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Sonication effect on cellular material in sedimentation and gravitational field flow fractionation

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Abstract

Sonication procedures are generally used prior to field flow fractionation (FFF) separation in order to produce suspensions without aggregates. Yeast cells manufactured in active dry wine yeast (ADWY) were placed in an ultrasound water bath in order to disrupt possible clumps and to obtain a single-cell suspension to be used in optimal conditions during fermentation processes. In order to determine whether this sample preparation procedure meets absolute needs, different yeast samples before and after sonication were analysed by two field flow fractionation techniques. It is shown that 2 min of sonication in the sample preparation process is sufficient to obtain an optimal dispersion of the yeast cells, that is, without critical percentage of aggregates. To demonstrate this effect, photographs of the yeast cell suspensions were performed with non-sonicated and sonicated yeast sample dispersion. The resulting data are compared with the elution profiles obtained from the two different FFF techniques. It is demonstrated that fractogram profiles prove the effectiveness of sonication methodologies.

Keywords: Field flow fractionation; Sonication; Yeast cells; Wine; Gravitational field flow fractionation

1. Introduction

In wine manufacturing processes, biocatalysts such as yeast cells are currently in wide use and are commonly stored as active dry wine yeast (ADWY) in the form of dehydrated granules. The living yeast cells are in a latent state due to the lack of moisture. They become active when dissolved in heated water (35–40 °C). Conservation of the ADWY type has originated a large number of different yeast strains, and these are now becoming commercially available. However, when reactivated, these strains require effective and particular dispersion states in the media for correct use. Thus, dispersion as a single-cell suspension is required, with limited mass or volume percentage of aggregates or clusters. If activated dry yeasts in aqueous suspension are analysed, low percentages of single cells are obtained due to cell clumping. In order to limit the clustering of aggregates specific treatments must be performed to assure a complete single cell dispersion of the active wine yeast suspension [1]. In general, the fermentation

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process generated by active yeasts in suspensions, converts cell nutrients into alcohol and carbon dioxide. The rate of this transformation depends on the percentage of single cells.

Sonication is a simple and efficient tool to disrupt cell clumps, and to make single cell suspensions available for the fermentation process. However to reduce the loss of cell viability when the yeasts are subjected to ultrasonic standing wave fields [1], minimised sonication times are required. Such single cell diagnostics can be achieved using granulometric particle size distribution methods. Yeast cluster assessments by flow cytometry and coulter counters are limited by the strong hydrodynamic shear forces inherent to the technologies used in analyses. Microscope-based technologies require time-consuming measurements or lead to biased results (cluster/aggregates formation, optic bias, specialised cluster/aggregates image analysis softwares). Fast and accurate alternative methods can be used, however, and field-flow fractionation (for example) can provide the particle size distribution of the yeast sample in suspension.

Field flow fractionation, invented by Giddings 35 years ago, encompasses versatile elution-driven separation techniques [2]. The general FFF principle [3] consists of the separation of the sample components inside a narrow, ribbon-like channel with the form of a parallelepiped by the action of an external field applied perpendicular to a flow passing through the empty channel. The laminar flow creates a parabolic flow profile in the ribbon thickness. Different interactions of the sample components with the field make it possible to concentrate or drive these components toward one wall (usually designated as the accumulation wall) in carrier flow streamlines of different velocities. The migration along the channel of sample components at different velocities produces the separation. The versatility of the FFF separations resides in the variety and intensity of the external field, in the separator geometry as well as in the experimental conditions. In the literature, the field type generates the FFF sub-technique [3,4]. In this work, two types of FFF techniques were used. One of these employs the gravitational field and is known as gravitational FFF (GFFF). It is low-cost, simple, non-destructive and easy to implement in a standard HPLC system. The other technique that employs multigravitational fields is known as sedimentation FFF (SdFFF). This technique permits the reduction of the analysis time, allows the reduction of carrier phase consumption and improves the enhancement of the sample separation. SdFFF, however, requires much more sophisticated instrumentation [3–5]. Sedimentation and gravitational FFF techniques have proved to be remarkably well suited for the separation of colloids and micron-sized species, mainly those of biological origin, like cells [6–19].

It has been demonstrated recently [20] that FFF associated to particle size distribution techniques can separate yeast populations with very high selectivity. Active dry wine yeasts are now used throughout the industry, and optimisation of the sample treatment of the cells is mandatory in any industrial use. Previous studies reported the suitability of SdFFF and GFFF for the analyses of this kind of sample [20–23].

The objective of this report is to demonstrate that FFF applied to different commercial yeast strains can be used to monitor and to optimise yeast cell suspensions during the sonication step, thereby producing suspensions of controlled characteristics.

2. Experimental

2.1. GFFF system

The GFFF system employed here was described in a previous study on yeast [23]. Briefly, the dimensions of the ribbon-like channel were 0.0151 cm thick, 2 cm wide and 30 cm long. Total void volume V_o was 831 μl determined by eluting Na_2CrO_4 (Aldrich, WI, USA) at 373 nm. The detection was performed using a HP Model 1050 (Hewlett-Packard, Palo Alto, CA, USA) with a variable wavelength UV–Vis detector. The injected amount was always 20 μl, and the injection time 45 s at an injection flow-rate of 0.2 ml min^{-1}. For sample relaxation, stop-flow time was always 6 min. The carrier liquid was 0.5% Triton X-100 in MeOH–H_2O (20:80, v/v) and all the fractograms were obtained at 330 nm at room temperature. These experimental conditions were found most suitable for GFFF of wine-making yeast [21–23].

2.2. SdFFF system

The sedimentation FFF system used in this work was the same as described in a previous work [20]; briefly, the dimensions of the ribbon-like channel were 743 mm long, 10 mm wide and 0.08 mm thick. The channel volume was 555 μl and the channel diameter was 27.6 cm. Total void volume, i.e. channel volume+connection tubes+((injection and detection device volumes)/2), was 695±4 μl (n= 15) measured using acetone 1% (v/v).

The mobile phase was phosphate-buffered saline (PBS), pH 7.4. Sample introduction was performed via a 7525 Rheodyne (Cotati, CA, USA) injector with a loop of 20 μl. The detector was a UV–Vis Spectroflow 757 (ABI-Kratos, Ramsey, NJ, USA) operating at 254 nm. These experimental conditions were found most suitable for SdFFF of wine-making yeast [20]. Channel decontamination was performed by injecting solutions of a mixture of ethanol–water when the field was stopped.

2.3. Scanning electron microscopy (SEM) and optical microscopy

SEM photographs of the sonicated and non-sonicated yeast dispersions of Killer D-47, Bourgoblanc and L1033 were obtained with a microscope Model S-2300 (Hitachi, Tokyo, Japan) at 15 or 10 kV. Samples were dry-frozen with liquid N_2 and sputter-coated with ca. 500 nm of Au.

For L1033 yeast strain, photographs of the kinetics of sonication were performed by a Leica DMRB FLUO microscope (Leica Microsystems, Wetzlar, Germany) with an optical lens of NPlan L 20×/0.4 Ph1. Photos were digitalised and analysed by Metamorph. All the photographs were performed at the Serveis Científico-Tècnics of the Universitat de Barcelona.

2.4. Yeast samples

Three types of active dry wine yeast from *Saccharomyces cerevisiae*: L1033, Bourgoblanc and Killer D-47 were used. They were supplied by Dr. Guasch (Group of Química Analítica Enològica i dels Aliments, Universitat Rovira i Virgili, Tarragona, Spain). All the samples were dispersed at 0.1% (w/v) in the carrier liquid by sonication at a frequency of 35 kHz (Sonorex RK 100, Bandelin Electronic, Berlin, Germany) and at different times. The sample was then injected into the sedimentation or gravitational FFF system. All injections were made at room temperature.

3. Results and discussion

Active dry wine yeasts are now used throughout the industry, and an optimisation of the sample treatment of the cells is mandatory before any industrial use. The major goal in managing yeasts for the wine fermentation process is to obtain single cell suspensions based on sonication procedures, the time of which must be optimised to limit loss viability. Monitoring of such a process can be performed using a non-destructive technique such as granulometry for the clusters and/or aggregates. The strong hydrodynamics of flow cytometry or coulter methods and the long lag time of microscopy reduce cluster/aggregate monitoring accuracy. FFF allows the experimenter to discriminate the critical existence of residual clusters/aggregates in a yeast sample by eluted signal profile analyses. Previous studies reported the suitability of SdFFF and GFFF for the analyses of this kind of sample [20–23]. The methodology developed earlier [21] was improved by studying the sample preparation, and the new conditions were applied to different yeast strains. The SdFFF and GFFF peak characteristics were used to monitor elution profiles containing single yeast cells, while photographs were also used to evaluate the sample treatment.

The yeast models used in this report are L1033, Killer-D47 and Bourgoblanc. Suspensions were made directly from ADWY strains. L1033 and Killer-D47 single cell suspension (after sonication) characteristics have been already published, with the purpose of defining, among other parameters, their particle size distribution [20]. The elution of these cells by field flow fractionation is possible, and it was demonstrated that FFF could lead to the isolation of yeasts' sub-populations according to a qualitative elution model described as the ''Steric Hyperlayer'' [20].

From the general ''Steric-Hyperlayer'' elution

model [24–27], it can be deduced that in identical experimental conditions, particles of identical shape and density are retained in order of their size, with the bigger particles being eluted first. Moreover, spherical particles of identical size are separated according to their density; the densest being more retained. However the size-dependent elution pattern of yeasts observed in SdFFF [20], and the reduced channel thickness (150 to 80 μm) used in this report, led to a single major hypothesis. It is highly suspected that yeast separations in SdFFF or GFFF are mainly driven by size differences. In any event, much larger yeast aggregates or clusters will appear and will therefore be less retained than single cells in both techniques. With FFF elution, separations from suspensions made of single particle doublets, triplets, or higher order aggregates [28,29] are possible. Using proper elution conditions it is possible to isolate single cells from doublets and triplets, clusters/aggregates elute close to the void volume [30], and tailing peaks can also be observed [31]. If single cell characteristics (single particles size distribution) are to be analysed from the FFF elution pattern, aggregates can generate biased results. Pazourek et al. [28] have shown for GFFF that the optimisation of the sample preparation procedure (reducing and limiting aggregates) can be carried out with adequate sonication. Battu et al. [29] have demonstrated that micron-sized bacteria aggregates produce channel contamination, reducing the recovery and modifying elution characteristics. As a consequence, proper cell sample preparation and decontamination processes were developed. Sonication was already applied with success to bacteria [29] and prokaryotic cells [16] in order to eliminate or reduce clumping, which suggests effects on yeasts.

Killer D-47 models were used in a first step. They appeared to be associated with large percentages of aggregates when suspended, as shown in Fig. 1A. The complexity of these aggregates, varying mainly in cell number (chains) or shape, was evidenced by microscopy at high magnification as shown in Fig. 1B. Sonication procedures were known to reduce these supra-cellular architectures leading to single cell populations as shown in Fig. 1C. A low number of clusters evidenced in Fig. 1C (I, II) are artefacts created by the sedimentation of the cell suspension. When comparing Fig. 1A with Fig. 1C, yeast cluster/

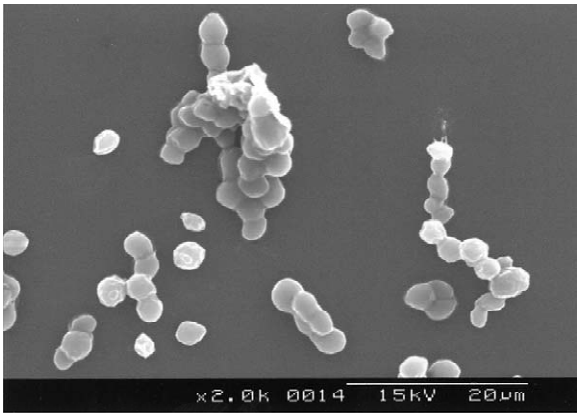

Fig. 1. Scanning electron microscopy (SEM) photographs of Killer D-47. (A) Non-sonicated sample. Magnification: ×2 at 15 kV. (B) Non-sonicated sample. Magnification: ×6 at 15 kV. (C) 2 min sonication. Magnification: ×2 at 10 kV. I and II are possible cluster artefacts.

aggregate patterns are very different if they are sonicated or not. Therefore, using FFF it is possible to control the Fig. 1A and C pattern in terms of whether it will lead to different fractogram profiles. SdFFF elution of Killer D-47 suspension after sonication or not were performed and fractograms are shown in Fig. 2. Fig. 2A corresponds to a sample suspended in the carrier phase without sonication, while Fig. 2B shows the injection of sonicated suspension in identical experimental SdFFF elution conditions. Both fractograms had common features. Firstly, a large void volume signal is observed, considering the ultra thin channel used, and as already demonstrated for bacteria [29], it contains clusters and aggregates. However, void volume corresponding signals cannot be considered as a specific cluster/aggregate signature. On the contrary, retained signals at a retention ratio of 0.313 of both fractograms specifically indicate the elution of single yeasts cells. In Fig. 2A, the single cell peak is of very low intensity while that of Fig. 2B demonstrated the elution of a higher percentage of single yeast cells. The complex balance of flow-rate/external field/accumulation wall characteristics needed to obtain yeast cell retention was also associated with field intensity of the dependent signal, indicating the reversible release of adsorbed cells, an effect already described [20]. However, such release can be attributed specifically to yeast cells. When the release peak area of Fig. 2A and B are compared, the reversible release, even qualitatively, is reduced if sonicated sample is eluted, thus enhancing single cell recovery.

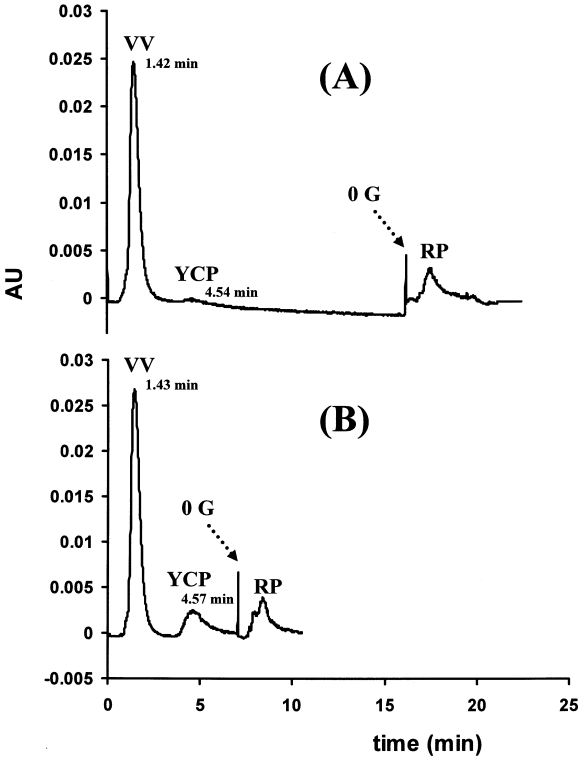

Fig. 2. SdFFF fractograms of Killer D-47. (A) Injection of non-sonicated yeast. (B) Injection of sonicated yeast (2 min). Experimental conditions: field strength, 40 G; flow-rate, 0.5 ml/min; other conditions are given in Section 2.2. YCP, yeast cell peak; VV, void volume peak; RP, release peak; 0 G, stop field mark. Retention times are indicated in the figure.

The conclusions of the first step are: (a) that SdFFF fractograms can monitor qualitatively and quantitatively (after proper calibration or using fraction collection and subsequent granulometric/image analysis) the single retained cells, and, (b) sonication is associated with a higher single cell recovery.

The previous experiments were performed using a quite arbitrary sonication time of 2 min, known empirically to offer proper cluster/aggregates destruction. However, with the purpose in mind of maximum viable yeast production, the sonication time must be minimised. Optimisation of this type of process was performed by using other types of active dry wine. L1033, was studied by gravitational field flow fractionation and microscopy analyses to optimise the sonication time required for a single cell with limited damage. In order to observe retention of clusters/aggregates, a wider channel thickness was used. The leading supported hypothesis of this experiment is that the percentage of clusters/aggregates released is of higher impact on fermentation than that of damaged single cells caused by sonication [1]. Five different yeasts with pretreatment sonication times where chosen: (A) non-sonicated, (B) 30 s, (C) 1 min, (D) 2 min and (E) 3 min; samples where then eluted immediately in GFFF and the recorded fractograms are shown in Fig. 3. Fractogram (A), corresponding to the non-sonicated sample, is bimodal, and a fraction collection microscopy at 10 min elution time showed a mixture of clumps of different size, Fig. 3A-I, while at 15 min single cells were predominant, Fig. 3A-II. High FFF channel contamination can be observed, as well,

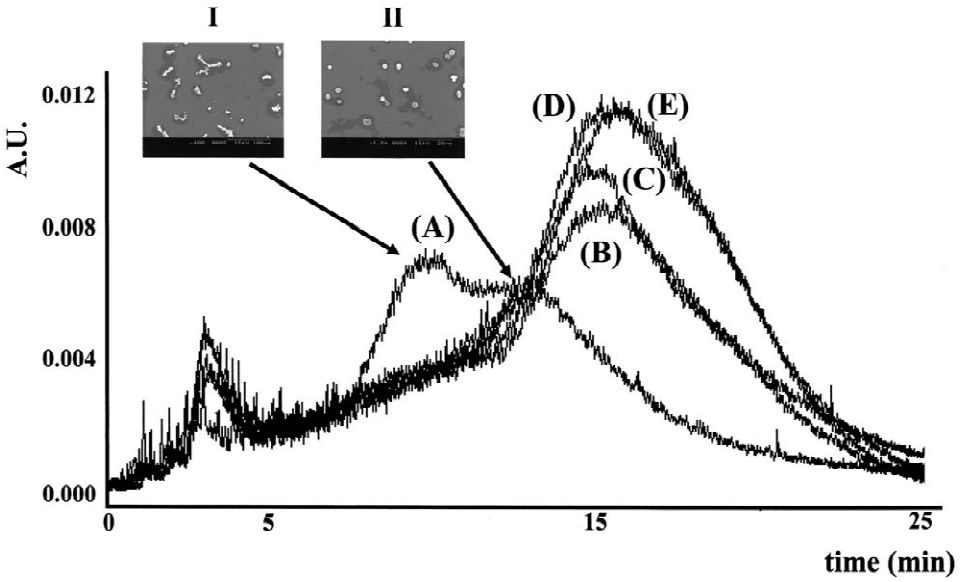

Fig. 3. GFFF fractograms of L1033 at different sonication times: (A) non-sonicated. (I) SEM photograph of the collected fraction at 10 min elution time. (II) SEM photograph of the collected fraction at 15 min elution time. (B) 30-s sonication. (C) 1-min sonication. (D) 2-min sonication. (E) 3-min sonication. Experimental conditions: field strength, 1 G; flow-rate, 0.2 ml/min; other conditions are given in Section 2.1.

leading to specific channel cleaning procedures. Fractograms B, C, D and E, obtained after increased sonication times, showed sonication time-dependent peak characteristic modifications (the area being increased from fractogram B to D). These modifications are correlated to the increased number of single cells (optical microscopy measurements).

The considerable peak profile differences observed when fractogram A is compared with fractogram B, confirm the conclusions obtained from Killer-D47 strain. Fractograms D and E appeared almost analogous compared to fractogram C. It can be concluded that for the L1033 strain, the optimised sonication time is in the 1- to 2-min range. However, the 2-min sonication time determined here is in complete agreement with previous experiments [20–23]. In order to control such analyses with independent methods, yeast image characteristics were monitored as shown in Fig. 4. The objective of such monitoring is to determine the cluster/aggregate residuals. For each yeast sample, two images were taken from very different localisations in the yeast film prepared for microscopy.

Fig. 4A shows photographs of the non-sonicated sample. It can be observed that a high percentage (in volume and in number) of the sample is aggregated forming clusters of large size distribution. This pattern should be compared to the fractogram A of Fig. 3, and will be seen to confirm the hypothesis that FFF can monitor the presence of clusters or aggregates of high order. Fig. 4B corresponding to a 30-s sonication shows a considerable reduction of aggregate size, which must be compared to Fig. 3 fractogram B. Reductions of cluster/aggregate size are associated with the withdrawal of bimodality of the signal and with the decreased retention ratio of fractogram B compared to fractogram A. Fractograms B, C, D, E show analogous characteristics in terms of retention ratio and peak profile, suggesting that the monomodal peak is effectively made of single cells. Fig. 4C–E image analyses show clearly cluster/aggregate size reduction, D and E images being made only of single cells.

In order to confirm the kinetics of sonication on another yeast strain, Bourgoblanc was assayed in SdFFF according to procedures already described [20]. The sequence is shown in Fig. 5. The procedure of sample injection (sonicated/not sonicated) con-

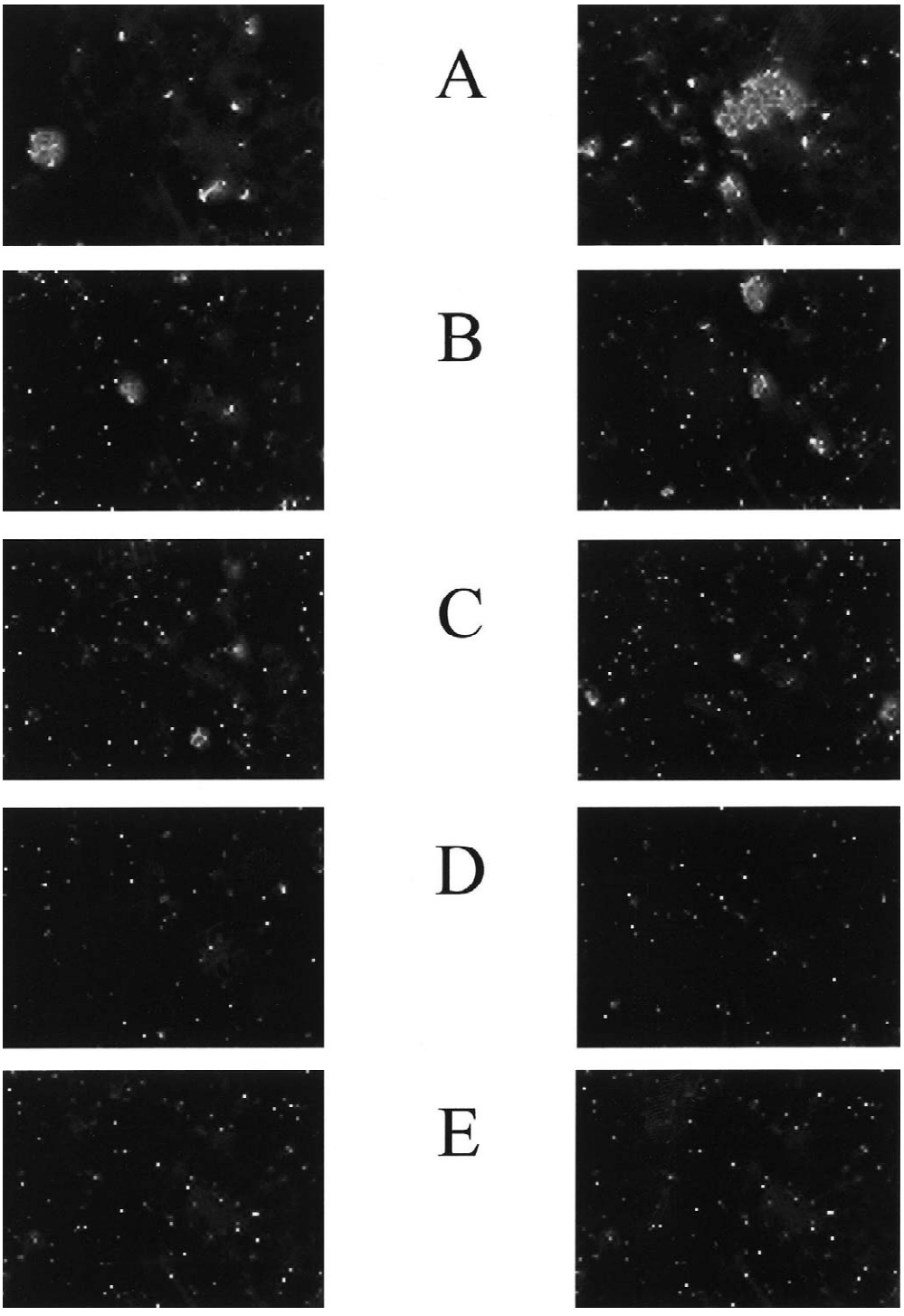

Fig. 4. Photographs obtained for the kinetic process of sonication for L1033 yeast strain. For each sonication time, two photographs were taken: (A) non-sonicated sample. (B) 30-s sonication. (C) 1-min sonication. (D) 2-min sonication. (E) 3-min sonication. Experimental conditions are given in Section 2.

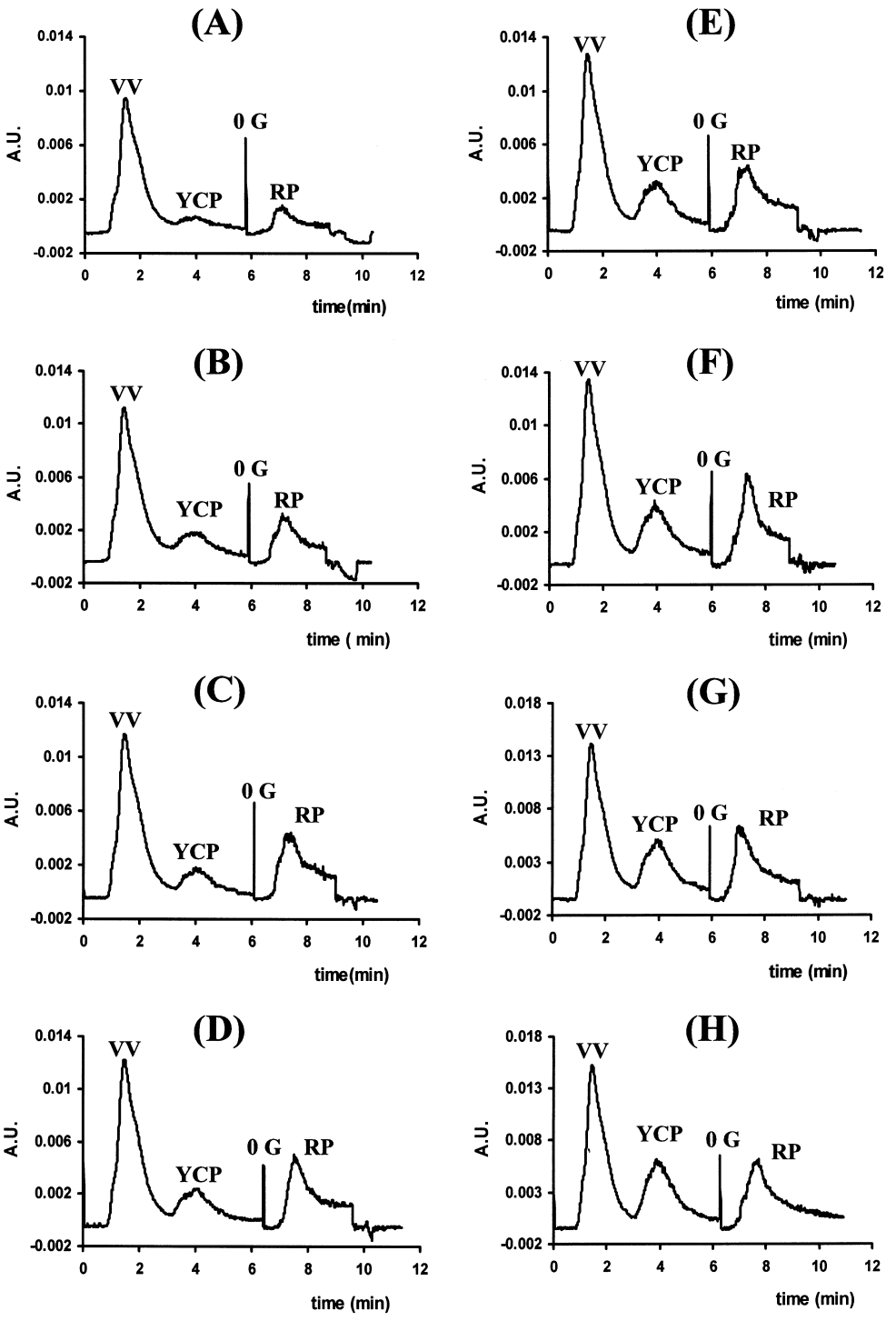

Fig. 5. Sonication time-dependent sequence of sedimentation FFF fractograms for Bourgoblanc strain. (A) 0 s. (B) 15 s. (C) 30 s. (D) 45 s. (E) 60 s. (F) 120 s. (G) 180 s. (H) 240 s. Flow-rate, 0.4 ml/min; field strength, 35 G; mobile phase, PBS pH 7.4; other experimental conditions are described in Section 2. YCP, yeast cell peak; VV, void volume peak; RP, release peak; 0 G, stop field mark.

Table 1
Data obtained for the Bourgoblanc sonication time-dependent sequence by the analysis of the SdFFF fractograms of the sample and release peaks

Sonication time (s)	Yeast peak area (cm^2)	Yeast retention ratio	Release peak area (cm^2)
0	3.48	0.363	22.41
15	5.68	0.376	30.80
30	6.12	0.368	36.75
45	9.75	0.367	41.41
60	12.04	0.365	49.95
120	18.63	0.372	52.54
180	17.68	0.372	56.87
240	19.36	0.376	83.12

sists in introducing yeast in the separator without stopping flow once the elution flow and the external field were established. After elution, the field is systematically stopped to allow reversibly trapped species to be eluted. Data calculated from Fig. 5 sequences are given in Table 1. Retention ratio values are almost constant for 15 to 240 s sonication time, showing that most eluted yeasts in FFF were single cells. Only at 0 s sonication time did the retention ratio shift toward void volume, showing that the retention value obtained was compatible with the large size and proportion of clusters shown in Figs. 4A and 5A for the two different yeast strains. As can be seen in Table 1, single cell peak area is increased with sonication time, indicating a higher recovery. Surprisingly, the release peak area does not decrease with increasing sonication time. It therefore demonstrated that sonication time enhances single cell recovery and reduces irreversible cell trapping, also reducing the time consumed and the need for decontamination processes.

4. Conclusions

Sonication treatment has been shown to be mandatory to disrupt yeast aggregates. Correlations between FFF and single cell elution characteristics such as the peak area, showed that FFF can be used as a rapid and low cost single cell probing tool. The 2-min sonication time widely used appears to be the optimised one. Sonicated and eluted yeast populations were single-cells that have morphological integrity. So far, it has not proved possible to predict what type of ADWY needs sonication or why some strains aggregate or not. Systematic use of FFF for granulometric/image analyses control procedures is therefore recommended.

Acknowledgements

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