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Study of morphological and physiological parameters of cultures of Yarrowia lipolytica undergone electrochemical stress

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Yarrowia lipolytica is an yeast that differs from most cellular models studied in relation to physiology, genetics and cell biology. Since it is not considered pathogenic, was used in industrial applications as the single-cell protein production. Moreover, secretes enzymes such as proteases, esterases, phosphatases and lipases. The lipases produced may be extracellular and/or intracellular and are both commercially and industrially important. Biochemical processes crucial to cell viability depend on the electrical potential across the cell membrane, therefore it is proposed that the application of electric field to a living cell promotes metabolic responses similar to those linked to other types of stress. Additionally, the electric potential application may increase the permeability of the cell membrane and might thus promote the excretion of lipase.
This work aims to characterize the effects of electrical potential in the cultivation of Y. lipolytica on physiological (glucose consumption, lipase activity, pH) and morphological responses (digital image analysis and cell viability). Two different voltage values were applied: 0,75 V and 1,00 V in comparison to control experiments.
The application of electric potential was beneficial for the production of lipase. The results express that with the increase of electric potential lipase was excreted for the medium earlier leading to a higher productivity. Besides, with the electrochemical stress there was an increase in glucose consumption rate and no influence in cell morphology.

1. Introduction

Yarrowia lipolytica is a strictly aerobic yeast with high ability to produce products of high added value (large industrial interest) as lipases, citric acid and single cell proteins, and various enzymes such as lipases, proteases, esterases and phosphatases.

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Currently the most important use of this yeast is the production of lipase due to the wide applicability of this product, such as degradation of oils and fats. *Y. lipolytica* cells may produce lipases linked to the cell (OTA *et al.*, 1982).
Eukaryotic cells can respond to a variety of environmental stresses such as nutrient availability, pressure, temperature, etc. Cellular responses to electric stress have been studied in Biotechnology (Bartlett *et al.*, 1997), bioanalytical techniques (Ci *et al.* 1997; Gheorghiu and Asami, 1998) and for medical purposes (Veiga *et al*., 2005).
Araujo *et al.* (2004) found oscillations in the culture of *Saccharomyces cerevisiae* when it was subjected to electrical stimuli. The application of electric field to a living cell promotes metabolic responses similar to those linked to other types of stress. The application of this type of stress in yeast *Y. lipolytica* can lead to increased membrane permeability and may thus promote the production of lipase.

2. Materials and Methods

2.1 Materials

The components of culture media and preservation were supplied by the following companies: Merck (peptone), Oxoid (yeast extract), Isofar (Glucose) and Vetec (Agar). The substrate of serum lipase, p-nitrophenyl laurate, was provided by SIGMA and enzymatic kit for determination of glucose was supplied by HUMAN GmbH

2.2 Microorganisms and culture medium

The yeast used in this work is a wild strain of *Yarrowia lipolytica* IMUFRJ 50682 selected from an estuary of the Guanabara Bay in Rio de Janeiro, Brazil (Hagler and Mendonça-Hagler, 1981) and identified by the Institute of Microbiology, Center for Health Sciences Federal University of Rio de Janeiro stored at 4 ° C in medium YPD-agar.
Initially, the cells were grown in YPD medium (1% yeast extract, 1% peptone and 2% glucose) for 48 hours under orbital agitation (160 rpm) and temperature of 28 ° C.

Experiments

Cells previously grown as described above, were inoculated in culture medium YPD (1% yeast extract, 2% glucose and 0.64% peptone) in a bench top bioreactor in order to obtain an initial cell concentration average 1 g / L. The electric potential was applied in the middle of the exponential phase (after 4 hours of experiment). A control experiment, ie without application of electric potential, was carried out under the same conditions.
A bench top bioreactor (Multigen, New Brunswick Scientific Co., USA) with a capacity of 2 L was used. Equipment containing aeration system, magnetic stirring and temperature control was provided with two turbines and two chicanes. A polarographic oxygen probe (DO-Lutron 5510, Lutron Electronics Co., Inc) was used to measure the concentration of dissolved oxygen within the bioreactor. The oxygen supply was achieved with room air with the aid of a submerged aeration system, the air passed through an air filter (Advantec, Toyo Roshi mμKaisha, Japan) with porosity of 0.2 The experimental conditions were: 28 ° C, agitation of 550 rpm and an aeration of 1.5 vvm. The experiments were performed in triplicate which ensures greater reliability in the experiments.
The electric potential applied to the study was 0.75 V (Araújo *et al*., 2004) and 1,0V. The potentiostat used was PG-01 Ohnimetra Instruments, with the working electrode

and counter electrode of platinum and reference electrode of saturated calomel as the diagram in Figure 1.

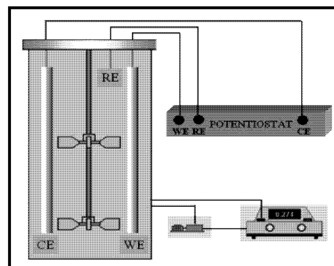

Figure 1: Reactor bioelectrochemical. RE = reference electrode (SCE), CE = counter electrode (Pt), WE = working electrode (Pt).

Biochemical Methods

Cell growth was followed by optical density measurements at 570 nm. Absorbance values were converted to mg d.w./mL using a factor previously determined experimentally.

Lipase activity was estimated by variations on absorbance at 410nm in spectrophotometer (Hach, DR4000UV) due to oxidation of p-nitrophenyl laurate (p-NFL) (supplied by SIGMA) with a concentration of 0.162 mg / mL in potassium phosphate buffer (0,05M), pH 7.0 (Pereira-Meirelles, 1997).

Glucose comsumption: Glucose was quantified by the method of glucose oxidase (enzyme kit, Human GmbH, Germany).

Microscopic methods

Morphological analysis

Cell morphology was observed along the batch run using a Nikon optical microscope with 1,000 X magnification, in both control and stress conditions. Traditional tools generally used for image enhancing were employed. The RGB image was decomposed into its respective channels, and the green channel was employed in the image treatment steps (Kawasse et al., 2003). A grayscale intensity image was created and a combination of bottom and top hat filtering was used to enhance image contrast. A binarization step was performed followed by the elimination of border structures and a hole-fill procedure was carried out. Application of morphological operations, such as erosion (to remove small debris) and reconstruction, gave the final image. This image treatment was performed in MATLAB v.6.1 (The Mathworks Inc., The Mathworks Inc.) environment (Freire *et al.,* 2005).

In order to extract cell's individual properties, their characteristics were determined using image analysis of the labeled objects: area is the area of the projected surface of the object on the plane of vision; hyphal length and hyphal width were determined as the maximum Feret diameter (FMax) and minimum Feret diameter (FMin), respectively. The Feret diameter is given by the distance between two parallel tangents in any given direction (Kawasse *et al*., 2003). Elongation is given by the ratio between hyphal length and hyphal width:

$$Elongation = \frac{F_{Max}}{F_{Min}}$$

Viability analysis

Fluorescent dye of acridine orange was used for the simultaneous distinction of live and dead cells (dead cells give out red or orange fluorescence and living cells yellow-green; 180 μM AO, pH 6). Cell counting was done in a Nikon optical microscope.

3. Results

3.1 Cell growth and glucose consumption

Specific growth rate (μ) and substrate consumption rate (-dS/dt), measured during the exponential growth phase, were used to evaluate the effect of the stress in growth kinetics. Table 1 presents the results for the the control experiments and the experiments with application of 0.75 V and 1.0 V.

Table 1: Specific growth rate (μ) and substrate consumption (-dS/dt) in control experiments and with 0.75 V and 1.0 V.

Stress	$\mu(h^{-1})$	-dS/dt
Control	0,37	2,34
0,75V	0,19	2,62
1,0V	0,23	1,87

The application of electric potential induces a decrease in the specific cell growth rate by approximately 40% in comparison to the control.

The electrochemical stress accelerates the consumption of substrate when the experiment is conducted with 0.75V. However, when 1.0V is applied there is a reduction in consumption.

These results may indicate that the electric potential increases the porosity of the cell and in experiments with 0.75 V facilitating the entry of substrate in to the cell, according to Fox (2006).

3.2 Lipase production

The extracellular lipase activity was monitored in the cultures by spectrophotometric method. Figure 2 shows the activity profiles obtained for all conditions studied.

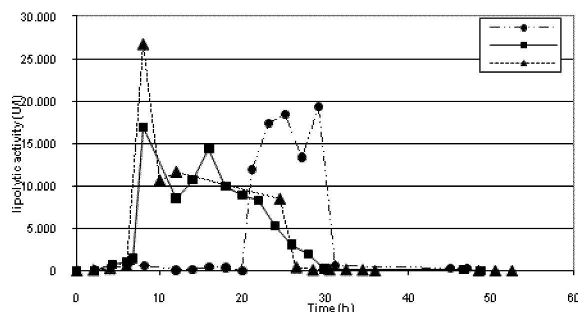

Figure 2: Lipolytic activity in the control experiments and with the application of 0.75V and 1.0V over time.

The production of lipase is facilitated by the application of electric potential which is easily visualized in the profiles obtained. This acceleration in the time of excretion is justified by the increase in cell permeability (Fox, 2006).

3.3 Morphologycal analisys

Figure 3 shows the histogram formation of hyphae during *Y. lipolytica* cultivation in control experiments and with the application of 0.75V and 1.0V; at last day (48h).

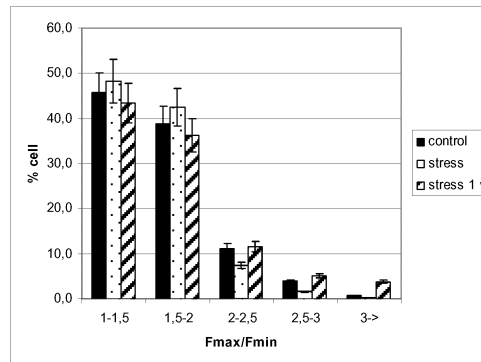

Figure 3: Histogram formation of hyphae in control experiments and with the application of 0.75V and 1.0V.

It is possible to observe that the application of stress did not influence cell morphology, the profile of hyphae formation is very similar for all experiments, including the control. Figure 3 also shows that the formation of hyphaes is not induced, with most cells in the round form (Fmax/Fmin = 1-1.5) or a little bit elongated (Fmax/Fmin = 1.5-2).

3.4 Viability analysis

In order to analyze the cells viable and throughout the process we used the technique epiflorescence with acridine orange. For the analysis were collected at the beginning of paragraphs first, second and third days of experiment.

The results of the analysis of cell viability with the application of electric potential in the control are shown in Figure 4 cells for active and inactive obtained during the experiments: control, 0.75 V and 1.0 V at third day.

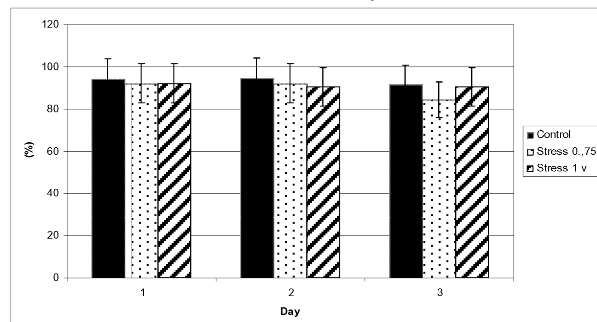

Figure 4: Histogram of cell viability obtained during the experiments: control, 0.75 V and 1.0 V.

The application of electric potential did not exert influence on cell viability.

4. Conclusion

The application of electric potential apparently has a strong influence on the cell membrane, thus increasing the porosity, which was observed with increasing substrate consumption and decrease the rate of cell growth. Additionally, lipase was excreted earlier to the medium, increasing productivity.

Through morphological analysis revealed that the application of stress does not influence the formation of hyphae, nor cell mortality. The viability of the use of electric potential for the release of lipase at large-scale may be an important drawback and there might be a need for particular designs for effective application.

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