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Morphogenetic Behavior of Tropical Marine Yeast *Yarrowia lipolytica* in Response to Hydrophobic Substrates

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The morphogenetic behavior of a tropical marine *Yarrowia lipolytica* strain on hydrophobic substrates was studied. Media containing coconut oil or palm kernel oil (rich in lauric and myristic acids) prepared in distilled water or seawater at a neutral pH supported 95% of the cells to undergo a transition from the yeast form to the mycelium form. With potassium laurate, 51% of the cells were in the mycelium form, whereas with myristate, 32% were in the mycelium form. However, combinations of these two fatty acids in proportions that are present in coconut oil or palm kernel oil enhanced the mycelium formation to 65%. The culture also produced extracellular lipases during the morphogenetic change. The yeast cells were found to attach to the large droplets of the hydrophobic substrates during the transition, while the mycelia were associated with the aqueous phase. The alkane-grown yeast partitioned more efficiently in the hydrophobic phases when compared with the coconut oil-grown mycelia. A fatty acid analysis of the mycelial form revealed the presence of lauric acid in addition to the long-chain saturated and unsaturated fatty acids observed in the yeast form. The mycelia underwent a rapid transition to the yeast form with n-dodecane, a medium-chain aliphatic hydrocarbon. Thus, the fungus displayed a differential behavior towards the two types of saturated hydrophobic substrates.

Keywords: *Yarrowia lipolytica*, dimorphism, hydrophobic substrates

Several fungi are able to switch between two morphological forms, namely yeast and mycelial, making them dimorphic. This phenomenon has been reported in pathogenic as well as nonpathogenic fungi, where *Yarrowia lipolytica*, *Benjaminiella poitrasii*, and *Mucor circinelloides* are examples of nonpathogenic dimorphic fungi [4, 11, 16]. Such fungi are often used as model systems for understanding eukaryotic cell differentiation, and are also significant in studies related to the adaptation of microorganisms to different environmental and ecological conditions. For example, the dimorphism of *Mucor circinelloides* is accompanied by a number of noticeable physiological and biochemical changes [16], whereas strains of *Y. lipolytica* undergo morphological changes under a variety of conditions, indicating the presence of different signaling pathways for dimorphic transition [28].

Y. lipolytica is often isolated from environments containing hydrophobic substrates, such as alkanes or triglycerides [5], leading to the use of alkanes for the production of organic acids [3] and manipulation of the genes for alkane degradation [15]. From a biotechnological point of view, this triglyceride-utilizing fungus has been used for the production of lipases and esterases [13, 14, 29], and single-cell oil [25], and in the treatment of wastewater [22]. In a previous study, a tropical marine strain of *Y. lipolytica* isolated by the current authors was found to utilize a variety of hydrophobic substrates, and was also applied to the degradation of aliphatic hydrocarbons and triglycerides in palm oil mill effluent (POME) [22, 34].

Furthermore, this *Y. lipolytica* isolate was found to undergo a dimorphic transition from a yeast form to a mycelial form in the presence of N-acetyl glucosamine (GlcNAc) under partially anaerobic conditions, indicating that such a transition may allow the isolate to scavenge oxygen [34]. The present authors also previously demonstrated that the fungus exists in the yeast form on alkanes. However, the present paper describes the unusual morphogenetic behavior of this *Y. lipolytica* strain in response to the presence of oils containing lauric acid and myristic acid. In previous literature, certain strains of *Y. lipolytica* have been reported to display a preference for fatty acids. Moreover, under such conditions, certain biochemical characteristics are also affected [18, 25, 27]. These *Y. lipolytica* strains

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have thus been used to modify the fat composition of substrates and storage of lipids [26, 32, 27]. In addition, a morphological analysis of *Y. lipolytica* cells grown on oleic acid has also been reported [17], where the cells showed the presence of specialized structures (protrusions and surface-bound lipid droplets) that were lacking in glucose-grown cells. However, there have been no reports on changes in the morphological form in response to the presence of specific fatty acids. Accordingly, the present study reports on the correlation of the fatty acid composition of the medium with mycelium formation, where saturated fats composed of medium-chain fatty acids resulted in enhanced mycelium formation. Therefore, the results indicate that this response may be a possible means of adaptation by the organism to its ecological niche.

MATERIALS AND METHODS

Microorganism, Maintenance, and Pre-Inoculum Preparation

A strain of *Y. lipolytica*, NCIM 3589, isolated from a tropical marine environment was used in all the experiments. The fungus was previously identified based on its morphological features, biochemical characteristics, fatty acid methyl ester profiles, the presence of a double-stranded RNA plasmid-like structure, and G+C content in the DNA [34]. The fungus is able to adapt to high salt concentrations and can grow in fresh water, as well as seawater-based media. The yeast cells were inoculated into a medium containing 7 g/l yeast nitrogen base (YNB) in distilled water with 5 g/l glucose, and incubated aerobically at 30°C for 24 h. The culture was then centrifuged at 6,000 ×*g* for 10 min at 4°C, and the harvested cells were washed and resuspended in saline. Thereafter, a standard inoculum containing 2×10^6 cells/ml was inoculated into the YNB media (50 ml in 250-ml Erlenmeyer flasks) to study the dimorphic transition. The flasks were incubated aerobically at 30°C for 72 h and the morphology was observed.

Monitoring of Cell Morphology

Aliquots were withdrawn at regular intervals and observed microscopically, as described earlier [34]. The sample examination was carried out on a hemocytometer grid using a Zeiss microscope (Axioskop 40). Single or budding cells were counted as one yeast morphological unit, and cells with germ tubes were considered as one hyphal morphological unit. All values are expressed as percentages.

Effects of Different Oils on Dimorphic Transition

A basal medium containing 7 g/l YNB in distilled water was used. All the carbon sources were tested at a concentration of 5 g/l and included peanut oil, sesame oil, olive oil, coconut oil, and palm kernel oil. The oils were previously emulsified in 5 g/l gum acacia and sonicated for three cycles of 30 sec. YNB media containing glucose or GlcNAc were used as the controls.

Effects of pH and Salinity on Morphology of *Y. lipolytica* NCIM 3589

The standard suspension was inoculated into the YNB medium prepared in a 50 mM citrate phosphate buffer (pH 4.0, 5.0, 6.0, and 7.0) containing different carbon sources, as different strains of *Y. lipolytica* display a varied response to changes in the medium pH. The flasks were then incubated at 30°C and 200 rpm for 72 h, while also observing the morphology microscopically at different time intervals. As a result, the oil emulsions were found to remain stable at all pH values. The ability of the yeast to undergo a transition in the presence of seawater was also studied. A medium containing YNB and coconut oil was prepared in defined seawater containing (g/l of deionized water) NaCl, 24.5; $CaCl_2{\cdot}2H_2O$, 1.54; KBr, 0.1; NaF, 0.003; KCl, 0.7; H_3BO_3, 0.03; Na_2SO_4, 4.09; $NaHCO_3$, 0.2; $SrCl_2{\cdot}6H_2O$, 0.017; and $MgCl_2{\cdot}6H_2O$, 11.1. Additionally, another experiment was performed using the YNB coconut oil medium prepared in natural seawater obtained from Mumbai, India. The pH was monitored by withdrawing aliquots under aseptic conditions, and maintained at a value of 7.0 with the addition of sterile 0.1 N NaOH at regular intervals, owing to the precipitation of the seawater salts in the citrate phosphate buffer.

Determination of Lipase Activity

The *Y. lipolytica* strain was cultivated in a buffered YNB medium (pH 7.0) containing coconut oil. Aliquots were then withdrawn, centrifuged at 6,000 ×*g* for 10 min at 4°C, and the washed cells resuspended in saline to estimate growth (absorbance at 600 nm, A_{600}) using a Jasco V-530 UV-visible spectrophotometer. The pH was also monitored over the incubation period. The colorimetric method [33] was used to determine the lipase activity in the cell-free supernatant. Specifically, stock solutions of *p*-nitrophenyl palmitate (20 mM) were prepared in isopropanol, and the assay was carried out in a Tris-HCl buffer (0.05 M, pH 7.5) at 37°C for 20 min with appropriate controls. The absorbance of the *p*-nitrophenol was measured at 410 nm (Jasco V-530 UV-visible spectrophotometer). Each assay was performed in triplicate and the mean values presented. One unit of lipase activity was defined as the amount of enzyme releasing 1 μM of paranitrophenol from para-nitrophenyl palmitate per milliliter per minute under the assay conditions.

Effect of Potassium Salts of Fatty Acids on Morphology

Potassium salts of fatty acids, such as lauric acid, myristic acid, palmitic acid, and stearic acid, were added as sole carbon sources at varying concentrations (0.5 to 1.5 mg/l) and the cell morphology was determined. These values were chosen, as higher concentrations (more than 1.5 mg/l) are known to inhibit growth. Potassium laurate and myristate were also added at a ratio of 2.6:1 (the averaged ratio of the two fatty acids present in coconut oil and palm kernel oil) in the buffered YNB medium and the mycelium formation was checked.

Microscopic Observations During Transition

The yeast to mycelium transition was checked in the buffered YNB medium containing coconut oil. Aliquots were withdrawn at regular intervals, while light microscopic observations were made using a Zeiss microscope (Axioskop 40) equipped with a photographic attachment. The mycelium to yeast transition was observed by adding mycelia from the coconut oil medium into the YNB medium containing alkanes (n-dodecane).

Partitioning of Yeast and Mycelial Cells

The ability of the yeast and mycelial cells to partition into hydrophobic phases was estimated by following the method described earlier [21]. Cells were harvested from the buffered YNB medium

containing coconut oil or n-dodecane and washed twice with saline. To 3 ml of the suspension (A_{600}=1.0), 30 μl of a hydrophobic substrate (n-dodecane or coconut oil) was added and the mixture vortexed for 1 min. After allowing the aqueous and organic phases to separate for 15 min, the absorbance of the aqueous phase was measured at 600 nm. The absorbance values for the cells without the addition of the hydrophobic substrates were used as the control. The relative partitioning was calculated as [(A_{600} of the control−A_{600} after the hydrophobic substrate overlay)/A_{600} of the control]×100. All the experiments were carried out in triplicate, and the results expressed as averages indicating a standard deviation.

Determination of Fatty Acid Composition

The yeast cells were obtained from the glucose or dodecane-containing media after 48 h, while the mycelial forms were harvested from the YNB coconut oil medium after 48 h and washed extensively with saline (no residual substrate fat was detected at this time). The fatty acid composition was then determined [19]. Specifically, the cell suspensions were saponified with methanolic NaOH in a tube sealed with a Teflon-lined screw cap at 100°C for 1 h. The saponified material was then cooled and neutralized. The free fatty acids were methylated with a boron trichloride methanol reagent for 5 min at 85°C. The methyl esters were then extracted in a 1:1 mixture of diethyl ether-hexane and dried over Na_2SO_4. The gas chromatography was carried out as described previously [19].

Results and Discussion

The dimorphism in *Y. lipolytica* is influenced by the interplay of carbon and nitrogen sources, pH, and anaerobic stress, all of which have been reported to regulate the transition and subsequent adaptation of the fungus to its natural ecological niche [9, 10, 31]. Table 1 shows the morphology of NCIM 3589 in the presence of different carbon sources. With hydrophilic sources, such as glucose or GlcNAc, the fungus remained in the yeast form under aerobic shake-flask conditions, as reported earlier [34]. In previous literature, there are discrepancies concerning the morphology of *Y. lipolytica* on different hydrophilic substrates under varied culture conditions. For example, the growth of *Y. lipolytica* was reported as being restricted to the yeast form with glucose [24], whereas other reports have indicated that mycelium formation is triggered with glucose and other hydrophilic materials [12, 28].

Table 1. Effects of carbon sources and pH on morphology of *Yarrowia lipolytica* NCIM 3589.

Carbon source	pH	Percentage of mycelia
Glucose, N-acetylglucosamine, n-dodecane	4.0 to 7.0	ND
Peanut oil, olive oil, sesame oil	4.0 to 7.0	40
Coconut oil, palm kernel oil	5.0	60
Coconut oil, palm kernel oil	6.0	90
Coconut oil, palm kernel oil	7.0	95

The medium contained 7 g/l YNB prepared in a 50 mM citrate phosphate buffer with the individual carbon source at 5 g/l at the respective pH. The incubation was at 30°C for 48 h.
ND: not detected.

In the present study, when using hydrophobic substrates, such as alkanes (n-dodecane), growth was promoted in the yeast form, which was consistent with an earlier report by the current authors on the dimorphism of this isolate [34]. Meanwhile, fatty materials, such as coconut oil or palm kernel oil, induced the formation of mycelia (60%) under the conditions of an uncontrolled pH (Table 1). Additionally, oils such as peanut oil, olive oil, or sesame oil were found to induce suboptimal (40%) mycelium formation.

Effect of Culture Conditions on Cell Morphology

The pH of the unbuffered YNB medium dropped from 7.0 to 3.0 after growth. Thus, since pH is known to play a significant role in the transition, a set of buffered media containing different carbon sources was prepared. Flasks containing the buffered media without an added carbon source did not support significant growth. Table 1 also shows the effect of pH on the morphology of the fungus with different carbon sources. The optimal mycelial formation (95%) was observed at pH 7.0 when the cells were grown with coconut oil or palm kernel oil. These results on the effect of pH were also in agreement with an earlier observation [31]. The authors also previously reported on the maximal mycelium formation at a neutral pH, with the minimal proportion of mycelia at pH 3.0. However, different strains of *Y. lipolytica* display a varied response to the media pH. For example, one strain of *Y. lipolytica* has been reported to display no relationship between transition and pH, thereby indicating the presence of distinct pathways for dimorphic transition [28].

Effect of Different Oils on Morphology

Coconut oil and palm kernel oil induced a unique yeast to mycelium transition. Both of these oils are rich in lauric and myristic acids as saturated fatty acids. Coconut oil is known to have the following approximate fatty acid composition: C8:0, 8%; C10:0, 7%; C12:0, 48%; C14:0, 18%; C16:0, 9%; C18:0, 3%; C18:1, 6%; and C18:2, 2%; palm kernel oil is composed of C8:0, 4%; C10:0, 4%; C12:0, 45%; C14:0, 18%; C16:0, 9%; C18:0, 3%; C18:1, 15%; and C18:2, 2% [7]. Most previous studies related to the growth of *Y. lipolytica* on oils have used olive oil, which has a totally different fatty acid composition as follows: C16:0, 10%; C18:0, 2%; C18:1, 78%; C18:2, 7%; and C18:3,1% [7]; few of such studies have reported on olive oil triggering a yeast to mycelium transition in certain isolates [20, 23]. Nonetheless, *Y. lipolytica* NCIM 3589 showed a suboptimal transition (40%) in the presence of olive oil.

Effect of Salinity on Transition

Since the fungus under investigation was originally isolated from a marine environment, its morphology was monitored in seawater, as well as natural seawater-based YNB media (pH 7.0). The results on mycelium formation in the seawater media were comparable to those obtained with freshwater-based media. There have also been some reports on salt-tolerant strains of *Y. lipolytica*. For example, a salt- and alkali-tolerant strain has already been used in salinity adaptation studies [1]. Moreover, *Y. lipolytica* strains have also been isolated from hypersaline and marine environments, implying that this fungus may play an important role in such environments [2, 13]. However, the present paper is the first report on an unusual morphological response of a marine strain to the presence of substrate fats. As discussed later, this may be relevant for the adaptation of the fungus to its ecological niche.

Growth Pattern and Lipase Production

Y. lipolytica NCIM 3589 was found to produce extracellular lipase activity in a growth-associated manner (Fig. 1). The growth pattern of the fungus is also shown in the figure. The initial lag period of 12 h was followed by the log phase, and the cells reached the stationary phase after 48 h. The extended lag period may have been due to the adherence of the cells to the triglyceride droplets. There was also a marginal drop in pH during the incubation period. The maximum lipase activity (33.71 units/ml) was observed after 48 h, at which point the yeast to mycelium transition was complete. Thereafter, there was a drop in lipase activity, as no residual substrate was detected. Such a pattern of extracellular lipase production has already been reported [29]. Thus, the fungus would appear to be able to utilize triglycerides, as it includes multiple gene coding for

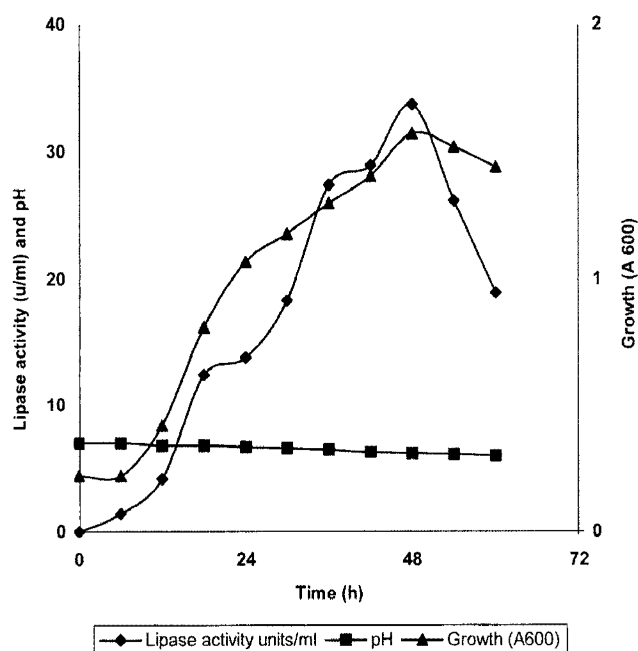

Fig. 1. Growth profile, medium pH, and lipase production of *Y. lipolytica* NCIM 3589 in YNB medium containing 5 g/l coconut oil prepared in 50 mM citrate phosphate buffer, pH 7.0, incubated at 30°C.

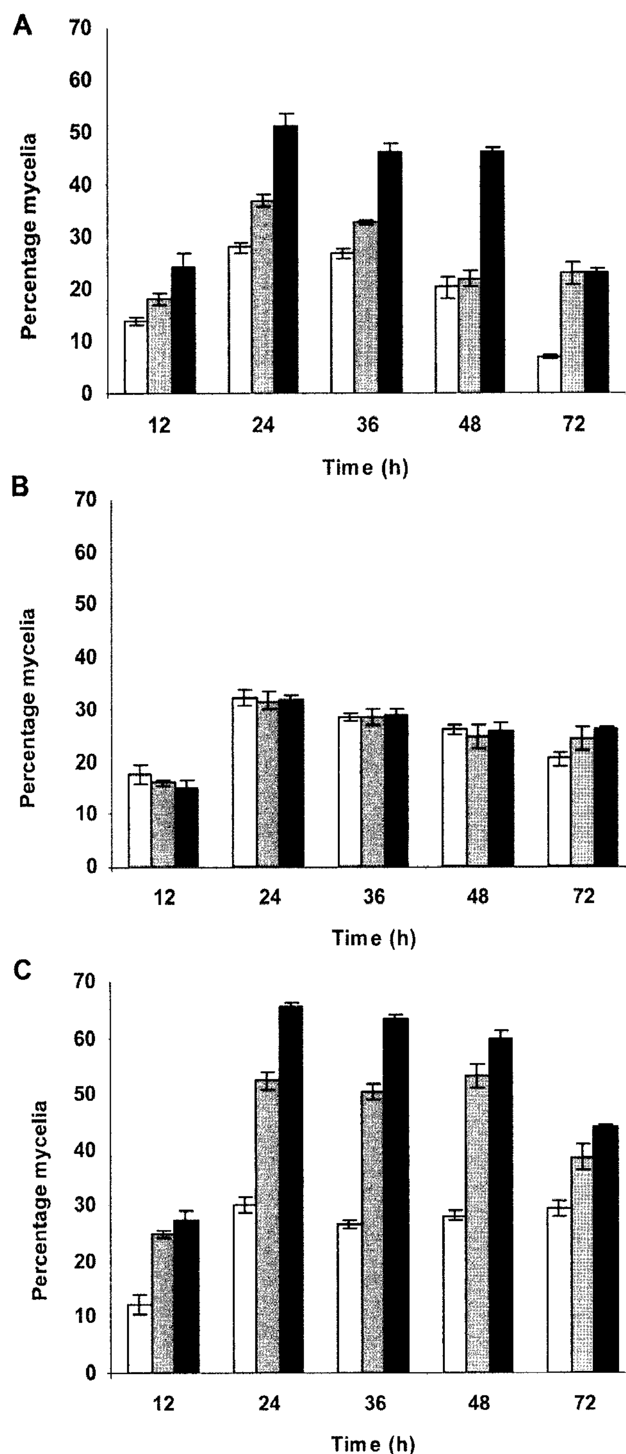

Fig. 2. Effect of potassium laurate and myristate on the morphology of *Yarrowia lipolytica* NCIM 3589.

A. Laurate (mg/l) 0.5 □; 1.0 ▒; 1.5 ■. B. Myristate (mg/l) 0.5 □; 1.0 ▒; 1.5 ■. C. Laurate and myristate (mg/l), respectively, 0.5 and 0.19 □; 1.0 and 0.38 ▒; 1.5 and 0.57 ■.

the essential enzymes, such as lipases [6, 14] and acyl coenzyme-A oxidases [17]. The strain under investigation was previously found to degrade POME containing high concentrations of saturated fatty acids and caused a 90% reduction in chemical oxygen demand [22]. However, *Y. lipolytica* is only able to utilize triglycerides after lipases have hydrolyzed them into free fatty acids [5, 6]. In the present study, it would seem that the lipases produced by the fungus hydrolyzed the triglycerides present in the coconut oil or palm kernel oil and produced lauric and myristic acids as the major free fatty acids. These fatty acids were then taken up by the yeast cells and possibly incorporated into the cells undergoing transition.

Effect of Potassium Salts of Fatty Acids on Morphology

Lauric acid (45–48%) and myristic acid (18%) constitute the major fatty acids in coconut oil and palm kernel oil [7], and their effect on the fungal morphology was investigated. Fig. 2A shows the effect of potassium laurate on the morphological transition of the fungus. In all cases, a transition was observed within 24 h, unlike the 48 h required with the oils. In the presence of 1.5 mg/l laurate, 51% of the cells were in the mycelial form. Thus, myristate was not as effective as laurate in promoting dimorphic transition (Fig. 2B). The maximum transition observed was 32%. A combination of laurate and myristate in a ratio of 2.6:1 (the averaged ratio present in coconut oil and palm kernel oil; 46.5:18) resulted in enhanced mycelium formation (Fig. 2C), as with 1.5 mg/l laurate and 0.57 mg/l myristate, 65% of the cells were in the mycelial form. Meanwhile, longer chain saturated fatty acids (potassium palmitate or stearate) or oleic acid promoted the yeast form. Several reports already exist on the morphological changes of *Y. lipolytica* grown in the presence of oleic acid [17]. In a previous detailed study carried out by the authors, specialized structures (protrusions and surface-bound lipid droplets) were only observed when the fungus was grown on oleic acid. However, unlike this report, the current authors did not observe any change in the morphology (mycelium formation) in response to the presence of fatty acids.

Microscopic Observations of Yeast-Mycelium Transition

Fig. 3 shows the pattern of transition that occurred when the fungus under investigation was inoculated into the buffered YNB medium containing coconut oil at a neutral pH. Initially, the yeast cells were seen to adhere to the triglyceride droplets, and then gradually the cells completely covered the oil drops (Fig. 3A). After 20 h of incubation in the presence of the saturated triglycerides, germ tube formation was initiated, which was clearly visible from the edges of the oil droplets (Fig. 3B). The mycelia tended to appear in the aqueous phase and became the predominant morphological form thereafter (Fig. 3C). Well-developed

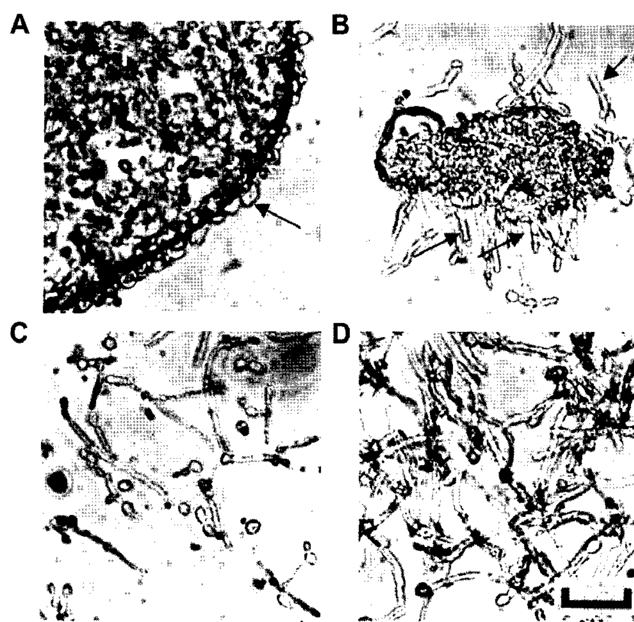

Fig. 3. Yeast to mycelium transition of *Yarrowia lipolytica* NCIM 3589 in YNB medium containing 5 g/l coconut oil prepared in 50 mM citrate phosphate buffer, pH 7.0, incubated at 30°C.

A. After 12 h, the arrow shows yeast cells adhering to oil drops. **B**. After 24 h, yeast to mycelium transition was observed, and the arrows show mycelia radiating away from oil drops. **C**. After 36 h, mycelia were dislodged from oil drops. **D**. After 48 h, mycelia were the major morphological form. Magnification 400×. Scale bar 25 μm.

mycelia were seen after 48 h, at which point the substrate was utilized (Fig. 3D). The characteristic attachment of the cells to the triglyceride droplets may have been relevant for the production of lipases in a growth-associated manner. The fungal cells associated at the oil-water interfaces produced lipases (Fig. 1), which are already shown to exhibit the phenomenon of interfacial activation [5]. Thus, the attachment of the yeast cells to the oil droplets would seem to be a characteristic feature of this fungus, as an earlier report by the current authors on the dimorphism of this fungus also revealed the same adherence of the cells to oil drops based on light microscopy [34].

Mycelium to Yeast Transition with Alkanes

When the mycelia that had been developed in the optimized coconut oil medium were inoculated into a medium containing n-dodecane (saturated aliphatic hydrocarbon with 12 carbon atoms), there was a rapid transition into the yeast form. Most of the hyphae budded off the yeast cells within 6 h of aerobic incubation (Fig. 4A). These yeast cells then adhered to the alkane droplets (Fig. 4B). The majority of the mycelia were observed to be associated with the aqueous phase (Fig. 4C). After this transition, yeast cells were the predominant form (Fig. 4D). The adherence of the yeast cells to the drops and dislodgement of the mycelia were also previously observed with coconut

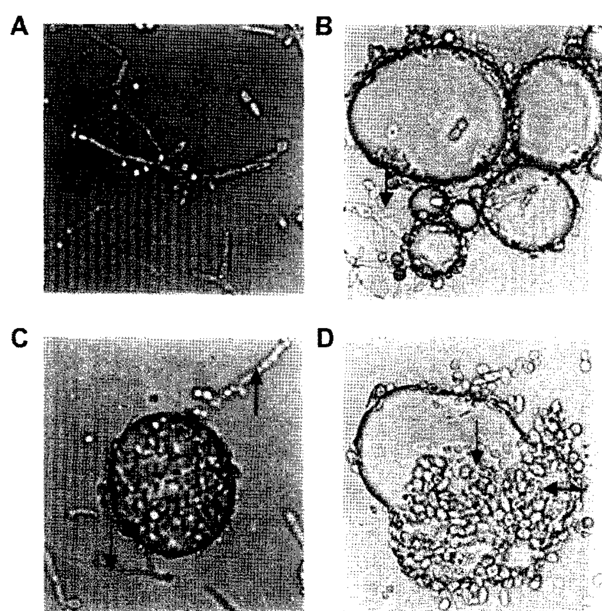

Fig. 4. Mycelium to yeast transition of *Yarrowia lipolytica* NCIM 3589 in YNB medium containing 5 g/l n-dodecane prepared in 50 mM citrate phosphate buffer, pH 7.0, incubated at 30°C.
A. After 6 h, the arrow shows free mycelia budding off yeast cells. **B**. After 6 h, the arrow shows yeast cells budding off and adhering to alkane droplets. **C**. After 12 h, yeast cells cover alkane droplets, and the arrows show mycelia associated with the aqueous phase. **D**. After 24 h, the arrows show that yeasts are the major morphological form. Magnification 350×.

oil during the yeast to mycelium transition (Fig. 3B). No extracellular lipase activity was detected during this transition.

This observation was relevant, as alkanes are known to be taken up in an intact form by attachment to large droplets, and alkane monooxygenase systems are the enzymes involved in the initial degradation of alkanes [5]. Although alkanes have been used in various investigations of *Y. lipolytica* strains as substrates for the production of different products [3], the mycelium to yeast transition patterns have not yet been studied in detail. Thus, the present light microscopic observations support earlier claims that yeast is the predominant form during alkane degradation [8, 30, 34].

Fatty Acid Composition of Cells

The fatty acid profiles of the yeast (grown on glucose or alkane) and mycelial forms (grown on coconut oil) were compared. All the cells showed the presence of C18 (major), C17, and C16 saturated and unsaturated fatty acids as the dominant cellular fatty acids. However, the mycelial form also showed the additional presence of lauric acid. Oleic and linoleic acids have also been reported to be the major cellular fatty acids in other strains of *Y. lipolytica* [27]. Although coconut oil mainly contains shorter-chain fatty acids (lauric acid, C12, and myristic acid, C14), the whole-cell lipid profile of the mycelial form showed the predominance of C18, C17, and C16 unsaturated and saturated fatty acids. One explanation for this is that *Y. lipolytica* strains include certain enzymes (elongases and desaturases) that cause chain elongation and fatty acid desaturation [5, 18], or it could be the result of a differential fatty acid specificity against substrate fatty acids [26, 27]. It has also been suggested that changes in the ratios of unsaturated and saturated fatty acids can lead to changes in the physical properties of the membranes and alter growth properties [17]. Therefore, a possible reason for the transition could lie in such a change in the substrate composition, causing a subsequent change in the morphology from the yeast form to the mycelial form.

In conclusion, the present study found that the marine *Y. lipolytica* strain underwent a unique transition from the yeast form to the mycelial form with substrates containing lauric acid and myristic acid at a neutral pH. Such a change in morphology may be relevant as regards the fungus adapting to its ecological niche. Oils such as coconut oil and palm kernel oil are routinely used in India and occur naturally in tropical environments. In wild strains, the ability to form mycelia can provide a selective advantage in the case of stress conditions [9, 10]. Thus, the formation of mycelia can be significant for the fungus in terms of providing a better opportunity to scavenge available nutrients from the marine environment. With saturated n-alkanes, there was a reverse transition, implying that the yeast form is the preferred form during the utilization of such substrates. New confocal laser scanning microscopy studies are currently under way on the formation of interfacial biofilms on these hydrophobic substrates.

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