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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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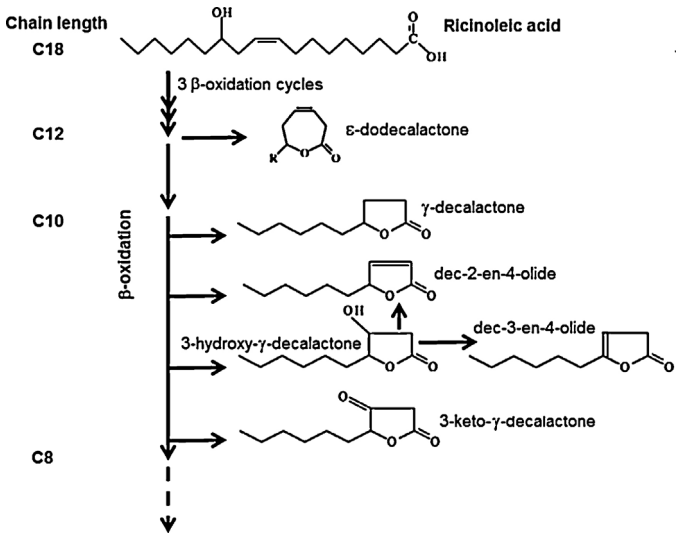

Fig. 1. Accumulation of lactones from the β-oxidation of ricinoleic acid. R: C6H13.

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

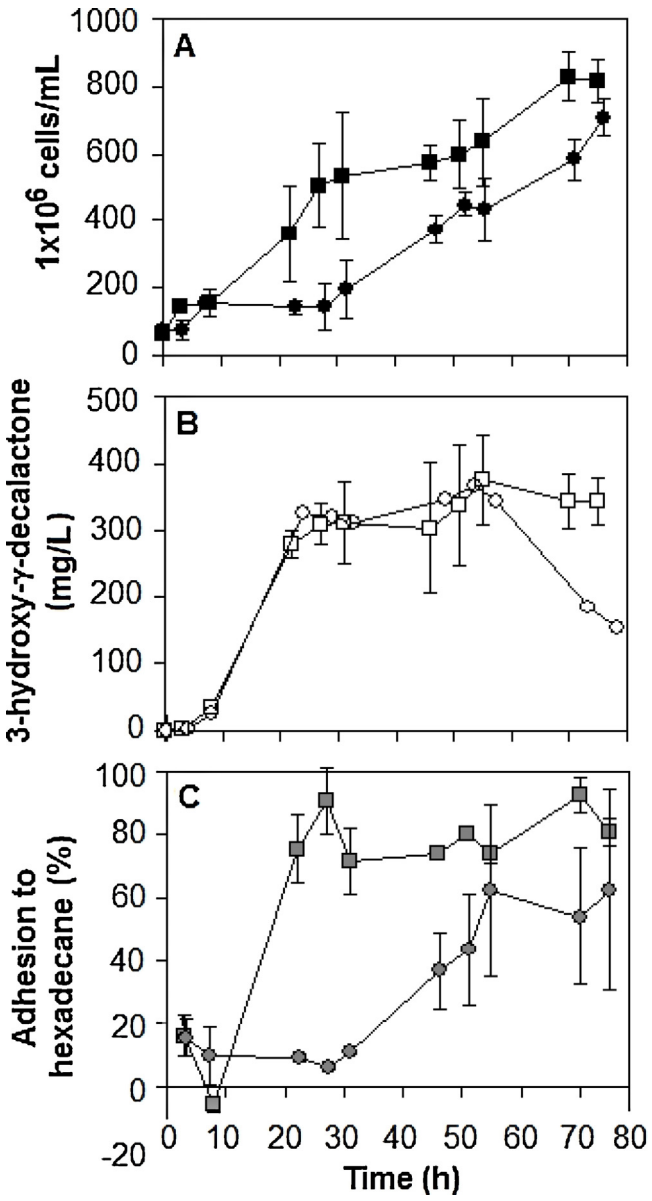

Fig. 2. Effect of agitation (140 rpm (circles) and 170 rpm (squares)) on growth, surface properties and 3-hydroxy-γ-decalactone accumulation by *Y. lipolytica* in glass Erlenmeyer. (A) kinetics of growth of *Y. lipolytica* cells in the biotransformation medium (black symbols) (B) 3-hydroxy-γ-decalactone accumulation (white symbols) and (C) percentage of adhesion to hexadecane (MATH-test) (grey symbols).

number were comparable (Fig. 2A). Moreover, the production of 3-hydroxy-γ-decalactone was similar in the two conditions with an amount of about 300 mg/L after 24 h, increasing to 350 mg/L after 50 h and declining afterwards for the 140 rpm condition (Fig. 2B). The specific production was almost similar and comprised between 0.6 and 0.8 mg/10^5 cells between 20 and 60 h for both conditions except between 15 and 30 h for 140 rpm for which, due to a delayed cell growth, the specific production reached 1.45 mg/10^5 cells. The affinity of cells to hydrophobic interfaces in the two conditions was investigated through the adhesion of cells to hexadecane. For both conditions, the adhesion was low at the beginning of the culture (inferior to 20%) and increased thereafter. For the 170 rpm-culture, the adhesion increased dramatically to about 80% after 24 h whereas it increased more slowly and to a lesser extent (around 60%) for the 140 rpm-culture (Fig. 2C).

3.2. Culture in a plastic reactor promotes the adhesion of hydrophobic cells

In the first part, an increase in the affinity of cells for hexadecane was observed. In this 2nd part, the objective is to evaluate the impact that adhesion could have on a culture. In that goal, cultures were carried out in glass (hydrophilic) or plastic (hydrophobic) Erlenmeyer flasks. During the cultures in plastic flasks, a thin film of cells was observed on the upper part of the reactor whereas no cells adhered to the glass reactor, as illustrated in Fig. 3.1. The film formation occurred in both conditions of agitation, at 140 rpm as well as at 170 rpm. In these culture conditions, the adhesion of a cell film on the plastic of the reactor did not change significantly the production of 3-hydroxylactone compared to production by non-adhering cells in glass reactors (results not shown). However, the morphological state of the cells in the reactor was investigated. Cells adhering to the surface were carefully harvested with a sterile Pasteur-pipette to be observed under the microscope (Fig. 3A2). They had an elongated form and were often present in aggregated groups. In contrast, cells harvested in the liquid phase of the glass reactor had an oval shape and were not aggregated (Fig. 3B2). The morphological difference between adhering and non-adhering cells was quantified by the measurement of the ratios of dimensions (length divided by width) which were equal to 2.5 (±1.0) for adhering cells in plastic bioreactors while the value for glass reactors was 1.6 (±0.3). The surface properties of cells in suspension in the different flasks were investigated (Fig. 3.3). Cells taken from the liquid culture medium in the glass flask were more hydrophobic than those taken from the plastic one.

3.3. The accumulation of 3-hydroxy-γ-decalactone depends on the plastic surface available for adhesion

To check whether the presence of adhering cells could modify the production of 3-hydroxy-γ-decalactone, cultures in presence of increasing surfaces of plastic as supports of adhesion were carried out. Plastic pieces of 1 cm^2 were added to the culture with a total surface varying between 0 to 80 cm^2. The effect of this addition on the production of lactones is shown in Fig. 4. With an added surface as low as 20 cm^2, the production of the hydroxylated lactone increased of about 90 mg/L (Fig. 4A). Then, for each 20 cm^2 further addition, the accumulation increased of about 200 mg/L up to 850 mg/L for 60 cm^2. Then, for the next addition of 20 cm^2, the production fell to a value similar to the one obtained for 20 cm^2. For γ-decalactone (Fig. 4B), the increase in the plastic-surface available was less important but exhibited a similar trend than for 3-hydroxy-γ-decalactone with 247 mg/L for 40 cm^2 and 264 mg/L for 60 cm^2.

3.4. In a plastic air-lift reactor, the increased cell adhesion subsequent to the increased aeration results in a dramatic drop in the accumulation of 3-hydroxy-γ-decalactone

The concentration of 3-hydroxy-γ-decalactone and dissolved oxygen levels during cultures in airlift reactor at various aerations are shown in Fig. 5. At 0.157 vvm, cells grew to reach about 7×10^7 cells mL^{-1} but the accumulation (per litre) of 3-hydroxy-γ-decalactone was very slow and poor, reaching only 122 mg at 63 h (Fig. 5B). In the meantime, a fast and almost total consumption of oxygen was observed in only 18 h of culture. For a higher aeration (0.368 vvm) (Fig. 5A), oxygen decreased gradually and only 5% remained at 33 h of culture. After this, a decrease in oxygen consumption occurred and about 16% oxygen remained for the next 33 h. Under this aeration, almost no growth was detected in the medium but we observed a layer of cells covering the superior slice of the bioreactor (as shown in Fig. 6C for a higher aeration).

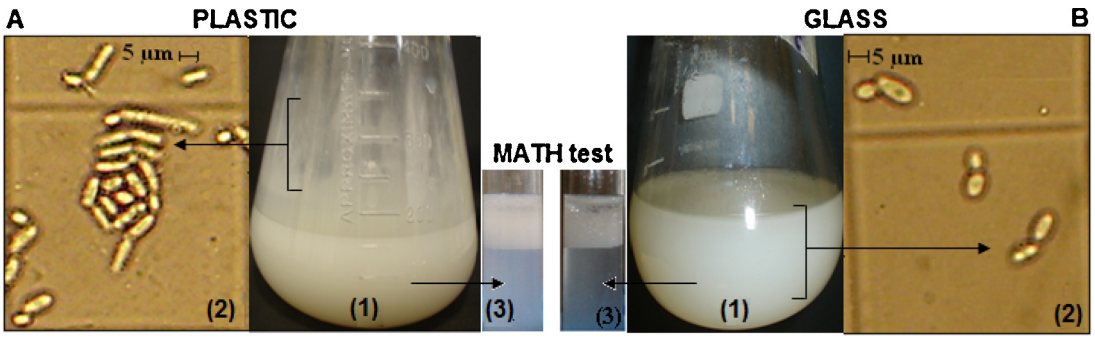

Fig. 3. Impact of reactor material on cell properties. (A) Culture in plastic or (B) glass Erlenmeyer. 1 pictures of Erlenmeyer, 2 micrographs of cells adhering to the reactor surface (A2) or in the medium (B2), 3 adhesion of cells to hexadecane. The hexadecane-phase is in the top and the aqueous-phase in the bottom.

3-Hydroxy-γ-decalactone accumulated slowly reaching 244 mg/L at 45 h of culture (Fig. 5B). When the aeration was set to 0.493 vvm, a higher level of dissolved oxygen remained in the medium (28.6% at 70 h) (Fig. 5A). In this condition, a long lag-phase was observed (13 h) followed by an exponential-growth-phase in which the cell amount reached 12.9×10^7 cells mL^{-1}. After 30 h of culture, the number of cells continued to increase, in correlation with a rapid accumulation of 3-hydroxy-γ-decalactone which reached 1.7 g/L at 61 h (Fig. 5B). Using aeration rates of 0.368 vvm and 0.493 vvm, a thick layer of cells and lipids adhering to the wall of the reactor was observed, particularly in the upper part (Fig. 6A–C). From these experiments carried out in a plastic bioreactor (PMMA material), it was deduced that the presence of an adhering layer of cells could explain the increase in the accumulation of 3-hydroxy-γ-decalactone.

4. Discussion

The production of lactones is a biotransformation difficult to manage. Studies have shown that it is very sensitive to aeration, pH and to the metabolic properties of the cells such as the acyl-CoA oxidase activity [11–13,30,31]. However, the oxygen availability appears to be a key parameter driving the extent of oxidation. With full oxygen availability, oxidation occurs until acyl compounds are degraded to acetyl moieties whereas a controlled oxygen limitation causes the stop of β-oxidation at the interesting C10-level [14]. It is thus similar to other metabolisms such as the microbial production of xylitol or to the one of citric acid for which there is an aeration optimum [32,33]. However, our results contrast with this rule as increasing aeration did not decrease lactone production and, in contrast, it increased the production of 3-hydroxy compounds which is normally a sign of insufficient NAD regeneration resulting from low aeration [6]. Although this phenomenon can be observed for the production of lactones by strains having a blocked β-oxidation metabolism for short-chain compounds such as *Candida antartica* (Alchihab, personal communication), this has never been reported for strains able to degrade completely acyl- to acetyl-moieties such as *Yarrowia lipolytica*. Our results suggest thus that, when increasing aeration in the presence of hydrophobic surfaces,

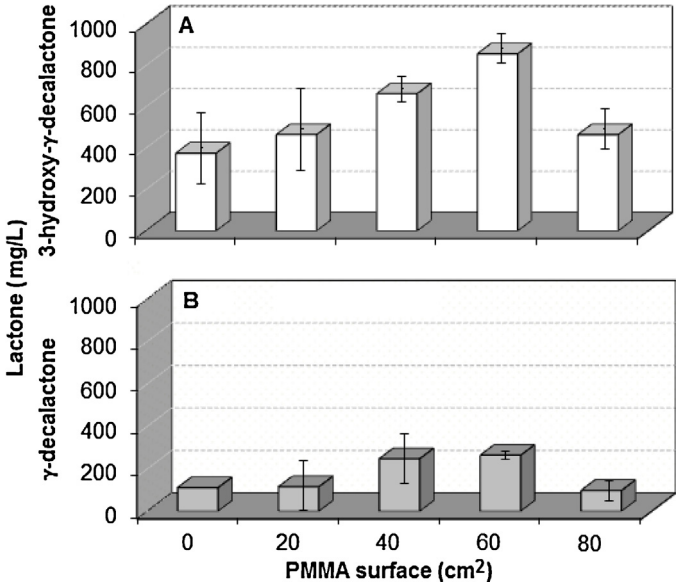

Fig. 4. Effect of the presence of PMMA surfaces in glass Erlenmeyer flasks at 170 rpm of agitation on (A) 3-hydroxy-γ-decalactone and (B) γ-decalactone accumulation by *Y. lipolytica*. Bioreactors with 0, 20, 40, 60 and 80 cm^2 PMMA surface.

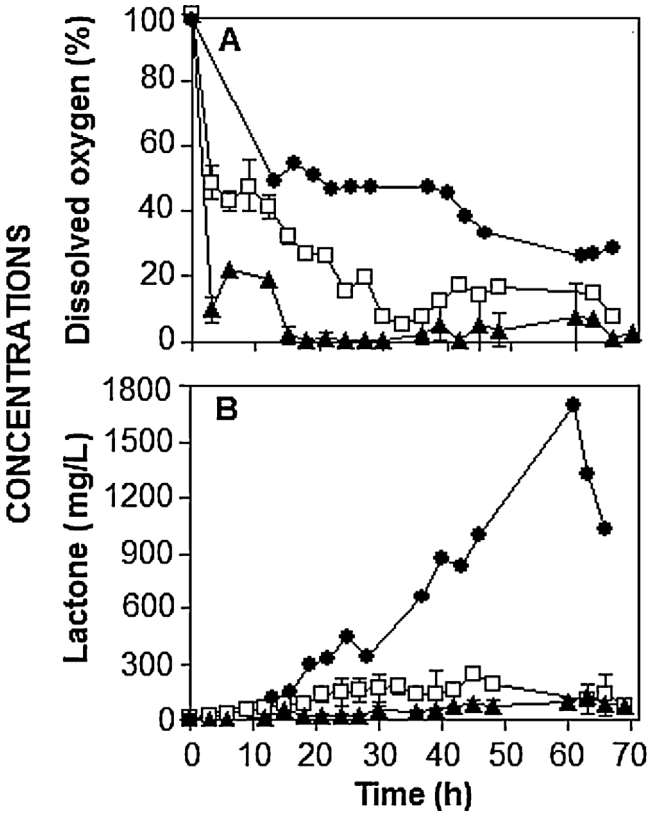

Fig. 5. Oxygen consumption and 3-hydroxy-γ-decalactone accumulation during cultures of *Yarrowia lipolytica* on the Biotransformation medium in airlift reactor at 0.493 vvm (●), 0.368 vvm (□) and 0.157 vvm (▲): (A), dissolved oxygen level and (B), accumulation of 3-hydroxy-γ-decalactone.

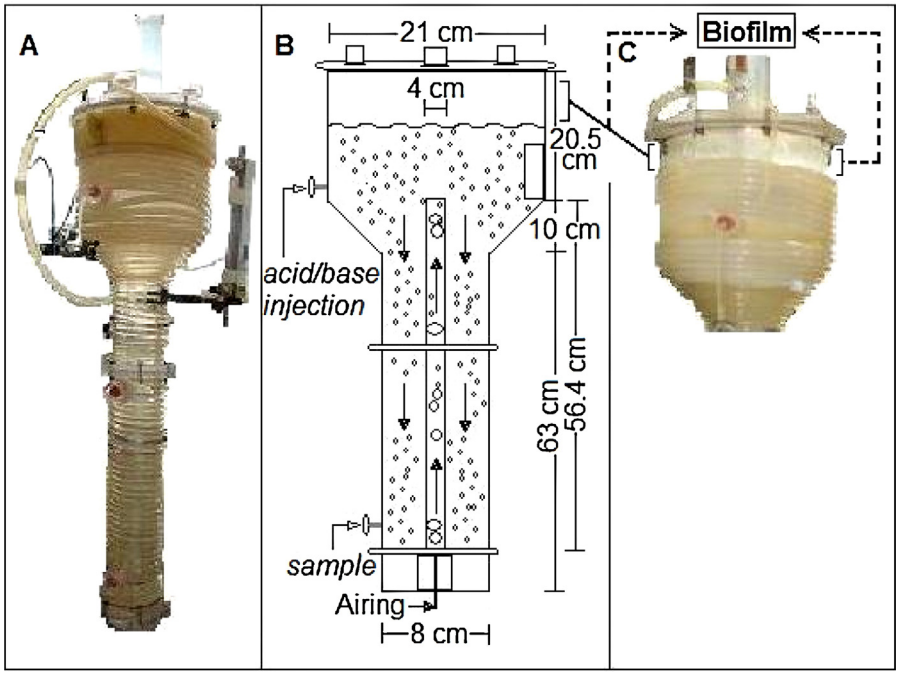

Fig. 6. Airlift bioreactor utilised for experiments (A), picture, (B), schematic view of the reactor and (C), localisation of adhering cells (at 13 h of culture at 0.493 vvm).

cells are in a particular physiological state exhibiting a metabolic shift. Modification in the cell physiology can be observed with the increased adhesion of cells to hexadecane when increasing agitation but this does not lead to any metabolic shift when cells are grown in glass reactors (Fig. 2). Indeed, modification of yeast surface properties induced by the presence of lipid substrate has already been observed at the entire population level [33–35] and, taking into account the importance of aeration in the lipid metabolism, this relationship between aeration and surface properties is not surprising. Our key result about surface properties changes is that, when grown in the presence of a hydrophobic interface, the subpopulation of cells exhibiting an affinity for hydrophobic interfaces adheres to this interface and, when this adhesion occurs, a metabolic shift is observed together with a morphological one: cells change their morphological properties to an original form with an intermediate length between the yeast-like [36] shape and the hyphal or pseudo-hyphal one. To our knowledge, this is the first description of such a morphology although the aeration level has also been described to make *Y. lipolytica* change between its yeast and pseudohyphal forms [37]. Interestingly, our results showing that these cells exhibiting a metabolic shift resulting in high proportions of 3-hydroxy-γ-decalactone in high aeration conditions [13] suggest that the biofilm structure would lead to a decreased oxygen availability resulting in a change in the intracellular redox balance.

Our results show that biofilm reactors, as an immobilisation mean, could be of great interest to improve biotransformation by *Y. lipolytica*. Microorganism immobilisation has been known for years to be a good way to improve rates in many systems [38] and in particular with yeast biocatalysts [39] and for the production of aromas [40]. For the production of lactone, immobilisation has not been much employed and did not result in high production [21,22]. As for other biotransformations carried out in heterogeneous media [41], the medium for this aerobic reaction comprises many phases between which transfers are an important driving force. So the introduction of a new immobilisation phase may decrease transfers to cells. Such an immobilisation-induced limitation has been used successfully to limit oxygen transfer and favour xylitol production in a process in which aeration has to be limited similarly to the production of lactones but where the medium is a homogeneous and aqueous one [42].

These artificial immobilisation methods are popular among researchers although they make the whole process more complex and are thus less utilised for industrial production. A natural biomimetic way of immobilising cells can be by mimicking natural adhesion of cells to a surface. Adhesion of microbial cells to an interface is the first step in biofilm formation. The evolution of surface properties leading to adhesion has already been observed as a response to the presence of a hydrophobic interface [34,35] and the relationship with changes in the cell wall composition has been evidenced (Romero-Guido et al., unpublished results). Biofilms are complex ecological systems in which cells are protected by extracellular components but they also change their phenotypic properties [36].

5. Conclusion

In conclusion, high aeration conditions increase the hydrophobicity of *Y. lipolytica* resulting in higher adhesion to plastic surfaces. The formation of adhering films of *Y. lipolytica* stimulates the production of 3-hydroxy-γ-decalactone and, in airlift reactor, a 10-fold increase has been obtained. We are currently studying the impact of this biofilm physiological state on the activity of the enzymes of β-oxidation but this result obtained in a simple process based on natural adhesion without additional immobilisation step may be developed for all applications of *Y. lipolytica* on hydrophobic compounds biotransformation or degradation. From the present work a novel and simple process is proposed for the production and accumulation of molecules of interest for food or pharmaceutical fields. Moreover, the discrimination of the subpopulations of *Y. lipolytica* by adhesion capacity offers new opportunities to increase their efficiency.

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