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Waste fat biodegradation and biomodification by *Yarrowia lipolytica* and a bacterial consortium composed of *Bacillus* spp. and *Pseudomonas putida*

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PRACTICAL APPLICATION

Fats, oils and greases (FOGs) are hardened and insoluble solid formations generated in significant amounts and their disposal and biodegradation presents a major problem for local authorities. Biodegradation of waste cooking fats, such as butter and oil, was performed by two different microbial populations, a yeast strain namely a *Yarrowia lipolytica* LFMB 20, and a bacterial consortium comprised of *Bacillus* spp. and *Pseudomonas putida*.

The degradation of these fats by both microbial populations indicates their ability to be effectively used for bioremediation of various FOGs found in several types of wastewaters. High biomass yield by *Y.*

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lipolytica and high cellular lipid yield of the bacterial consortium were observed. Both cellular and non-consumed lipids presented differences when compared with the fatty acid (FA) composition of the fat substrate employed. The ability of both microbial populations to produce lipids with altered FA content presents significant industrial, ecological and biotechnological interest, especially towards the biodegradation of waste cooking fats.

ABSTRACT

Fats, oils and greases (FOGs) are a particular environmental threat. Biodegradation of FOGs is a challenge and in this study the biodegradation of waste cooking fats, namely butter and olive oil, was studied using a non-conventional yeast, *Yarrowia lipolytica* strain LFMB 20, and a bioaugmentation product consisting of *Bacillus* spp. and *Pseudomonas putida* CP1 strain. The microorganisms were grown aerobically in shake-flask experiments in an enriched medium supplemented with *ca* 0.85% w/v of waste fat. Analysis of the remaining substrate showed a removal of *ca* 90% of the fat by the yeast at the end of the incubation, while the bacteria removed *ca* 95% of both fats. Growth rate, biomass production and biomass yield per unit of fat consumed were all higher for the yeast compared to the bacterial consortium. The bacterial consortium exhibited autolysis and a significant decrease in its DCW value at the late growth phases of both fat substrate cultures. The main fatty acids (FAs) present in both fats were linoleic (Δ9,12C18:2), oleic (Δ9C18:1), palmitic (C16:0), palmitoleic (Δ9C16:1) and stearic (C18:0) acid. Both the bacterial consortium and *Y. lipolytica* preferentially removed Δ9C18:1 from the medium, while a negative selectivity against C18:0 was reported. Both inocula produced microbial mass that contained intra-cellular lipid quantities, but the bacterial consortium gave significantly higher lipid in DCW values compared with the yeast (maximum values up to *ca* 63% w/w for the butter and *ca* 42% w/w for the olive oil while the respective values for both lipids were 22%±2% w/w for *Y. lipolytica*). In all cases, intra-cellular lipids in DCW values decreased during the late growth phases, while their FA composition differed with those of the substrate fat.

Keywords: *Yarrowia lipolytica*; *Bacillus;* bioaugmentation; biodegradation; FOG; *Pseudomonas putida*

1. INTRODUCTION

Fats, oils and greases, known as FOGs, are generated in significant amounts from food service establishments, industries and washing up activities, as well as from cooking and food preparation facilities [1]. FOGs can develop as hardened and insoluble solid formations. Therefore, their disposal presents a major problem for local authorities both in terms of municipal and industrial wastes as they can significantly reduce the flow of wastewater in sewer lines or even block it completely [2-4]. On the other hand, these waste FOGs are available for free and one consideration is the conversion of these waste materials into biodiesel or renewable diesel [1,4,5]. Another consideration is the transformation of such low- or negative-cost compounds into a range of value-added compounds using enzyme and microbial technologies [4-7]. Most common approaches for FOG management prevent FOG entering the sewer [8]. Where FOG does enter the system, the use of bioaugmentation for FOG treatment at source results in a reduction of sewer line blockages [4,9]. Oleaginous microorganisms have been investigated for many years because of their properties and their potential application in industry and wastewater treatment [10-14]. In several cases, these microorganisms have been used both to degrade fats and consume the released fatty acids (FAs) for growth, transforming both intra-cellular and extra-cellular fatty materials into “new” lipids presenting different FA composition compared with the initial substrate fat [5,7,15]. The incorporation of a fat substrate into the microbial cell and the intracellular changes of the FAs are defined by the enzymatic capabilities of the microorganisms.

The yeast *Y. lipolytica* is a model microorganism that has been studied extensively in relation to its potential to break-down and assimilate hydrophobic materials [5,15]. Therefore, strains of this microorganism have been shown to produce extra-cellular and intra-cellular lipases and also lipases connected to the cell membrane. This organism can use triacylglycerols (TAGs) as a sole carbon source which are hydrolyzed to FAs and glycerol. TAG hydrolysis is rather a selective process since

most microbial lipases exhibit a *sn*-1,3 regiospecificity and therefore FAs esterified at the external positions are firstly released from the TAGs structure [16], while the remaining *β*-monoacylglycerols are subsequently hydrolyzed after their isomerization to *α*-isomers [5,17]. Besides regiospecificity, microbial lipases may also exhibit FA specificity, recognizing specific aliphatic chains according to their number of carbon atoms and double bonds [16-19]. The released FAs will be further incorporated inside the yeast cells through a process that is not random [20] and will either be metabolized through the *β*-oxidation pathway, or will be stored in the lipid reserves unaltered or following reactions of elongation or unsaturation [5,7,15,16].

The ecological niche for *Y. lipolytica* encompasses lipid-rich food like margarine, cheese and meat or shrimp products and it is also found in sewage and oil plants [21,22]. *Y. lipolytica* has been widely studied due to its considerable biochemical properties, its ability to produce several biotechnologically important metabolites, its dimorphism and amenability to molecular techniques. On the other hand, the genus *Bacillus* is of considerable importance in relation to the biodegradation of fatty materials, as it includes lipase- and biosurfactant-producing species. Members of this genus produce endospores that render them very resistant to adverse environmental conditions. They play an important role in the biological treatment of pollutants and have been widely used as additives in supplements [3,24,25]. *Bacillus* spp. along with other fat-degrading microorganisms such as *Acinetobacter* spp. and/or *Pseudomonas* spp. have been isolated from a variety of sources and have been studied for their degradative ability in laboratory-scale experiments [24,26,27]. This study focused on the use of *Yarrowia lipolytica* LFMB 20 and a bacterial consortium consisting of *Bacillus* spp. and *Pseudomonas putida* CP1 for the degradation of a “soft” and a “hard” waste fat used in cooking. Moreover, the FAs’ metabolism was investigated and critically discussed.

2. Materials and Methods

2.1 Microorganisms and media. Yarrowia lipolytica LFMB 20 [28] was supplied by the culture collection of the Agricultural University of Athens. The yeast was stored on a culture medium PDA (Potato Dextrin Agar) at *T*=4 °C. It was incubated at *T*=29 °C for 4 days on PDA before use in the batch studies. The bioaugmentation product BFL (BioFuture Ltd, Ireland) was in powder form. The inoculum size used was 1%, w/v. The product comprised a mixture of *Bacillus* spp (*ca* 3×10^8 cfu/g). BFL-CP1 comprised BFL and 1%, v/v, *Pseudomonas putida* CP1 which was grown overnight in nutrient broth and washed in phosphate buffer saline (pH=7.0) (OD=1.0 at 660 nm). *Pseudomonas putida* CP1 was obtained from the culture collection of the Microbial Ecology Group, School of Biotechnology, Dublin City University.

For the degradation studies of both the yeast and the bacterial consortium, a basal medium was used supplemented with initial substrate fat (S_0) concentration 8.5±1.5 g/L of either cooked olive oil or cooked butter (wastes deriving from local restaurant facilities) for the batch fermentation studies. The free acidity of the used oil was 5.0±1.5%, w/ w, while the peroxide number of the residue was around 150±10 mg/kg of oil. The free acidity of the used butter was 4.5±1.0%, w/ w, while its peroxide number was around 140±10 mg/kg of butter. All cultures for both microorganisms were incubated at *T*=29±1 °C in an orbital shaker (Zhicheng ZHWY 211C; PR of China) at 190±5 rpm. Erlenmeyer 250-mL flasks were used containing 50±1 mL of the medium, incubated by a 24-h pre-culture (see below). The salt composition of the basal medium for the case of *Y. lipolytica* was (in g/L): KH_2PO_4 7.0, Na_2HPO_4 2.5, $MgSO_4\times7H_2O$ 1.5, $FeCl_3\times7H_2O$ 0.15, $ZnSO_2\times7H_2O$ 0.02, $MnSO_4\times H_2O$ 0.06, $CaCl_2\times2H_2O$ 0.15. 0.5 g/L of yeast extract and $(NH_4)_2SO_4$, respectively, were used as nitrogen sources. Tween 80 (5%, w/w, of fat) was added to the culture medium as a surfactant reagent and an Ultra Turrax homogenizer was used for the dispersion of the fat [11]. The yeast was first

incubated in the pre-culture medium composed of glucose, yeast extract and peptone (concentrations 20 g/L, 10 g/L and 10 g/L respectively) for 24 h at *T*=29±1 C, 190 rpm. One mL from the 24h pre-culture was transferred aseptically into the culture medium. The pH was maintained at 6.0 by the addition (when needed) of 5M KOH [28].

For the degradation studies by the bacterial consortium BFL-CP1, an enriched nutrient medium (ENM) was used reported by Brooksbank et al. [3]. The medium contained (in g/L): yeast extract 0.2, glucose 0.1, KNO_2 1.0, $MgSO_4 \times 7H_2O$ 0.2, NaH_2PO_4 0.1, $CaCl_2 \times 2H_2O$ 0.001, $MnSO_4 \times H_2O$ 0.01, ferric ammonium citrate 0.005. Butter and olive oil, were added and emulsified as previously. The powder was dissolved in tap water (10%) and 1% of the consortium was used in each flask.

2.2 *Dry biomass determination.* The whole content of the 250-mL flasks was collected and cells were harvested by centrifugation [9000 × g/15 min at *T*=10 °C; centrifugation performed in a Hettich Universal 320R (Germany) centrifuge]. The pellet was then washed once with ethanol and once with hexane applying centrifugation (9000 × g/15 min at *T*=10 °C) each time as previously described [11]. The biomass was dried at *T*=85 °C for 24 hours to obtain the dry cell weight (DCW). Specific growth rate μ (h^{-1}) was calculated by fitting the curve $\ln(X/X_0) = f(t)$ on the experimental points for the exponential growth phase. Biomass yield, $Y_{X/S}$, was expressed as the grams of cell dry weight (DCW) produced (X, g/L) per grams of substrate consumed (S, g/L).

2.3 Fat substrate extraction. Fat substrate (S, g/L) was monitored using an extraction-gravimetric method based on a modified method described by Brooksbank et al. [3] and Papanikolaou et al. [29]. The percentage removal of the fat was calculated according to the following equation:

$$Efficiency\ \%\ of\ fat\ removal = \frac{Initial\ fat\ level - Final\ fat\ level}{Initial\ fat\ level}\ x\ 100$$

2.4 Cellular lipid extraction. Total cellular lipid (L, g/L) was extracted from dry biomass with a chloroform-methanol mixture (30 mL, 2:1 v/v). The samples were filtered in a pre-weighted flask and the solvent was evaporated in a Buchi rotavapor R-114. The intracellular lipid was determined gravimetrically and was expressed as g/L [11]. Total lipid in dry weight ($Y_{L/X}$) was expressed as % (w/w) of accumulated lipid per DCW (X, g/L).

2.5 Fat analysis. Cellular and non-consumed lipid was converted to the respective methyl-esters in a two-step reaction with methanolic sodium and hydrochloric methanol and analyzed by gas chromatography (8000 series FISONS) as described by Papanikolaou et al. [11]. Thin layer chromatography (TLC) was used to analyze the hydrolyzed products of the extra-cellular non-consumed fat. The analysis was performed on Silica Gel TLC plates using hexane-diethyl ether-acetic acid (70:28:2, v/v/v) for the mobile phase, as described by Čipinytė et al. [30]. The mobile phase used was 20 mL and covered an 8 cm distance. The spots of oil and hydrolysis products, diluted in 3 mL hexane, were visualized under UV light.

2.6 Bacterial identification. Microbial identification to genus level was conducted by Gram-staining, spore-formation, cell morphology, catalase and oxidase production tests [31] followed by identification using 16S rRNA sequencing. The bacterial genomic DNA was extracted from an overnight culture (OD600=0.5-1.0, incubated in 10 mL nutrient broth at *T*=30° C and agitated at 150 rpm) according to a modified method described by Gevers et al. [32] and Chen and Kuo [33] for the Gram positive and Gram-negative bacteria, respectively. The universal primers (Sigma), pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pH (reverse) 5'-AAG GAG GTG ATC CAG CCG CA-3' [34] were to amplify the 16S rRNA gene from the bacterial isolates. The amplification was held in a thermal cycle GeneAmp PCRsystems 9600 as described by Gomaa and Momtaz [35]. The 16S rRNA gene sequencing was performed by Eurofins MWG Operon sequencing service (Germany)

(http://www.eurofinsdna.com) using the pA primer. The isolates were identified by their obtained sequences using the online BLAST searches on the NCBI website (www.ncbi/nlm/nih/gov).

2.7 Lipase activity. Lipase production from each individual bacterial isolate was investigated using 1% tributyrin agar plates and incubations were carried out at *T*=30 °C for 86 h. Enzyme production was indicated by clear zones surrounding the colonies [36]. Lipase activity was quantified measuring the diameter (mm) of the halo formed and it is expressed as: "–" for no lipase production, "+" for diameter < 0.5 mm, "++" for diameter between 0.6 and 1.1 mm, "+++" for 1.2 to 1.7 mm and "++++" for diameter > 1.7 mm.

2.8 Data Analysis. Each experimental point of all of the kinetics presented in the tables and figures related to all cultures of all microbial consortia are the mean value of two independent determinations. Each culture was performed in duplicate by using different inocula. The standard error of all determinations of the kinetics presented is ±15%.

3. Results

3.1 Kinetic results of microorganisms growing on waste butter and waste olive oil employed as carbon substrates. When *Yarrowia lipolytica* LFMB 20 was grown in an enriched medium supplemented with either waste cooking oil or waste cooking butter, fat removal was observed in both cases (Table 1). Nearly 70-80% of substrate fats were removed within the first 40-50 hours. The remaining fat was removed more slowly between days 2 and 5, while further incubation (up to 205±15 h) resulted in an overall fat removal of *ca* 90% w/w for both fat substrates. Fat removal was accompanied by significant cell growth both in waste cooking oil and butter. The organism grew rapidly in both cases, reaching a stationary phase when *ca* 80% of the fat had been removed. A higher maximum specific growth rate (μ_{max}), evaluated at the early exponential growth phase (0-24 h

after inoculation) was observed with the olive oil (μ_{max}=0.14 h^{-1}) compared to the butter (μ_{max}=0.11 h^{-1}). Maximum DCW values obtained were 8.0 g/L and 7.5 g/L, respectively. On the other hand, the representative maximum biomass yields on consumed fat ($Y_{X/S}$) obtained when the microorganism was grown on oil seemed lower than the ones obtained when the organism was grown on waste-cooked butter (*ca* 1.1 g/g against *ca* 1.5 g/g, respectively). When waste cooking olive oil was employed as substrate, a maximum value of 24.0% w/w of lipid in DCW was obtained at the early growth phase (at t=20 h, the L value was ≈1.1 g/L at the respective fermentation point). The L_{max} value obtained for the trial on olive oil was =1.32 g/L and was achieved later in the fermentation (at t=40 h, nevertheless at that point $Y_{L/X}$ was =17.8% w/w). In the case of butter, the L_{max} value was 0.89 g/L at t=48 h (at the respective fermentation point, the $Y_{L/X}$ value was 19.5% w/w). Maximum lipid in DCW value obtained ($Y_{L/Xmax}$) was =20.0% w/w, achieved at a relatively early stage of the fermentation (at t=25 h; see Table 1). It is concluded, therefore, that moderate cellular lipid quantities were produced by *Y. lipolytica* LFMB 20 during growth on both types of waste fats and in both instances the yeast re-consumed its previously accumulated lipids while entering the stationary growth phase. During the cellular lipid degradation (turnover) period, $Y_{L/X}$ values dropped for both fats used as substrates to values ranging between 1.9 and 7.0% w/w (Table 1).

The growth pattern of the mixed culture BFL-CP1 was similar on both fats, achieving the same growth rate (μ_{max}=0.10±0.01 h^{-1}). In both cases, significant cellular lipid autolysis was seen at the later stages of fermentation (see Table 1). The bacterial consortium, BFL-CP1, degraded both fats, achieving *ca* 95% w/w of fat removal at the end of the run. Interestingly, the mixed culture BFL-CP1 was capable of performing fat removal even during the late growth phase when cellular autolysis was observed, showing the potential for this consortium for applications in environmental processes. Although the mixed bacterial consortium and the yeast assimilated both fats even at the late growth phases of the runs, the rate of fat assimilation was comparable for these two types of

fermentations (see Figs 1a; b and 2a; b – in Fig. 1 growth of *Y. lipolytica* on olive oil while in Fig. 2 growth of the bacterial consortium on butter are illustrated). Moreover, noticeably higher quantities of fat were assimilated by both types of microorganisms during the initial stages of growth (i.e. within the first 3 days after inoculation). BFL-CP1 grew rapidly on both fats reaching a stationary phase at day 3 which continued until day 7, when a significant quantity of fat had been removed from the medium for both fat substrates (see Fig. 2a for the trial on butter). After the 7^{th} day of incubation (*ca* 170-180 h), cell lysis was observed for the bacterial consortium, irrespective of the fat employed as substrate (see Table 1 and Fig. 2). Compared with the trials performed with *Y. lipolytica*, the bacterial consortium demonstrated much lower DCW production and a much greater lipid accumulation capacity. The $Y_{L/Xmax}$ values achieved for the mixed culture BFL-CP1 were 62.7% w/w and 41.7% w/w for the trials performed on waste butter and waste olive oil respectively (respective L values 1.38 and 1.00 g/L). Cellular lipids were broken down rapidly following the point when maximum accumulation of cellular lipids was achieved. This was followed by further assimilation of the extra-cellular fat substrate (see Figs 2a; b).

3.2 Bacterial identification and lipolytic activity. Nine strains (BFL1-BFL9) were isolated from the bioaugmentation product BFL. All nine isolates grew well aerobically, were spore-formers, Gram-positive rods and oxidase and catalase positive indicating membership of the genus *Bacillus*. Identification to species level was conducted using 16S rRNA sequencing showing up to 100% homology with the respective species. The identified species were *B. circulans*, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. cereus* and *B. megaterium*. Lipase activity of each bacterial isolate was monitored and it was shown that the *Bacillus* species displayed satisfactory and varied lipolytic activity while the added strain, *Pseudomonas putida* CP1, did not produce lipase (Table 2). *Y. lipolytica* is a well-known, widely studied lipase-producing microorganism. To this end, the remaining non-consumed substrate fat was analyzed for its hydrolyzed products using TLC. Activity of

extracellular lipases led to hydrolysis of the fat (triacylglycerols – TAGs exclusively found on t=0 h) to diacylglycerols, monoacylglycerols and free fatty acids (FAs) from the first day of incubation. By the end of the incubation, TAGs were almost completely hydrolyzed. The pattern of hydrolysis by both lipase-producing consortia was similar for both fatty substrates (Fig. 3).

3.3 Cellular lipid and fat substrate analysis. The main FAs present in butter and oil were linoleic acid (Δ9,12C18:2), oleic acid (Δ9C18:1) palmitic acid (C16:0) stearic acid (C18:0) and palmitoleic acid (Δ9C16:1) and their concentration was monitored during the fermentation of *Y. lipolytica* LFMB 20 (Table 3) and BFL-CP1 (Table 4). C18:0 and Δ9C16:1 were at low concentrations comprising less than 4% of the total FAs. Following yeast growth on the oil, Δ9C18:1 continued to be the main FA. However, its concentration decreased whereas the percentages of the other FAs increased, indicating a selective utilization of Δ9C18:1 by *Y. lipolytica*. The relative FA composition of the extracellular fat after *ca* 190 h of incubation of *Y. lipolytica* LFMB 20 on butter did not change significantly with time. However, preferential degradation of oleic acid was observed, suggesting once more, a preferential utilization of Δ9C18:1 by *Y. lipolytica.* On the other hand, a decrease in the degradation of FA C18:0 was observed, in agreement with previous results reported for other *Y. lipolytica* strains cultivated on blends of saturated and unsaturated FAs [5,20]. The profile of the cellular lipids of *Y. lipolytica* resembled the FA composition of the medium. However, over the course of time, the strain tended to produce lipids with higher concentrations of C18:0 compared with the fat substrate employed as carbon source.

A similar response was observed for the bacterial consortium that preferentially degraded Δ9C18:1 (Table 4). However, the decrease in the concentration of oleic acid following 168 hours incubation was significant when growth of the bacterial consortium on both fats occurred. Also in the case of the bacterial consortium, the same trend was observed intracellularly, suggesting that Δ9C18:1 was rapidly incorporated by the bacterial cells, and was equally rapidly assimilated through

the *β*-oxidation process for energy production [7]. The principal cellular FA observed in the bacterial consortium in both waste butter and olive oil cultures was C18:0.

Discussion

The yeast *Yarrowia lipolytica* LFMB 20 performed high fat degradation, characterized by faster removal of the cooked oil than the cooked butter, achieving 90% and 88% fat removal, respectively. Very similar patterns were obtained by the bacterial consortium of *Bacillus* spp. and *Pseudomonas putida* CP1, degrading both waste cooked fats, with slightly slower removal rates than the yeast. However, at the end of the incubation period, the bacterial consortium resulted in a higher percentage fat removal (95%). The results are quite promising, especially by taking into account the high initial fat concentration in the growth media, which are regarded as higher than the levels associated with wastewater containing fatty materials from slaughterhouses and food processing facilities [3,5,6]. Considerable removal of fat by *Y. lipolytica* was observed by Davin and Quilty [37] who reported 75% beef tallow removal by a newly isolated *Y. lipolytica* strain under optimal fermentation conditions. Dominguez et al. [38] reported the potential application of *Y. lipolytica* CECT 1240 to degrade waste cooking oils. The initial concentration of the oil was lower than in our study, namely ~2 g/L, and they observed a 93.3% w/w removal of salad oil and 85.1% w/w removal of grease under optimum conditions. Other fungi have also been reported to degrade fats but not as effectively as *Y. lipolytica* LFMB 20. Tzirita and Quilty [39] have reported 94% biodegradation of butter (S_0=7.5 g/L) and 92% of olive oil (S_0=8 g/L) in an enriched medium by the fungus *Mucor circinelloides* after 13 days of incubation. Bednarski et al. [40] studied the growth of *Aspergillus niger*, *Geotrichum candidum* and *Mucor meihei*, on animal fats, tallow and poultry, and reported 18±4% and 36±4% beef tallow and poultry fat removal, respectively, after 5 days. Tan and Gill [41] reported 90% removal of 2.2 g/L beef tallow by *Saccharomycopsis* (=*Yarrowia*) *lipolytica*

after 8-12 hours. However, they used a high concentration inoculum (12%, v/v) and the S_0 concentration was much lower than in our study. Čipinytė et al. [30] investigated the fat degradation ability of lipase-producing strains in an enriched medium supplemented with 0.1% of animal fats and vegetable oils. The pure isolates degraded the 25-45% of the "hard" fat (tallow and lard) and 40-58% of the "soft" fat (sunflower oil and olive oil). Those findings suggest that the more highly saturated the fat, the greater the challenge for biodegradation [5].

When a fat medium is used as microbial carbon substrate, a series of events that are linked with the presence of TAGs into the culture medium and the total FA composition of the lipids employed as substrate are related with the efficiency of the biodegradation by the microbial population implicated in the process; in the case in which TAGs are employed as substrates, TAG hydrolysis, that is a rather selective process is carried out, since most microbial lipases exhibit noticeable stereo- and typo-selectivity [5,6,16,17]. Thereafter, the various FAs, that had previously been released from TAG structures through lipase-catalyzed hydrolysis, or exist as initial substrates (in several cases fatty by-products composed of free FAs have been employed as microbial substrates [11,12,20]), are incorporated inside the microbial cells or mycelia with different incorporation rates depending on their structure and the sufficiency of cell membrane in specific FA carriers [5,7,11,15,16]. In most cases, saturated FAs (and most often the FA C18:0) do not rapidly enter inside the cells, most probably due to their linear configuration, whereas the relevant unsaturated FAs (in most cases presenting a -*cis* configuration; i.e. Δ9C18:1, Δ9,12C18:2) are incorporated and metabolized into the cells or the mycelia much more rapidly [11,12,19,20,29]. Apparently thus, on a biochemical point of view, the above-mentioned fact is the main reason for the high challenge of biodegradation of saturated (and solid) fatty materials that can be generated in several food-processing activities.

The values of yeast DCW production were similar to those reported by Papanikolaou et al. [11] who obtained X_{max} values of 8.7 g/L and 7.7 g/L and $Y_{X/S}$ yields of 0.97 and 0.86 g/g for another *Y. lipolytica* strain (ACA-DC 50109) growing on stearin (an industrial derivative of animal fat composed of *ca* 100% w/w saturated FAs) and hydrolyzed oleic rapeseed oil in shake flasks. It is noted that, at least for a number of *Y. lipolytica* strains, although "hard" fats are not considered as appropriate for microbial growth [41] and despite all previous considerations related to the theoretical difficulty of biodegradation of media composed of saturated FAs, in submerged cultures carried out where fat substrates in which saturated FAs (e.g. C16:0, C18:0) were predominant, high DCW values (i.e. up to *ca* 31 g/L) accompanied by high $Y_{X/S}$ yields (up to *ca* 1.6 g/g) have been recorded [12]. On the other hand, Kajs and Vanderzant [42] reported lower yield values (0.76 g/g) for *S. lipolytica* and *Candida utilis* grown on tallow. Ota and Kushida [43] reported a yield of 0.88 g/g for *Candida* sp. grown on sardine oil, while *S. lipolytica* grown on beef tallow obtained a yield half this value. These results again demonstrate the challenge of various microbial strains belonging to *Y. lipolytica* to degrade "hard" fats, composed mainly of saturated FAs, that, as stressed are not easily assimilated by several types of microorganisms [7,15]. In general, in our study, the values of $Y_{X/S}$ obtained for both fats were very satisfactory when compared with the average value (*ca* 1.0 g/g) reported in the literature for *Y. lipolytica* [5,18-20,44-46] or for other microorganisms capable of growth on several fatty materials employed as substrates (i.e. *Apiotrichum curvatum*, *Wickerhamomyces anomalus* and *Aspergillus* sp.) [14,29,47].

The pH of the media was monitored regularly and it was observed that cell growth on both fats was not accompanied by significant pH drop throughout the culture (a drop of 0.4-0.6 units), indicating minor release of organic acids into the medium. The medium pH, therefore, always remained within the range of 5.2-6.0, which represents the most commonly used optimum pH value for the growth of yeasts, when cultivated on fats [11,12,48,49]. On the other hand, during growth on

glucose and/or glycerol under nitrogen-limited conditions (i.e. that promote the synthesis of organic acids or the *de novo* synthesis of microbial lipid [7,15]), the current *Y. lipolytica* strain produced noticeable quantities of citric acid (up to *ca* 58 g/L) [29].

Accumulation of intra-cellular lipid during the exponential phase was observed in all treatments, in agreement with the relevant literature which indicates that during growth on hydrophobic materials (the so-called "*ex novo*" lipid accumulation process), lipid production is a process associated with biomass production, occurring irrespective of nitrogen exhaustion from the growth medium [5,7,13,15]. Lipid in DCW values for *Y. lipolytica* were higher when growth was carried out on olive oil compared with growth on butter. The differences of the yield on the different substrates indicate that the process of lipid accumulation from hydrophobic substrates is critically influenced by the FA composition of the utilized fat [5,10,11,15,20]. Papanikolaou et al. [11] studied the growth of *Y. lipolytica* on blends of stearin (a "hard" fat composed of *ca* 100% w/w saturated FAs) and hydrolyzed oleic rapeseed oil (a "soft" fat principally composed of the FA Δ9C18:1) and found that lipid accumulation was positively related with the presence of the FA C18:0 in the medium [5,11,20]. When noticeable accumulation of cellular lipid occurred (i.e. lipid in DCW>30% w/w), the concentration of C18:0 into the medium was noticeably higher than that in the initial fat substrate composition, which explains the low levels of accumulated lipids observed during growth on olive oil and butter substrates (see Table 1). The lipid yield by the bacterial consortium was higher for the butter than for the oil and significantly higher than the yields obtained by the yeast, reaching the highest values ever reported in the literature. To the best of our knowledge there are no literature reports for such high yield values by *Bacillus* spp. and *Pseudomonas* sp.. Interestingly, the concentration of the accumulated saturated fatty acids (%, w/w), which was higher than 50%, resembled that of cocoa-butter or other exotic fats [11,15,50,51], indicating, the potential of the

bacterial consortium for the production of specialty lipids. The values obtained using both inocula indicate the considerable potential of these microorganisms to produce added-value compounds.

When fat substances are employed as microbial substrates, the pathways that could be involved in the fat degradation process, irrespective of the use of prokaryotes or eukaryotes, are as follows: *a*) Lipase-catalyzed hydrolysis of the TAGs employed as substrates; *b*) Incorporation of the released extra-cellular aliphatic chains inside the cells or fungal pellets and mycelia (as previously indicated, the rate of incorporation of the various extra-cellular FAs can be different and is related to the FA composition of the fatty blend employed); *c*) Potential reactions of biomodification of the FAs previously incorporated inside the microbial cells (i.e. reactions of elongation and/or desaturation); *d*) Reactions of incorporation of several FA chains inside the stored microbial TAGs; *e*) Partial or total assimilation of the acyl-CoA units, *via β*-oxidation pathway; *f*) Catabolism of the generated acetyl-CoA through the Krebs cycle (creation of ATP and energy); *g*) Anabolism of portion of the acetyl-CoAs generated after *β*-oxidation through the glyoxylic acid by-pass (reactions of glyconeogenesis or synthesis of amino-acids); *h*) Biodegradation ("turnover") of the previously stored TAGs when the extra-cellular carbon source is no longer available, yielding in the synthesis of acetyl-CoA, that will further be catabolized through Krebs cycle or will be used for anabolic reactions through the glyoxylic acid by-pass [5,7,11,13,15].

Butter as a "hard" fat (a fatty material that is relatively highly saturated) and olive oil as a "soft" fat (a highly unsaturated lipid), both deriving from a restaurant facility, were selected as suitable substrates that represented waste FOGs. The main FAs were palmitic, palmitoleic, stearic, oleic and linoleic acids, which have also been shown as principal long chain fatty acids of FOGs or other fatty wastes [9,24,50,51]. Montet et al. [52], Papanikolaou et al. [11], Papanikolaou and Aggelis [20] and Lopez et al. [46] noted the selective and rapid assimilation of the FA Δ9C18:1 as opposed to the FA C18:0 by strains of the yeast *Y. lipolytica*, a fact that was also validated in this

study. As with *Y. lipolytica* LFMB 20, the bacterial consortium did not assimilate FA (C18:0) and similar findings have been reported for *A. curvatum* and *Starmerella bombicola* [47,53]. However, in general, the FA composition of the intra-cellular lipid of the yeast grown on fatty materials, resembles that of the culture medium [10,13,15,29,54]. Williams et al. [51] observed a transformation of FOG FAs from unsaturated to saturated forms perhaps due to microbial transformations by bacteria. Finally, Tang et al. [9] reported that after the application of Bio-Amp, the unsaturated FAs may be transformed to their saturated intermediates before complete degradation takes place.

Conclusion

Through fat fermentation led by both *Y. lipolytica* and the consortium composed of *Bacillus* spp. and *Pseudomonas putida* CP1, FA profiles of both reserve lipids, as well as the remaining non-consumed fat substrate can be modified as the fermentation proceeds in comparison with the FA profile of the initial substrate. These modifications can be exploited to develop sustainable, mild, "green" and eco-friendly processes that are demanded and imposed by society and drive the research of oleochemical industries. The notable degradation of both "soft" and "hard" waste cooking fats at high initial concentrations by *Y. lipolytica* LFMB 20 and the bacterial consortium comprised of *Bacillus* spp. and *P. putida*, indicate their equal ability to be effectively used for the bioremediation of used-cooking oils and FOGs found in several types of wastewaters, including wastewater from restaurant facilities, slaughterhouses and food processing plants. Of particular interest is the fact that although the mixed culture BFL-CP1 was subjected to cellular autolysis at the later stages of growth, and despite this non-favorable stage for the microbial consortium, fat continued to be successfully removed indicating the potential of this consortium in environmental processes. Simultaneously, the high lipid yield values of both consortia reveal their potential for the

production of added-value compounds. Concluding, the ability of both *Y. lipolytica* yeast and *Bacillus* spp. and *P. putida* consortium to produce lipids with altered FA content presents significant industrial, ecological and biotechnological interest, since low- or negative cost fatty materials can be successfully "upgraded" and their FA composition can be substantially ameliorated.

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Table 1. Quantitative data deriving from kinetics of *Yarrowia lipolytica* LFMB 20 and the bacterial consortium BFL-CP1 on waste-cooked fats (at initial substrate fat S_0 concentration ≈8.5 g/L) concerning DCW (X, g/L) and total cellular lipids (L, g/L) production, substrate fat consumption (S_{cons}, g/L), total cellular lipids in DCW ($Y_{L/X}$, % w/w) and total DCW yield (g of DCW per g of substrate fat consumed) at different fermentation points: (a) when the maximum total lipid in DCW quantity ($Y_{L/Xmax,}$ % w/w) was achieved; (b) when the maximum total DCW quantity (X_{max}, g/L) was achieved; (c) when the maximum efficiency of substrate fat removal was achieved. Fermentation time is also presented for the mentioned points. Culture performed in shake-flask trials. Each point is the mean value of two independent measurements (SE<15%).

Strain/Substrate	Time (h)		S_{cons} (g/L)	Fat removal (%, w/w)[1]	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	$Y_{X/S}$ (g/g)
LFMB 20 Olive oil	20	a	4.0	42.6	4.4	1.06	24.0	1.10
	220	b,c	8.5	90.6	8.0	0.15	1.9	0.94
LFMB 20, Butter	25	a	2.9	29.0	4.3	0.86	20.0	1.48
	100	b	7.5	75.0	7.5	0.22	2.9	1.00
	191	c	8.8	88.0	7.0	0.49	7.0	0.80
BFL-CP1, Olive oil	72	a	3.8	47.5	2.4	1.00	41.7	0.63
	216	b	7.1	88.8	2.5	0.15	6.0	0.35
	312	c	7.6	95.0	1.1	0.11	10.4	0.14
BFL-CP1, Butter	48	a	3.3	44.0	2.2	1.38	62.7	0.67
	71	b	5.1	68.0	2.6	0.76	29.2	0.51
	312	c	7.1	94.7	1.0	0.04	4.0	0.14

1: $Efficiency\ \%\ of\ fat\ removal = \frac{Initial\ fat\ level - Final\ fat\ level}{Initial\ fat\ level}\ x\ 100$

Table 2 Bacterial identification and indication of lipolytic activity of the microorganisms comprising the bacterial consortium.

Bacterial Isolate	ID	% similarity	Lipase production
BFL1	*Bacillus circulans*	99%	++++
BFL2	*Bacillus megaterium*	99%	++
BFL3	*Bacillus subtilis*	100%	+++
BFL4	*Bacillus subtilis*	99%	+++
BFL5	*Bacillus amyloliquefaciens*	99%	+++
BFL6	*Bacillus licheniformis*	99%	++
BFL7	*Bacillus subtilis*	99%	++++
BFL8	*Bacillus licheniformis*	100%	++
BFL9	*Bacillus cereus*	99%	+++
Pseudomonas putida CP1	*Pseudomonas putida*	99%	–

Table 3**. Extra-cellular and intra-cellular fatty acids (%, w/w) when *Yarrowia lipolytica* LFMB 20 was grown on waste-cooked butter and olive oil. Cultures performed as in Table 1.**

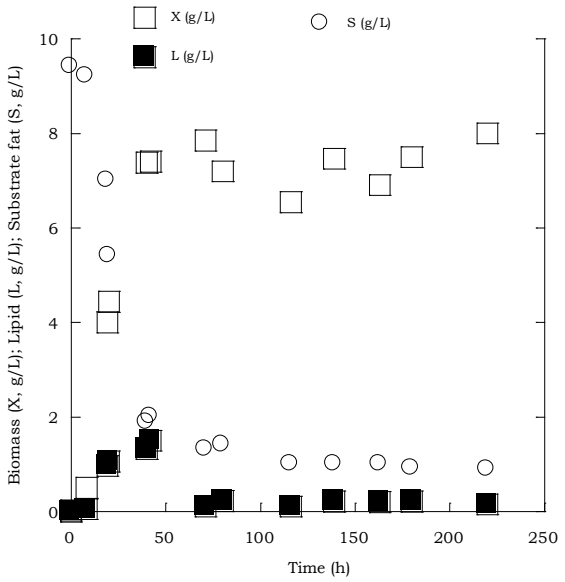
	EXTRA-CELLULAR FATTY ACIDS (%, w/w)						
	Fat removal (%, w/w)	(h)	16:0	Δ916:1	18:0	Δ918:1	Δ9,1218:2
OLIVE OIL		0	11.5	0.8	2.8	73.7	11.0
	80.0	40	12.8	1.0	6.7	66.6	12.7
	86.1	71	10.7	5.8	2.6	60.7	19.9
	89.4	116	10.3	6.2	2.8	60.2	20.2

	89.4	139	10.6	6.8	8.0	58.8	15.6
	89.5	163	15.2	4.6	8.2	55.5	13.2
			INTRA-CELLULAR FATTY ACIDS (%, w/w)				
	Cellular lipid (g/L)	(h)	16:0	Δ916:1	18:0	Δ918:1	Δ9,1218:2
	1.06	20	15.2	0.9	2.6	70.1	9.4
	1.32	40	10.0	0.9	1.9	72.7	12.6
	0.11	71	7.0	3.7	8.1	66.9	12.1
	0.21	139	10.8	2.8	15.1	59.3	7.7
			EXTRA-CELLULAR FATTY ACIDS (%, w/w)				
BUTTER	**Fat removal (%, w/w)**	(h)	16:0	Δ916:1	18:0	Δ918:1	Δ9,1218:2
		0	34.4	0.3	4.6	37.4	21.1
	54.6	48	39.9	0.3	5.6	35.5	18.4
	75.0	100	33.7	3.0	5.3	34.8	23.0
	88.0	191	36.0	0.5	7.0	31.0	24.0
			INTRA-CELLULAR FATTY ACIDS (%, w/w)				
	Cellular lipid (g/L)	(h)	16:0	Δ916:1	18:0	Δ918:1	Δ9,1218:2
	0.74	25	42.1	2.2	4.9	38.5	12.1
	0.22	100	38.4	1.3	6.1	37.4	16.5
	0.49	191	47.7	T.	7.7	34.5	9.9

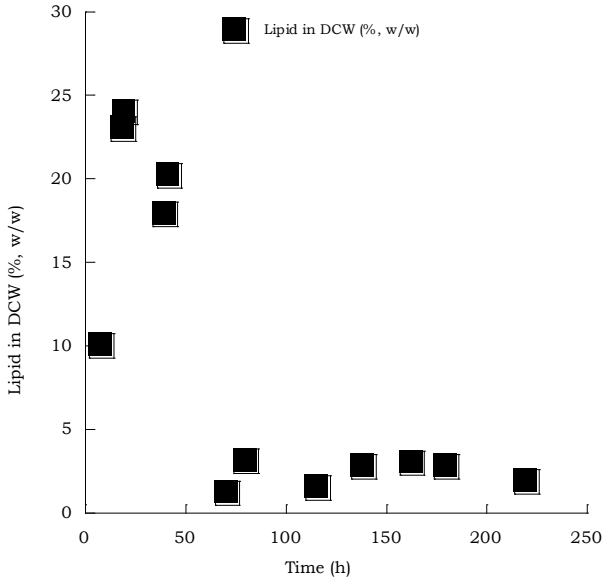
Table 4. Extra-cellular and intra-cellular fatty acids (%, w/w) when the bacterial consortium (BFL-CP1) was grown on waste cooked butter and olive oil. Cultures performed as in Table 1.

			EXTRA-CELLULAR FATTY ACIDS (%, w/w)				
OLIVE	**Fat removal (%, w/w)**	(h)	C16:0	Δ9C16:1	C18:0	Δ9C18:1	Δ9,12C18:2

		0	12.0	0.8	2.6	77.6	7.0
	47.5	72	10.6	0.4	10.0	77.4	1.7
	68.4	120	10.4	0.3	10.6	76.2	1.6
	88.1	168	10.4	T.	22.3	65.3	1.5
				INTRA-CELLULAR FATTY ACIDS (%, w/w)			
	Cellular lipid (g/L)	(h)	C16:0	Δ9C16:1	C18:0	Δ9C18:1	Δ9,12C18:2
	1.00	72	T.	T.	T.	54.1	45.0
	0.68	120	31.4	T.	37.5	27.0	3.8
	0.16	168	30.5	T.	39.1	26.5	3.3
				EXTRA-CELLULAR FATTY ACIDS (%, w/w)			
BUTTER	**Fat removal (%, w/w)**	(h)	C16:0	Δ9C16:1	C18:0	Δ9C18:1	Δ9,12C18:2
	0	0	45.5	2.4	16.5	33.7	1.9
	44.0	48	40.1	2.0	32.1	24.0	1.0
	68.0	71	41.8	2.1	29.2	25.6	1.1
	82.6	120	32.8	1.3	54.3	9.3	2.2
	82.8	168	34.3	0.8	55.6	8.3	1.7
				INTRA-CELLULAR FATTY ACIDS (%, w/w)			
	Cellular lipid (g/L)	(h)	C16:0	Δ9C16:1	C18:0	Δ9C18:1	Δ9,12C18:2
	1.38	48	31.1	0.7	42.5	24.1	0.8
	0.76	71	30.7	T.	55.0	14.2	T.
	0.07	168	29.1	T.	53.1	12.9	1.0

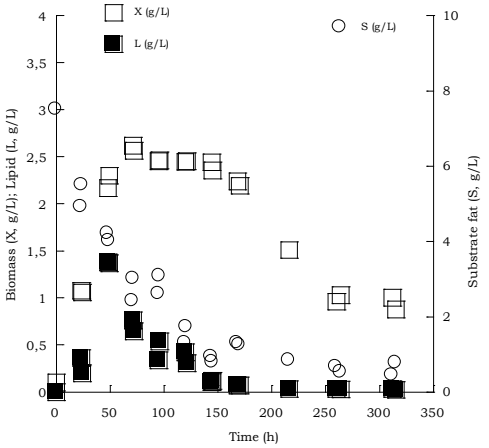


a

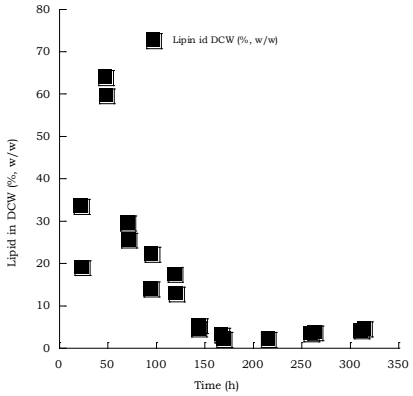


b

Figure 1. Biomass (X) intra-cellular lipid (L), substrate fat (S) (a) and cellular lipid in DCW (%, w/w) (b) evolution by *Yarrowia lipolytica* LMBF 20, growing on waste-cooked olive oil in shake-flask experiments. Each point is the mean value of two independent measurements (SE<15%).



a



b

Figure 2. Biomass (X) intra-cellular lipid (L), substrate fat (S) (a) and cellular lipid in DCW (%, w/w) (b) evolution by and the bacterial consortium BFL-CP1 on waste-cooked butter in shake-flask experiments. Each point is the mean value of two independent measurements (SE<15%).

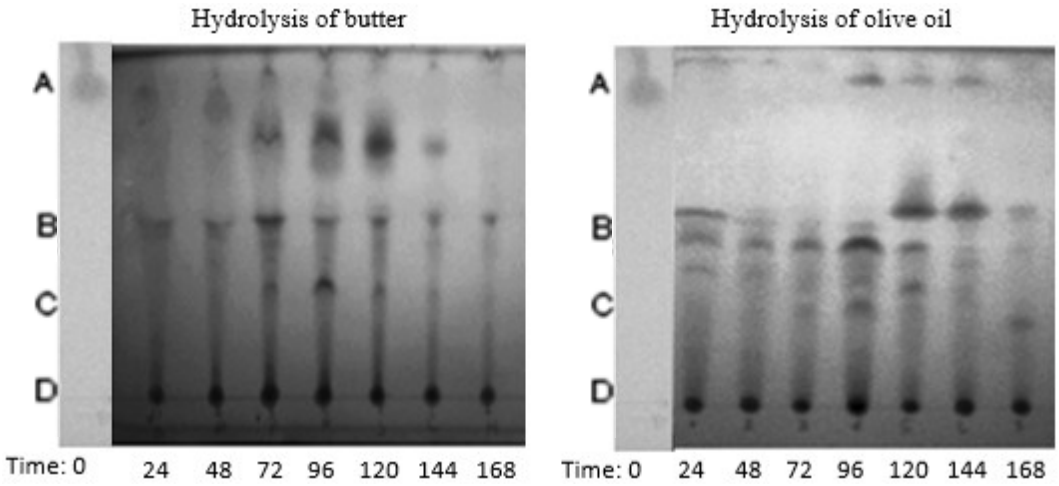


Figure 3. TLC analysis showing hydrolysis of both fat substrates, viz. waste butter and waste olive oil (A – triacylglycerols; B – free fatty acids; C – diacylglycerols; D – monoacylglycerols) by the bacterial consortium BFL-CP1.