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S. *cerevisiae* fermentation activity after moderate pulsed electric field pre-treatments

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ABSTRACT

The batch fermentation process, inoculated by Pulsed Electric Field (PEF) treated wine yeasts (*Saccharomyces cerevisiae* Actiflore F33), was studied. PEF treatment was applied to the aqueous yeast suspensions ([Y] = 0.012 g/L) at the electric field strengths of $E = 100$ and 6000 V/cm using the same treatment protocol (number of pulses $n = 1000$, pulse duration $t_i = 100$ μs, and pulse repetition time $\Delta t = 100$ ms). Electrical conductivity was increasing during and after the PEF treatment, which reflected cell electroporation. Then, fermentation was run for 150 h in an incubator (30 °C) with synchronic agitation. Electro-stimulation was revealing itself by the improvement of fermentation characteristics, and thus increased yeast metabolism. At the end of the lag phase ($t = 40$ h), fructose consumption in samples with electrically activated inoculum exceeded that of the control samples by ≈2.33 times for $E = 100$ V/cm and by ≈3.98 for $E = 6000$ V/cm. At the end of the log phase (120 h of fermentation), ≈30% mass reduction was reached in samples with PEF-treated inocula ($E =$ 6000 V/cm), whereas the same mass reduction of the control sample required approximately 20 extra hours of fermentation.

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1. Introduction

The fermentation of yeasts, stabilization of their multiplication and enhancement of the process productivity are industrially important [1]. Recently, various emerging technologies were demonstrated to have a stimulating effect on the microbial flora multiplication process. E.g., it was shown that continuously supplied low-power ultrasound in the range of 20–30 kHz could enhance ethanol production via stimulation of *Saccharomyces cerevisiae* M30 performance [2]. The optimum ultrasonic treatment at the frequency of 25 kHz resulted in a 15.6% ethanol concentration while ethanol concentration in the control system was lower ([Y] ≈ 12.0%). The positive influence of static magnetic fields (SMF) on the growth of *S. cerevisiae* biomass was also demonstrated [3]. The biomass (g/L) increment in eight samples, exposed to SMF treatment, was 2.5 times greater after their 24 h fermentation at 23 °C than that of the control cultures. The differential biomass growth rate (135%) in SMF-treated samples was slightly higher than glucose consumption rate (130%), which indicates an increase in biomass production of the magnetized cells.

Application of pulsed electric fields with restrictions of electric field strength and treatment time within certain reasonable ranges allowed preservation of the functionality of cell membranes [4]. PEF techniques have been known to facilitate the efficient transformation of cells [5,6], their fusion (electrofusion) [7], transport of plasmid DNA [8], destruction of the bacterium [9] and electrostimulation of plant protoplast division [10]. The stimulation of living cells under the impact of PEF treatment has recently attracted great attention. For instance, a stress response analysis of *S. cerevisiae* has shown that PEF-induced expression of the oxidation genes and glutathione played an important role in the stress resistance [11]. The electrical stimulation was verified in order to alter the *S. cerevisiae* culture cycles and to promote synchrony in division of cells [12]. Moreover, electrical stimulation resulted in smaller size of yeast cell populations. Application of continuous direct current (DC) or alternating current (AC) treatments to a culture broth after inoculation of yeast suspension allowed significant increase in the cell growth and alcohol production rates [13]. The positive role of electrical current (alternating current or pulsed direct current) as a tool for stimulation of microbe reactions (i.e., fermentation) was also reported [14]. However, the effects of PEF treatment on microbial activity, metabolism, and microbe reactions, practically, were not yet studied.

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The main purpose of the present work was to investigate PEF-induced effects on *S. cerevisiae* growth in synthetic media. PEF pretreatment was applied to aqueous suspensions of the cells of wine yeast *S. cerevisiae*. The electric field strengths were $E = 100$ V/cm and 6000 V/cm, the pulse duration was $t_i = 100$ μs, and the number of pulses was $n = 1000$. The conditions of inoculation, incubation, and harvesting of cell populations were carefully kept constant in order to obtain identical fermentation properties. Comparative studies of the kinetics of fermentation were carried out in order to characterize the PEF-induced stress responses of *S. cerevisiae*.

2. Material and methods

2.1. Preparation of yeast suspensions

The wine yeasts, *S. cerevisiae*, strain Actiflore F33 (Laffort, Bordeaux, France), were used throughout this study. The industrial dry powder (rod-shaped particles [15]) was mixed with distilled water with initial electrical conductivity of 4 μS/cm at 25 °C, the concentration of yeasts was [Y] = 0.012 g/L. The yeast suspension was subjected to vortexing (for 2 min, rotation speed 150 rpm and amplitude 4.5 mm) using Top Mix (Bioblock Scientific, Germany). Note that more vigorous mixing can lead to a drop in viability of cells due to their breakage, therefore it was avoided. The initial suspension conductivity after the vortexing was $\sigma_i \approx 18 \pm 3$ μS/cm. Then the suspension was gently agitated (100 rpm) at 30 °C using magnetic agitator. The swelling process was monitored by means of conductivity measurements (Inolab Level 1, Germany) at the frequency of 50 Hz. In PEF treatment experiments, the suspension was initially agitated for 15 min, then treated by PEF (total time of keeping suspension in PEF treatment chamber was 100 s) and agitated again. The total time of agitation of the PEF-treated and untreated suspensions was the same ($t_{in} = 30$ min). The pH of the samples was also measured before and after PEF treatments using a pH meter (Consort C931, Belgium); it was found to be always constant and equal to 4.16 ± 0.01. After agitation, the treated and untreated yeast suspensions were immediately inoculated into fermentation substrates.

2.2. Fermentation substrate

The fermentation process may reflect the composition of the reaction medium, which is not stable for a natural product that contains a multitude of components (e.g., grape juice). In order to make comparison of the fermentation effects in untreated and PEF treated inocula, a synthetic fermentation medium was used in this work.

The composition of the fermentation medium was the following: 5 g/L of yeast extract (Sigma Aldrich Steinheim), 6.36 g/L of ammonium sulfate (as a source of mineral nitrogen), 6.36 g/L of monoammonium phosphate (as a source of phosphoric content), and 142 g/L of sugar (as source of carbohydrates), including 68 g/L of glucose, 73.6 g/L of fructose, and 0.4 g/L of sucrose [1]. For sterilization purposes, all reagents and apparatuses were heated for 15 min at 121 °C in an autoclave (Lequeux, France). Their sterility was maintained throughout the experiments and monitored by microbiological control tests.

2.3. PEF-treatment

The PEF generator, Hazemeyer 5 kV 1 kA (Hazemeyer, Saint Quentin, France), providing monopolar pulses of near-rectangular shape, was used in this work. The treatment chamber consisted of a propylene container, into which 2 stainless electrodes ($A_{(electrode)} = 143$ cm^2) separated by 7 mm gap, was installed. The volume of the treatment chamber was 100 mL. The PEF experiments were carried out at two different electric field strengths of $E = 100$ and 6000 V/cm using the same protocol of pulses: the number of pulses $n = 1000$, pulse duration $t_i = 100$ μs, and pulse repetition time $\Delta t = 100$ ms. The time of PEF treatment was 0.1 s and the total time of keeping suspension in PEF treatment chamber was 100 s. The current and voltage data were measured and then collected using a data logger and specific software, developed by Service Electronique UTC, Compiègne, France. The temperatures before and after PEF treatment were monitored by a thermocouple. The use of aqueous suspension with low initial electrical conductivity (≈ 18 μS·cm^{-1}) allowed avoiding of a noticeable Joule heating during PEF treatment. The initial temperature was 30 °C. The temperature elevation ΔT, resulting from PEF treatment, was insignificant (≈ 0) at $E = 100$ V/cm. In order to avoid overheating the PEF treatment at $E = 6000$ V/cm was applied using two trains of pulses with pause between them for cooling of suspension. After the first train with $n = 500$, the temperature elevation was $\Delta T \approx 5$ °C, then suspension was cooled down to the initial temperature of 30 °C and the next train with $n = 500$ was applied. The treatment chamber was disinfected before and after each experiment by 80% ethanol solution.

2.4. Batch fermentation

Batch fermentation experiments were initiated by transferring 10 mL of 0.012 g/L yeast suspension to 300 mL of sterilized synthetic medium. The final concentration of yeasts in the fermentation substrate was [Y] ≈ 0.0039 g/L. The small-scale fermentations were carried out in six different vessels (4 with PEF treated yeast suspensions and 2 control ones) for 150 h under the controlled temperature (30 °C) with synchronic agitation at 150 rpm (HT Inforsag Bottmingen).

2.5. Analytical methods

Fermentation performance was estimated throughout the fermentation period using periodic (each 6 h) measurements of the mass of the fermentation substrate m and soluble matter content (°Brix). °Brix value was used as an approximate measure of the content of dissolved solids in aqueous solution and was represented as percentage by weight (% w/w). The values of m were obtained with the help of an analytical balance (Mettler PM6100, Switzerland). The values of °Brix were measured using a digital refractometer AR 200 (Leica Microsystems Inc., Buffalo, USA). The initial values of mass m_i and °Brix$_i$ were 310 ± 0.1 g and 14.0 ± 0.1, respectively.

Samples were also harvested for offline chemical analyses. UV absorption spectra were measured in the spectral range of 190–900 nm by UV spectrophotometer (Spectronic 20 Genesys, Spectronic Instruments, Rochester, NY). The path width of the Suprasil quartz cuvette was 10 mm (Hellma, Mullheim, Germany). The concentration of sugars was determined enzymatically using glucose and fructose analysis kit (Enzytec fluid Glucose/Fructose, R-Biopharm). The concentration of proteins [P] / (μg/mL) was determined using the Bradford procedure [16]. The protein content calibrations were done using measurements of absorbance at 595 nm with Bovine serum albumin (BSA) (Sigma A7030) as reference substance [16].

2.6. Statistical analysis

All the experiments were done in duplicates, and respective analyses were done, at least, in triplicate. Means and standard deviations of data were calculated. One-way analysis of variance was used for statistical analysis of the data using the Statgraphics plus (version 5.1, Statpoint Technologies Inc., Warrenton, VA, USA). For each analysis, a significance level of 5% was assumed. The error bars presented on the figures correspond to the standard deviations.

3. Results and discussion

During the swelling, the electrical conductivity σ of the inocula grew with time and reached a stable value of 25 ± 5 μS/cm after, approximately, 60 min of agitation (Fig. 1).

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