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Research article

Encapsulation of flavours into *Yarrowia lipolytica* active yeast cells. Fluorescence study of the lipid droplets morphology and steryl/sterol balance during the shock

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Abstract: Yeast are a powerful material for the encapsulation of compounds. Usually, yeast used as capsules are inactivated by the encapsulation treatment, which is stressful to cells. However, if kept active, cells can bring their own activity in addition to the encapsulated compound. We have observed previously that lipid-grown *Yarrowia lipolytica* were more resistant to encapsulation. The objective of the present study was to identify physiological markers involved in this resistance. Cells were cultured in the presence of glucose or methyl-oleate as the sole carbon source and submitted to a γ-dodecalactone stress. This paper focuses on the role of intracellular lipid droplets (LDs) and of the ergosteryl content to protect cells during the lactone treatment. Lipid-grown cells were more resistant to lactone and the presence of LDs before the shock increased significantly the resistance. The ergosteryl esters from the LD pool were hydrolysed to release ergosterol able to strenghten the plasma membrane during the shock. For cells devoid of LDs, membrane ergosterols were esterified concomitantly with LDs growth, resulting in a membrane weakening. By using *pox3*-mutant strains,

which possesse numerous and small-sized LDs, we observed the original behaviour: these mutants showed no increased resistance and their LDs exploded in the cytoplasma during the shock. These results point out the role of LDs in cell resistance to amphiphilic stresses as a storage compartment as well as in ergosterol homeostasis.

Keywords: *Yarrowia lipolytica*; encapsulation; active cells; lipid droplets; sterol-steryl ester conversion; lactone; acyl-CoA oxidase

1. Introduction

Capsules are widely used in medicine, food, environment, textiles etc to protect actives and target them to a special place or time. Yeasts are becoming a popular material for this application as they possess efficient protective properties and as they can encapsulate more than 50% w/w of actives [1]. Moreover, their biological properties can bring interesting characteristics compared to inert structures [2]. It is sometimes important to have inactive cells to keep the product stable, for instance for some food applications. However, active yeasts can be precious in some cases as yeasts can adhere to the target cell, which increases metabolisation of the compound, or living yeast capsules can be used as a starter in fermented foods. Unfortunately, the step of encapsulation is a drastic shock resulting usually in a loss of activity, especially when amphiphilic or hydrophobic actives are used. These compounds, as for instance alkanes, alkanols and lipids have a high affinity for biological membranes. Most of them are known as molecules causing a certain perturbation in the microorganism membrane functions [3,4]. However, some of them, like decanol or methyl oleate, have been reported to intercalate into membranes without perturbation of cell activity [3]. Results obtained in our laboratory have confirmed that methyl oleate causes a membrane fluidisation in the yeast *Yarrowia lipolytica* but does not perturb cell viability [5]. On the contrary, growth on methyl oleate as the source of carbon makes cells of *Y. lipolytica* more resistant to environmental stresses such as those caused by the presence of amphiphilic compounds like lactones [5]. This latter chemical family is known for its toxicity towards *Y. lipolytica* cells as reported by Aguedo et al. [6–8]. The molecule targets the lipid-rich cellular membranes and perturbs their function by increasing the fluidity of the membrane, decreasing its integrity and dissipating its energy.

It has been shown that, for encapsulation in yeast cells, the presence of cytosolic lipid particles called lipid droplets (LDs) improves significantly yields. Apart from encapsulation processes, LDs are involved in neutral lipid storage [9]. These particles are made of a highly hydrophobic core formed from neutral lipids (triacylglycerol and steryl esters with approximately 50% each in the stationary phase of *Saccharomyces cerevisiae*) surrounded by a phospholipid monolayer in which only a few proteins are embedded [10–12]. In the *n*-alkane-assimilating yeast *Y. lipolytica,* there are five acyl-coenzymes A oxidase isozymes (AOX1 through AOX5), encoded by *POX1* through *POX5* genes (Wang et al., 1999). The deletion of these latter genes leads to changes in the number and size of LDs [13].

Several observations suggest that sterols, mainly ergosterol in yeast, could be included in the mechanisms of cellular response against extracellular stresses [14] and toxic compounds [15,16]. Sterols can be present in cells under two forms. The free form contributes to the membrane structure and can be included into sterol sphingolipid-enriched micro-domains (rafts) that are involved in membrane trafficking and cell signalling [17]. Sterol is also present under the form of steryl esters

which are sequestered together with triacylglycerol (TAG) in lipid droplets [9]. These two forms of sterol in cells can be converted one to the other when needed [18]. In *S. cerevisiae,* this implies several enzymes such as steryl ester hydrolases Tgl1p, Yeh1p, Yeh2p and sterol acyltransferases Are1p, Are2p [11,19]. This interconversion may contribute to the cellular response towards environmental stresses [18,20]. In *Y. lipolytica,* Are1p is, according to [21], the only esterase active on steryl esters and Tgl1p has been detected as being the steryl ester hydrolase.

Our past results have shown that *Y. lipolytica* cells grown in lipids contained in total more ergosterol (free and ester forms) than cells grown in glucose [5,22]. It was also shown that γ-dodecalactone, a flavouring amphiphilic active, provoked the cellular depletion of ergosterol in a way depending on the medium on which cells were grown, highlighting thereby the role of the carbon source in the resistance to extra-cellular stresses. Between the different sources of carbon tested, glucose and oleate, the sterol depletion by lactone was more important in glucose-grown cells, which expressed also a more important sensitivity towards the lactone encapsulation stress. In glucose grown cells, an incubation with 3 g L^{-1} γ-dodecalatone during 150 min could deplete about 27% of cellular sterol corresponding to a decrease of 56% in cellular viability. In oleate grown cells, there was about 16% of sterol depleted and no cells were detected as dead [5]. This depletion in glucose-grown cells could explain the loss of activity but also, depletion and loss of activity might be two results of a membrane structure less adapted to the presence of amphiphilic products. In addition to the source of carbon, a second factor could be important as observed for cells grown on oleate. These cells, if grown on a minimum medium (YNBO, without peptones), underwent a sterol depletion which was also important in the presence of lactones. If grown on a rich medium (YPO, containing peptones and yeast extracts), Almost no sterol depletion was observed.

The objective of the present study was to investigate the role of lipids during encapsulation and its physicochemical stress provoked by dodecalactone. Our attention was given to the morphology of LDs, organelles that serve as the structure of encapsulation. In particular, we studied the potential of loading this organelle to extract lactone from the cell and its potential to make ergosterol available for the structuration of membranes as well as to extract membrane ergosterol for the structuration of LDs.

2. Material and methods

2.1. Strain, media, culture conditions and lactone treatment

The strain *Y. lipolytica* W29 (ATCC20460: CLIB89) and its mutants (table 1) were cultured in YPDA medium (Yeast extract Peptone Dextrose Agar: yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l, agar 15 g/l) at 27 °C for 48 h and used to inoculate the liquid media YPD (YPDA without agar), YNBO (yeast nitrogen base 6.7 g/l, methyl oleate 5 g/l, Tween 80 0.2 g/l), YPO (yeast extract 10 g/l, peptone 20 g/l, methyl oleate 5 g/l, Tween80 0.2 g/l) and grown at 27 °C, 140 rpm in a baffled erlenmeyer flask as previously described [5]. Cells in the mid-logarithm growth phase were treated with γ-dodecalactone at different concentrations up to 3 g/l and incubated at 27 °C, 140 rpm. The treated cells were harvested after different times for viability estimation, fluorescent staining or sterol analysis. As lactone was prepared in ethanol, a control was made with 1% v/v ethanol.

Table 1. *Y. lipolytica* strains used in this study.

Strain	Genotype	Reference
W29	Wild type	(Barth and Gaillardin 1997)
MTLY37	*Δpox2Δpox3Δpox5 pox4::URA3*	(Wang et al. 1999)
MTLY40-2p	*Δpox2Δpox3Δpox4Δpox5POX2-URA3*	(Mlickova et al. 2004)
MTLY40-3p	*Δpox2Δpox3Δpox4Δpox5POX3-URA3*	(Mlickova et al. 2004)

2.2. Cell viability

Cell viability was estimated in triplicate through the count of colony forming unit (CFU): successive decimal dilutions of cell suspensions were plated on YPDA and the colonies were counted after 48 h incubation at 27 °C.

2.3. Fluorescent observation of lipid droplets (LDs)

Cells were observed after staining with nile red and calcofluor white as described earlier [22].

2.4. Lactone extraction and analysis by gas chromatography

To evaluate the concentration of non-metabolised lactone, γ-dodecalactone was extracted and analysed as previously described [5]. Whole cells comprising the cell wall were extracted with ethyl ether.

2.5. Ergosterol analysis

Cells exposed to lactone at different concentrations were harvested after 150 minutes for ergosterol extraction.

Total intracellular sterol was extracted after saponification as reported by [23] with modification as previously described [5]. Contrary to this KOH-based extraction, free sterol was extracted as described earlier [24] with modifications. Yeast cells were collected, washed twice with sterilised distilled water and weighted. Washed cells (< 60 mg of cell dry wt or about 1 g of wet cells) were immediately resuspended in 3 ml of 80% (v/v) ethanol, and incubated at 80 °C for 15 min, to inactivate lipolytic enzymes. The ethanol extract was separated by centrifugation as above, and cells were frozen overnight. Frozen cells were then resuspended in 3 ml PBS buffer (20 mM, pH 7.0) in a glass tube containing 3 g glass beads (diam. 0.425–0.6 mm, Sigma) and broken by vortex for 3 min. 3 ml of *n*-heptane were then added into the same tube and vortexed for 3 min for sterol extraction. The heptane layer was transferred to a clean borosilicate glass screw-cap tube and stored at -20°C for 24 h before analysis. Analyses and concentration calculation were carried out as previously described [5].

The quantity of steryl esters was calculated from the difference between the quantity of total and of free sterol.

3. Results

3.1. Lactone toxicity towards cells depends on the medium where cells have been cultured but not lactone encapsulation

In a previous work [5], we have observed a different toxicity of γ-dodecalactone towards cells of *Y. lipolytica* depending on the culture medium used to grow cells. The cell uptake of a lactone series was also shown to depend on the lactone physical properties [25]. Here we wanted to check whether this difference in toxicity was related to a difference in the lactone uptake by cells. Cells were thus submitted to the lactone encapsulation shock in PBS buffer in standardised conditions of cells and lactone concentrations. The lactone uptake into the cells began instantaneously for cells grown on all three media. The accumulation was observed in the same amplitude for cells grown either on glucose or on lipids (Figure 1). However, the cells grown on the minimal lipidic medium (YNBO) were very rapidly loaded with about 60 mg of lactone/g of cells while for the two peptone media, the initial loading was slower. For an encapsulation concentration of 100 mg/g of cells, corresponding to an Encapsulation Yield (EY%: ratio of the mass of active material in the capsule to the mass of the capsule) of 10%, an Encapsulation Efficiency% (EE%: total amount of product encapsulated (≈400 mg/L) to the initial input of this product) of around 14% was observed. However, the cell viability after the shock depended on the culture media. The cell resistance was thus not dependent on the amount of lactone accumulating intracellularly but on the original culture medium.

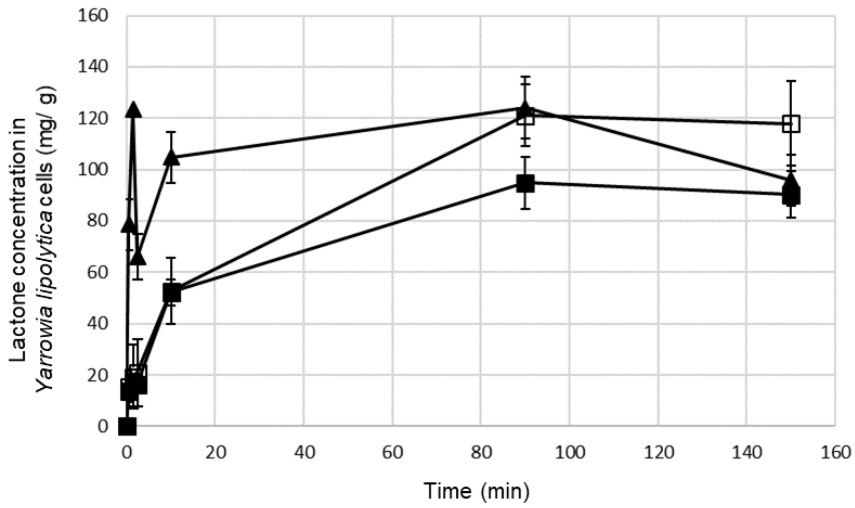

Figure 1. Lactone uptake into cells when incubated in PBS buffer with 3 g L^{-1} γ-dodecalactone. Prior to this incubation, cells were cultured in different media: (□) YPD; (■) YPO; (▲) YNBO.

3.2. The lactone shock provokes morphological changes in lipid droplets

Observation under light microscopy showed differences in morphology between cells from different culture media (Figure 2). We have observed cells with the Nile red fluorescent probe that stains neutral lipids in the cells. Normally, lipid reserves accumulate during the stationary phase of

growth. Here, we observed big LDs present in the cytoplasm of cells grown in the lipid rich medium (Figure 2C). In contrast, only one or two small red points corresponding to lipid storage spots were observed in cells grown in glucose (Figure 2A). For the minimal lipid medium YNBO only, beside some small cytosolic LDs, we have also observed several lipid droplets on the cell surface (Figure 2B). These observations were typical of cells coming from each culture medium and about 90% of the cells exhibited these characteristics. For YNBO cells, the presence of lipid droplets adsorbed on the cell surface may explain the rapid initial loading of lactone.

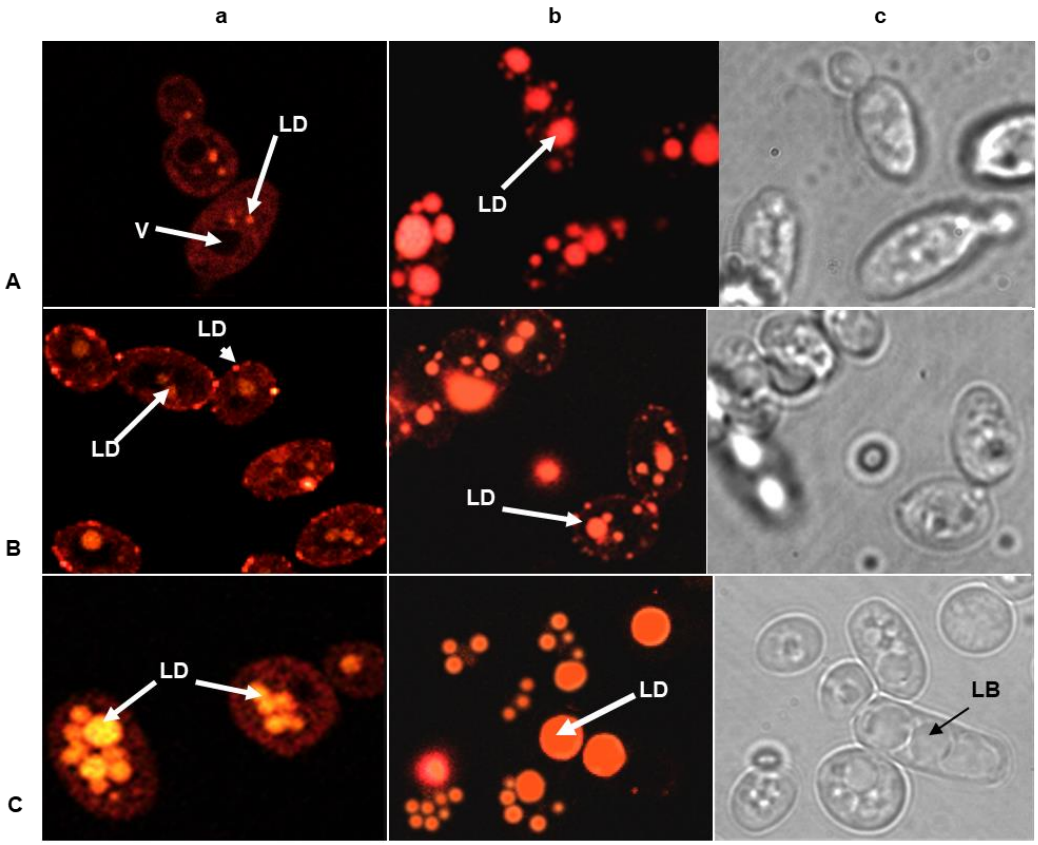

Figure 2. Nile red stained-lipid bodies observation under confocal (a,b) and light (c) microscopy of cells cultured in different media (A) YPD, (B) YNBO and (C) YPO before (a) and after a 60-min-incubation with lactone (b,c). V: vacuole; LD: lipid droplets.

Despite this initial difference, once cells were shocked with lactone, several big LDs could be observed for all three media (Figure 2). For lipid media, in about 60% of the cells, LDs were significantly bigger although in some cells a huge LD cohabitated with smaller ones.

3.3. Sterol homeostasis in response to the lactone encapsulation stress

Our previous results pointed out differences in total sterol concentrations depending on the culture medium. As sterol is an important component of membranes whereas steryl ester is present in LDs, it seemed interesting to investigate sterols during encapsulation. The concentration and ratios of the two forms of sterol in cells when incubated with different concentrations of lactone and different times

were investigated. They are given in Figure 3 and Table 2 with ratios depending on lactone concentrations (Figure 3A), times (Figure 3B) and sterol concentration (Table 2). The concentration of free sterol slightly increased during incubation with lactone in lipid media while it strongly decreased for the glucose medium in comparison with controls without lactone incubation. This diminution of free sterol in glucose-grown cells was correlated with lactone intracellular accumulation (Figure 1 and Table 2). With our attention given to LDs, the results showed that cells expressed in different ways their response to the lactone shock depending on their culture medium. For glucose grown cells, which exhibited only one or two small LD, the concentration of free sterols decreased while the one of steryl esters increased (Figure 3 and Table 2) corresponding to the transformation of small LDs into big ones in the cytoplasm (Figure 2A). This supposes a conversion from free sterols into steryl esters. Lipid grown cells exhibited an opposite behaviour: for both rich (YPO) and minimal (YNBO) media, steryl esters decreased and free sterols increased concomitantly with the coalescence of LDs, which supposes a conversion from steryl esters into free sterols (Figure 2B, 2C, 3 and Table 2). This increase in the free sterol ratio occurred from low lactone concentrations and was very rapid for YPO-grown cells whereas it was slower for YNBO-grown cells.

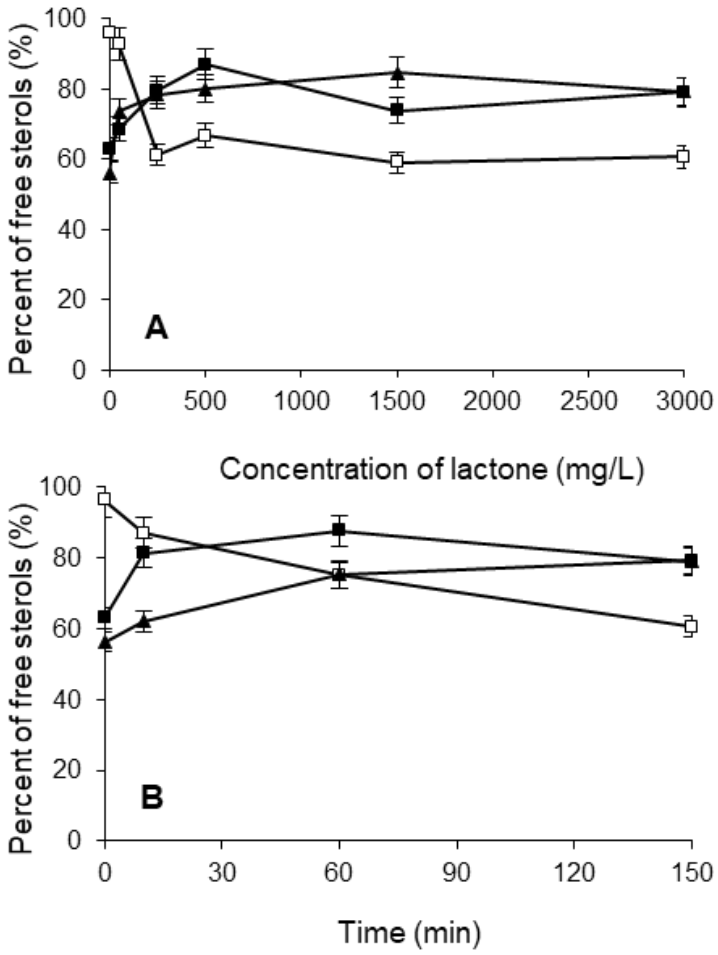

Figure 3. Evolution of the ratios of free sterol (A, B) to total sterol (free sterol + steryl esters) when incubating cells with lactone: (A) after 150 min at different lactone concentrations; (B) at 3 g L^{-1} lactone during 150 min. Prior to this incubation, cells were cultured in different media: (□)YPD; (■) YPO; (▲) YNBO.

Table 2. Concentration of sterol and steryl ester in the three media after 150 min incubation with or without 3 g/L dodecalactone.

C/	Sterol (µg/g of cell)	steryl ester (µg/g of cell)
YPD	52.7 ± 3.3	0.1 ± 3.3
YPD+3 g/L lactone	22.9 ± 4.1	15.4 ± 4.1
YNBO	38.6 ± 1.2	22.8 ± 4.1
YNBO +3 g/L lactone	30.0 ± 1.5	8.4 ± 3.9
YPO	31.4 ± 1.6	25.4 ± 2.8
YPO+3 g/L lactone	37.8 ± 3.8	10.2 ± 2.4

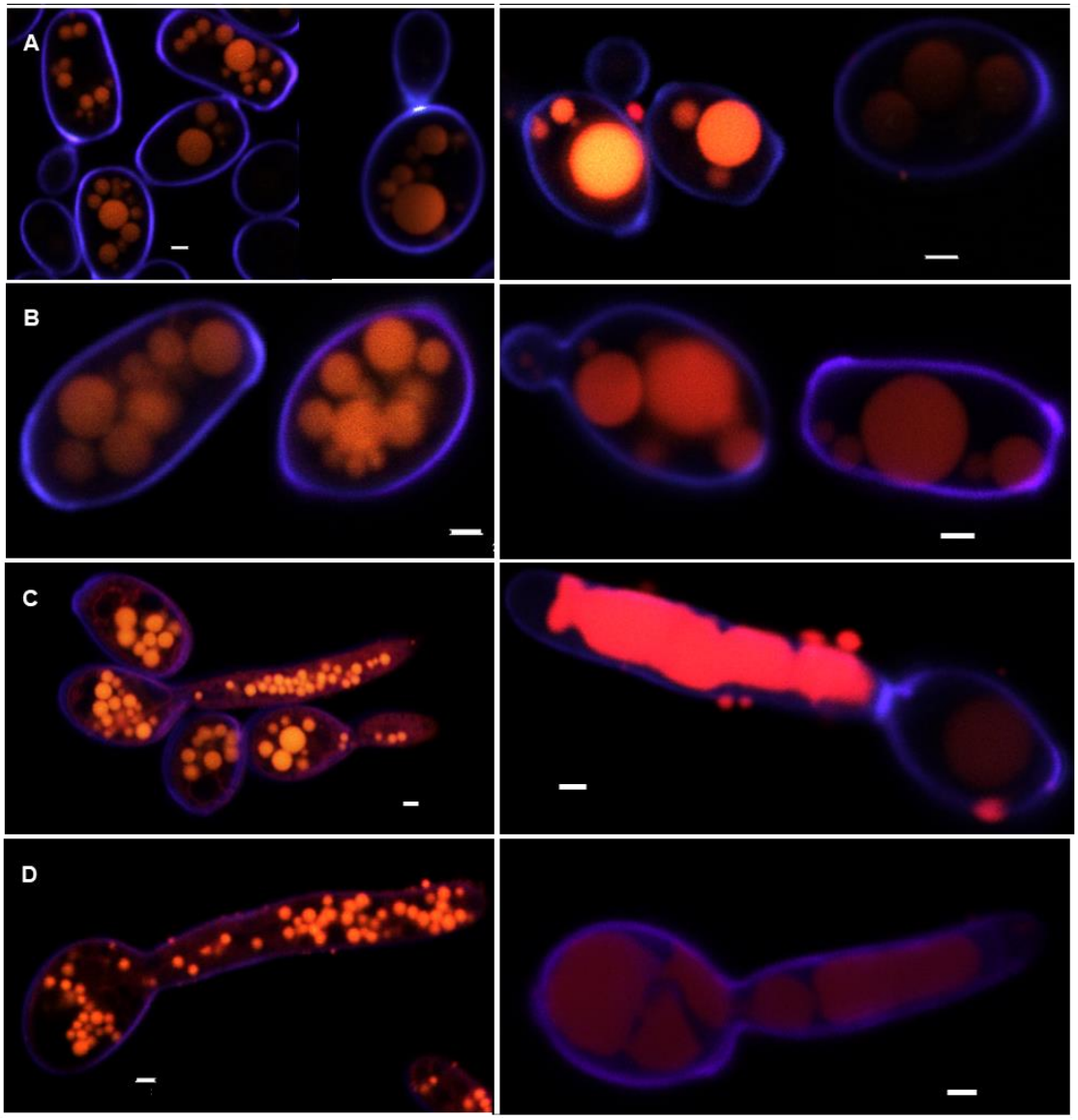

Figure 4. Nile-red-stained lipid bodies observation in different mutants of *Y. lipolytica* cultured in YPO medium: before (left side) and after a 60-min-incubation with lactone (right side). (A) W29; (B) MTLY40-2p; (C) MTLY40-3p; (D) MTLY37. Bar represents 1 µm; Cells were stained with calcofluor for easier visualisation.

The results above highlight the effect of the presence of lipid droplets prior to the lactone shock. It seemed thus important to investigate the effect of the lipid droplets structure as it has been shown to be impacted by the presence of acyl-CoA oxidase encoding genes (*POX*) [13]. In that goal, we have investigated the effect of the culture medium on the lipid droplets morphology of strains of *Y. lipolytica* exhibiting different *POX* genotypes and their evolution during the γ-dodecalactone stress (Figure 4).

After growth on glucose, the LDs of the various strains (not shown) were comparable to the one of the wild type, with only one or two very small visible particle. After growth or contact with methyl oleate, *POX2* possessing strains exhibited some medium size droplets whereas △*pox2* strains had a lot of very small droplets. For all of these △*pox2* strains, the lactone shock provoked the coalescence of LDs. The resulting hydrophobic phase flooded through most of the cell and the lipid phase form adopted the cytoplasmic form (4C and D, right). Contrasting with them, *POX2* strains had big spherical droplets (Figure 4A and B) Interestingly, all the mutant strains exhibited a higher resistance to lactone when grown on glucose (about 60% cells kept their growing capacity compared to 23% for the WT, Figure 5). When grown on lipid, the cell resistance was significantly higher for *POX2* strains but remained unchanged for △*pox2* strains.

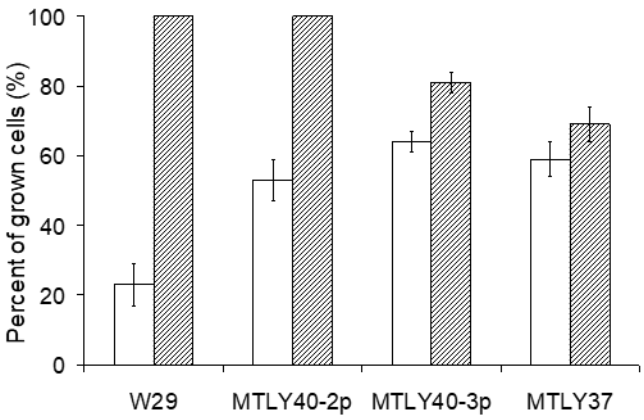

Figure 5. Effect of the culture medium on lactone resistance of different *pox* mutant strains of *Y. lipolytica* evaluated using CFU. For each mutant, the left bar corresponds to YPD medium and the right bar to YPO medium.

4. Discussion

The present paper deals with the encapsulation of a medium chain-length active, γ-dodecalactone, in yeast lipid droplets. We studied the implication of the lipid droplets in the mechanism of response of the yeast *Y. lipolytica* to the stress generated by this compound. As shown earlier, cells grown in glucose exhibit a higher sensitivity to lactone than cells grown in lipids as the carbon source [5]. Using a lipid fluorescent stain, we observed that the morphology of lipid droplets changed during the incubation with lactone. This change occurred rapidly (in only 10 min, results not shown) and was observed for cells cultured on all types of media regardless of the nature of the carbon source. Normally, the formation of LDs takes place in the stationary phase and includes the synthesis of steryl esters. It depends also on the carbon source [26]. This carbon-source dependent synthesis of LDs together with the medium dependent yeast behaviour in response to a lactone shock strengthened our hypothesis that

LDs could be involved in the response to this stress. Moreover, we have also observed changes in sterol homeostasis–interconversion between free sterols and steryl esters when cells underwent such a shock. For glucose grown cells, there were almost no steryl esters before the shock (Figure 3D). So, a rapid increase in steryl esters in glucose grown cells in contact with lactone supposes a conversion from free sterol into steryl esters that would thus be involved in LD formation and structure (Figure 2A). This conversion may be stronger than observed as a significant part of the cell population was inactivated by the shock (the CFU decreased of 77% but cells unstained with methylene blue or propidium iodide (active cells) decreased to a lower extent of only 31 and 38%, respectively [5]). It is unknown how long cells keep their ability to convert sterol during the sequence of events leading to the loss of activity caused by the stress (loss of growing ability (CFU) => loss of membrane integrity (propidium iodide) => loss of methylene blue excretion/metabolisation). Moreover, it is not known whether this transformation of membrane protecting sterols into LD structurating steryl esters increases or decreases cell activity.

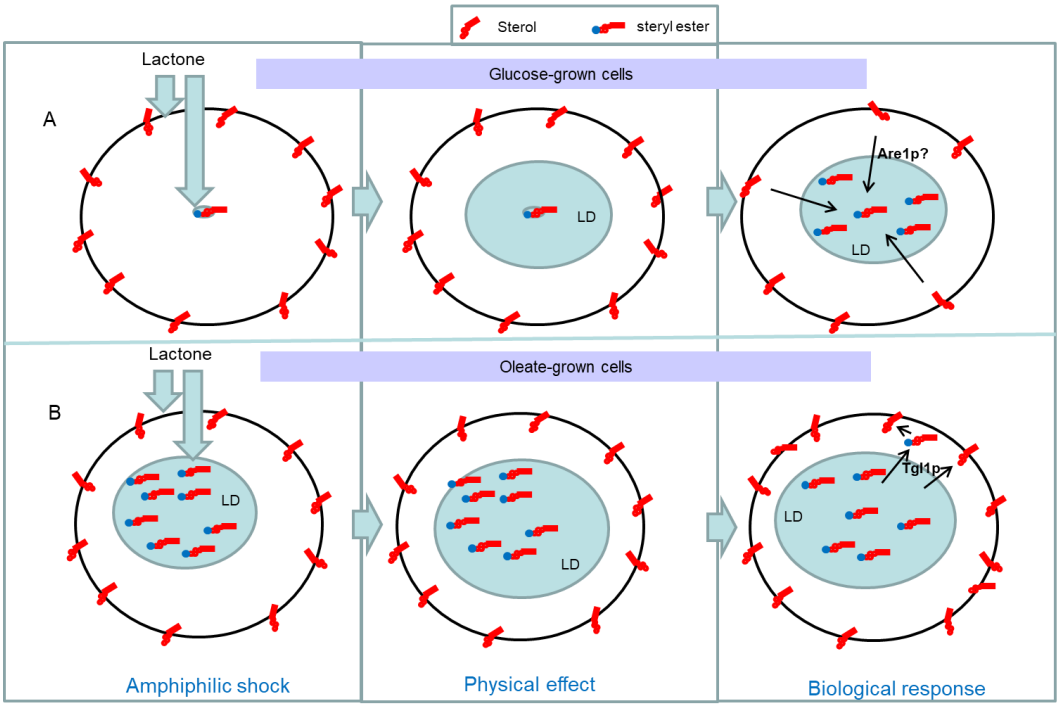

Figure 6. Putative mechanism of response of ergosterol homeostasis to a shock provoked by the medium chain length amphiphilic compound γ-dodecalactone. Left: shock, centre: lactone invades the plasma membrane and make the lipid droplets (LDs) grow, right: cell response to the shock. A: cells grown on glucose have almost no LDs and they use their membrane ergosterol to structure the growing LDs therefore making their plasma membrane more sensitive to lactone. B: cells grown on lipids have already LDs possessing steryl esters (SE). Despite the growing of LDs, SE are hydrolysed to give free sterols to protect membranes.

On the contrary, for lipid-grown cells, a decrease in steryl ester in parallel with an increase in free sterol suggests an inverse conversion. In yeast, steryl esters and free sterols are interconvertible

depending on the conditions. Under sterol depletion caused by the incubation of yeast cells with terbinafine, an inhibitor of erg1 required for squalene formation, steryl esters are mobilised very rapidly even in exponential growth phase to respond to the free sterol demand for membrane formation [20]. Lactone depletes also sterols in membrane when cells are incubated in their presence [5]. We suppose that the target of the lactone is the plasma membrane. This molecule focuses on the membrane lipids/sterols rich structure. But the way cells react to respond to this stress is not the same for glucose and lipid grown cells. Lipid grown cells use a sterol precursor stored as steryl ester in the LDs to furnish free sterol to protect membranes from damages. This response is not possible in glucose grown cells which have almost no LDs and thus, no steryl reserves. This explains the higher sensitivity of these cells to the lactone shock. Surprisingly, these cells still decrease their amount of sterol in presence of lactone as they convert their free sterol into steryl esters to form LDs. This result suggests that there is not only one mechanism implicated in the cell response to a lactone shock, and the presence of LDs seems to be the first requirement for cell resistance to this amphiphilic compound. The putative mechanism is presented in Figure 6. The presence of lactone induces probably the enzymes implicated in sterol/steryl interconversion which are the steryl esters hydrolase Tgl1p, and the sterol esterase Are1p. In *S. cerevisiae*, the steryl esters hydrolases are located in LD [27] while the sterol esterases are located in the endoplasmic reticulum [19]. [28] have analysed protein extracts of *Y. lipolytica* grown in glucose and methyl oleate. The extracts of lipid-grown cells showed more proteins (or putative proteins) implicated in sterol synthesis, such as Tgl1p or Erg6p, than the extracts of glucose grown cells. This may explain how oleate grown cells could maintain their free sterol level by converting from their steryl esters reserves to respond to the lactone shock.

In *S. cerevisiae*, the steryl/sterol pool regulation has been studied showing a feedback regulation of steryl ester formation by steryl ester hydrolysis [29]. However, although hydrolysis enzymes were regulated at the transcriptional level, sterol esterases were regulated through an enzyme inhibition at the enzyme level.

From the results obtained, we have observed that the presence of LDs played a role in preserving cells during the encapsulation of lactone. Moreover, structured LDs coalesced less then small LDs and they kept their integrity. MTLY37 (Δ*pox2*Δ*pox3*Δ*pox5 pox4*::*URA3*) cells and wild type glucose grown cells exhibited also an accumulation of lipid droplets but these coalescent LDs were broken during the lactone treatment which resulted in a high cell mortality (Figure 5C, D). MTLY40-2p (Δ*pox2*Δ*pox3*Δ*pox4*Δ*pox5POX2-URA3*) and wild type lipid grown cells accumulated many LDs that coalesced but kept their structure during the lactone shock. For them, cell viability was unchanged (Figure 5A, B). Such a LDs behaviour has already been observed for cells lacking a perilipin in *S. cerevisiae* [30] and can have interesting applications in encapsulation.

Lipid droplets have been known for long time in yeast and they are now the subject of a huge research effort in biotechnological projects of oleaginous yeasts as well as in encapsulation. Their implication into some stress responses has also been reported [31] but our study shows for the first time a role of LDs in cell response to a stress driven by the active to be encapsulated. The acyl coA oxidase isozymes Aox2p and Aox3p are also proposed to be implicated in cell response mechanisms to the lactone stress due maybe to their low capacity of lactone degradation on one hand, but mainly to their role in LD formation on the other hand [32,33]. The homeostasis of sterol is an interesting point explaining the survival of *Y. lipolytica* during encapsulation and this phenomenon has to be further studied to develop applications of encapsulation into active yeast cells.

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Conflict of interest

All the authors declare that they have no conflict of interest.

Author contributions

TMNT, CRG and YW conceived and designed research. TMNT, CRG, HPT, HDT and HTD conducted experiments. TMNT, CRG and YW analysed data and wrote the manuscript. All authors read and approved the manuscript.

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