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An air-lift biofilm reactor for the production of γ-decalactones by *Yarrowia lipolytica*

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ABSTRACT

Decalactones are interesting flavouring compounds that can be produced from ricinoleic acid. In this study, the production of lactones in biofilms using *Yarrowia lipolytica* is investigated. The hydrophobia of cells increased for increased aeration rates resulting in higher adhesion when the reactor wall was hydrophobic (plastic). To increase adhesion, sheets of methyl-polymethacrylate (PMMA) were added in the reactor and the production of lactones increased with the surface of plastic added, reaching 850 mg/L of 3-hydroxy-γ-decalactone for 60 cm^2. In an Airlift bioreactor made of PMMA, biofilms were present at the top of the reactor for increased aeration. In the meantime, a metabolic shift occurred resulting in high amounts of 3-hydroxy-γ-decalactone. At 0.493 vvm and 61 h of culture, the dissolved oxygen ratio was of 28.6% and cells grew to only 1.29×10^6 cells/mL in the liquid medium but 3-hydroxy-γ-decalactone accumulated to 1.7 g/L instead of less than 0.3 mg/L for lower aeration. Adhering cells had a particular elongated shape intermediate between the yeast and the pseudofilamentous forms. It is concluded that adhering *Y. lipolytica* cells are in a specific physiological state changing their structure but also their metabolic properties and these properties make them good candidate for simple immobilisation process.

1. Introduction

Lactones are aroma compounds encountered in fruits and fermented food. The C10-γ-decalactone accumulates during the degradation of a C18-hydroxylated fatty acid, ricinoleic acid, by some yeast species [1–4]. During this catabolism, other lactones accumulate such as dodecen-6-olide, two decen-4-olides and their precursor, 3-hydroxy-γ-decalactone (Fig. 1) [3,5,6]. The production of lactones has been widely investigated [2,6,7] and some authors have focused on the metabolic flux with attempts to modify it by deleting or overexpressing genes coding for β-oxidation enzymes [8–12]. Recent works have emphasised the importance of aeration and redox in the process. They showed that a low aeration perturbs the β-oxidation pathway which is involved in the degradation of fatty acids, thereby promoting the accumulation of incompletely degraded fatty acids such as lactones [4,13–15] (Fig. 1). However, it has also been shown that lactones are toxic to the producing cells [16,17] and works to decrease the contact between lactones and cells have been made by adsorbing lactones on polymeric materials [18–20] or immobilising cells in a protecting material [21,22].

This latter solution is a potential way of increasing the reactor density, the cell stability and of protecting cells from toxic compounds present in the medium. However, the addition of an immobilisation step can be seen as prohibitive by industrial biotechnology which is interested in simple ways of production. This could be improved if a universally applicable technology could be employed but so far many technologies have been developed, each responding to a specific demand and many requiring heavy preparation steps. Moreover, entrapped cells are often submitted to nutritional limitations and high concentrations of products. Their activity is thus reduced compared to planktonic cells. To overcome

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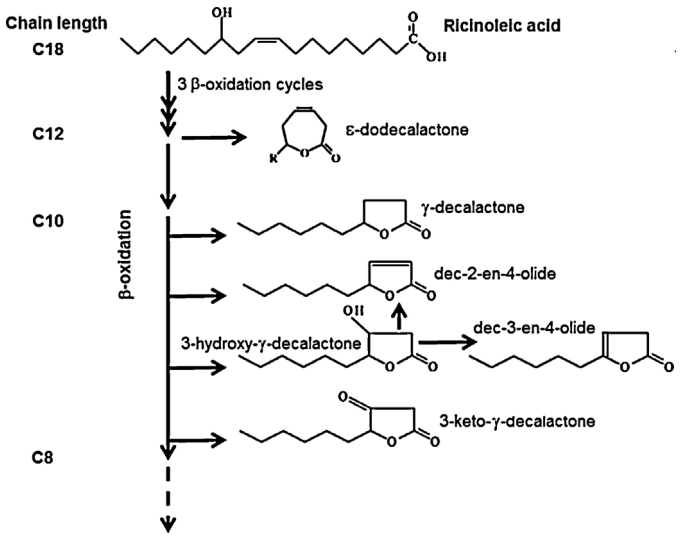

Fig. 1. Accumulation of lactones from the β-oxidation of ricinoleic acid. R: C6H13.

these drawbacks, some biotechnologists have proposed a natural way of immobilisation based on natural microbial structures known as biofilms [23]. In these structures, cells are protected by the matrice but in addition, they show increased physiological resistance to environmental stresses and they can carry out biotransformation reactions. However, biofilm immobilisation is a process requiring cells able to adhere to surfaces in the conditions of the biotransformation process. To our knowledge, such conditions have been investigated neither with yeast nor with lipidic emulsion media. In the present study, we propose to investigate the adhesion of cells of *Yarrowia lipolytica* to an air-lift reactor wall to favour the production of lactones. Conditions of high aeration increased the hydrophobicity of cell surfaces and favoured the formation of biofilms. In these conditions, a metabolic switch led to the production of 1.7 g/L of 3-hydroxy-γ-decalactone.

2. Materials and methods

2.1. Strains and culture conditions

The strain used in this study was *Y. lipolytica* W29 (ATCC 20460). Preculture was carried out for 48 h on YPDA [Yeast Peptone Dextrose Agar: 20 g/L glucose, 20 g/L peptone of casein, 10 g/L yeast extract (both products from Merck Mikrobiologie, Germany) and 15 g agar (agar obtained from Himedia, Laboratory India)] at 27 °C and cells were used to inoculate a 500 mL baffled Erlenmeyer flask containing 200 mL of YPD medium, to an A_{600nm} = 0.2 (6×10^6 cells mL^{-1}). Flasks were shaken at 140 revolutions per minute (rpm) for 19 h until the culture reached the late logarithmic growth phase. Cells were harvested by centrifugation (5 min at 27 °C and 9000 × *g*) washed twice with 0.9% NaCl (w/v), and recovered in 6.7 g/L YNB (filtered yeast nitrogen base, Sigma Aldrich, Portugal), to inoculate to an A_{600nm} = 1.57 (41×10^6 cells mL^{-1}) a 8.5-L Airlift bioreactor of the concentric draught tube type, with an enlarged degassing zone (manufactured in sheets of methyl polymethacrylate (PMMA) with a thickness of 4 mm in the Centre for Biological Engineering, Universidade do Minho, Portugal [24]), containing 5.5 L of biotransformation medium [15 g/L of methylricinoleate (M.R.) (Stéarinerie Dubois; Boulogne, France), 5 g/L NH_4Cl (Himedia) and 0.2 g/L Tween 80 (Polyoxyethylenesorbitan mono-oleate), from Sigma–Aldrich, Saint-Quentin Fallavier, France]. Numeration of cells was carried out on samples harvested from homogenously emulsified culture media which were counted in a cell of Malassez after sedimentation of yeast cells.

2.2. Characterisation of cell adhesion

To evaluate the adhesion of cells depending on the bioreactor material, small model Duran glass (Schott, Germany) or polycarbonate [25] (Nalgene, New York, USA) bioreactors were used consisting of non baffled 500-mL Erlenmeyer flasks. Cultures were carried out as previously described except that 5 g/L methyl ricinoleate were used instead of 15 g/L. The agitation of flasks was set to 140 or 170 (rpm) (Thermoshake THO5/THL5, Gerhardt, France). To increase the solid/liquid interface: 0, 20, 40, 60 or 80 pieces of 1 cm^2 of PMMA sheet with a thickness of 4 mm were added in the flask bioreactor. These pieces have been cut from the same material used to make the Airlift bioreactor. To evaluate the difference in adhesion of cells between each surface of PMMA sheet, the pieces were taken from bioreactors and put in a sterile plastic tube containing 25 mL of a sterile saline solution. The tubes containing the pieces were agitated for 2 min using a vortex. 50–100 μL of cells in the liquid phase were taken and then observed on a Neubauer cell (Improved CE, Marienfeld, Germany).

2.3. Physicochemical properties of cells surface

The hydrophobicity of cells of *Y. lipolytica* was evaluated by Microbial Adhesion to Hydrocarbons (MATH-Test) [26]. This study evaluates the affinity of cells towards a non-polar solvent like hexadecane. For the experiments, yeast cells were harvested by centrifugation (5 min at 27 °C and 7000 × *g*), washed twice with sterile saline solution (V_1) and resuspended in it. The absorbance was fixed to A_{600nm} = 0.4 (A_1). 2.4 mL of this suspension were added to 0.6 mL of hexadecane contained in a test-tube, and then the tube was mixed by vortex for 30 s in order to form an emulsion. This mixture was then left for 20 min until the separation of the two phases. The absorbance of the aqueous phase was measured (A_2), and the adhesion was expressed in percentage units (%):

$$\%\,\text{adhesion} = \left(1 - \frac{A_2}{A_1}\right) \times 100$$

2.4. Regulation of Airlift bioreactor parameters

The temperature of the medium was set to 27 °C by a shaker bath (Neslab, Ex-600). The culture pH was set to 4.45 (±0.05) with NaOH and HCl. It was regulated with a pH sensor (InPro 3030/225, Mettler Toledo, Switzerland) and a pH controller (BIOLAB, B. Braun, Germany). The gas output was condensed at 4 °C. Cultures were subjected to different aeration conditions: 0.86 L/min, 2.026 L/min and 2.71 L/min (equivalent to 0.157, 0.368 and 0.493 volume of air per volume of reactor per minute (vvm), respectively). The K_La was evaluated by the static gassing-out method [27]. Dissolved oxygen concentration was measured with a polarographic-membrane probe (12/220 T, Mettler Toledo), and a software package (LABtech Notebook, Datalab Solution) was used to follow dissolved oxygen during the time of culture. The experiments were carried out in duplicate.

To estimate the probe response time (τ), a method that describes the response of the probe to a step in dissolved oxygen concentration by a first-order system was used. The obtained $\tau\psi$ value of 7 s at 27 °C was used to correct K_La values [28].

2.5. Lactones analyses

Analyses were carried out on 1.5 mL samples collected from the culture medium in 4 mL glass vials. These samples were prepared as follows: 10 μL HCl (36% (w/v)) were added to stop the metabolism and to achieve the complete lactonisation of 4-hydroxy acids, 10 μL of γ-undecalactone (internal standard, Sigma–Aldrich) solubilised in absolute ethanol were added to reach a final concentration of 100 mg/L. The mixture was extracted with 1.5 mL of diethyl ether, by gentle shakings during 30 s. The organic phase was analysed in a HP6890 gas chromatograph (Agilent Technologies, Lyon, France) with a HP-INNOWax capillary column (Agilent-UptiBondInterchim: 30 m × 320 μm × 0.25 μm) with N_2 as a carrier gas at a linear flow rate of 4.3 mL/min. The split injector (split ratio, 7.1:1) temperature was set to 250 °C and the flame ionisation detector, to 300 °C. The oven temperature increased from 60 °C to 145 °C at 5 °C/min, and finally at 2 °C/min to reach 215 °C [29].

2.6. Image analyses

Microscopic imaging was carrying out with an Axioskop Optical Microscope (Carl Zeiss, Portugal) with the AxioVision 3.1 software. Micrographs were taken with AxioCam HRc-A. Cells were counted in a Neubauer cell.

2.7. Statistic analysis

All experiments have been carried out at least three independent times.

3. Results

3.1. Culture at higher aeration increases the affinity of cell surfaces to hexadecane

As a first step, the increase in adhesion of cells of *Y. lipolytica* to hydrophobic surfaces at higher agitation was checked. Cells were grown in Erlenmeyer flasks agitated at 140 or 170 rpm and the metabolic and surface properties were monitored (Fig. 2). For growth at 170 rpm, the lag-time was reduced compared to growth at 140 rpm but the maximum growth rate and the maximum cell