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Surface properties of *Yarrowia lipolytica* and their relevance to γ-decalactone formation from methyl ricinoleate

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Abstract

The surface of the lipid-degrading yeast, *Yarrowia lipolytica*, was characterized by contact angle and zeta potential (ζ) measurements. The cells were mainly hydrophilic with a negative charge that was only affected from pH 2 to 4. To study the effects of the surface charges on the biotransformation of methyl ricinoleate into the aroma compound, γ-decalactone, the ζ values of the substrate droplets were modified by adding a cationic surfactant into the medium at concentrations that did not diminish cell viability: the adhesion of the lipid substrate to the cells was increased but not the overall performance of the process, therefore the adhesion is not the rate limiting here. Our methodology offers interesting perspectives for further applications.

Introduction

Numerous biotechnological processes are based on the development of microorganisms within a biphasic medium formed by an oil-in-water emulsion. The complexity of such media makes difficult the effective control of the overall reactions. During the degradation of a hydrophobic substrate, direct contact between the microorganisms and the emulsion occurs. Physico-chemical factors, such as surface charges or hydrophobicity, are important in such adhesion phenomena. This has been observed using bacteria able to degrade oil or alkanes (Bredholt *et al.* 2002). The multiple factors involved deserve a more detailed study, notably in the case of yeasts of biotechnological interest. *Y. lipolytica* is one of these species, since it is used at industrial scale and studies for new potential applications are in course (Lanciotti *et al.* 2004). A current utilization of *Y. lipolytica* is the production of the aroma compound, γ-decalactone, from methyl ricinoleate, which is a well-documented process (Waché *et al.* 2003).

The cells' surface characterization has often been done in a relative manner by determining the percentage of adherence of a whole cell population to hydrocarbons (van der Mei *et al.* 1995) or solvents (Bellon-Fontaine *et al.* 1996), as we also did in previous work: correlations between the lactone productivity (Aguedo *et al.* 2004) or the growth lag phase of *Y. lipolytica* (Waché *et al.* 2000) and the interfacial surface of the substrate were evidenced. However, it was then hypothesized that the yeast assimilates the lipids through mechanisms requiring polar interactions, since an increase in the adhesion lipids/cells was associated mainly with an increase in Lewis acid–base interactions (Aguedo *et al.* 2003). Thus the surface charges may be of great relevance in the assimilation of hydrophobic substrates.

The aim of the present study was to go further into the above described aspects and to bring some

new insights in the characterization and in the role of surface properties and charges during degradation of lipids by yeast cells.

The cells' surface was characterized by contact angle measurements; the electrokinetic properties of the cells and of the substrate droplets were obtained from their zeta potentials (ζ). The ζ values of the lipid droplets were then artificially modified in order to increase their adhesion to the cells. The data enabled us to specify the relevance of surface properties in the degradation of lipids by yeast.

Materials and methods

Microorganism and media

Yarrowia lipolytica W29 (ATCC20460; CLIB89) was grown at 27 °C in 500 ml baffled Erlenmeyer flasks containing 200 ml medium and agitated at 140 rpm. After inoculation with 5×10^6 cells ml^{-1}, the pre-culture was carried out in a pH 5.6 adjusted medium containing per liter: 15 g glucose, 2.5 g NH_4Cl, 2.1 g KH_2PO_4, 3.6 g Na_2HPO4, 0.2 g $MgSO_4$ 7 H_2O, 0.1 g NaCl, 5 mg $FeSO_4$, 1 mg $CuSO_4$, 0.5 mg $ZnCl_2$ and 0.1 g yeast extract.

For γ-decalactone production, the cells in late growth-phase (19 h) were washed (6000 *g*, 5 min) three times in 9 g NaCl l^{-1} and transferred to the biotransformation medium at an initial concentration of 10^8 cells ml^{-1}. This medium was composed of methyl ricinoleate (purity ~80%, Stéarinerie Dubois, Boulogne, France) at 10 g l^{-1}, 2.5 g NH_4Cl l^{-1}, 6.7 g YNB l^{-1}, 1 g Tween 80 l^{-1} and made up to 200 ml with distilled water. The biotransformation was then conducted in 500 ml baffled Erlenmeyer flasks (27 °C, 140 rpm).

γ-Decalactone quantification

For extraction and analysis of γ-decalactone, 2 ml samples were extracted with diethyl ether and were analyzed by GC. All the technical details were already described (Aguedo *et al.* 2004).

Zeta potential measurement

The zeta potentials were determined by electrophoretic mobility with a Zeta-Meter System 3.0 (Zeta-Meter, Inc.; USA).

For the ζ values of the yeast, the cells were harvested by centrifugation at 2000 *g* for 10 min and then resuspended in 1 mM KNO_3 at various pH values (adjusted with KOH or HNO_3). When indicated, the cells were washed once (2000 *g* for 10 min).

For the measurement of the ζ of the emulsion droplets, the biotransformation medium was prepared as described above. It was then agitated during 1 h (140 rpm, 27 °C) without the cells; a 2 ml sample was then diluted to 20 ml in KNO_3 solutions at various pH values to achieve the measurements. When indicated, the cationic surfactant, cetyltrimethylammonium bromide (CTAB), was added into the medium at 0.1 mM in order to modify the ζ values of the lipid droplets.

Evaluation of the lipid droplets adhesion to the cells

This was done through optical microscopy, with the aid of a Neubauer improved slide. The yeast cells were first counted, then were the total lipid droplets adhering on their surface. For each slide, a minimum of 200 cells was counted. The results were expressed as %, the reference (medium without CTAB added) being equal to 100%.

Contact angle measurements

The surface tension and hydrophobicity of the yeast were determined by sessile drop contact angle measurements on cells lawns. The cell lawns were obtained by filtration of the cell suspension on a 5 cm diameter filter with a pore diameter of 0.45 μm. The filters were then cut into stripes and mounted on glass slides, and in order to standardize the moisture content, the slides were kept inside Petri dishes containing a solution of 20 g agar l^{-1} and 10% (v/v) glycerol. The determination of the contact angles was made with the aid of an image analysis system (Kruss-GmbH, Hamburg, Germany). The measurements were carried out at room temperature using three different liquids with known surface tensions: water, formamide and α-bromonaphthalene. The angles obtained on the lawns with these three liquids enabled to calculate, according to the Young–Good–Girifalco–Fowkes equation (van Oss *et al.* 1987), the total surface tension (γ^{tot}) and their components: Lifshitz–van der Waals (γ^{LW}) and Lewis acid–base (γ^{AB}) which in its turn has a positive (γ^+) and a negative (γ^-) component. The values of the free energy of interaction between cells and water

(ΔG^{tot}) were then calculated according to van Oss (1997).

Statistical analysis

All the reported data are from at least three independent experiments and the error bars on the graphs or the error values in the tables represent the standard deviations obtained with the values of these minimal three replicates.

Results and discussion

The surface characteristics of the lipid-degrading yeast, *Y. lipolytica*, were measured from the contact angles formed by sessile drops of three different liquids (two polar and one apolar) on uniform and homogeneous cells lawns to give an evaluation of the degree of hydrophobicity of the cells. These values then enabled surface tension and free energy of the cells to be calculated. As shown by the values in Table 1, the cells' surface is overall rather hydrophilic. After washing with distilled water, the angles formed by the polar liquids (water and formamide) increased, indicating that this treatment decreased slightly the cells hydrophilic character; however in this case the cells had no clearly defined character, as the angle formed by water was around 50° (Henriques *et al.* 2002).

The calculated total free energy of interaction between the cells (ΔG^{tot}) was in both cases higher than 0, meaning that the cells had more affinity for water than among themselves: this confirms the overall hydrophilic character of the cells.

Washing the cells with distilled water, on the one hand, decreased the total surface tension (γ^{tot}) but, on the other hand, increased their apolar character: the Lifshitz–van der Waals component, i.e. the apolar component, of the surface tension (γ^{LW}) increased and the Lewis acid–base component, i.e. the polar component (γ^{AB}), decreased (Table 1). In both tested conditions, the negative component of γ^{AB} (γ^-) was much higher than the positive one (γ^+), indicating that the cells are predominantly electron donors. The same characteristic was reported for the yeasts *Candida albicans* (Henriques *et al.* 2002) and *Saccharomyces uvarum* (Bránayik *et al.* 2004).

The fact that the water-washed cells presented a γ^+ value equal to 0, and were more apolar than non-washed ones indicates that washing the cells may remove some positively charged hydrophilic compounds from their surface. Moreover, the rather hydrophilic character of the cells and their negative charges, form a repulsive barrier and the cells do not aggregate spontaneously in the medium (not shown).

As the global surface charge of the cells is a fundamental parameter to understand the interactions within a culture or a biotransformation medium, the zeta potential of the cells was determined as a function of the suspension medium pH (Figure 1).

The obtained ζ values indicate that the yeast possess a net negative and almost constant surface charge (around −20 mV) at pH values higher than 2.5, which is the value where the isoelectric point of these cells stands. This pH value corresponds to the same isoelectric point as the one reported elsewhere for *S. cerevisiae* (Mozes *et al.* 1987). In fact, the medium pH exerted an important influence on ζ values for a pH values range from 2 to 4.

Table 1. Values of the contact angles, of surface tension (γ^{tot}) and its components (γ^{LW}), (γ^{AB}), (γ^+) and (γ^-), and free energy of interaction between cells and water (ΔG^{tot}), for *Yarrowia lipolytica* grown on glucose medium until late growth phase and then washed or not.

Treatment	Contact angle (°)			Surface tension (mJ m^{-2})					
	Water	BN	Formamide	γ^{LW}	γ^+	γ^-	γ^{AB}	γ^{tot}	ΔG^{tot}
No washing	35.3 ± 2.6	62.7 ± 3.2	16.3 ± 5.2	23.6	8	33.4	32.6	56.2	6.4
Washing with distilled water	54.9 ± 4.2	37 ± 6.4	53.4 ± 4.3	35.9	0	33.7	0.2	36.1	11.7

BN = 1-Bromo-naphtalene.

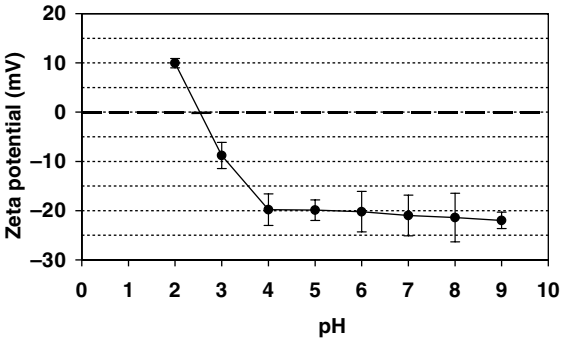

Fig. 1. Zeta potential of *Y. lipolytica* as a function of the medium pH (determined by electrophoretic mobility in 1 mM KNO_3).

Washing the cells with distilled water resulted in ζ values more negative, which was in accordance with what was previously obtained from the contact angle measurements. However, washing the cells with milder solutions (phosphate buffer, 50 mM pH 7.4, or 9 g NaCl l^{-1}) did not modify the global charge of the cells (Table 2).

According to van der Mei *et al.* (1993), the use of potassium phosphate buffers at various pH values (as in the present case), results in various degrees of protonation of surface groups and thereby affects the zeta potentials and then possibly the magnitude of the electrostatic interactions with the lipid droplets.

So far, it has been shown that yeast cells are hydrophilic and negatively charged for pH values higher than 4 and that washing the cells with distilled water may significantly alter their surface properties. This being so, it was decided to check on substrate droplets properties. The ζ values of the methyl ricinoleate droplets from the biotransformation medium were determined (before the introduction of the cells into the medium): they presented ζ values equal to 0 whatever the pH of the medium (not shown). It can therefore be assumed that the methyl ricinoleate droplets have a neutral and hydrophilic surface due to the coating with Tween 80: they are dissolved in the aqueous medium and so their attachment on the cells may occur through hydrophilic interactions and notably through electrostatic forces.

Table 2. Zeta potentials (ζ) of *Yarrowia lipolytica* after various treatments. The cells were previously cultured on glucose.

Treatment of the cells	ζ (mV)
No washing	−15.3 ± 1.7
Washing with phosphate buffer, 50 mM pH 7.4	−15.1 ± 2
Washing with 9 g NaCl l^{-1}	−16 ± 2.1
Washing with distilled water	−19.3 ± 1.4

The methyl ricinoleate droplets were initially neutral, in theory the addition of an ionic surfactant into the medium (already containing Tween 80), should modify the surface charges of the droplets and thus these could be altered in order to favor the contacts between the cells and the substrate, as it was already reported with animal cells (Dan 2002). Here, the cationic surfactant, CTAB, was added into the medium at pH 5.6. The ζ values obtained as a function of the CTAB concentration are reported in Figure 2. This surfactant gave positive ζ values to the oil droplets, reaching 30 mV for a concentration equal to 1 mM.

As CTAB can be toxic to yeasts, the viability of the cells was determined after 10 h spent in media containing different concentrations of this surfactant. The viabilities of the cells were comparable to that of the reference cells up to 0.1 mM CTAB, so these concentrations were tested on the biotransformation process (Table 3). First, the efficiency of the adherence between the cationic lipid droplets and the cells was checked: the presence of CTAB improved indeed the quantity of small methyl ricinoleate droplets adhering to the cells surface: from a factor 3 or 2, for, respectively, 0.02 or 0.1 mM of CTAB in the medium (Table 3). The adhesion did not occur in a proportional manner, which is not a paradox since it was reported elsewhere that the work of adhesion of cationic liposomes to negative cells as a function of the charge ratio of both entities is not described by a linear function (Dan 2002).

Aroma compound production was then determined in the presence of various concentrations of

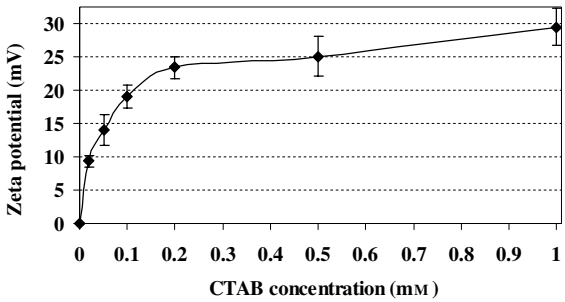

Fig. 2. Zeta potential of methyl ricinoleate droplets as a function of the CTAB concentration added to the biotransformation medium.

Table 3. CTAB concentrations tested and cell viability obtained in the different media; also the number of lipid droplets adhering to the cells, the initial γ-decalactone productivity and its maximum concentration, all three expressed as percents, reported to the reference (value equal to 100%), i.e. the medium containing no CTAB.

CTAB (mM)	Cell viability (%)[a]	% reported to the reference (without CTAB)		
		Lipids/cells adhesion	Initial lactone productivity	Lactone C_{max}[b]
0	99	100 ± 19[c]	100[d]	100 ± 15[e]
0.02	99	307 ± 14	86	70 ± 11
0.05	98	277 ± 44	66	50 ± 8
0.1	98	195 ± 39	19	19 ± 3

[a]Determined by methylene blue staining on cells samples after 10 h in biotransformation media.
[b]C_{max} = maximal concentration of γ-decalactone in the media after 10 h (Aguedo *et al.* 2004).
[c]100% corresponds to 4.3 lipid droplets per cell.
[d]100% corresponds to 8.3 mg l^{-1} h^{-1} with our biotransformation conditions.
[e]100% corresponds to 101.5 mg l^{-1}.

CTAB: the relative initial productivity of γ-decalactone and the maximal concentrations reached in the medium are presented in Table 3. In fact, increasing concentrations of CTAB brought about a decrease in the biotransformation yields, diminishing the initial productivity of γ-decalactone and also the maximal concentration of that compound: a fivefold decrease is obtained in both cases with 0.1 mM CTAB.

The interfacial surface of the lipid droplets can influence the lactone productivity (Aguedo *et al.* 2004): here, according to microscopy observations, no modification of the droplets size was observed in the presence of the different concentrations of CTAB (not shown); so this could not be a cause for the decrease in lactone production. These elements indicate that the modification of the charges and as a consequence, of the adhesion between the cells and the substrate does not favor the production of γ-decalactone, i.e. also the degradation of the hydrophobic substrate.

Conclusions

The interactions between microorganisms and oil-in-water emulsions are relevant in several biotechnological domains (food, environment or fine chemicals production). The interactions of *Y. lipolytica* with methyl ricinoleate droplets were studied here.

Y. lipolytica can use hydrophobic substrates, however, the cells are hydrophilic and negatively charged in the same range as some other yeasts unable to grow on lipids, as *S. cerevisiae*.

Washing the cells with distilled water decreased their surface hydrophilicity. Increasing the cells/substrate contacts did not improve the biotransformation, therefore it is not a main factor in the assimilation of the hydrophobic substrate. The uptake of dissolved lipids by a transport system in the cell's membrane or cell's wall may be a more relevant point for the process.

Controlled modifications of the surface charges of lipids can be used to alter the interactions between cells and the substrate. Thus, the measurement of the ζ values of the cells and of the substrate droplets prior to starting a process could be envisaged when the control of the interactions can lead to the optimization of the overall reaction rate.

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