Studies on the Correlation Between Biosurfactant and Lipid Synthesis in *Candida tropicalis* CMGB114 Using Hydrocarbons and Vegetable Oil Wastes

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During past decades there is an augmented interest regarding the possibility of biosurfactant and lipid production using microorganisms able to assimilate various polluting substrates represented by hydrocarbons and industrial or household wastes such as glycerol and fried vegetable oil. The yeast strain Candida tropicalis CMGB114 produced hhigh rates of biosurfactants when grown on yeast extract and peptone supplemented with 1% petroleum, fried sunflower oil or n-hexadecane, respectively, on yeast extract and NH_4NO_3 with 40 g/L glycerol. Proliferating lipid bodies were observed in the cells from all the tested culture media, indicating an active lipogenesis and a correlation between biosurfactant and lipid synthesis. Future research will focus on developing strategies for practical applications of C. tropicalis CMGB114 in bioremediation and biofuel production.

Keywords: biosurfactants, bioremediation, vegetable oil wastes, lipids, biofuel, Candida tropicalis

Candida tropicalis is one of the most versatile yeast species with strains isolated from various environments, from normal human mucocutaneous flora and immunocompromised patients[1], to natural environments such as polluted soil or water and industrial effluents [2]. *C. tropicalis* is one of the main yeast species with high abilities of degrading hydrocarbons represented by *n*-alkanes with long carbon chain (C_{10}-C_{18}), benzene and phenolic derivates, through monoterminal oxidation, respectively, the 3-oxoadipate pathway, being also able to assimilate by ω-oxidation, the fatty acids from vegetable oil wastes [3-6]. Long chain fatty acids and α,ω-dicarboxilic fatty acids resulted from this metabolism are further used in industry for obtaining cosmetics, lubricants, adhesives or plastics [7].

Microbial fatty acids became of large interest for biofuel production, an ecological response to the growing world crisis due to depletion of conventional fuel resources and alarming augmentation of emission of greenhouse gases. Biofuels are biodegradable and non-toxic, while the mixtures of biodiesel and diesel proved to determine a reduction of polluting emmissions, being also used in recovery technologies for the agricultural sector [8]. Biofuels are classified in: primary - pellets and firewood used as raw materials for heating, cooking and electricity, and secondary - such as ethanol, coal, biodiesel and biogas which are obtained by processing the primary fuels and are used in industry and for vehicles. Liquid secondary biofuels obtained from cellular biosynthesis processes from yeasts, fungi or microalgae are known as the third generation of biofuels [9].

The yeasts able to produce and accumulate high levels of lipids and fatty acids (*single cell oil*) used for biofuels are denominated as oleaginous yeasts and belong mainly to *Candida, Rhodotorula, Yarrowia, Cryptococcus* and *Trichosporon* genera. They present many advantages: high biomass production using relatively cheap growth media, the knowledge on genome structure and function of many yeast species, the possibility of improving the lipid metabolism. During the lipid metabolism in the yeast cell, the lipid bodies proliferate. The key steps are represented by citrate, malate and triacyl glycerols synthesis and the tricarboxylicacids cycle (TCA)/Krebs [10, 11].

On the other hand, the ability of some *C. tropicalis* strains to use hydrocarbon substrates, justify the interest in elaborating studies for their use in bioremediation of oil / petroleum-polluted environments. The mechanisms used by the yeast cells to assimilate oil, respectively, petroleum compounds, are: direct adhesion and transport through the cell wall, or using biosurfactants to solubilize the hydrocarbon droplets by reducing the surface tension. The biosurfactants present many advantages compared to the chemical surfactants: low toxicity, high biodegrability, selectivity and specific activity. Therefore, obtaining enhanced rates of biosurfactants represents one of the main strategies used in bioremediation and depletion of pollutants (petroleum compounds, fried oils, heavy metals) from the environment [12-15].

In the present article, we study the ability the yeast strain *Candida tropicalis* CMGB114 to degrade hydrocarbon substrates and the mechanism of their assimilation in the yeast cells. We present the optimal conditions for biosurfactant production using various carbon sources such as *n*-hexadecane, petroleum, glycerol, olive oil and fried sunflower oil, also describing the correlation between biosurfactant and lipid synthesis.

Experimental part

Strain

The yeast strain *Candida tropicalis* CMGB114 (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Romania) was maintained in a Revco Legaci™ Refrigeration System (Copeland, U.K.) at -70°C on Yeast Peptone Glucose (YPG: 5 g/L yeast extract, 10 g/L peptone, 2 g/L glucose) supplemented with 200 g/L glycerol (Sigma-Aldrich, Germany). The strain was taxonomically characterized in the Laboratory of Microbial genetics and Biotechnology, Department of Genetics (Csutak et al., pers. comm.).

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Biodegradation of hydrocarbons

The strain *C. tropicalis* CMGB114 was grown on YPG for 20 hours at 28°C, 150 rpm in a Certomat BS-T rotary shaker (B. Braun Biotech International, Germany). Then, 5 mL of culture were centrifuged for 6 min at 6500 rpm and the inoculum (0.3 x 10^8 cells /mL) was cultivated in flasks in a final concentration 1% for 19 days, at 28°C and 150 rpm in liquid Bushnell-Haas mineral medium (B-H: 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4 \times 7H_2O$, 0.05 g/L $FeCl_3$, 0.02 g/L $CaCl_2 \times 2H_2O$) supplemented with carbon source represented by 1% *n*-hexadecane (Sigma-Aldrich, Germany) or 1% petroleum (Fluka, Sigma-Aldrich, Germany). The samples were collected at time 0 and after 2, 5, 9, 13 and 19 days. The biodegrading ability of *C. tropicalis* CMGB114 was evaluated by monitoring the cell counts using a Thoma counting chamber and the *p*H [16, 17].

Biosurfactants assays. Emulsification index

For determination of biosurfactant production, the strain *C. tropicalis* CMGB114 was cultivated for 168 h at 28°C and 150 rpm, in five different media: three media based on Yeast Peptone medium (YP: 10 g/L yeast extract, 10 g/L peptone) with 1% *n*-hexadecane, petroleum or fried sunflower oil commercialy available, respectively, two biosurfactant production media (BS) adapted after [18, 19] (BS: 1 g/L NH_4NO_3, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \times 7H_2O$, 3 g/L yeast extract) supplemented with 40 g/L glycerol (Sigma-Aldrich, Germany) (BS-Gly) or 40 g/L olive oil (Carl Roth, Germany) (BS-olive oil).

After 72, respectively, 168 h of incubation, the production of biosurfactants was determined using the emulsification activity. On this purpose, equal volumes of cell-free broth and petroleum, *n*-hexadecane or fried sunflower oil (table 1) were mixed in tubes by vortexing at 2500 rpm (BioCote vortex, Stuart, U.S.A.). After resting 24 h at 22°C, the emulsification index (E24%) was determined according to [20].

Lipid synthesis

The 72 and 168 h samples from the cultures used for biosurfactant production were screened for lipid synthesis by Sudan Black B staining with using a method adapted after [21, 22]. We used a 0.08% Sudan Black B (Merck, Germany) solution prepared in 97% ethanol under shacking, then cooled at room temperature and filtered (Rotilabo filter papers, Carl Roth, Germany). Modification of the cell aspect, cell-substrate interactions, the appearance and frequency of dark blue intracellular lipid droplets were observed at optic microscope (Micros, Austria).

Results and discussions

Biosurfactant production. The mechanism of assimilation of hydrocarbon substrate in the yeast cell

Hydrocabon metabolism in yeast cells involves a complex series of processes from their uptake in the yeast cells, to the synthesis of compounds with essential role in the cellular metabolism, such as acyl-coenzyme A, lipids and ATP [23-25]. The assimilation of the hydrocarbons in the yeast cells as well as certain steps from the biodegradative pathway are related with the production of biosurfactants under specific culture conditions, mainly in the presence of various hydrocarbon substrates [26- 28].

The biodegradative ability of the strain *C. tropicalis* CMGB114 was evaluated by monitorization of the yeast culture in terms of cell growth and *p*H variation. The presence of *n*-hexadecane induced a constant growth of the culture after the first two days of incubation (fig. 1a), compared to petroleum which determined cell proliferation short after incubation, within four days, followed by a decreasing phase and a plateau (fig. 1b). The results are indicating an augmented ability of the strain *C. tropicalis* CMGB114 to degrade the *n*-hexadecane and other *n*-alkanes from petroleum (containig aproximately 82% aliphatic compounds). The descending curve and the plateau from figure 1b most probably suggestes that *C. tropicalis* CMGB114 can also assimilate the aromatic hydrocarbons from the petroleum, but with a reduced rate.

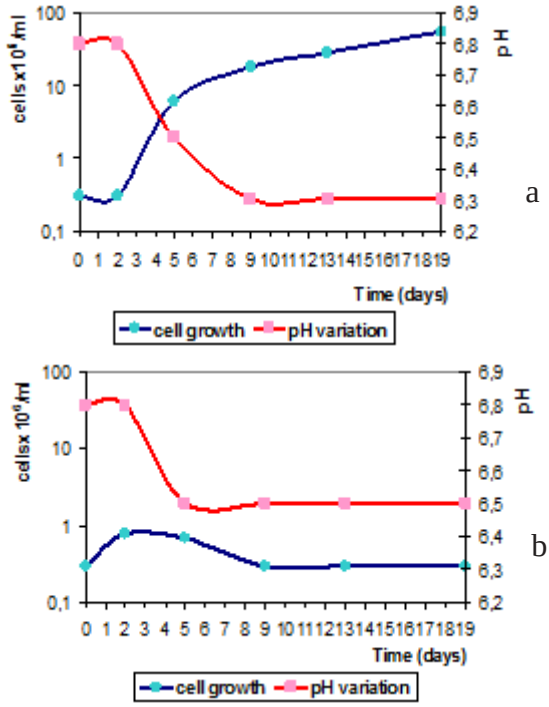

Fig. 1 Biodegradation of (a) *n*-hexadecane and (b) petroleum by *C. tropicalis* CMGB114 recorded as growth curves and pH variation

C. tropicalis is described as being able of degrading petroleum hydrocarbons, the saturated fractions (long carbon chain *n*-alkanes) as well as aromatic derivates [29], the processes being related to the production of acidic compounds which leads to a decrease of *p*H values (fig.1) [17, 30]. Meanwhile, biosurfactant production seems to determine higher *p*H values [19]. This observation can be correlated with our results for the emulsification index. The data obtained for emulsification of fried sunflower oil from table 1, show that the cells grown in YP with petroleum produced biosurfactants with constant and much higher rates (E24 is 26% after 72 h and 23% after 168 h) than cells grown in YP with *n*-hexadecane (11%, respectively, 17%). Moreover, the reduced *p*H variation from figure 1b (from 6.8 to only 6.5) indicates that, in the presence of petroleum, there is a balance between biosurfactant production and degradation of hydrocarbons leading to release of acidic compounds. This suggets that biosurfactant synthesis in *C. tropicalis* CMGB114 is active, representing the main mechanism involved in petroleum assimilation in the cells.

In comparision, the *p*H value drops rapidly when *n*-hexadecane is used, from 6.8 to 6.3, indicating accumulation of acidic compounds. However, the biosurfactants obtained by growing *C. tropicalis* CMGB114 during 168 h on YP with *n*-hexadecane, resulted in a 50% emulsification index against petroleum (table 1), comparable with data mentioned in other studies [31]. In our opinion, this suggests that the *n*-hexadecane is assimilated in *C. tropicalis* CMGB114 cells not only in the pseudo-emulsified form due to biosurfactant production, but also by direct adhesion and transport throughout

Growth media	Emulsified substrate	E_{24}% after	
		72 h	168 h
BS-Gly	*n*-hexadecane	15	42
	fried sunflower oil	-	41
BS-olive oil	*n*-hexadecane	-	5
YP-petroleum	*n*-hexadecane	20	11
	fried sunflower oil	26	23
YP-hexadecane	petroleum	32	50
	fried sunflower oil	11	17
YP-fried sunflower oil	*n*-hexadecane	10	33

Table 1
EMULSIFYING ACTIVITY OF BIOSURFACTANTS FROM CELL-FREE BROTH OF *C. TROPICALIS* CMGB114

channels within the cell wall described for other yeast species [32, 33].

While the synthesis of biosurfactants was representative after 168 hours when glycerol was used as sole carbon source, an E_{24} of only 5% was obtained using the olive oil (table 1). Our results are comparable to those reported by [34] who obtained good rates of biosurfactants for *C. antarctica* and *C. albicans* isolates from hydrocarbon polluted soil, when using 4% glycerol and similar growth medium.

Biosurfactants were determined at low levels and only after a long period of time, 168 h, in the presence of olive oil as sole carbon source. The data are supported by the experiments of [19] on *C. glabrata* grown on 5% cotton seed oil. They obtained higher yields of biosurfactant synthesis by using a mixture of vegetable oil and other carbon substrates - 7.5% cotton seed oil and 5% glucose. Also, [35] obtained biosurfactants that lowered the surface tension within the first 16 h of incubation of *C. lipolytica*, using a culture medium similar in composition to the one we used, supplemented with a combination of 6% soybean oil refinery residue and 1% glutamic acid.

Lipid synthesis and correlation with biosurfactant production

The lipid droplets from the cytosol of the yeast cells contain neutral lipids consisting of triacylglycerols, sterols and steryl esters and have important functions in protein storage and degradation. Oleaginous yeasts, comprising some *Candida* species, have the ability to use vegetable oils and animal fats to produce high amounts of fatty acids or triacylglycerols and to accumulate lipids at approximately 20% of their biomass, representing thus a valuable source for biofuel production [36]. The lipid metabolism and accumulation in yeast cells are highly influenced by the carbon source (glucose, glycerol, xylose, vegetable oils or their combinations), the nitrogen source (organic, inorganic), temperature, *p*H and culture conditions.

Lipid droplets were bserved for the cells from all five growth media, showing that *C. tropicalis* CMGB114 has an augmented lipid synthesis (fig. 2 panels A).

The microscope analysis of the *C. tropicalis* CMGB114 cells grown during 72 h on BS-Gly showed large vacuoles and lipid droplets which stained blue with Sudan Black B (fig. 2 BS-Gly: panels A and B), indicating active metabolic processes. The situation did not change after 168 h (fig. 2 BS-Gly: panel C).

As shown by [10] lipid synthesis in *Cryptococcus curvatus* and *Yarrowia lipolytica* was induced by using minimal media supplemented with glycerol as sole carbon source or mixed with industrial lipids. Glycerol and ammonium salts were also described as inducing lipid synthesis in yeast strains isolated from soil [37]. Moreover, *C. freyschussii* accumulated in short time (30 h), lipids with similar composition as plant oil, when grown on same concentrations of glycerol (40 g/L) and yeast extract (3 g/L) as those used during our experiments [38]. These facts would explain the rapid lipogenesis observed also in *C. tropicalis* CMGB114.

It is interesting to notice that, on BS-Gly medium, after the first 72 h, biosurfactant production in *C. tropicalis* CMGB114 was very low and grew dramatically at the end of the interval (168 h) (table 1). One explanation might reside in the fact that, after a longer period of time, a high ammount of the fatty acids resulted from glycerol metabolism in *C. tropicalis* CMGB114 cells are incorporated in biosurfactants [39]. The biosurfactants produced by *C. tropicalis* were identified as sophorolipids [40] or carbohydrate-protein-lipid-complexes [31], their structure depending on the strain used during the experiments.

The presence of the olive oil determined continuous accumulation of lipid droplets in *C. tropicalis* CMGB114 cells during the entire experiment (fig. 2 BS-olive oil: panels B and C). This is in correlation with other studies showing that combination of glucose, soil-molasses and oil with yeast extract and urea gave good results for *Candida bombicola* [10], while the oleic acid, main component of the olive oil, along with glucose, yeast extract and urea were successfully used for sophorolipid production in *C. tropicalis* [40]. In contrast, biosurfactant synthesis was insignificant on olive oil medium (table 1). In order to understand these results we need to take a closer look to the data recorded for the YP-fried sunflower oil medium. While the lipogenesis (fig. 2 YP- fried sunflower oil: panels B and C) was similar to the one observed for the BS-olive oil medium, we obtained good levels of biosurfactants with an E_{24} against *n*-hexadecane of 10, respectively, 33% after 168 h, in contrast with the olive oil case (table 1). The difference might be due to the composition of olive oil (up to 70-80% oleic acid and around 15% linoleic acid) compared to sunflower oil (approximately 30% oleic acid and 50-60% linoleic acid). It is possible that the biosurfactant produced by *C. tropicalis* CMGB114, include as hydrophobic component, mostly linoleic acid residues, which might explain the preference for the sunflower oil as carbon substrate for biosurfactant production. Similar results were mentioned for sophorolipids from *Candida bombicola* [41].

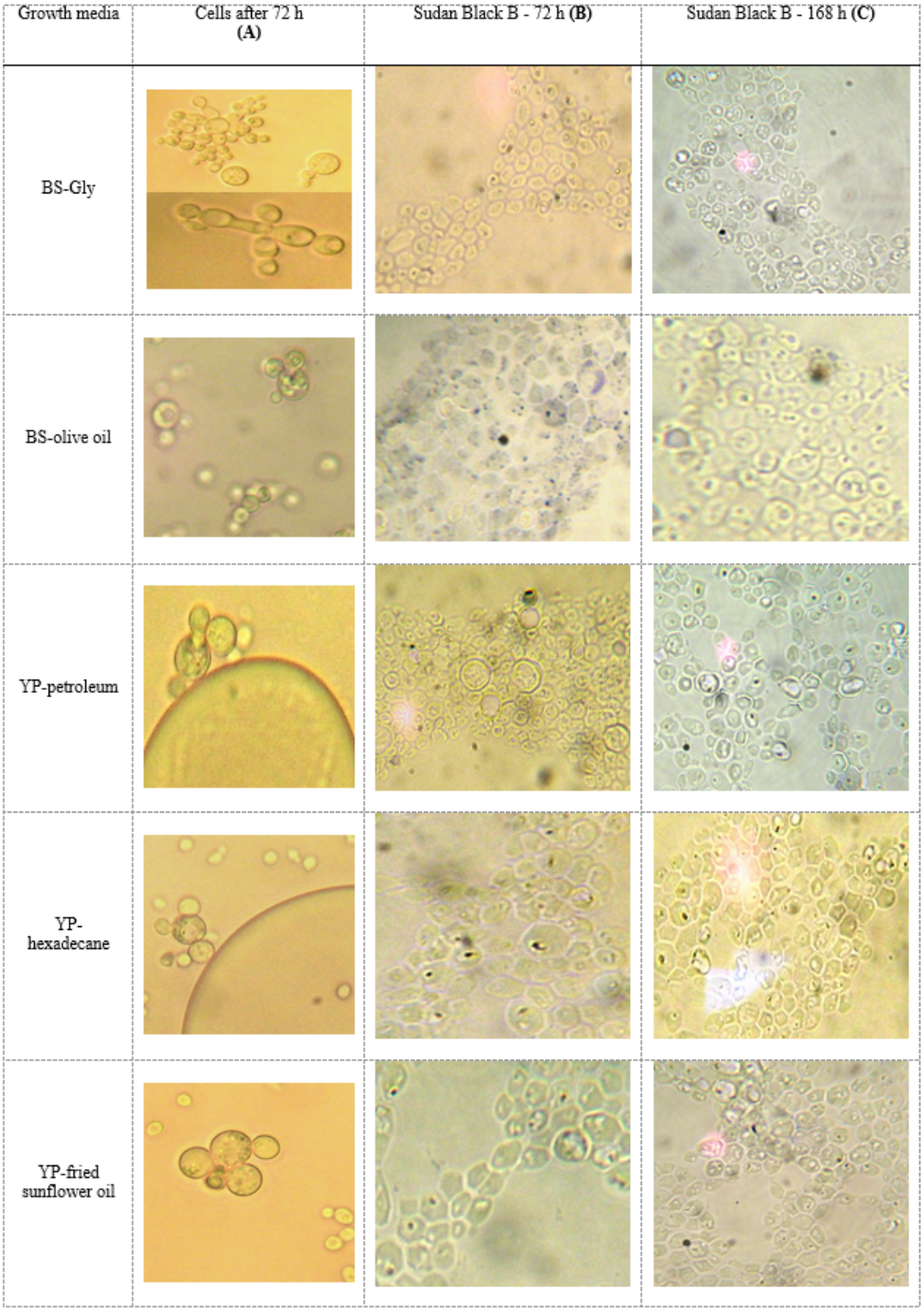

Fig. 2. Microscopical appearance of *C. tropicalis* CMGB114 cultures

Acyl-coenzyme A and fatty acids represent main metabolic points both in lipogenesis and hydrocarbon degradation in yeasts [42, 43]. This explains the ability of some oleaginous yeasts, such as *C. tropicalis* CMGB114, to produce biosurfactants when grown on substrates such as *n*-hexadecane or petroleum which also induce high lipid proliferation (fig. 2 YP- petroleum and YP-hexadecane: panels B and C). It is interesting to notice, under these conditions, the presence of yeast cells attached to the hydrocarbon droplets (fig. 2 YP- petroleum and YP-hexadecane: panels A) indicating active biosurfactant synthesis.

In present, there is little information concerning lipid synthesis in yeasts using alkanes as sole carbon source. Early studies mentioned a high content (51%) of triglycerides and free fatty acids for *C. tropicalis* cultivated on hexadecane [44], while [45] observed an increase of fatty acids content for alkane-grown *C. tropicalis* cells (12%) compared to glucose-grown cells (6.9%). Furthermore, the accumulated fatty acids had the same number of carbon atoms as the substrate in the case of longer chane alkanes.

Conclusions

The strain *C. tropicalis* CMGB114 was able to assmiliate hydrocarbons, especially *n*-hexadecane, with a high rate over a long period of time. Best results for production of biosurfactants were obtained on YP medium containing yeast extract and peptone with 1% petroleum, fried sunflower oil or *n*-hexadecane, and on basic media with yeast extract and NH_4NO_3 supplemented with 40 g/L glycerol. Numerous lipid droplets were observed in *C. tropicalis* CMGB114 cells from all the culture media tested for obtaining biosurfactants, indicating an active lipogenesis and the existence of a correlated mechanism between biosurfactant and lipid synthesis. To our knowledge, the present work on *C. tropicalis* is the first dealing with biosurfactant production using petroleum as

sole carbon source, and one of few reports to describe optimized conditions for synthesis of biosurfactants from glycerol and of lipids from hydrocarbon substrates. In conclusion, the strain *C. tropicalis* CMGB114 shows a great potential for practical applications in bioremediation of polluted environment and for future research on biofuel production.

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