than the gas hold-up time in both sections l and l'. When the flow of the carrier gas is restored to its original direction, sampling peaks are recorded. The height h of each sample peak is proportional to the concentration c (l, t) of the solute in the sampling column at $x = l'$ and at time t, when the reversal occurs [28] (Fig. 1):

$$h = 2c(l',\ t) \tag{1}$$

The height of the sample peaks reaches a maximum value $h = H_x$, after which it remains constant.

The relation between the maximum height H_x and the liquid phase concentration $c(l)$ at the solute was given by [28,29]:

$$c(g) = \frac{c(l)}{K} = \frac{uH_x}{2}\left(\frac{L_1}{D} + \frac{1}{k_c}\right) \tag{2}$$

where $c(g)$ is the gaseous concentration of the solute vapor in equilibrium with the bulk liquid phase with concentration $c(l)$, K is the partition coefficient, u is the linear velocity of the carrier gas, L_1 is the length of the diffusion column (Fig. 1), D is the diffusion coefficient of the solute in the carrier gas, k_c is the mass transfer coefficient for the evaporation of the solute from the liquid and H_x is the maximum value of sample peak's height.

The above procedure was repeated in different fermentation periods (t_x) from the initial to the final phase of the fermentation process and the corresponding values of H_x were calculated. After a mathematical model we conclude to the following equation [27]:

$$\ln(H_x^0 - H_x) - \ln H_x^0 - kt_x \tag{3}$$

where H_x^0 is the value of the height of the sampling peak, H_x, at the end of the fermentation and t_x the fermentation time. By plotting $\ln(H_x^0 - H_x)$ against t_x the values of the rate constants for the fermentation process (k), can be calculated. Finally, solutions with different ethanol concentration (%, v/v) into triply distilled water were placed into the glass vessel at the end of the diffusion column and the values of H_x were measured. These values and their corresponding ethanol concentrations were plotted and a calibration curve (slope = 1.202 cm M^{-1}, intercept = 0.041 cm and $R^2 = 0.999$) was obtained. From the calibration curve and by measuring the height of the sample peaks during the fermentation process we can find the unknown ethanol concentrations of each fermentation mixture.

2.5.3. High-performance liquid chromatography (HPLC)

A Shimadzu chromatograph (Kyoto, Japan) equipped with a SCR-101N stainless steel column, a LC-9A pump, a CTO-10A oven at 60 °C and a RID-6A refractive index detector was used for residual sugar determination. The mobile phase was triply distilled water and the flow rate was adjusted at 0.8 mL min^{-1}. Samples of the fermentation product (0.5 mL) were diluted into 50 mL of triply distilled water, containing 2.5 mL of 1% (v/v) butanol-1, and 40 μL were injected directly into the column. Standard solutions of various glucose concentrations (0–130 g L^{-1}) were prepared for the determination of the residual sugar with the corresponding ratio of residual sugar peak areas/1-butanol peak areas to residual sugar concentrations.

The ethanol (99% purity) and sugar used for identification purposes were purchased from Merck, Germany, while the carrier gas used was helium of 99.999% purity from Aeroscopio (Athens, Greece).

The pH value of wine at the end of each fermentation process was determined by using a 744 Metrohm pH Meter Model.

3. Results and discussion

As it is well known, there are four phases to fermentation once the yeast has been added: *the initial lag phase* where little appears to be happening, as the yeast is adjusting to its new environment and beginning to grow in size, *the growth phase* during which the yeast begin to reproduce rapidly and the growth rate is maximum and stable, *the stationary phase* where cell mass is retained stable and growth and death rates are in equilibrium and the last one is *the decline phase* where nutrients are becoming scarce and death rate prevails over growth rate.

The estimation of the duration of each of the above phases is of great importance as it allows the determination of the rate constants for each phase of the fermentation process.

In the present work the GrFFF and the RFGC techniques were used for the kinetic study and the distinction of the growth phases of AXAZ-1 cell proliferation during the alcoholic fermentations conducted at different environmental conditions.

3.1. GrFFF fractionation of yeast cells

In all GrFFF analysis it is assumed that the elution mechanism of yeast cells correspond to the steric/hyperlayer mode [32]. According to this, the interplay of steric interactions of particles with the channel walls and the hydrodynamic lift force of various origins, leads to the larger particles move more quickly than the smaller ones, and the separation mechanism is based on the inherent particle size than on the ability of particle to undergo Brownian diffusion [13,31]. Due to the fact that, GrFFF technique has a significant number of applications on yeast cells, different yeast samples were tested at various time intervals, in order to examine cell proliferation, during the alcoholic fermentations conducted at different temperature and pH values. Fig. 2 shows the fractograms of the analyzed cell populations at different time periods for fermentation, since the yeast has been added in the culture medium, obtained by GrFFF at pH 2.0 and 3.0 and at 30 °C.

As it was noticed from the fractograms (Fig. 2), no variations were observed at peak profiles obtained at 30 °C, as a unimodal distribution was observed from the beginning to the end of the fermentation process at both pH values. The same action was observed at the temperatures of 25 °C, 20 °C and 15 °C (data not shown), where a unimodal distribution was observed as well. As a result the distinction of the phases of yeast growth cycle was impossible to be done. It is obvious, that at this pH values the metabolic activity of yeast cells was significantly diminished as they were subjected to hyperosmotic shock due to the low pHs of the culture media. Fig. 3 shows the peak profiles obtained during the cell proliferation at pH 4.0 and at 30 °C and 15 °C. For fermentations at pH 4.0, a growth phase was observed at 6.5 h < t < 12.0 h for all the temperature values.

The growth phase was followed by a steady stationary phase (unimodal distribution) which lasted until the end of the reaction. No decline phase was observed at the fermentation processes conducted at pH 4.0.

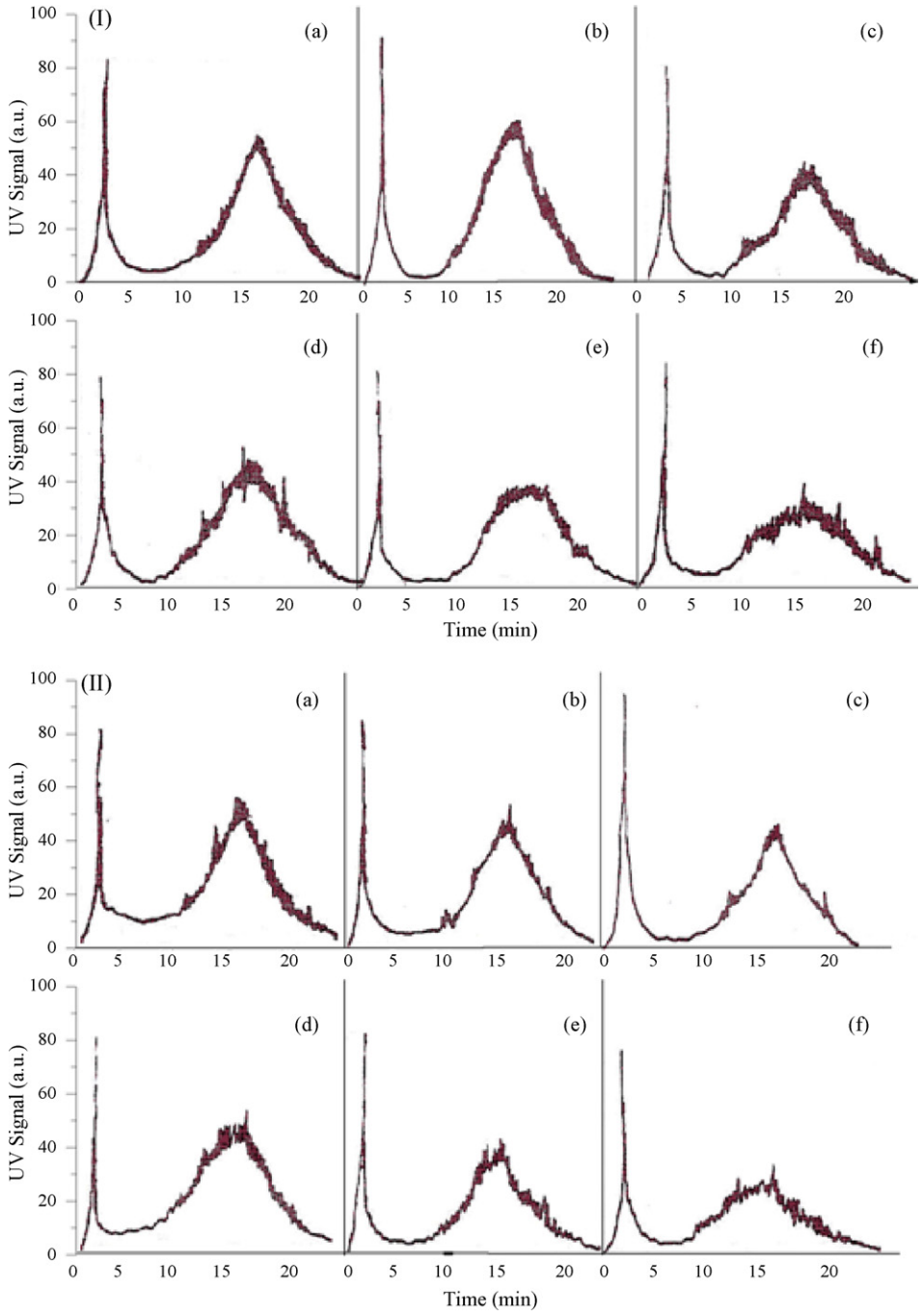

Fig. 2. Fractograms obtained by GrFFF for AXAZ-1 cells at 30 °C (I) pH 2.0: (a) 0 h, (b) 6.5 h, (c) 12.5 h, (d) 22.5 h, (e) 39.5 h, (f) 55.5 h and (II) pH 3.0: (a) 0 h, (b) 6.5 h, (c) 12.5 h, (d) 22.5 h, (e) 28.5 h, (f) 37.5 h.

When the fermentations were conducted at pH 5.0, at the same range of temperatures, differences in the behavior of yeast cells were observed during their proliferation, as it was noticed from Fig. 4.

At 30 °C, at the beginning of the reaction (t_x = 0 h), one peak was observed at the fractogram which suggests that yeast cells lie in the lag phase. The same happens at 2.5 h where a unimodal cell size distribution was also observed. At 4.5 h and 6.5 h the growth of yeast cells has already been started and a bimodal distribution was observed in the corresponding fractograms (Fig. 4). This behavior could be explained by taking into account that daughter cells were separated from mother cells and were eluted in the last fraction at higher retention volumes. As it has been demonstrated [33] during the cell cycle densities were highest for cells with small buds and lowest for mother cells. Moreover, Merino-Dugay et al. [18] indicated that selective elution takes place when differences in the shape and density of cells exist. We realize that the first peaks, which show low V_r values, correspond to large cells with low density, whereas the peaks with high V_r values correspond to cells smaller in size with high density. The next fractograms of Fig. 4 show a stable bimodal distribution at 8.5 h and 10 h which reveals the appearance of the stationary phase. The decline phase follows, from 12 h until the end of the reaction, with a monomial distribution taking place again testifying the decrease of the number of yeast cells. Fermentations at 25 °C proceeded almost similar with that at 30 °C whereas fermentations at 20 °C and 15 °C began

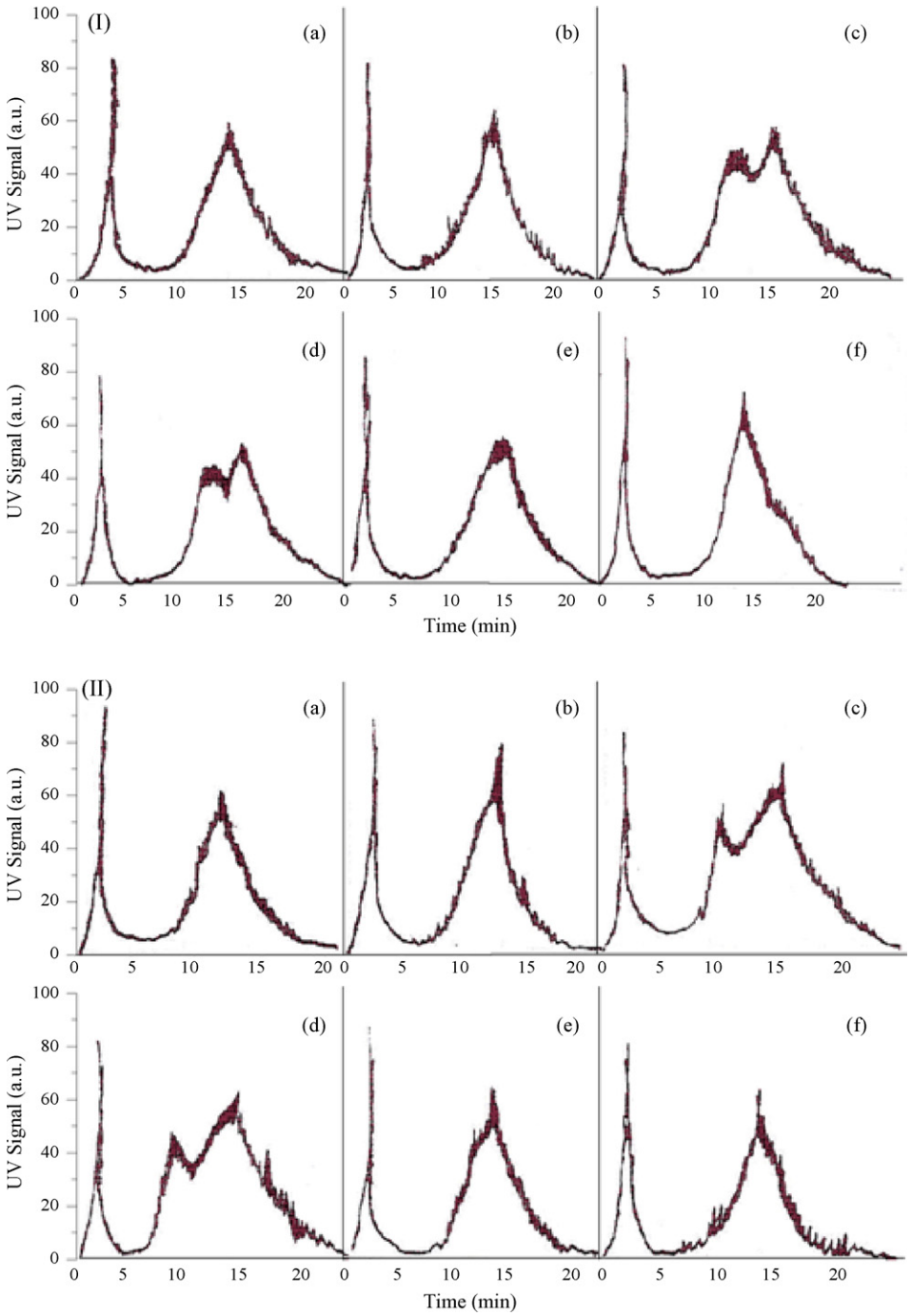

Fig. 3. Fractograms obtained by GrFFF at pH 4.0 for AXAZ-1 cells at (I) 30 °C: (a) 0 h, (b) 4.5 h, (c) 8.5 h, (d) 12.5 h, (e) 18.5 h, (f) 22.5 h and (II) 15 °C: (a) 0 h, (b) 4.5 h, (c) 8.5 h, (d) 18.5 h, (e) 22.5 h, (f) 38.5 h.

more slowly as it was observed by their longer lag phase. At 15 °C after the lag phase (0 h < t < 6 h), a bimodal size distribution was observed at 6.5 h < t < 22 h. After the growth phase a long stationary phase took place at 22.5 h until the end of the reaction with a stable unimodal distribution in comparison with 30 °C where a stable bimodal distribution appeared. No decline phase was observed during the alcoholic fermentation at these temperatures.

3.2. Effect of pH and temperature on biomass concentrations

There are several reports in the literature analyzing the effect of temperature and pH values on the population dynamics of *Saccharomyces* strains [34]. The usual growth curve, consisting of lag, growth, stationary and decline phases was not observed at all cases and as expected the growth in yeast cells varied according to the changing parameters.

The distinction of the four phases, analyzed above, can also be confirmed by the determination of cell concentrations. The effect of fermentation temperature and pH values on yeast populations was evident in Fig. 5.

At pH 2.0 (Fig. 5a), the initial cell population (1.5×10^6 cfu g^{-1}) did not change significantly at 30 °C and 25 °C despite the temperature variations. AXAZ-1 cells were not able to grow and proliferate at these conditions and they produced stuck fermentations with higher sugar contents. At 20 °C and 15 °C, at the same pH value, yeast cells did not follow their growth cycle, as they were dimin-

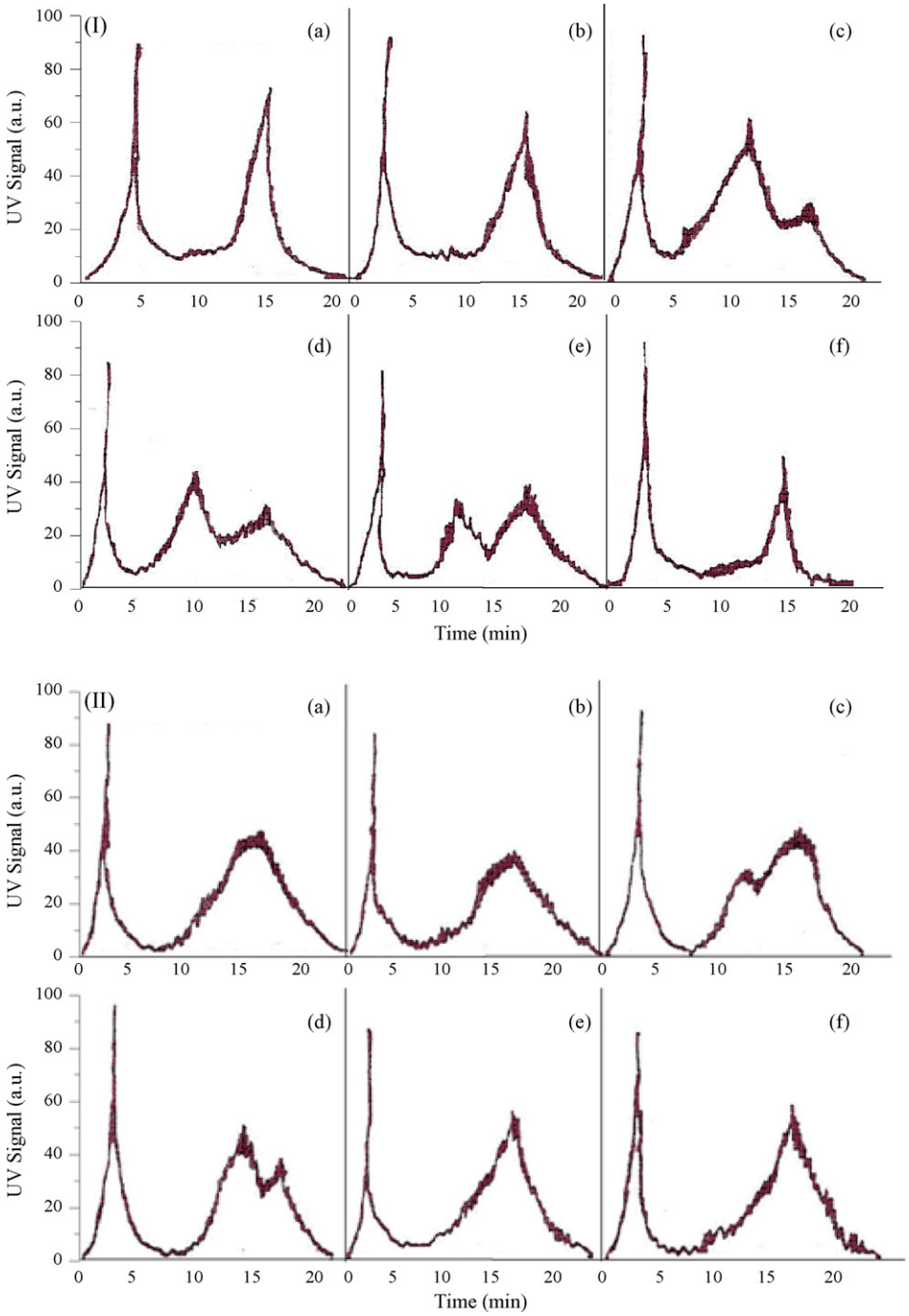

Fig. 4. Fractograms obtained by GrFFF at pH 5.0 for AXAZ-1 cells at (I) 30 °C: (a) 0 h, (b) 2.5 h, (c) 4.5 h, (d) 6.5 h, (e) 8.5 h, (f) 12 h and (II) 15 °C: (a) 0 h, (b) 6 h, (c) 6.5 h, (d) 22 h, (e) 22.5 h, (f) 34 h.

ished to a great extent from the early hours of the fermentation processes. These results agree with the literature, as low pH values deviate from the optimum pH value for the activity of plasma membrane-bound proteins (pH 5.0), including enzymes and transport proteins [35].

At pH 5.0, however, the distinction of the phases is obvious in Fig. 5b. Fermentations at 30 °C and 25 °C appeared a short lag phase, with almost stable populations, and reached similar maximal populations (1.93×10^8 cfu g^{-1} and 1.50×10^8 cfu g^{-1}, respectively) at 8 h and 13 h, respectively (growth phase). After the growth phase the cell concentrations remained stable for a short period (stationary phase), after which they decreased down to values 10^5 cfu g^{-1}. At 20 °C and 15 °C, at the same pH value, fermentations began more slowly, as it was observed by their longer lag phases (Fig. 5b). This caused a delay in reaching the maximal cell populations (3.50×10^7 cfu g^{-1} and 9.0×10^6 cfu g^{-1}, respectively), but since they reached they remained almost stable until the end of the reactions. This indicates a long stationary phase and the absence of decline phase as it was also observed from the fractograms of Fig. 4, obtained by GrFFF technique. Another worth-mentioning result is the decrease of yeast viability at the end of the reactions as the temperature increased, which according to the literature, can be owed to the greater accumulation of intracellular ethanol at higher temperatures. This will enable cell toxicity [36] and alter the mem-

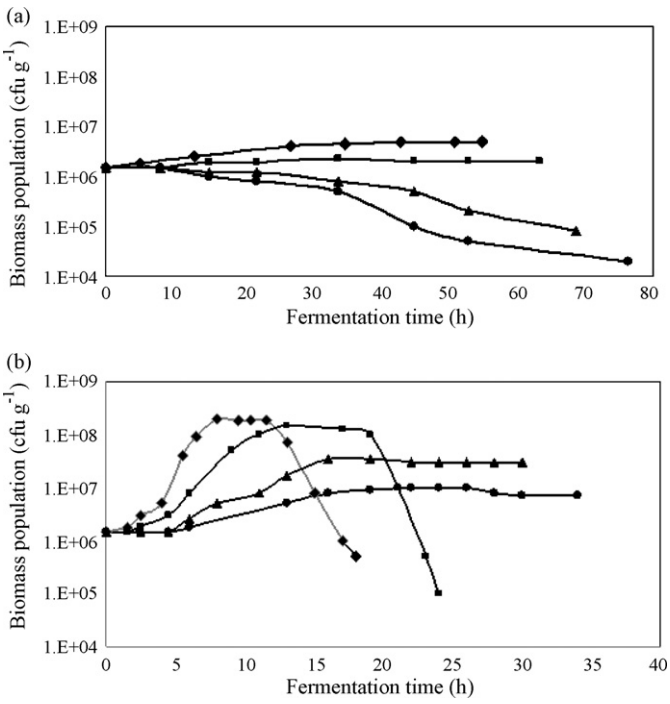

Fig. 5. Variation of AXAZ-1 cell populations during the alcoholic fermentation processes conducted at (a) pH 2.0 and at 30 °C (♦), 25 °C (■), 20 °C (▲) and 15 °C (●) and (b) at pH 5.0 and at 30 °C (♦), 25 °C (■), 20 °C (▲) and 15 °C (●).

brane structure decreasing its functionality [37]. However, lower temperatures reduce cell toxicity but also growth rate [38], as a result we observed lower yeast cell populations and the absence of the decline phase.

3.3. Confirmation of GrFFF technique

In order to validate the GrFFF technique for the distinction of the phases of AXAZ-1 cells growth cycle, another method was used, called reversed-flow gas chromatography. Through the determination of ethanol with the RFGC technique and with the aid of Eq. (3) (cf. experimental part), the graphical representation of Fig. 6 arises, showing the variation of $\ln(H_x^0 - H_x)$ vs t_x.

From the slopes of the above-mentioned straight lines we can determine the reaction rate constants (k), of the alcoholic fermentation processes conducted at pH 5.0 and at 30 °C, 25 °C, 20 °C and 15 °C. Each slope corresponds to a different fermentation phase. The existents of the above-mentioned slopes were verified by the R^2 values which are between 0.94 and 1.00.

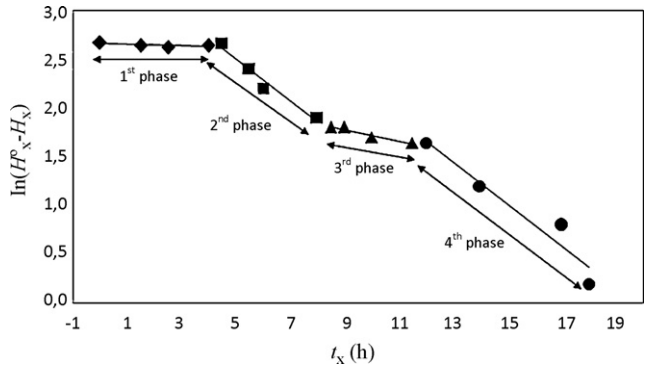

Fig. 6. Variation of $\ln(H_x^0 - H_x)$ with time, according to Eq. (1), for AXAZ-1 cells at pH 5 and T = 30 °C for the 1st (♦), 2nd (■), 3rd (▲) and 4th (●) alcoholic phases.

Table 1
Rate constants (k), with their standard deviations for the fermentation phases of AXAZ-1 cells at pH 5.0 and at 30 °C, 25 °C, 20 °C and 15 °C, obtained by RFGC.

Temperature (°C)	k ($\times 10^3$ h^{-1})			
	1st phase	2nd phase	3rd phase	4th phase
30	8.2 ± 0.4	200.0 ± 0.8	45.0 ± 0.9	200.0 ± 0.9
25	6.7 ± 0.3	130.0 ± 0.8	30.3 ± 1.2	120.2 ± 0.7
20	6.4 ± 0.1	70.0 ± 0.8	15.2 ± 0.4	70.2 ± 0.7
15	5.5 ± 0.1	30.3 ± 0.2	8.0 ± 0.2	30.3 ± 0.8

The distinction of the phases of AXAZ-1 cell cycle with the GrFFF, presented similar times with the distinction of the alcoholic fermentation phases conducted with the RFGC technique. This is obvious from Fig. 6 as the first phase (lag phase) lasts from 0 h to 4 h, the second (growth phase) from 4.5 h to 8 h, the third (stationary phase) from 8.5 h to 11.5 h, the fourth (decline phase) from 12 h to 18 h.

As above-mentioned, by using Eq. (3) of the RFGC technique, we can calculate the reaction rate constants and the activation energies at each phase of the alcoholic fermentation process.

The greatest values of the reaction rate constants were observed at the second and the fourth phase (growth and decline phase, respectively), whereas the lowest and almost stable values were observed at the first phase (lag phase), during which the total biomass concentration does not show any change. It is noteworthy that a rate constant was also calculated for the third phase which indicates that this phase is not stationary [39] but the biomass concentration changes slowly during the fermentation process. Rate constant values for this phase were one order of magnitude smaller than those given for the second and fourth phase. A comparison of these values (rate constants for the second and fourth phase) found in the present work by RFGC with those given in the literature [40] shows a good agreement, as all of these values are of the same order of magnitude. The activation energies of the alcoholic fermentation phases were calculated from the Arrhenius equation:

$$\ln\ k = \ln A - \frac{E_a}{R}\frac{1}{T} \quad (4)$$

where k is the reaction rate constants of the alcoholic fermentation phases, A is the pre-exponential factor, E_a is the activation energy, R is the gas constant and T is the temperature values. Using the calculated values of rate constants for each alcoholic fermentation phase (Table 1) and plotting $\ln k$ vs $1/T$, the activation energies at each phase of the fermentation process were determined from the slopes of the graphical representations. The activation energy for the first phase of the alcoholic fermentation process was 18.4 ± 0.4 kJ mol^{-1}, whereas for the remaining phases (second, third and fourth) higher values were observed: 90.5 ± 0.2 kJ mol^{-1}, 94.5 ± 0.3 kJ mol^{-1} and 95.6 ± 0.5 kJ mol^{-1}, respectively.

By the combination of the GrFFF (elution curves and retention volumes), RFGC and HPLC techniques, through which ethanol and sugar were determined, respectively, the following results are obtained which are summarized in Table 2.

The phase and time that maximal cell populations were observed, at different values of temperature and pH 5.0, were determined through the fractionation of yeast cells by GrFFF at various times. With several counts of cell populations, as described in the experimental part, the maximal cell population was obtained, by the method of dilution series, for each fermentation process. The maximal fermentation rate was expressed as the concentration of consumed sugars per day ((g/L)/day), and was calculated from the maximal slope of the graphical representation of sugar consumption vs fermentation time (Fig. 7a). The maximal ethanol productivity was obtained from the graphical representation of ethanol production vs time and expressed in (g/L)/day (Fig. 7b).

Table 2
The phase and time of maximal population detected by GrFFF, the maximal fermentation rate determined by HPLC, the ethanol productivity measured by RFGC, and the maximal cell population found by the method of dilution series, at pH 5.0 and 30 °C, 25 °C, 20 °C and 15 °C.

Temperature (°C)	Phase of maximal population	Time of maximal population (h)	Maximal cell population (cfu/mL)	Maximal fermentation rate (g/L)/day	Maximal ethanol productivity (g/L)/day
30	Growth	6.5	1.93×10^8	31.8	12.2
25	Growth	8.5	1.50×10^8	10.2	8.2
20	Growth	12.5	0.35×10^8	7.4	5.5
15	Growth	15.5	0.10×10^8	5.3	3.0

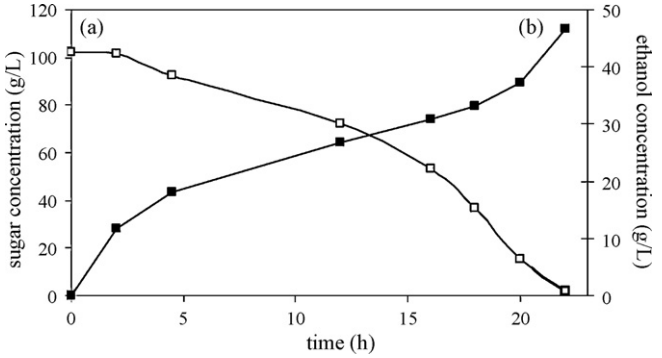

Fig. 7. Variation of (a) sugar consumption (□) and (b) ethanol production (■) with time.

The maximal fermentation rate and ethanol productivity at all temperature values and at pH 5.0, were observed at growth phase, detected with GrFFF. It is obvious that temperature affected not only yeast metabolism, as mentioned above, but also the fermentation rate and ethanol productivity. The slow rates of maximal fermentation observed at 20 °C and 15 °C can explain the longer lag phase and the absence of decline phase at these temperature values. The maximal ethanol productivity decreased with the reduction of temperature. This can be related to the reduced metabolic activity of *S. cerevisiae* strains at low-temperature fermentations [41].

4. Conclusions

Gravitational field-flow fractionation (GrFFF) has proved to be a useful tool for the study of the effect of experimental conditions (pH and temperature) on AXAZ-1 growth kinetics. As it was observed, pH values effect significantly growth and proliferation of AXAZ-1 cells. At low pH values no variations were observed at elution curves fractionated with GrFFF. Yeast cells seemed to grow better in pH 5.0 than in pH 2.0, due to the pH of the plasma membrane, which regulates yeasts' internal pH. At 30 °C and pH 5.0, yeast cells showed a normal functionality, as it was observed from the peak profiles obtained by GrFFF. For lower temperatures, the enzymes suffer from inactivation which results in a slow advance of the phases of AXAZ-1 cell cycle and the absence of decline phase, in some cases, as at 15 °C. The distinction of the phases of AXAZ-1 cell cycle with the GrFFF, was confirmed with the reversed-flow gas chromatography (RFGC) technique, which presented similar times for the alcoholic fermentation phases, but also with determination of cell counts. The same technique was also used for the determination of the rate constants and the activation energy for each phase of the fermentation process.

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