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Valorization of lipid-rich wastewaters: A theoretical analysis to tackle the competition between polyhydroxyalkanoate and triacylglyceride-storing populations

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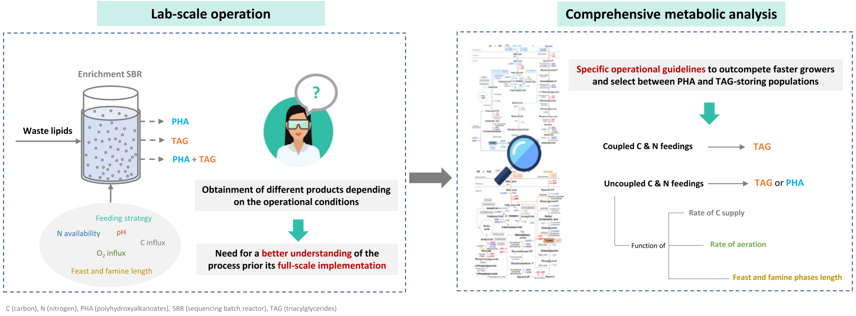
HIGHLIGHTS

- Operational conditions select for preferent TAG or PHA storage from waste lipids.
- Coupled C and N supply promotes TAG production.
- Uncoupled C and N feedings along with limited C excess favours PHA over TAG storage.
- Pathways analysis unravelled the experimentally slower synthesis of PHA than TAG.

GRAPHICAL ABSTRACT



C (carbon), N (nitrogen), PHA (polyhydroxyalkanoates), SBR (sequencing batch reactor), TAG (triacylglycerides)

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ABSTRACT

The lipid fraction of the effluents generated in several food-processing activities can be transformed into polyhydroxyalkanoates (PHAs) and triacylglycerides (TAGs), through open culture biotechnologies. Although competition between storing and non-storing populations in mixed microbial cultures (MMCs) has been widely studied, the right selective environment allowing for the robust enrichment of a community when different types of accumulators coexist is still not clear. In this research, comprehensive metabolic analyses of PHA and TAG synthesis and degradation, and concomitant respiration of external carbon, were used to understand and explain the changes observed in a laboratory-scale bioreactor fed with the lipid-rich fraction (mainly oleic acid) of a wastewater stream produced in the fish-canning industry. It was concluded that the mode of oxygen, carbon, and nitrogen supply determines the enrichment of the culture in specific populations, and hence the type of intracellular compounds preferentially accumulated. Coupled carbon and nitrogen feeding regime mainly selects for TAG producers whereas uncoupled feeding leads to PHA or TAG production function of the rate of carbon supply under specific aeration rates and feast and famine phases lengths.

1. Introduction

Food industries are global generators of large volumes of solid and liquid wastes. According to the Food and Agriculture Organization of the United Nations (FAO), one-third of the global food (about

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130 million tonnes) is wasted adversely affecting the natural environment. Besides, conventional handling and treating methods (mainly composting, incineration, and landfilling) involve the generation of air pollutants and/or are energy-intensive and expensive further slowing down sustainable development (Jin et al., 2021; Kumar et al., 2021). In particular, fish-canneries are characterized by producing high strength liquid effluents rich in fat, oil, and grease (FOG) (Cristóvaõ et al., 2015; Monteiro et al., 2018), with concentrations that can reach up to 5 g FOG/L (Klaucans and Sams, 2018; Mannacharaju et al., 2020). These streams present fundamental operational challenges first at their collection and transportation (as they cause blockages in the sewer system), and later at their treatment (affecting oxygen transference) (Frkova et al., 2020; Wallace et al., 2017). For this reason, FOG is normally separated at a pre-treatment stage in the wastewater treatment plant (WWTP) employing different techniques (i.e. dissolved air flotation units, skimming tanks, grease traps, etc.), concomitantly generating a secondary high-loaded and complex waste stream that has to be properly managed (Klaucans and Sams, 2018). Towards the advancement of the circular economy, this stream can be valorized rather than considered an undesirable residue (Dahiya et al., 2018; Wallace et al., 2017).

Lipids can be hydrolyzed by microbial activity producing mainly free fatty acids and glycerol (Becker, 2010), which are excellent substrates for the biological cytoplasmatic storage of carbon in the form of two different intracellular compounds, polyhydroxyalkanoates (PHAs) and triacylglycerides (TAGs) (Morya et al., 2018; Patel and Matsakas, 2019; Sangkharak et al., 2020). PHAs are biodegradable polymeric molecules with great interest due to their potential to replace conventional petroleum-based plastics (Sabapathy et al., 2020). On the other hand, TAGs recovered by certain oleaginous microorganisms from waste lipids present a fatty acid profile suitable for biodiesel production (Sawangkeaw and Ngamprasertsith, 2013; Tamis et al., 2015). Traditionally, the microbial valorization of lipid-rich streams involved expensive methods focused on the use of pure strains with a very high accumulation capacity and edible plant oils as a substrate leading to the obtainment of uncompetitive biofuels and biomaterials (Basnett et al., 2018; Pérez-Arauz et al., 2019). This fact motivated the use of residual lipid-rich streams for the production of both TAGs (Herrero et al., 2018; Lopes et al., 2018) and mostly PHAs (Mohapatra et al., 2017; Pernicova et al., 2019; Surendran et al., 2020), but it was not enough to render their production more economically feasible. To increase competitiveness, the use of waste substrates must be combined with mixed culture biotechnology and engineering designs with reduced costs and easily maintained operational conditions (Gujjala et al., 2019; Kourmentza et al., 2017; Kumar et al., 2020; Yadav et al., 2020). This solution considers engineering the ecosystem rather than specific strains to maintain a characteristic or functionality in the culture (Kourmentza et al., 2017; Mooij et al., 2013). Mooij et al. (2013) showed how to obtain a stable, open system enriched in storing populations inspired by Darwin's Theory of evolution by natural selection. To design better biotechnologies, they proposed that imposing the right selective environment can emphasize the ecological role of storage compounds, allowing for the robust enrichment of a complex culture with optimal storage capacity. However, what about competition between individuals in the community that store different intracellular compounds, and how could we target them by applying ecological selection principles? Although both PHAs and TAGs are valuable, potential applications of their mixture have not been identified, and downstream extraction and purification towards the separation of both compounds would probably have an important incidence in the process costs, reducing its competitiveness. Therefore, identification of the right selective pressures that maximize the accumulation of one storage compound and minimize the other, is fundamental towards increasing the value of the product recovered.

In this research work, we analyze the experimental shifts observed in the dominance between PHA and TAG storing populations in a 4-L enrichment sequencing batch reactor (SBR) fed with waste residual oil from the cooking water of the industrial production of canned tuna removed in the primary treatment of the factory WWTP (Argiz et al., 2021). It was the first time that the fed of a non-pretreated oily waste stream to an open mixed microbial culture (MMC) was explored and different types of storage compounds (PHAs and TAGs) were accumulated. However, although the experimental results obtained gave some light on lipid-rich waste streams valorization and certain operational conditions seemed advantageous for one or another type of accumulators (Argiz et al., 2021), there is still an important lack of understanding concerning culture and hence product selection that limits the further development of the biotechnology.

The objective of this research was to better understand the process towards a future larger-scale implementation. For that purpose, comprehensive metabolic descriptions supported by experimental observations were used to define specific operational guidelines towards imposing suitable selective pressures for not only outcompete faster-growers but select between storing populations.

2. Materials and methods

2.1. Experimental procedure

A 4-L SBR was fed with waste fish oil rich in oleic acid (Table S1 of Supplementary Material (SM)). It was inoculated with activated sludge from an urban WWTP. The SBR was aerobically operated in 12 h cycles selecting strains with high storage ability. The enrichment was carried under aerobic dynamic feeding (ADF) selection strategy, which relies on subsequent feast/famine (F/F) cycles in which the MMC is initially subjected to an excess of carbon source (feast) and then submitted to carbon deficiency (famine) (Kourmentza et al., 2017). At the end of each cycle, the reactor exchanged half of its volume (see dilution water composition in Table S2 of SM) resulting in hydraulic (HRT) and solid (SRT) retention times of 24 h.

Three operational periods were considered: I) simultaneous C (carbon) and N (nitrogen) (excess, about 1.2 g NH_4Cl/cycle) addition at the beginning of the cycle (conventional ADF, single growth limitation strategy); II) C supply at the beginning of the cycle and N addition (limited, about 0.6 g NH_4Cl/cycle) after 3 h to avoid its availability during the feast phase and restrict the growth of non-storing microorganisms (double growth limitation strategy (DGL)) (Kourmentza et al., 2017; Lorini et al., 2020); period III) same conditions as period II but feeding the N source after 2 h. The amount of carbon added to the system, expressed as chemical oxygen demand concentration (COD), was 4.48 g COD/cycle in the three operational periods studied.

More details concerning the SBR configuration and operation can be found in Argiz et al. (2021), and information regarding analytical methods and calculations is summarized in Table S3 of SM.

2.2. Metabolic analysis

The pathways for oleic acid and glycerol metabolic activities were constructed supported by literature assuming oleic acid as the only fatty acid present in the substrate (see Table S1).

Biotransformation of oleic acid was considered as: synthesis and degradation of P3(HB), assuming that a unit of PHA biopolymer comprised three hydroxybutyrate (HB) units (Fig. 1); triolein synthesis and degradation, describing a unit of TAG as three oleic acid units and one molecule of glycerol (Fig. 2); and its direct respiration (Fig. 3).

For the three catabolic pathways considered (Figs. 1, 2, and 3), the number of reactions involved, the maximum ATP produced, and the oxygen required was calculated. Also, the bioenergetics of specific reactions of interest called 'branching points', where bifurcation between two pathways occurs, were analyzed. It is to note that P3(HB) synthesis from acetyl-CoA is not thermodynamically favorable in comparison with acetyl-CoA oxidation in the TCA cycle (Fig. 1). Therefore, it was assumed that all P3(HB) synthesized from oleic acid occurred via hydroxyacyl-CoA.

The net yield of ATP was calculated as a combination of the ATP produced via substrate-level phosphorylation and the ATP yielded as a

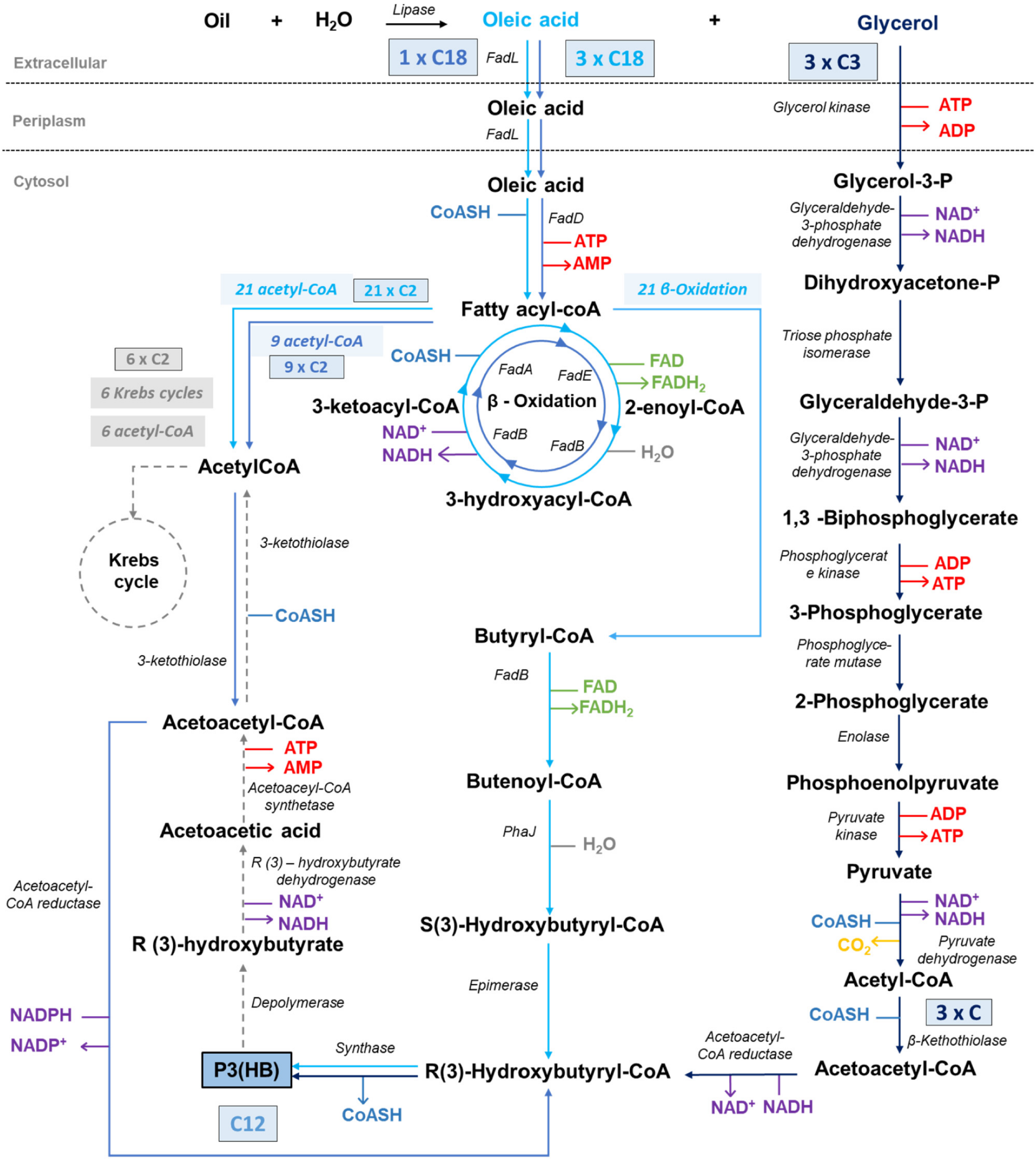

Fig. 1. Metabolic pathways and calculations of P3(HB) synthesis from three mol of oleic acid via 3-hydroxyacyl-coA (-), from one mol of oleic acid via acetyl-CoA (-), from three mol of glycerol (-), and degradation of 1 mol of P3(HB) (--). Abbreviations: fadA (β-kethotiolase), fadB (3.hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydrase), fadD (acyl-CoA synthetase), fadE (acyl-CoA dehydrogenase), fadL (long chain fatty acid transport protein), PhaJ (R)-3-enoyl-CoA hydratase. P3(HB) storage (basis: 3 mol oleic acid): 2.15 steps/Cmol, 1.58 ATP/Cmol, 0.42 mol O_2/Cmol. P3(HB) degradation (basis 1 mol P3(HB)): 5.92 steps/Cmol, 5.13 ATP/Cmol, 1.13 mol O_2/Cmol.

consequence of NADH (2.5 mol ATP/mol NADH) or $FADH_2$ (1.5 mol ATP/mol $FADH_2$) oxidized per mole of substrate consumed (Shestov et al., 2013). To determine the amount of oxygen reduced to water per substrate consumed, the moles of NADH and $FADH_2$ reduced along the pathway were calculated knowing that these reduced forms release two electrons to the electron transport chain eventually consuming 0.25 mol of O_2 per electron. To have comparable data, all results were expressed per mole of carbon (Cmol).

Details regarding pathways analyses are described in SM section "*2 Metabolic pathways analysis*" and calculations are exemplified in the xlsx file "*Oleic acid metabolism calculations*".

2.3. Microbial analysis

DNA from biomass samples was isolated using the FastDNA-2 mL SPIN Kit for Soil and the FastPrep24 apparatus (MP-BIO, USA). Two

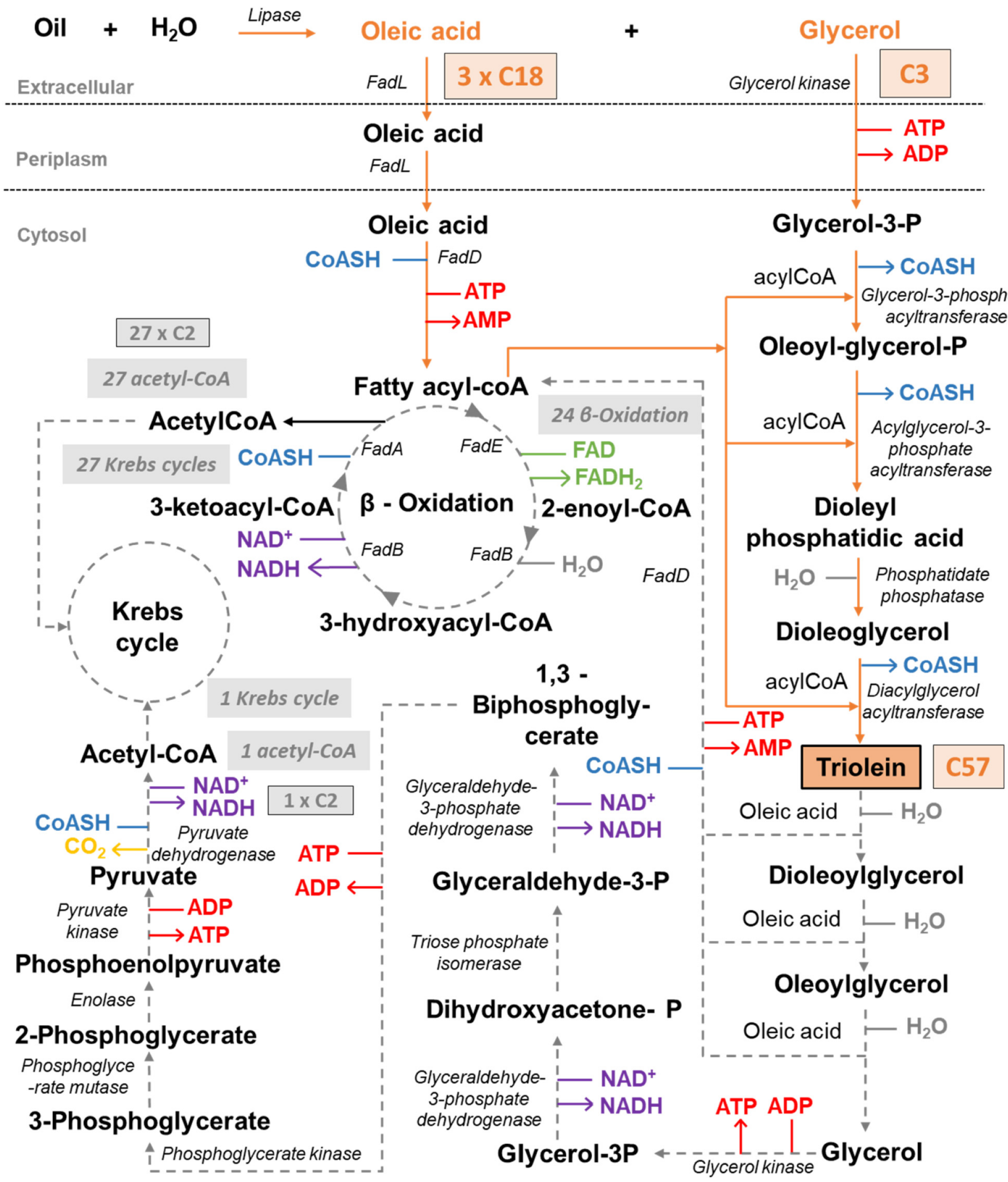

Fig. 2. Metabolic pathways and calculations regarding triolein synthesis from three oleic acid units and one mol of glycerol (-), and its degradation (--). Abbreviations: fadA (β-kethotiolase), fadB (3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydrase), fadD (acyl-CoA synthetase), fadE (acyl-CoA dehydrogenase), fadL (long chain fatty acid transport protein). Triolein storage (basis: 3 mol oleic acid + 1 mol glycerol): 0.35 steps/Cmol, - 0.12 ATP/Cmol, 0.00 mol O_2/Cmol. Triolein degradation (basis 1 mol triolein): 6.98 steps/Cmol, 6.64 ATP/Cmol, 1.43 mol O_2/Cmol.

independent biological replicates were used from each sampling time.

Bacterial and fungal Illumina sequencing was made using the primers Pro341F/Pro805R (Takahashi et al., 2014) and QuantF/FungiQuantR, respectively. The pipeline analysis was performed following MothurMiSeq guidelines in the software Mothur v1.44.1 (Schloss et al., 2009). The resulting operational taxonomic units (OTUs) (97% similarity threshold and abundances higher than six sequences (relative abundance (RA) > 0.001%)) were taxonomically classified through the blast suite of the Geneious 2021.1.1 software (Biomatters, New Zealand) against the bacterial 16S rRNA and fungal 18S rRNA NCBI database (www.ftp.ncbi.nlm.nih.gov/blast/db/). Bacterial and fungal sequences were deposited in GeneBank (accession numbers SUB9840624 and SUB9074010, respectively).

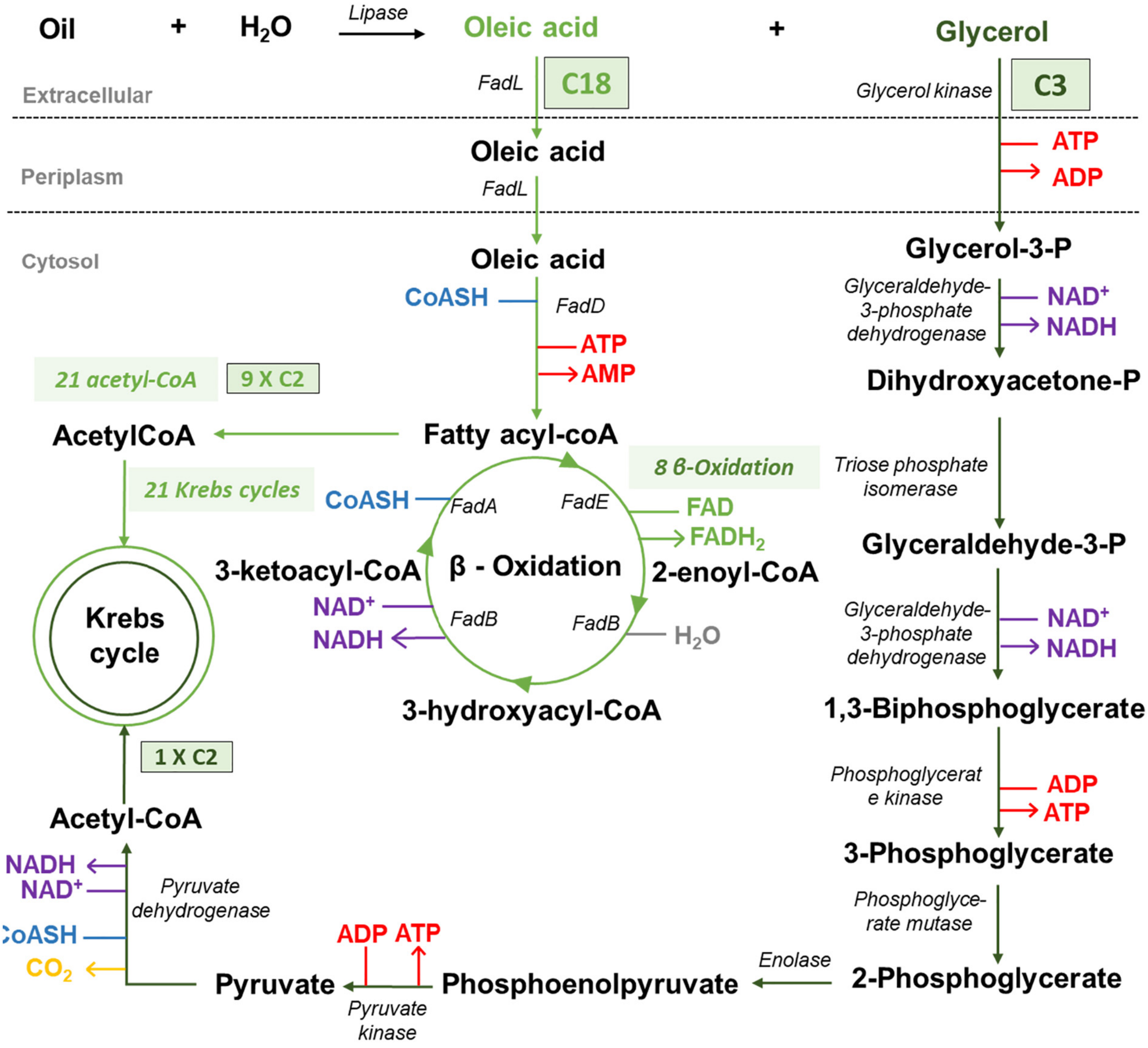

Fig. 3. Metabolic pathways and calculations concerning the respiration of one mol of oleic acid (▬) and one mol of glycerol (▬). Abbreviations: fadA (β-kethotiolase), fadB (3.hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydrase), fadD (acyl-CoA synthetase), fadE (acyl-CoA dehydrogenase), fadL (long chain fatty acid transport protein). Respiration (basis: 1 mol glycerol): 7.11 steps/Cmol, 6.67 ATP/Cmol, 1.44 mol O_2/Cmol. Respiration (basis: 1 mol oleic acid): 6.33 steps/Cmol, 5.33 ATP/Cmol, 0.50 mol O_2/Cmol.

3. Results and discussion

3.1. Selection of storing compounds and microbial populations correlates with changes in operational parameters

Fig. 4 shows the evolution of the main parameters controlling the process (pH, dissolved oxygen concentration (DO), PHA and TAG storage, COD, and total nitrogen (TN)) during three representative SBR enrichment cycles (all at stationary state after more than 50 cycles) of the three periods defined. As it can be observed, the dominance of TAG or PHA as storing compound correlates with specific changes in the operational parameters maintained during operation. When C and N sources were added together at the beginning of the cycle, and the N source was fed in excess (period I), only TAG storage was observed (Fig. 4A). After shifting the feeding strategy in period II (Fig. 4B) (C and N supply is uncoupled maintaining the same organic load of period II but limiting N availability), PHAs were preferentially accumulated. In period III, nitrogen source supply was brought forward 1 h maintaining the rest of the operational parameters. This shifted the type of dominant storage compound obtained from PHA to TAG and allowed for the obtainment of higher intracellular storages in comparison to period I (Fig. 4C).

The operational changes made not only led to a change in product yielding but also important variations in the composition of the dominant fungal and bacterial genera present within the community. Between periods I and II fungal genus *Geotrichum* (OtuF0003) and *Mortierella* (OtuF0004) decreased their relative abundances (RAs) from 26.0% and 22.8%, to 6.21% and 0.33% respectively, whereas *Apiotrichum* (OtuF0001) increased its RA from 37.8% to 87.2% (Fig. 5A). This correlates with the lower degree of hydrolysis observed and the decrease in TAG storage between periods I and II (Fig. 4A, B). *Geotrichum* (OtuF0003) has been reported to be cultivated on hydrophobic substrates being capable of secreting extracellular lipases (Hlavsová et al., 2009) and assimilating carbon sources such as fats and oils to use them for growth and storage (Papanikolaou et al., 2017; Patel et al., 2019). The same occurs with *Mortierella* (OtuF0004), also reported being able to secrete lipases (Jermsuntiea et al., 2011; Kotogán et al., 2018) and produce lipids from hydrophobic substrates such as triolein or sesame oil (Papanikolaou and Aggelis, 2019). However, *Apiotrichum* (OtuF001) has been only identified as capable of growing and storing lipids on hydrophilic substrates (Papanikolaou and Aggelis, 2011a; Park et al., 1990; Qian et al., 2021; Ykema et al., 1989) and it has not been reported as a lipase producer.

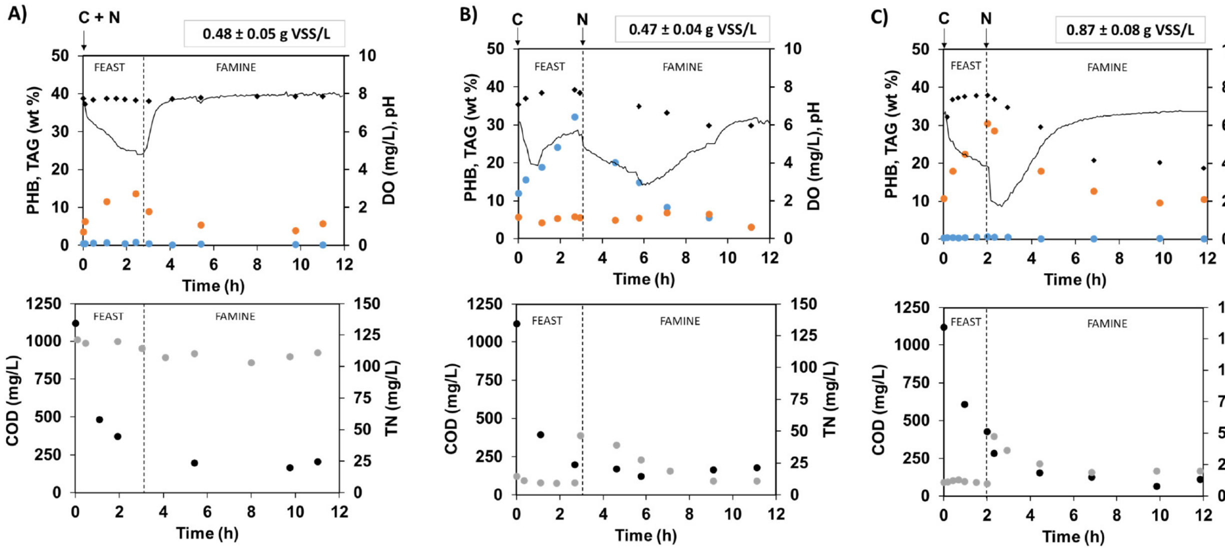

Fig. 4. PHB (●), TAG (●), pH (♦), DO (-), TN (●), and COD* (●) profiles evolution in representative SBR cycles (steady-state) during periods I (A), II (B) and III (C). *Initial COD values (1120 g/L) are referred to the amount of non-hydrolysed oily substrate added to the system at the beginning of the cycle. These were calculated dividing the mg of COD added at the beginning of the cycle (determined from the substrate COD (mg/g), its density (g/L) and the volume fed (L)) by the bioreactor volume (L). The discontinuous vertical line represented in this figure defines the end of the feast phase and the beginning of the famine.

For the bacterial community, *Chryseobacterium* (OtuB0003, OtuB0005) (38.5%), *Acidovorax* (OtuB0008) (12.2%) and *Acinetobacter* (OtuB0009) (9.0%) were the main bacterial genera during period I (Fig. 5B). However, after changing the feeding strategy between periods I and II, genera *Chryseobacterium* (OtuB0003, OtuB0005) reduced its abundance from 38.5% to 17.2%, and *Acidovorax* (OtuB0008) virtually disappeared (RA < 1%). Nonetheless, *Acinetobacter* (OtuB0007, OtuB0009, OtuB0010) raised its RA from 9.0% to 21.2%, and genera *Azospirillum* (OtuB0001), *Pandoraea* (OtuB0006), and *Klebsiella* (OtuB0014) went from being minority OTUs to present RAs of 21.06%, 13.0%, and 8.2%, respectively (Fig. 5B). Those genera that notably raised their RAs after changing the feeding strategy were all identified as PHA storing populations, which correlated with the higher PHA storage capacity observed in period II (Fig. 4B). Bacteria belonging to genera *Azospirillum* have been reported as capable of storing high amounts of PHA from substrates like acetate, pyruvate, lactate, and malate (Alves et al., 2017; Itzigsohn et al., 1995; Martínez-Martínez et al., 2019). Regarding *Pandoraea*, it was observed to accumulate PHA from waste frying oil and other industrial by-products (de Paula et al., 2017; Kumar et al., 2017, 2018; Liu et al., 2019). Also, *Klebsiella* has been widely identified as a very promising PHA producer (Ferreira et al., 2016; Wong et al., 2002) due to the high production yields that can be obtained on low-cost substrates (Valdez-Calderón et al., 2020; Wong et al., 2002). Besides, *Klebsiella* species present an active lipase system that makes them capable to hydrolyze and assimilate hydrophobic substrates such as waste frying oil (Tufail et al., 2017). Regarding *Acinetobacter*, several research works reported the high PHA synthetic yield from a wide variety of substrates (VFAs, glucose, glycerol, etc.) including cooking oils (Giraldo-Montoya et al., 2020; Li et al., 2020; Muangwong et al., 2016; Sabapathy et al., 2020) due to their ability to secrete lipolytic enzymes (Liu et al., 2013; Sharma et al., 2019). Nonetheless, *Acinetobacter* species are also capable of storing TAG from both hydrophilic (Alvarez and Steinbüchel, 2003; Manilla-Pérez et al., 2010; Salcedo-Vite et al., 2019; Wältermann et al., 2005) and hydrophobic substrates including olive oil (Alvarez et al., 1997; Alvarez and Steinbüchel, 2003), which explains their presence when TAGs were preferentially stored.

Between periods II and III, the fungal population shifted as *Apiotrichum's* (OtuF0001) RA decreased from 88.2% to 2.1%, and *Candida* (OtuF0002) (66.4%), followed by *Fonsecaea* (OtuF0005) (18.7%) and *Capronia* (6.9%) were then the dominant fungal populations (Fig. 5A). This community shift correlates with the observed increase in the degree of substrate hydrolysis and TAG concentration (from PHA to TAG between periods II and III, Fig. 4B and C respectively). Thus, oleaginous yeasts belonging to the genus *Candida* (OtuF0002) have been widely recorded to use not only hydrophilic but also hydrophobic substrates (i.e. triolein, olive oil, linseed oil) via the *ex novo* pathway (Dias et al., 2021; Papanikolaou and Aggelis, 2011b) and stand out by their ability to hydrolyze lipids (Theerachat et al., 2017). *Fonsecaea* (OtuF0005) has not been identified as an oleaginous fungus although it was demonstrated its ability to produce high levels of secretory lipases (Okeke and Gugnani, 1989). Besides, certain species belonging to genera *Capronia* (OTUF0007) were identified as capable of degrading lipids and exhibiting a strong lipase activity although there is no information concerning lipids storage (Untereiner and Malloch, 1999). For the bacterial community between periods II and III, *Azospirillum* (Otu0001) abundance was almost maintained (21.1% versus 25.3%) but PHA-storing genera *Pandoraea* (OtuB0006), *Chryseobacterium* (OtuB0003, OtuB0005), and *Klebsiella* (OtuB0014) became minority OTUs (RAs <0.5%). More details of the microbial community composition can be found in SM section "*3. Microbial analysis*".

3.2. Uncoupling C and N supply shifts preferential product stored: from TAG to PHA synthesis

Simultaneous carbon and nitrogen supply (period I) does not limit microbial growth in the feast phase (Silva et al., 2017). Under these conditions, O_2 consumption for ATP production supporting growth is expected to be preferential as long as carbon is available in the reaction medium (N is supplied in excess in period I).

Selection between P3(HB) synthesis from oleic acid or its oxidation is a function of the metabolic state of the cell (Ren et al., 2009), and only high [NADH]/[NAD$^+$] ratios, expected under limiting O_2 conditions (De Graef et al., 1999; Sun et al., 2012), will inhibit β-Oxidation favouring S(3)-Hydroxybutyryl-CoA conversion into R(3) Hydroxybutyryl-CoA to be further polymerized into P3(HB) (see details in SM section "*2. Metabolic pathways analysis*"). For P3(HB) synthesis, out of the 18 mol of carbon in one mole of oleic acid, 14 are oxidized meanwhile 4 mol are stored in P3(HB) form (Fig. 1). In presence of nitrogen excess, only if O_2 is limiting it will be more advantageous to synthesize P3(HB) from oleic acid than to oxidize it.

Overall, TAG accumulation from hydrophobic carbon sources follows the *ex novo* metabolic pathway, a growth-associated process in which intracellular lipids accumulation and respiration occur simultaneously

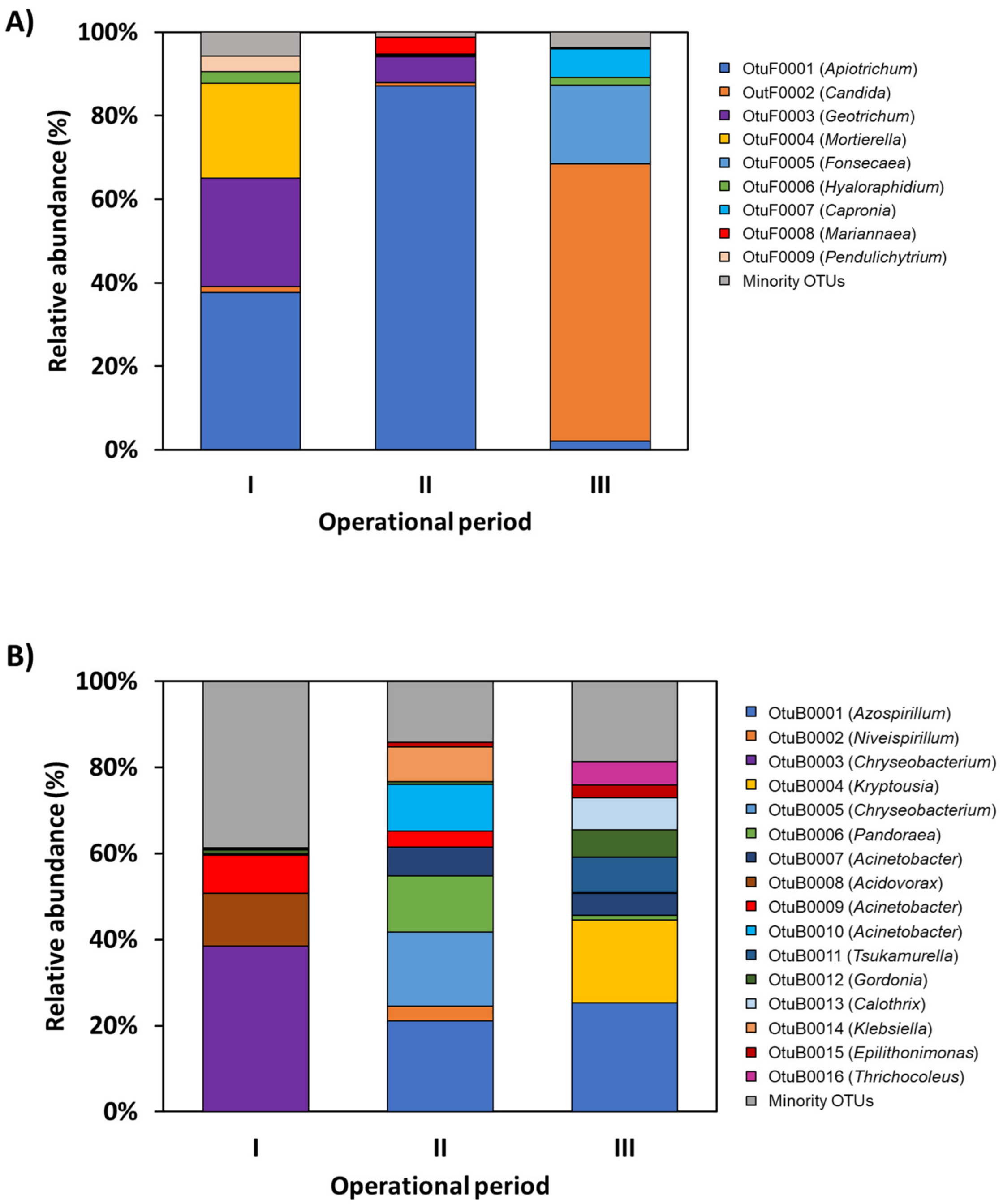

Fig. 5. Average relative abundances of dominant (RA > 1%) A) fungal, and B) bacterial OTUs identified by high throughput Illumina sequencing in enriched activated sludge samples (n = 2) during periods I – III.

when the carbon source is added in excess (Athenaki et al., 2018; Vasiliadou et al., 2018). Therefore, under coupled C and N feedings in excess, with no limiting O_2 conditions, COD oxidation is directed towards ATP production for growth and activation of TAG accumulation via *ex novo* pathway. This accumulation, although not preferential (maximum TAG concentrations in period I are low, 13.7 wt%), might help yeasts survive under the famine conditions. However, external COD is not fully depleted at any point of the cycle (Fig. 4A), therefore the bacterial community (Fig. 5B) can survive only through total carbon oxidation. PHA accumulation is not observed (Fig. 4A) as carbon use for growth is not nitrogen or oxygen limiting, and therefore internal [NADH]/[NAD^+] ratios are not expected to be high not enabling P3(HB) synthesis.

The uncoupling of carbon and nitrogen supply (starting period II) resulted in the observation of a shifted steady-state with the dominance of PHA storage over TAG. The feast phase without nitrogen feeding limits growth (Silva et al., 2017), which diminishes the ATP requirements of the cell. This implies that pressure towards carbon oxidation for ATP production is reduced and, under the feast famine strategy, storing populations will be pushed towards the production of storage compounds. Indeed, O_2 consumption in period II lowers in the feast phase (positive DO concentration slope, Fig. 4B), with faster COD consumption if its concentration at the end of the feast period is to be compared with the one measured in period I. This allows us to predict higher internal [NADH]/[NAD^+] ratios and therefore the unblocking of PHA production pathways.

The dominance of PHA production in the system promotes the longest O_2 consumption in the famine phase when compared with the ones observed in Fig. 4A and C. If in this system O_2 can be considered a proxy for ATP production, we can conclude that PHA accumulation allows longer periods of growth in the famine phase being a more successful strategy for survival in conditions of carbon starvation. The analysis of the pathways has shown us that mobilization of stored PHA is in terms of complexity, ATP production, and O_2 consumption is similar to TAG mobilization (Fig. 1, 2). However, previously reported observations have shown that TAG mobilization seems not to be preferential over external COD depletion but PHA consumption is (Aggelis et al., 1995; Vasiliadou et al., 2018). Thus, the LCFAs that were not consumed during the feast phase will be available as free fatty acids in the reaction medium during the famine (hydrolysis, transport, and activation were already performed in the feast phase). However, stored TAGs need to be intracellularly hydrolyzed and activated, which requires ATP (Fig. 2). A similar conclusion can be drawn from our observations, as is only in period II where O_2 consumption continuous beyond external COD exhaustion (Fig. 4).

3.3. Faster carbon supply shifts again the preferential product stored maintaining an uncoupled C and N feeding: from PHA to TAG synthesis

Period III starts when nitrogen feed was advanced 1 h maintaining the rest of the operational parameters. The feeding of sodium bicarbonate buffer was lower in this period (see SM section "*4. pH decrease in the famine phase*"), which decreased the pH value observed at the end of each cycle (Fig. 4C). These changes seem to dramatically select for TAG accumulators (Fig. 4B versus Fig. 4C, Fig. 5). TAG concentrations at the end of period III were the highest, and the culture was enriched in TAG producers (Fig. 5).

Yeasts (TAG accumulators in our SBR) are well-known to possess and express an active lipase system able to hydrolize complex fatty substrates (see SM section "*3. Microbial analysis*"). The highest substrate hydrolysis was achieved in this period (it was estimated a 35% and 75% hydrolysis in periods II and III respectively, see SM section "*5. Substrate hydrolysis estimation*"), which led to a notably higher carbon bioavailability increasing the amount of metabolizable carbon in the system per unit of time (Carsanba et al., 2018). This can explain the higher biomass concentration observed once reached the steady-state operation (from 0.47 ± 0.04 g VSS/L to 0.87 ± 0.08 g VSS/L in periods II and III). Remarkably, higher biomass concentrations than in period II were achieved with similar nitrogen consumptions. This is an indicator that a significantly higher abundance of yeast versus bacterial populations was achieved: yeasts and fungi require almost half of N to produce one mole of biomass than bacteria (see SM section "*6. Nitrogen balance*") (Milo and Phillips, 2015; Popovic, 2019).

The lower pH values reached at the end of the cycle in period III in comparison to period II (Fig. 4B, C) have limited the bacterial survival and enhanced the enrichment of the culture in TAG-storing microorganisms (Donot et al., 2014). Once steady-state conditions were reached, the higher carbon excess during the feast phase if compared to period II was evidenced by the evolution of COD profiles. When PHAs were preferentially stored, the carbon source was depleted before N source addition (Fig. 4B). However, in period III, extracellular carbon was still available during the famine phase (Fig. 4C). Therefore, as in period I, systematic O_2 consumption for external carbon oxidation and growth was concomitant to TAG degradation in the famine phase. This is evidenced by the sharp decrease of dissolved oxygen after nitrogen addition (Fig. 4C). Nevertheless, under uncoupled feeding, the maximum TAG accumulation obtained was much higher than when the feeding was coupled (period I). This led to a longer period of oxygen consumption compared to the one observed in period I.

According to metabolic pathways analysis, TAG storage is a substrate-limited process in comparison with PHA. Thus, fatty acids cannot be intracellularly accumulated as reserve materials until dioleoyl phosphatidic acid dephosphorylation. Therefore, to obtain an accumulative compound (dioleoglycerol), at least two oleic acid moles and one mole of glycerol are needed (Fig. 2). On the contrary, just one mole of oleic acid is needed to produce an HB unit that can be stored and further polymerized (Fig. 1).

The calculated stoichiometric ratios of carbon to oxygen (see detailed calculations in SM section "*7. C/O_2 ratios for PHA and TAG synthesis*"), showed that although to favor the selection of storing populations against non-storing ones always an excess of carbon is required. As a function of the carbon excess, one or another storage compound will be preferred. Substantially higher carbon influxes promote TAG (105 mg COD/(L·h), maximum 31.8 wt% at the end of the feast phase, Fig. 4C) over PHA accumulation (33 mg COD/(L·h)), maximum 32.1 wt% PHA at the end of the feast phase (Fig. 4B).

It was also observed that while PHAs were produced at a rate of 0.182 Cmol PHA/(Cmol substrate·h) reaching the maximum accumulation capacity after 3 h (2.15 steps/Cmol and 2.92 steps/Cmol via hydroxyacyl-CoA and acetyl-CoA, respectively) (Fig. 4B), TAG storage rate was almost the double (0.349 Cmol TAG/(Cmol substrate·h)) obtaining the maximum intracellular storage after only 1.5 h (0.35 steps/Cmol) (Fig. 4C). PHA synthesis appears as a more complex process than triolein accumulation (Fig. 1; Fig. 2), which can explain why the maximum storage capacity for PHA was achieved later than for TAG (Fig. 4B versus Fig. 4C). It can also explain why under fast rates of carbon feed, the conversion of carbon into PHA becomes rate-limiting, giving an advantage to the faster synthesis of TAGs. Triolein yielding only requires oleic acid transport and acylation in the cytoplasm of the cell (Fig. 2) whereas P3(HB) production involves the transformation of oleic acid via β-oxidation cycle (Fig. 1) in a pathway 1.80 steps/Cmol longer than the one required for triolein production using the same substrate.

In our experiment, the decrease of pH must play a role in the observed enrichment in yeast happening during period III. This probably enhanced hydrolysis of the substrate, allowing for higher carbon availability despite maintaining the same feed regime. The maximum TAG accumulation observed in our experiments was reached in this period. It was associated with remarkable higher hydrolysis and an earlier nitrogen feeding, which supports our hypothesis that a high carbon supply is necessary to systematically select for TAG production when C and N feeds are uncoupled.

4. Conclusions and recommendations

Long-chain fatty acids and glycerol present in lipid-rich streams serve as precursors for the microbial production of two different added-value compounds, PHAs and TAGs. Through this research, guidelines to engineering the competition between PHA and TAG-storing populations in an open community aiming for the preferent and most efficient obtainment of one or another storage compound were defined. Towards further process optimization and future large scale implementation of the biotechnology, the following outcomes should be taken into consideration:

- Avoid simultaneous oleaginous carbon and nitrogen feedings to select for PHA accumulators as it seems highly unlikely under these conditions (period I). Carbon consumption will be preferentially used for respiration and because PHA accumulation is a rate-limiting process compared with TAG synthesis, the latter will be more efficient when competing for the external carbon available. Moreover, TAG accumulation happens simultaneously to respiration processes while PHA synthesis requires a metabolic decision between ATP production or internal carbon storage.
- To increase intracellular accumulation uncouple carbon and nitrogen sources supply. This feeding strategy promotes higher concentrations of storage compounds under similar length of famine periods. It was observed maximum intracellular accumulations (as a sum of PHA and TAG) of 38 wt% and 32 wt% in periods II and III (uncoupled), and 15 wt% in period I (coupled). Switching between preferential PHA or TAG accumulation depends on the operational conditions (period II and period III, respectively) and comes together with the

measurement of higher relative abundances of PHA storing populations or oleaginous microorganisms (responsible for substrate hydrolysis and TAG storage; mainly yeast).

- Higher rates of hydrolysis and nitrogen supply in presence of external carbon, seems to favor preferential TAG production (period III). This can be explained by faster rates for TAG synthesis than for PHA (0.349 Cmol TAG/(Cmol substrate·h)) vs. 0.182 Cmol PHA/(Cmol substrate·h), which correlates with the theoretical analysis that shows PHA synthesis to be a more complex process. Besides, yeasts present the capacity to concomitantly use external carbon for respiration and accumulation.
- To select PHA producers, the carbon excess has to be limited (in our experiments, low hydrolysis), and preferentially, the external substrate should be depleted before nitrogen addition (period II). This can be explained by the lower carbon to oxygen stoichiometric ratio calculated for PHA synthesis than for TAG (0.60 and 2.02, respectively).

CRediT authorship contribution statement

Lucía Argiz: Investigation, Writing – original draft, Formal analysis, Conceptualization. **David Correa-Galeote:** Formal analysis, Visualization. **Ángeles Val del Río:** Validation, Visualization, Supervision, Funding acquisition. **Anuska Mosquera-Corral:** Validation, Supervision, Project administration, Funding acquisition. **Rebeca González-Cabaleiro:** Conceptualization, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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