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ORIGINAL PAPER

The Effect of the Electric Field on Lag Phase, β-Galactosidase Production and Plasmid Stability of a Recombinant *Saccharomyces cerevisiae* Strain Growing on Lactose

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Abstract Ethanol and β-galactosidase production from cheese whey may significantly contribute to minimise environmental problems while producing value from low-cost raw materials. In this work, the recombinant *Saccharomyces cerevisiae* NCYC869-A3/pVK1.1 flocculent strain expressing the *lacA* gene (coding for β-galactosidase) of *Aspergillus niger* under *ADHI* promoter and terminator was used. This strain shows high ethanol and β-galactosidase productivities when grown on lactose. Batch cultures were performed using SSlactose medium with 50 gL^{-1} lactose in a 2-L bioreactor under aerobic and microaerophilic conditions. Temperature was maintained at 30 °C and pH 4.0. In order to determine the effect of an electric field in the fermentation profile, titanium electrodes were placed inside the bioreactor and different electric field values (from 0.5 to 2 Vcm^{-1}) were applied. For all experiments, β-galactosidase activity, biomass, protein, lactose, glucose, galactose and ethanol concentrations were measured. Finally, lag phase duration and specific growth rate were calculated. Significant changes in lag phase duration and biomass yield were found when using 2 Vcm^{-1}. Results show that the electric field enhances the early stages of fermentation kinetics, thus indicating that its application may improve industrial fermentations' productivity. The increase in electric field intensity led to plasmid instability thus decreasing β-galactosidase production.

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Introduction

People that are lactose-intolerant cannot eat several milk and milk-derived products. When the intestine produces little or no lactase, lactose (the milk sugar) is not digested and moves into the colon, where bacteria ferment it, producing hydrogen, carbon dioxide and organic acids. The results of this fermentation are diarrhoea, flatulence (gas) and abdominal discomfort. β-Galactosidase (β-GAL) (EC 3.2.1.23) is able to cleave β-linked galactose residues from various compounds and is commonly used to cleave lactose into galactose and glucose. β-GAL preparations are widely used for hydrolysis of lactose in milk, milk products and whey (Panesar et al. 2006; Oliveira et al. 2011).

Domingues et al. (2000, 2002) have previously reported that cheese whey permeate could be used as substrate for β-GAL and ethanol production by the recombinant strain used in the present work. The production of ethanol and β-GAL from cheese whey and other sub-products from industries can significantly contribute to minimise environmental problems and to obtain valuable products from low-cost raw materials. Moreover, the downstream processing is simplified due to the cell self-flocculation characteristics of the culture that reduce the costs of the separation and purification processes. In addition, the feasibility of using a high-cell-density continuous system for production of β-Gal (Oliveira et al. 2007) and simultaneous production of β-Gal and ethanol has been proven (Domingues et al. 2005).

Responses of living cells and biochemicals to electrical signals have been used in biotechnology in general (Bartlett et al. 1997), in bioanalytical techniques and for medical purposes (Stacey et al. 2003). Depending on the intensity of the applied electrical potential and on its time function (constant, pulsed, etc.), distinct effects in metabolic processes may occur. Such exogenous stimulation determines the appearance of an electric potential across the cell membrane, which depends on the strength of the external field, cell shape, cell radius and membrane properties. Among the several types of stress reported in the literature, thermal stress is the most widely studied. It was shown, for example, that thermal treatment (freeze–thaw treatment) of *Saccharomyces cerevisiae* results in an increased tolerance to ethanol (Wei et al. 2007), which pointed out to industrial applications of induced cell stress. Electric field-promoted stress presents industrial advantages over other types of stress because there is no addition of external chemicals, which may increase product separation and waste treatment costs (Pereira and Vicente 2010). The inactivating effects of moderate electric fields (ohmic heating) on different microorganisms have been widely studied mainly for food product pasteurisation (Icier and Bozkurt 2009; Zell et al. 2009) and sterilisation (Yildiz and Baysal 2006; Pereira et al. 2007; Sun et al. 2008; Baysal and Icier 2010; Machado et al. 2010). In addition, the effects of sub-lethal moderate electric fields were studied on growth kinetics and metabolic activity of lactic acid bacteria, and some changes in the lag phase duration and bacteriocin production were reported (Cho et al. 1996; Loghavi et al. 2007; Loghavi et al. 2008). However, no other studies on the sub-lethal effect of electricity in microorganisms during fermentative processes could be found, therefore justifying the opportunity of presenting the results reported herein.

Materials and Methods

Microorganism

The microorganism used in this work was the recombinant *S. cerevisiae* NCYC869-A3 flocculent strain transformed with pVK1.1 plasmid (Fig. 1) (Kumar et al. 1992). This strain secretes *Aspergillus niger* β-GAL (encoded by the *lacA* gene) under the control of the *ADHI* promoter and terminator (Domingues et al. 2002). The initial concentration of yeast used in the bioreactor inoculum was 2×10^8 cfu/ml.

Culture Media

The recombinant yeast was maintained at 4 °C on slants or at −80 °C in permanent culture of YNB selective medium [6.7 gL^{-1} yeast nitrogen base (w/o amino acids), 20 gL^{-1} lactose]. SSLactose medium was used for fermentation (5 gL^{-1} KH_2PO_4, 2 gL^{-1} $(NH_4)_2SO_4$, 0.4 gL^{-1} $MgSO_4{\cdot}7H_2O$, 2.0 gL^{-1} yeast extract and 50 gL^{-1} lactose).

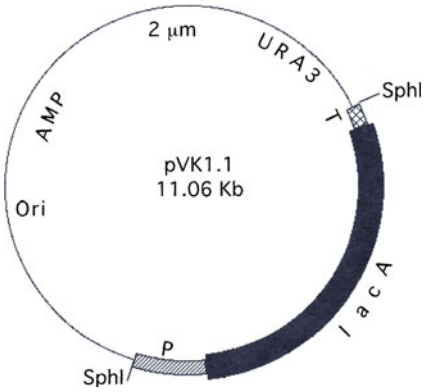

Fig. 1 Map of pVK1.1 plasmid (Kumar et al. 1992). The cDNA for *A. niger* secretory β-galactosidase (lacA) is flanked by the *ADH1* promoter (P) and terminator (T)

Bioreactor Operation

Batch culture experiments were performed using a 2-L glass bioreactor provided with a temperature control jacket (Vidrolab, Portugal) (Fig. 2). All the experiments were conducted with agitation, temperature and pH measurement and control. Temperature was monitored using type K thermocouples, with a Teflon coating, placed inside the reactor; the Teflon coating ensured the necessary electrical insulation once the medium was subjected to an electric field. The temperature was kept at 30±1 °C by developing a control program in Labview 7.0 Express (National Instruments, USA), operating on a water bath with a heater, connected in closed-circuit to the fermentation vessel and a circulation pump. The initial pH was 5.4, which was allowed to drop to 4.0 during the fermentation and then kept at 4.0± 0.15 by automatic addition of ammonia solution (30 % *v*/*v*). The agitation was made by a magnetic stirrer, and the bioreactor was aerated with filtered air at a flow rate of 2.5 vvm. These conditions guarantee a minimum of 20% of dissolved oxygen throughout all stages of the fermentation. When needed, microaerophilic conditions were attained by an initial sparging of filtered air followed by the suppression of the air supply. For sterilisation, both vessel and silicone tubing were autoclaved for 30 min at 121 °C.

Two stainless steel electrodes (made “in house”) were placed symmetrically inside the bioreactor with a distance of 7.5 cm between them. Each electrode has a surface area of 65 cm^2. The electric field, ranging from 0 to 2 Vcm^{-1}, was generated by an alternating current source of 50 Hz.

A data-logger (NI-USB9161, National Instruments, USA) was employed to record continuously and simultaneously current intensity, voltage and temperature.

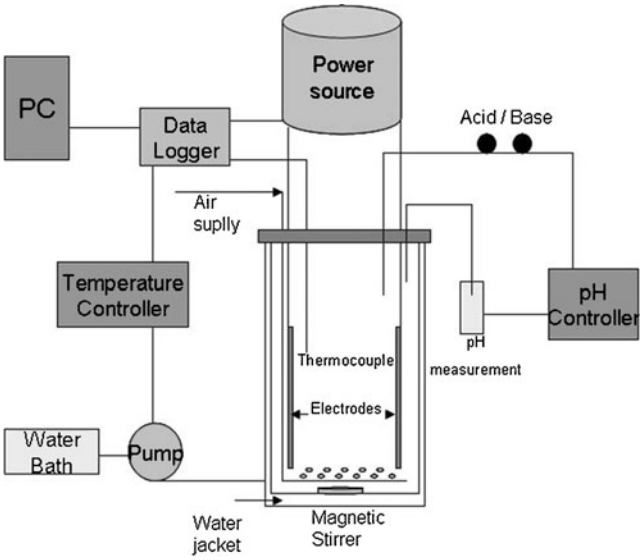

Fig. 2 Schematic diagram of the experimental setup

At least two independent runs were performed for each fermentation condition.

β-Galactosidase Activity Measurements

β-GAL activity was measured in triplicate directly from the fermentation broth without further purification. One unit of activity was defined as the amount of enzyme that hydrolyses 1 nmol of *p*-nitrophenil-β-D-galactopyranoside (*p*NPG) per minute, at 65 °C. The activity was measured as the amount of *p*-nitrophenol released from *p*NPG per minute. Samples were incubated with 1.7 mM substrate in 0.075 M Na–acetate buffer, pH 4.5. The pH was raised to 10 with 1 M of Na_2CO_3, and the activity was measured spectrophotometrically at 405 nm (Bailey and Linko 1990).

Biomass Concentration Determination

Biomass concentrations were measured as dry weight (DW) and/or using absorbance methods. The DW was determined by filtering the sample through 0.2-μm filter paper and then drying at 105 °C for 24 h. The absorbance was measured at 620 nm and compared to a standard curve for absorbance versus DW previously constructed. Before absorbance readings, cells were deflocculated with a solution of NaCl (1.5 % *w*/*v*, pH 3.0). In both methods, samples were analysed in duplicate.

Lactose, Glucose, Galactose and Ethanol Concentration Determinations

The total reducing sugars (RS) concentration was determined by the dinitrosalicilic acid method described elsewhere (Miller 1959). Sugars (lactose, glucose and galactose) and ethanol were quantified by high-performance liquid chromatography using an isocratic pump (model 880PU, JASCO, Tokyo, Japan), a refractive index (RI) detector (model 830-RI Intelligent RI Detector, JASCO, Tokyo, Japan), a Rheodyne manual injector 775i and Varian Star Workstation software, with a 300×6.5 mm ID organic acids column (model 257034, Chrompack, Middelburg, The Netherlands). The eluent was 0.005 M H_2SO_4. The analyses were made at an elution rate of 0.3 ml min^{-1} and an oven temperature of 60 °C. Under these conditions, the retention times of lactose, glucose, galactose and ethanol were 12.2, 14.3, 15.4 and 31.1 min, respectively. In all cases, samples were analysed in duplicate.

Plasmid Stability

Plasmid stability is the relative amount of cells containing the plasmid after several generations. This stability was determined by plating the biomass samples into Petri dishes containing SSlactose agar medium supplemented with 40 mg L^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The colonies stained blue still contain the plasmid, while the colonies stained white lost the plasmid. It is then possible to estimate the plasmid stability by dividing the number of blue colonies by the total number of colonies. The plasmid stability was determined at the end of each fermentation run.

Statistical Analysis

Each experiment was replicated at least twice to ensure the repeatability and the reproducibility of the results. The values presented in figures and tables are the mean values of replicates with average standard deviations <10%.

Results and Discussion

The fermentations were conducted under aerobic and microaerophilic conditions, and the initial lactose concentration was 50 gL^{-1}. For both conditions, different electric fields (ranging from 0 to 2 Vcm^{-1}) were applied. The time evolution of the reducing sugar consumption and biomass production is presented in Figs. 3 and 4.

Aerobic Fermentations

Figure 3 shows that lactose was totally metabolised by the recombinant strain in <22 h when an electric field of 2 V cm^{-1} was applied. This represents a reduction of about 6 h in the fermentation time when comparing to the other aerobic conditions tested and may be due to the increase in the biomass production. Biomass concentration increased

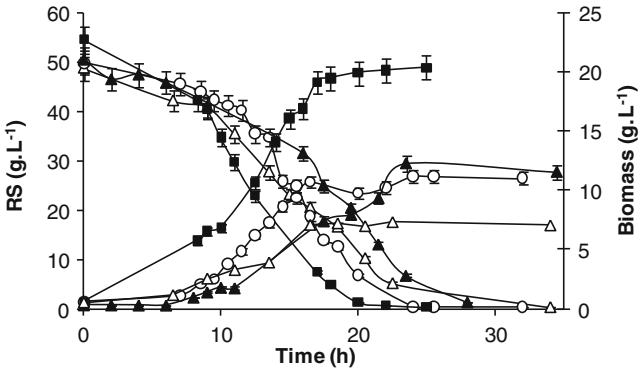

Fig. 3 Evolution of the reducing sugars (RS) and biomass concentration during aerobic fermentations under different electric field values. Each data point corresponds to the average of at least two independently run fermentation experiments, and the *error bars* correspond to the standard deviations. Symbols: *empty triangle* 0 V cm^{-1}; *empty circle* 0.5 Vcm^{-1}; *filled triangle* 1 Vcm^{-1}; *filled square* 2 Vcm^{-1}

188% when the electric field was changed from 0 to 2 V cm^{-1} (Table 1). In fact, the biomass yield increased linearly with the applied electric field (Fig. 5). Stone [cited by Rowley (1972)] also reported that current generated through electrochemical reactions stimulated the growth of bacteria, resulting in bacterial counts 100-fold greater than for controls. However, moderate electric field did not have a significant effect on the final fermentation biomass of a lactic acid bacterium (*Lactobacillus acidophilus*) (Loghavi et al. 2007, 2008).

The lag phase was significantly affected by the intensity of the electric field (see Table 1), increasing for the lower values of electric field and then decreasing when using higher voltages. In fact, while for an electric field of 2 V cm^{-1}, the lag phase is shorter than for the conventional fermentation (absence of electric field, 0 Vcm^{-1}), for 0.5 and 1 Vcm^{-1}, the lag phase actually increased 21% and 53%, respectively when compared to the conventional fermentation. The calculation of the growth rate points at a decrease of that parameter when the lag phase is shorter. Similar results were found when applying an electric current to *L. acidophilus* (Cho et al. 1996; Loghavi et al. 2008). Cho et al. (1996) reported significant changes in the lag phase when using ohmic heating during fermentations at sub-optimal temperatures and some minor changes when operating near the optimal temperature. As in the present work, the lag phase also increased slightly when using lower voltages and operating near the optimal temperature. However, in our work, significant changes could be found even when the fermentations were performed at the optimal temperature. It is possible that these changes would have been even more evident if operating at sub-optimal conditions.

Additional research is needed to explain the changes observed in the duration of the lag period under the influence of an electric field. However, the hypothesis that the use of higher electric fields may improve nutrients absorption due to two different phenomena may be advanced. Such phenomena would be: improved transport through the cell flocs and improved transport through the cell membrane due to pore formation or activation of transport proteins. Castro et al. (2004) reported some influence of the electric field in several food-processing enzymes, and these results may indicate that such influence occurs also over other enzymes; this hypothesis, however, is yet to be proven. Indeed, Loghavi et al. (2009) have shown direct evidences that lag phase reduction during moderate electric field fermentation of *L. acidophilus* is due to cell permeabilisation, using a dye staining technique to identify intact and permeabilised cells.

The substrate conversion yield on β-GAL ($Y_{P2/s}$) increased with increasing electric field values (from 0.5 to 2 Vcm^{-1}; Table 1), and the extracellular β-GAL activity increased during the exponential and stationary growth phases, but the global yield (calculated with the initial and final fermentation values) decreased when the electric field increased from 1 to 2 Vcm^{-1} (data not shown). This result may be due to plasmid instability, as we shall see further ahead in the text.

The presence of oxygen is important to foster yeast growth. In a previous work, oxygen concentration did not significantly affect β-GAL production (Domingues et al. 2004). Also in the present case, oxygen limitation had no effect on the final amount of β-GAL produced. Domingues et al. (2004) reported that if oxygen is in excess, an ethanol consumption phase is expected, increasing further the β-GAL production after the extinction of the original carbon source (sugar). This ethanol consumption phase was not observed in any of the fermentations performed in the present work. In addition, ethanol yields in lactose and biomass decrease with

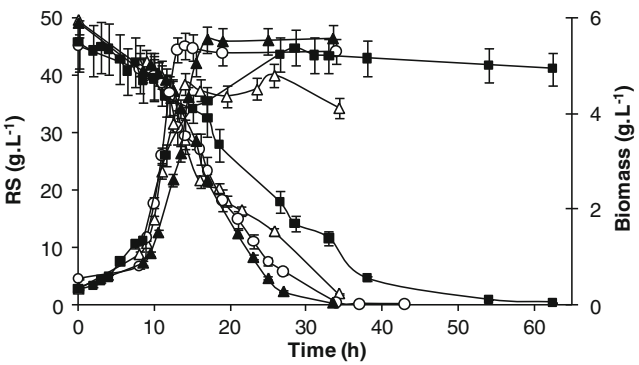

Fig. 4 Evolution of the reducing sugars (RS) and biomass concentration during microaerophilic fermentations under different electric field values. Each data point corresponds to the average of at least two independently run fermentation experiments, and the *error bars* correspond to the standard deviations. Symbols: *empty triangle* 0 V cm^{-1}; *empty circle* 0.5 Vcm^{-1}; *filled triangle* 1 Vcm^{-1}; *filled square* 2 Vcm^{-1}

Table 1 Percentage of variation of the kinetic parameters for aerobic and microaerophilic fermentations conducted under different electric field values in relation to data obtained in the absence of electric field for the same aeration condition

	Electric field (V cm^{-1})					
	Aerobic			Microaerophilic		
Kinetic parameters	0.5	1.0	2.0	0.5	1.0	2.0
μ (h^{-1})	85	24	−15	39	−11	−40
t_{lag}(h)	21	53	−100	65	21	−84
t_D (h)	−46	−19	17	−28	12	66
Final biomass (g L^{-1})	50	81	188	59	59	29
$Y_{x/s}$ ($g_{biomass}/g_{lactose}$)	42	68	195	25	29	26
$Y_{P1/s}$ ($g_{ethanol}/g_{lactose}$)	123	50	1	50	13	28
$Y_{P1/x}$ ($g_{ethanol}/g_{biomass}$)	−1	2	−46	−42	−22	85
$Y_{P2/s}$ ($U/g_{lactose}$)	−37	−8	25	206	114	16
$Y_{P2/x}$ ($U/g_{biomass}$)	−16	−89	24	−2	76	3
qs_{p1} ($g_{ethanol}/g_{lactose}h^{-1}$)	−17	−17	−15	−7	−21	−53
qs_{p2} ($U/g_{lactose}h^{-1}$)	193	35	−32	−78	−58	−93

Yields are given by the slope of the straight line of biomass values versus lactose values ($Y_{x/s}$) and product values versus residual lactose or biomass values ($Y_{P/s}$ and $Y_{P/x}$, respectively) over fermentation time. Each value in the table has been obtained from at least two reproducible fermentations (standard deviations below 10%)

μ specific growth rate, t_{lag} lag phase time, t_D duplication time, X biomass, S lactose, $P1$ ethanol, $P2$ β-galactosidade, $Y_{x/s}$ biomass yield relative to lactose, $Y_{P/s}$ product yield relative to lactose, $Y_{P/x}$ product yield relative to biomass, qs_p specific product yield relative to lactose

increased electric field values. This decrease is possibly related with the correspondent increase of final biomass concentration (Table 1 and Fig. 5). This might indicate a shift toward pure respiratory metabolism, where biomass build-up is preferred in relation to ethanol production. However, this hypothesis needs further confirmation.

Microaerophilic Fermentations

Figure 4 presents the evolution of the reducing sugars and biomass concentration during microaerophilic fermentations.

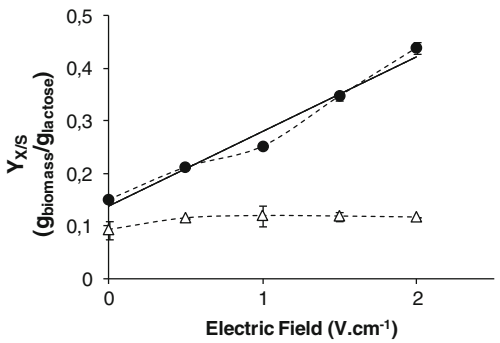

Fig. 5 Biomass yield as a function of the applied electric field, for aerobic and microaerophilic fermentations. Each data point corresponds to the average of at least two independently run fermentation experiments, and the *error bars* correspond to the standard deviations. Symbols: *circle* aerobic; *triangle* microaerophilic. The *straight line* for the aerobic conditions corresponds to the linear correlation obtained ($Y_{X/S}$=0.143×electric field+0.136; R^2=0.98)

The analysis of data indicates that the effect of electric field is not as evident as when air is provided. For most of the electric field strength values tested, the fermentation ended at approximately 35 h, exception made for 2 V cm^{-1}, where more than 50 h were needed to completely metabolise lactose. This electric field had a significant negative effect on the yeast specific growth rate under microaerophilic conditions. As a consequence, lactose metabolisation was slower under 2 V cm^{-1} electric field. The presence of the 2 V cm^{-1} electric field has been reported to enhance biomass growth under aerobic conditions; this has been confirmed in the present work (see "Aerobic Fermentations" above), and has been attributed to cell permeabilisation occurring for electric field values above a certain limit (Loghavi et al. 2009). On the other hand, the reduction in biomass growth rate under anaerobic conditions (without electric field) has been reported for the same system (Domingues et al. 2004). Our results in the presence of a 2 V cm^{-1} electric field are in line with these findings, indicating that when oxygen is suppressed in the presence of an electric field, a reduction in the biomass growth rate is observed. This effect is possibly due to a (negative) synergy between the stress provoked by the presence of the electric field (e.g. inducing cell permeabilisation) and the stress provoked by the oxygen limitation. This hypothesis needs to be confirmed with further work.

The biomass yield remained approximately constant (Fig. 5), meaning that under microaerophilic conditions, there is no stimulatory effect of the electric field (for the

applied frequency and intensities) on growth. This behaviour is the opposite from the one observed for aerobic conditions, but reinforces Rowley's (1972) statement that the presence of an electrical current will either enhance or inhibit the growth of the microorganisms, depending on the experimental conditions (in this case, the presence or suppression of oxygen). On the other hand, the lag phase was significantly reduced when using an electric field of 2 Vcm^{-1} (Table 1).

As previously observed for aerobic conditions, the substrate conversion yield on β-GAL decreased when using 2 Vcm^{-1}. This decrease is, once more, probably due to the loss of plasmid therefore affecting the production of β-GAL (see next section). This also explains the extended fermentation time. Lactose was more slowly hydrolysed in glucose and galactose by the lesser excreted amounts of β-GAL, and this reduced the amount of sugars available for consumption. Domingues et al. (2004) reported that under anaerobic conditions, the plasmid stability was lower, which can explain the more pronounced (negative) effect on β-GAL productivity when air is suppressed.

Effects of Electric Field on Plasmid Stability

Plasmid stability is a major concern in industrial cultures using recombinant microorganisms to produce heterologous proteins. Segregational instability during cell division and changes in the plasmid structure, such as deletion, insertion and rearrangement, are the main causes of plasmid instability. Considering the use of a recombinant strain (transformed with a non integrative plasmid), this is one of the important issues to be addressed. This strain was transformed with a 2-μm based plasmid (pVK1.1), which is a native plasmid present in several *Saccharomyces* strains in a number of 30–100 copies per cell (high copy number). The modified plasmids have, in general, lower stability than the original 2 μm, but they are the most frequently used in yeast transformation (Futcher and Cox 1984; Ludwig and Bruschi 1991).

Plasmid stability was determined at the end of each fermentation, and the results are shown in Table 2. The values obtained in the absence of the electric field are in accordance with the ones presented by Domingues et al. (2004), who reported, for the same yeast strain, when using a similar experimental setup and culture conditions, a plasmid stability of approximately 70% and 60% for aerobic and anaerobic conditions, respectively. Note that different blue patterns were observed in the X-gal Petri plates, indicating population heterogeneity in regard to plasmid copy number. Nevertheless, all blue colonies were counted as β-galactosidase-producing cells. This heterogeneity has been observed for all fermentation conditions and may be attributed, as previously reported, to plasmid copy number variation among the population (Oliveira et al. 2007).

Table 2 Plasmid stability measured at the end of the fermentations conducted under different electric field values, for aerobic and microaerophilic conditions

Electric field ($V\ cm^{-1}$)	% Plasmid stability under aerobic conditions	% Plasmid stability under microaerophilic conditions
0	72.00±4.24	60.00±2.83
0.5	57.10±2.32	51.00±2.83
1.0	40.24±1.41	44.42±2.00
1.5	31.07±1.82	40.12±3.46
2.0	31.43±0.91	39.20±–[a]

The data are the average (± standard deviation) of two independent fermentation runs

[a] For this particular data point no duplicate was available

It can be clearly concluded that the increase in electric field causes a significant decrease in plasmid stability. In the experiments performed using a continuous electric field, the plasmid stability decreased sharply for both aerobic and microaerophilic experiments. However, a more pronounced decrease is observed under aerobic conditions (Table 2). This is consistent with the fact that plasmids can be lost during cell division. Under aerobic conditions, a higher number of cell divisions occurred with the increase in the applied electric field (as final biomass values were higher for higher electric fields). Furthermore, Araújo et al. (2004) reported that the presence of an electric field in batch *S. cerevisiae* cultures induces electrochemical stress in the cells and affects the cellular cycle, cell size and also cell division synchrony. These results are in line with the ones obtained in this research regarding plasmid stability. Herein, it is hypothesised that changes in the cell cycle and division, namely, membrane instability and more fluidity of the lipid bi-layer, due to electrical interferences at the cellular micro-environment, contribute to an increased plasmid loss. Being so, when using recombinant strains, under ohmic conditions, the use of integrative plasmids may be more adequate to keep the desired characteristic introduced by genetic engineering. In alternative, the use of electric fields in discontinuous mode may also result in a process optimisation.

Conclusions

The use of sub-lethal moderate electric fields to maintain the temperature during fermentations affected the growth and metabolic activities of the studied recombinant *S. cerevisiae* strain growing on lactose. The use of higher electric fields resulted in a shorter lag phase and a higher biomass yield,

under aerobic conditions. When microaerophilic conditions were used, the lag phase was also significantly reduced, but the effect on biomass yields was not so clear.

Plasmid instability increased with the raise of electric field intensity for both aerobic and microaerophilic conditions.

The use of ohmic heating in industrial fermentative processes may be useful in the early stages of the fermentation (reduction of lag phase); however, further research must be carried out to assess and explain the effect of ohmic heating on the metabolic pathways of microorganisms and, in the case of recombinant organisms, on plasmid stability.

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