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Increased Electron Donor and Electron Acceptor Characters Enhance the Adhesion between Oil Droplets and Cells of *Yarrowia lipolytica* As Evaluated by a New Cytometric Assay

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The adhesion of methyl ricinoleate droplets to cells of the yeast *Yarrowia lipolytica* was investigated. A new cytometric method, relying on the double staining of fatty globules with Nile Red and of cells with Calcofluor, enabled us to quantify methyl ricinoleate droplet adhesion to cells precultured on a hydrophilic or on a hydrophobic carbon source. In this last case, droplet adsorption was enhanced and a MATS (microbial adhesion to solvents) test revealed that this increase was due to Lewis acid−base interactions and not to an increase in the hydrophobic properties of the cell surface. These preliminary results demonstrate that the developed cytometric method is promising for various applications concerning the study of interactions between microorganisms and an emulsified hydrophobic substrates.

KEYWORDS: Adsorption; methyl ricinoleate; surface properties; *Yarrowia lipolytica*; cytometry

INTRODUCTION

The yeast *Yarrowia lipolytica* is frequently associated with dairy products, notably with milk products, and it has been shown to be active as a cheese-ripening agent (*1*). This species has been currently used in many processes involving a hydrophobic carbon source such as the production of organic acids, of single-cell proteins (*2*), of fatty acid-derived aroma compounds (*3*), and the bioremediation of diesel-contaminated soils (*4*) or of olive mills wastewater (*5*). In a previous paper, we have shown that due to its surface properties, this species was able to reduce the mean diameter of lipid droplets within an oil-in-water emulsion culture medium, during the biotransformation of methyl ricinoleate (MR) to γ-decalactone (*3*). However, the nature of the interactions between these yeast cells and the hydrophobic substrate within a biphasic medium is still poorly understood.

Generally, lipids assimilation by microbial cells requires a contact between the oil phase and the cells. This contact can occur through a direct adsorption of hydrophobic droplets to the cell surface, or it can be mediated by a surfactant (*6*, *7*). In the case of direct adsorption, several mechanisms can be involved including hydrophobic, Lewis (acid or base), electrostatic, or van der Waals interactions. Some simple tests have been developed to characterize the hydrophobic properties (MATH, microbial adhesion to hydrocarbons) (*8*) or the electron donor or electron acceptor (Lewis acid−base) properties (MATS, microbial adhesion to solvents) of microorganisms (*9*). These tests and some others such as contact angle or ζ-potential measurement (*10*) or hydrophobic fluorescent beads adherence (*11*) elucidate information on the surface properties of the cells. This can be useful to predict the microbial behavior toward a surface. However, tests to investigate what really happens within a biphasic culture medium are lacking. Microscopic observations are hardly convenient to quantify the adhesion of the lipids to the cells, and granulometric particle size evaluation (*3*), which is suitable to analyze an entire culture medium, does not give direct information about the adhesion.

In the present study, we have investigated the surface properties of *Y. lipolytica*, and to evaluate the interaction between cells and fatty droplets in the medium, we have developed a cytometric assay to evaluate the droplet adhesion. The results showed that *Y. lipolytica* possesses adsorption properties resulting in fatty acid droplets adsorption on their surface. The adhesion was increased after a preculture on a hydrophobic carbon source as compared to a preculture on glucose. According to MATS tests, this increase was due to an increase in the electron donor and electron acceptor character and not to a modified surface hydrophobicity.

MATERIALS AND METHODS

Strain, Media, and Culture Conditions. The strain *Y. lipolytica* W29 (ATCC 20460; CLIB89) was cultured for 48 h on malt extract agar (Difco, Osi, Paris, France) at 27 °C and used to inoculate a 500 mL baffled Erlenmeyer flask containing 200 mL of a glucose medium (*3*), YPD, or of MR medium (*3*) to an $OD_{600} = 0.25$ (6×10^6 cells/mL). MR medium contained 10 g/L MR, 6.7 g/L yeast nitrogen base,

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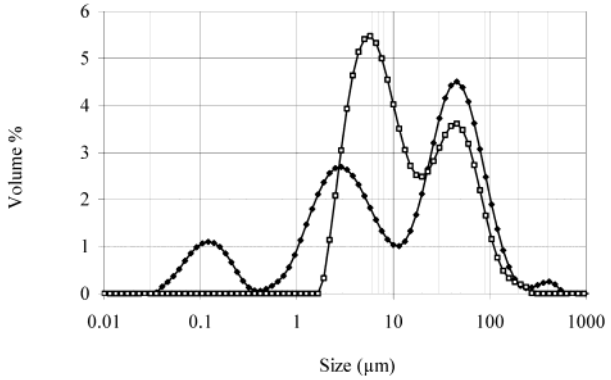

Figure 1. Granulometry evaluation of the particles size distribution in an emulsified MR medium without cells (●) or containing yeast cells (□).

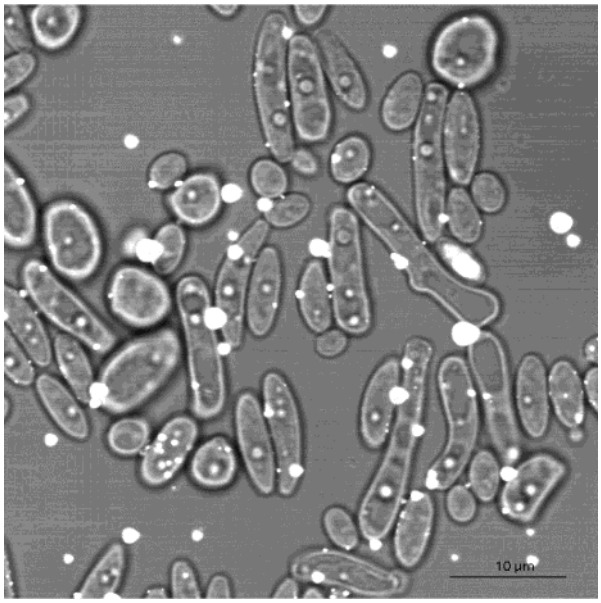

Figure 2. Variable size MR droplets on the surface of *Y. lipolytica* cells, observed by confocal microscopy. In this case, cells were cultured without ethanol and both yeast and pseudo-filamentous forms were present.

and 5 g/L NH_4Cl and was emulsified by agitation (140 rpm, 27 °C) in the presence of 0.2 g/L Tween 80. In all culture media except for **Figure 2**, ethanol (0.75 g/100 mL) was added to induce the yeast form of the cells vs the pseudo-filamentous one. Flasks were shaken at 140 rpm for 19 h until the cultures reached the late logarithmic growth phase. Cells were harvested (6000 g, 5 min), washed twice with phosphate buffer (50 mM, pH 7.4), and resuspended in the MR medium for the various analyses. All chemicals were purchased from Sigma/Aldrich (Saint-Quentin Fallavier, France) except MR (Stearinerie Dubois, Boulogne, France).

Laser Granulometry. The size of the fatty acid droplets was evaluated using a Malvern Mastersizer-S2-1 laser granulometer (Malvern Instruments, Malvern, U.K.). MR media were analyzed without or with cells precultured for 19 h on a glucose medium. The media without cells were shaken for 1.25 h, and the media with cells were shaken for 1 h before inoculation and then 15 min with the cells before analysis.

MATS Tests. Lewis acid−base, i.e., electron donor/electron acceptor, interactions were evaluated using the MATS method (*9*). This technique is based on the microbial cell affinity to a monopolar and to an apolar solvent. The monopolar solvent can be acidic (electron acceptor) or basic (electron donor), but both solvents must have similar surface tension Lifshitz−van der Waals components. The solvent pairs used were chloroform (electron acceptor) and hexadecane and ethyl acetate (electron donor) and decane.

Cells grown for 19 h on the glucose or on the MR medium were harvested (6000*g*, 5 min), washed twice, and resuspended in a phosphate buffer (0.1 M, pH 7) to an OD_{600} of 0.70 ± 0.02. Five milliliters of this suspension was added to a glass tube containing 1 mL of solvent. The tube was gently inverted 10 times, and after 4 min, 2 mL of the aqueous phase was removed and its OD_{600} was measured. Results are given in percent of bound cells: % adherence $= 1 - A/A_0$, where A_0 is the OD_{600} of the aqueous microbial suspension before mixing and A is the OD_{600} of the aqueous microbial suspension after mixing. Each experiment was performed in triplicate by using three independently prepared cultures.

Cell and Fatty Acid Staining. Cells grown on the glucose or MR medium were harvested (6000*g*, 5 min), washed three times, and stained with Calcofluor M2R (20 mg/L for a cell suspension of $OD_{600} = 6$, stock solution 1 mg/mL in distilled water containing one drop of NaOH) for 20 min at 27 °C in a phosphate buffer (0.1 M, pH 7). MR emulsions were stained for 30 min (27 °C, 140 rpm) with Nile Red (8 μg/mL; stock solution, 4 mg/mL in acetone). The emulsion and the cells were stained separately, and then, cells were added to the emulsion under agitation at room temperature. Confocal microscopy observations were done as previously described (*3*).

Flow Cytometry Measurements. Flow cytometry analysis and sorting were performed with a Beckman-Coulter EPICS-ELITE/ESP Cytometer, equipped with an Innova 90C Ion Laser. The laser was tuned to 350 nm and to an output of 150 mW. Forward scatter at linear amplification was used as the threshold parameter to reduce electronic and small particle noise. The side scatter was detected with a 360 nm Dichroic Long Pass filter. The Calcofluor and Nile Red fluorochromes were detected with a 440 nm Band Pass filter and a 610 nm Band Pass filter, respectively. The results were performed on a list mode of 10^4 events and analyzed with Expo2 software. There was some spectral overlap between the two emitted fluorescence; therefore, the system's compensation was set up where appropriate to eliminate interference.

Sorting was carried out throughout a 100 μm nozzle. Double fluorescent events were sorted at a flow rate of 1000 cells/s with high purity (>95%). Samples were collected on a polylysine-covered glass slide in order to confirm the quality of sorted cells in epifluorescence microscopy. The microscopic observations were carried out using a Nikon E400 microscope equipped with a camera (Nikon DXM 1200); the images acquisition was done using ACT-1 software (distributed by Nikon).

RESULTS

Granulometric Characterization of the Medium. The granulometric analysis of the emulsified MR medium showed that it contained three distinct droplet populations, with a mean size of 0.1, 3, and 43 μm. When the cells were present in the medium at an OD_{600} around 4, two populations with a size of 5.5 and 43 μm were detected (**Figure 1**). It appeared that the detection of the smallest oil droplets in the medium disappeared for a yeast cell population higher than an OD_{600} equal to 1.5 (not shown). The cells alone in water containing NaCl (9 g/L) were detected, and they presented a mean size around 5.5 μm. The size of the biggest MR droplets (43 μm) was not modified by the presence of the cells. The disappearance of the smallest MR droplets when the cells were present may be due to their adhesion to the cell surface; in that case, the method may not be sensitive enough to detect the increase in particle size due to the presence of lipid droplets on cells surface. It could also be due to the massive cell presence that would overshadow the smallest droplets.

Microscopic Observation of the Medium. Observations by confocal microscopy revealed that the cells had numerous small MR droplets bound to their surface. Their size varied from 0.1 to 2.5 μm (**Figure 2**), which corresponds to the size of the smallest droplets previously detected by granulometric measurements. However, many unbound small droplets were still visible in the medium, indicating the limitation of the granulometric measurements, which did not enable the detection of these droplets populations.

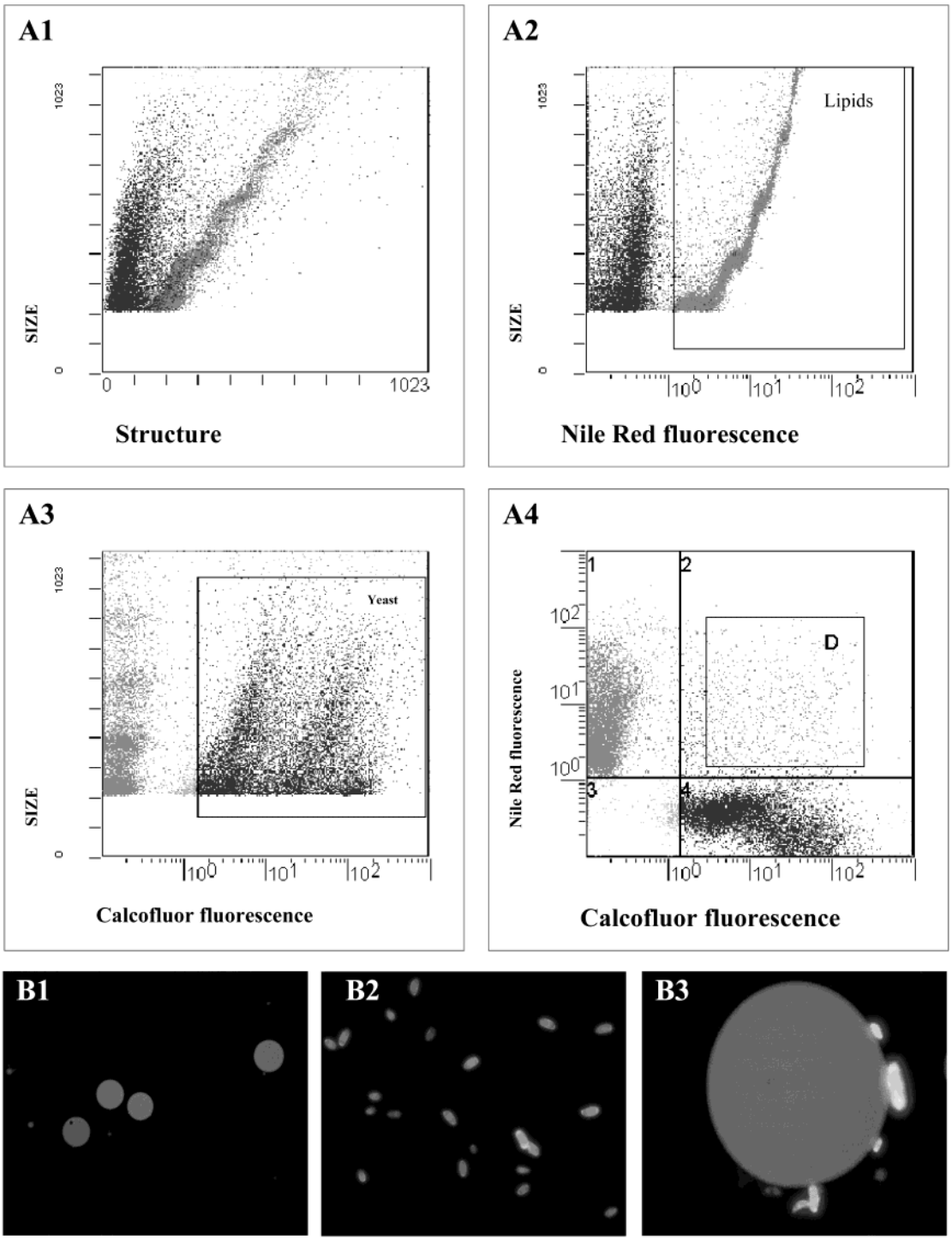

Figure 3. Two-dimensional scatterplots obtained by flow cytometric analysis of a MR medium containing yeast cells (**A**) and fluorescence microscopy observations (**B**). (**A1**) Discrimination of MR droplets and cell populations by size vs structure. (**A2**) Detection of Nile Red stained MR droplets in the presence of unstained cells and (**A3**) of Calcofluor stained cells in the presence of unstained MR. (**A4**) Detection of the double stained events (defined by D region) in a medium by using the two fluorochromes. Representation of samples obtained after cell sorting: (**B1**) MR droplets stained with Nile Red (from region 1 of **A4**), (**B2**) yeast cells stained with Calcofluor (from region 4 of **A4**), and (**B3**) yeast cells on the surface of a MR droplet with double staining (from region D of **A4**) (× 40).

Cytometric Assay to Evaluate the Adhesion. To quantify the adsorption between MR droplets and the cells within the biphasic medium, we developed a cytometric method. The principle of this original method was based on an in situ double fluorescence staining, using one fluorochrome for the oil droplets and another one for the yeast cells.

For the staining of MR droplets, different concentrations of Nile Red were used. With 8 μg/mL, 100% of the fatty droplets were stained (**Figure 3A2**,**A4**), as observed in fluorescence microscopy, whereas no yeast cells were stained. For the staining of yeast cells, various dyes were tested including fluorescein diacetate, carboxyfluorescein diacetate, Hoechst 33342 (*12*), and Calcofluor. The staining obtained with the three first fluorochromes was low and hardly reproducible; only a small proportion of the cells was stained with the fluorescein derivatives and a variable percentage (between 30 and 70%) with

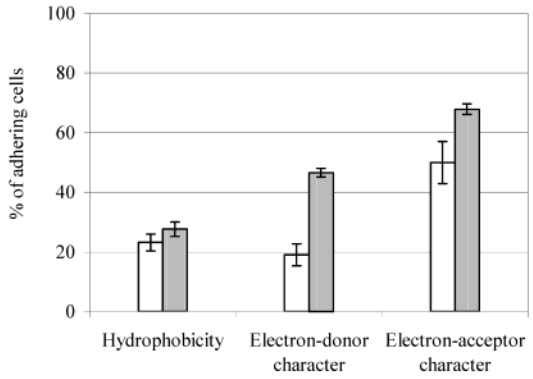

Figure 4. MATS tests carried out on *Y. lipolytica* cells grown in YPD (white) or in MR (gray) media. Hydrophobicity was evaluated by the cell adhesion to hexadecane; the electron donor character was calculated from the adhesion to chloroform minus that to hexadecane; the electron acceptor character was calculated from the adhesion to ethyl acetate minus that to decane.

Hoechst (not shown). As such compounds have to enter the cell, the staining may depend on some physiological factors. A permeabilizing chemical treatment should improve cells staining by these compounds, but with the goal of studying the surface properties of the cells, such a treatment was not appropriate. The cell wall dye Calcofluor, for its part, was very efficient as almost 100% of the cells were stained with 20 μg/mL (**Figure 3A3**,**A4**). This hydrophilic compound did not stain any fatty acid droplet; furthermore, when Nile Red stained fatty acids and Calcofluor stained cells were simultaneously analyzed, no energy transfer was detected. The double stained events, corresponding to the cells/lipids adhesion, could be discriminated and so quantified; these were defined within the D region in **Figure 3A4**, whose delimitation comprised a margin to ensure the exclusion of stained cells or lipids alone. The different populations were sorted and then observed by fluorescence microscopy, as illustrated by **Figure 3B**. These analyses followed by cell sorting and microscopic observation were carried out three independent times giving similar results, but **Figure 3** corresponds to only one representative experiment.

The method was then applied to the quantification of the double stained events obtained with yeast cells precultured in YPD or MR media and then transferred to a Nile Red stained MR medium. For the yeast cultured in YPD medium, after a 15 min contact time with the MR medium, 5% of the total events detected by cytometry corresponded to double stained events; the percentage was around 10 for the yeast cultured in MR medium. The adhesion between the yeast cells and the MR droplets was thus twice as high for the cells precultured in MR medium than for the cells precultured in YPD medium.

MATS Tests. To characterize yeast cell surface properties and to evaluate the factors involved in lipid droplets adhesion, MATS tests were conducted with yeast cells precultured in YPD or in MR media (**Figure 4**). The cell surface hydrophobicity, which was determined by the partitioning of the cells between water and hexadecane, was not significantly different for both cell populations. The affinity of the cells to an acidic or a basic solvent (corrected for the partitioning between water and an apolar solvent with similar hydrophobic properties) expresses, respectively, the electron donor or electron acceptor character of the cell surface. Thus, when compared to the cells cultured in YPD medium, the cells cultured in MR medium exhibited increased electron donor character and, to a lesser extent, electron acceptor character (**Figure 4**). In conclusion, the cell growth in the hydrophobic MR medium did not induce an increased hydrophobic character of the yeast cell surface but rather influenced its Lewis acid−base properties.

DISCUSSION

The degradation of hydrophobic compounds through β-oxidation or other peroxisomal oxidation pathways requires the entry of the compounds inside the cell. One of the mechanisms proposed is direct interfacial transport. In this case, the adsorption of apolar droplets to the cell wall occurs first (*6*, *13*). As shown in the present study by microscopy observations, MR droplets are adsorbed to the surface of *Y. lipolytica* cells (**Figure 2**). The interaction enabling this contact is usually described as depending on the hydrophobicity of cells (*6*, *8*).

When using laser granulometry in an attempt to quantify the interaction between the cells and the lipids, limitations of the technique were evidenced; it enabled us to detect the particle size distribution in the medium, but for the monitoring of the fatty acid droplet adhesion to the yeast surface, the cell concentration strongly influences the interpretation of the results. Subsequently, the cytometric method that we developed appeared more convenient. This method, which can be of interest to other researchers studying microbial cultures on hydrophobic substrates, enabled fast analysis of a numerous cell population. It allowed us to show that there is an inducible phenomenon in the adhesion between fatty droplets and yeast cells. Different authors have observed a modification of the cell wall of *Candida* sp. grown on *n*-alkanes. For instance, Osumi et al. (*14*) have observed that the surface is smooth for cells grown on carbohydrates and turns rough when a mixture of alkanes (C10−C13) becomes the carbon source. The surface of yeasts observed in emission scanning electron microscopy revealed protrusions of 100−200 nm in diameter, which seemed connected to the cell membrane, forming a sort of channel. Such channels have also been observed by Meisel et al. (*15*, *16*), and Käppeli and Fiechter (*17*) have isolated a polysaccharide−fatty acid complex from the surface of *Candida tropicalis* grown on alkanes. These inducible protrusions or complexes could be involved in the fatty acid droplets adhesion to the yeast cell wall.

The developed cytometric technique requires only a simple staining step, which after an UV laser excitation, enables the quantification of cells interacting with fatty droplets. According to the detection threshold applied here for the stained lipid droplets (**Figure 3A2**) and to the delimitation of the region D (**Figure 3A4**), the cells with very small droplets adhering to their surface (as appearing in **Figure 2**) could not be discriminated within the region D, and the majority of the double stained events corresponded to the representation of **Figure 3B3**. This point could possibly be improved by lowering the detection thresholds.

Nevertheless, the cytometric method was useful in this study to evaluate the adsorption in the culture conditions. These conditions are very close to the conditions used in the biotransformation of MR to γ-decalactone (*18*). Interestingly, with only one additional step, this technique can include the evaluation of the size of the fatty droplets (the fluorescence intensity depends on the size of the globule) and thus enable us to investigate the question of which droplets are better adsorbed and assimilated. This question of the lipid globule size and its influence on the metabolism has been investigated only through the growth rate point of view suggesting the importance of the contact angle between yeast cells and droplets: with globules smaller or bigger than cells, growth was rapid but the rate decreased for similar sizes (*19*). Our technique could confirm if the growth rate is linked to the adhesion between droplets

and cells. Furthermore, the subsequent application of cell sorting may be useful for isolation of yeast mutants with increased lipid adherence properties and possibly an enhanced lipid assimilation capacity. Whatever, this preliminary application of the proposed cytometric method demonstrates that it can be applied for the optimization of biotechnological processes or for studying the interactions between yeast cells and fat globules, for example, in milk.

The originality of our results concerning the interactions between yeast and lipids is that *Y. lipolytica*, when grown on MR, does not increase its hydrophobicity but its electron donor and, to a lesser extent, electron acceptor character. In the meantime, the adsorption of fatty globules to the yeast surface increases the suggestion that the adhesion is linked to Lewis acid−base interactions and not, or at least not only, to hydrophobic/hydrophilic interactions. This could reflect that *Y. lipolytica* does not assimilate fatty acids through a direct interfacial hydrophobic contact but through a mechanism related to a surfactant-mediated assimilation requiring more polar interactions. It can be noted that other strains of *Y. lipolytica* have been shown to produce biosurfactants (*20*, *21*). We are currently investigating this aspect through the study of various transposition mutant strains.

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