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Open-culture biotechnological process for triacylglycerides and polyhydroxyalkanoates recovery from industrial waste fish oil under saline conditions

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ABSTRACT

Industrial waste fish oil streams contain high concentrations of medium and long-chain fatty acids suitable to produce value-added compounds. However, to process them dilution is required, and the water produced in the fish-canning industry commonly contains high salinity, which might limit its reuse as a dilution stream. Although NaCl is well-known to negatively affect biological activity, its effect on triacylglycerides (TAG) and polyhydroxyalkanoates (PHA) storage has not been well studied yet. Here, it was explored if intracellular TAG and PHA production can be efficient under saline conditions (10 g NaCl/L). For that purpose, waste fish oil was valorised using a mixed microbial culture (MMC) in a two-stage process (culture selection plus accumulation). Results showed that salinity influenced not only the activity but the structure of the microbial communities developed in the bioreactors. The bacterial genera *Acinetobacter* and *Rhizobium* and the mold *Candida glaebosa* clade were observed as the storing microorganisms which abundance increased under saline conditions whereas *Dipodascus* and *Mortierella* notably decreased. Nonetheless, despite the osmotic stress, promising results were obtained and maximum intracellular accumulations of 54.2 wt% (TAG:PHA = 28:72, 0.131 $Cmmol_{TAG}/Cmmol_S$, 0.303 $Cmmol_{PHA}/Cmmol_S$) and 50.9 wt% (TAG:PHA = 63:37, 0.291 $Cmmol_{TAG}/Cmmol_S$, 0.114 $Cmmol_{PHA}/Cmmol_S$) were observed when PHA and TAG were preferentially stored, respectively.

1. Introduction

The fish-canning industry is characterized by the generation of large volumes of liquid effluents containing high concentrations of organic compounds including, in many cases, hydrophobic lipids [1]. When the canneries have their wastewater treatment, comprising an aerobic biological step, these organic compounds usually reduce the efficiency of this treatment by limiting oxygen transfer [2]. For this reason, these lipid-rich compounds are mostly removed and rejected before full stream treatment in the wastewater treatment plant [2,3].

Recently, waste fish oil has started to be considered as a sustainable and low-cost source for the obtainment of storage compounds as triacylglycerides (TAGs) [4] and polyhydroxyalkanoates (PHAs) [5,6], value-added precursors for the production of biofuels and biomaterials alternative to fossil-based products [1].

Tamis et al. [4] have shown the feasibility of recovering lipids as TAGs when directly feeding soybean oil to a mixed microbial culture (MMC) in a process consisting of two steps: enrichment of the microbial culture in microorganisms with a high storage ability, and maximization of the storage compounds accumulation.

Regarding PHAs, its production from oily streams traditionally requires an additional pre-treatment unit for the obtainment of soluble compounds suitable for their bioconversion into PHAs (usually volatile fatty acids, VFAs) [7,8]. Nonetheless, Argiz et al. (2021) [9] recently demonstrated the possibility of performing in the same enrichment unit, the transformation of a lipid-rich waste stream produced in the fish-canning industry into long-chain fatty acids (LCFAs) and glycerol. Given that these compounds are precursors of both TAGs and PHAs, Argiz et al. (2021) [9] tried to identify those selective pressures that might control the selection of the dominant metabolic pathways. It was

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observed that uncoupling carbon and nitrogen feedings and limiting nitrogen availability in the medium allowed to maximise PHA accumulation. On the contrary, when this feeding strategy was combined with low pH in the famine phase, TAG storage was promoted [9].

The enrichment of MMCs in populations with high storage capacity is commonly carried in sequencing batch reactors (SBR) operated under the feast/famine (F/F) regime. In these reactors, half of their volume is exchanged with the feeding stream at the end of the operational cycle. Consequently, when using extremely high-loaded oily streams as a substrate (chemical oxygen demand (COD) concentration between 2000 and 2500 g/L), large volumes of water are required for dilution, severely reducing process sustainability. To enhance resource efficiency the possibility of reusing a water stream produced in the same industry for dilution could be considered. For this purpose, the fact that, in many cases, the effluents generated in the fish-processing sector contain relatively high salt concentrations needs to be considered.

The negative effect of salt concentrations above 5 – 8 g NaCl/L over the microbial activity has been reported for conventional biological treatment systems [3], but the influence of salt over the accumulation of TAGs [10–13] and PHAs [14,15] was mainly studied concerning the use of pure cultures and easily metabolizable carbon sources. These studies indicate that, in general, external stress caused by salinity promoted the intracellular accumulation of both compounds although different sensitivities were observed mainly depending on the strain used. Regarding the effect of salinity over TAGs storage in MMCs there is no available information and concerning PHAs accumulation, only [16,17] tested its influence when using non-acclimated and acclimated open cultures, respectively. Palmeiro-Sánchez et al. (2016) [16] considered concentrations up to 20 g NaCl/L and observed that maximum intracellular PHA storage decreased with the increase of NaCl in the medium. Wen et al. (2018) [17] demonstrated that the acclimation of the culture to salinity in the enrichment process allowed for the obtention of a higher PHA production under saline conditions although concentrations over 5 g NaCl/L caused a decrease of the process productivity.

The objective of the present study is to evaluate if identified selective pressures (C and N feeding regime and pH, [9]) that allowed for preferential PHAs or TAGs storage from waste oil in a two-stage system would also work under saline conditions (10 g NaCl/L). This research is focused on the analysis of the effect of NaCl over the operational parameters controlling the process, the culture enrichment and its microbial diversity, the maximum storage capacity, and the process productivity. Therefore, this study provides valuable information regarding the feasibility of reusing saline effluents as dilution water in the waste oil valorisation process for TAG and PHA production.

2. Materials and methods

2.1. Experimental set-up

To evaluate the effect of salinity over the industrial waste fish oil valorisation process, two lab-scale bioreactors were operated: an enrichment unit (SBR-S) and an accumulation fed-batch reactor (FBR-S). This two-stage system was analogous to the described by Argiz et al. (2021) [9], used as “control” (without salt) for comparative purposes. This consisted of an enrichment reactor (SBR-C) in which storing microorganisms were selected from activated sludge in the absence of salinity, and an accumulation reactor (FBR-C) inoculated with the biomass enriched in SBR-C.

2.1.1. Operation of the enrichment reactor (SBR-S)

The enrichment reactor (SBR-S) was inoculated with activated sludge from a full-scale reactor in operation at an industrial wastewater treatment plant treating fish-canning saline effluents (10–15 g NaCl/L). The SBR-S, with a working volume of 4 L, was operated under the F/F regime in cycles as described in Figure SI 1. At the end of each cycle of 12 h, half of the volume of the reactor (2 L) was exchanged, resulting in hydraulic and solid retention times of 24 h. The SBR-S was continuously aerated and completely mixed with air flowing through two air diffusers located at the bottom. The temperature was controlled at 30 ± 3 °C by a thermostatic bath (Techne Inc, USA), and the dissolved oxygen (DO) concentration was measured online using a portable multimeter (model HQ40d, Hach-Lange, USA). No pH controller was used.

The carbon source consisted of the oily fraction of a fish-canning industry effluent (waste fish oil) from canned tuna production (2 mL/cycle, equivalent to 114.5 Cmmol/cycle) (Table SI 1a), the same used in SBR-C [9]. To simulate its dilution with the effluent of the same factory, 10 g NaCl/L were added to a synthetic water stream (2 L/cycle) with the same composition as the one used in SBR-C (Table SI 1b).

2.1.2. Fed-batch accumulation experiments

The maximum intracellular (TAG and PHA) accumulation capacity of the culture was determined by the performance of accumulation fed-batch assays in a 4-L FBR-S inoculated with biomass from SBR-S obtained after the two last enrichment cycles previous to FBR-S start-up. Except for the feeding regime, which consisted of pulses (between 6 and 10) of 0.8 mL of oil (equivalent to 45.8 Cmmol/pulse) without nitrogen addition, the operational conditions were kept identical to those of the SBR-S (the salinity was maintained at 10 g NaCl/L). The microbial activity in the accumulation assays was monitored by the continuous measurement of DO concentration. A new substrate pulse was added once the DO concentration experienced an increment indicating that the waste fish oil from the previous pulse was consumed. Maximum accumulation capacity was assumed to be reached when DO concentrations remained close to saturation values after the addition of a pulse.

2.2. Operational periods

The SBR-S was operated for 122 days, subdivided into three operational periods (Table SI 2) defined by two nitrogen feeding strategies (period I vs. periods II and III) and two concentrations of sodium bicarbonate buffer ($NaHCO_3$) in the water stream used for dilution (periods I and II vs. period III). In each period, one enrichment cycle (days 22, 85, and 106) and one accumulation assay (days 29, 85, and 106) were characterized (Table SI 2). For comparative purposes, it was considered analogous operational periods from the systems operated under saline (SBR-S) and non-saline conditions (SBR-C) (I, II, III vs C-I, C-II, C-III, respectively).

Until day 62 (period I), C and N sources were fed simultaneously and in excess at the beginning of the cycle imposing a conventional aerobic dynamic feeding regime (ADF). In periods II and III (days 63 – 122), C and N feedings were uncoupled imposing the double growth limitation (DGL) strategy. C was still fed in excess at the beginning of the cycle but N was added limited at the end of the feast to assure its absence during this phase, and avoid the growth of non-storing populations. Until day 85 (period II), the amount of $NaHCO_3$ added in the nutrients solution was the same as in period I. From day 86 onwards (period III), its concentration was reduced (from 1.0 to 0.8 g $NaHCO_3$ added per cycle).

The operational cycles with ADF feeding (period I) and uncoupled DGL (periods II and III) are described in Figure SI 1.

2.3. Analytical methods and calculations

The pH value was measured with a pH and Ion-Meter (GLP 22 Crison, Spain) and the electrical conductivity with a conductivity meter (probe Sension + EC5 HACH, Spain). Total and volatile suspended solids (TSS and VSS) were measured according to APHA/AWWA/WEF, (2017). The COD was determined according to Taylor et al. (1989) [18]. Total COD was measured in the raw sample ($_tCOD$) whereas the soluble fraction ($_sCOD$) and the other analyses determined in the liquid fraction were performed in the centrifuged (Centrifuge 5430 Eppendorf, USA) and filtered sample (0.45 μm pore size, cellulose-ester membrane, Advantec, Japan). VFA were determined by gas chromatography (6850 Series II

Agilent Technologies, USA), ions (Li^+, Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, NO_2^-, Br^-, NO_3^-, PO_4^{-3}, SO_4^{-2}) by ion chromatography (861 Advanced Compact IC Metrohm, Switzerland), and total organic carbon, inorganic carbon and total nitrogen (TOC, IC, and TN, respectively) by catalytic combustion (TOC-L analyzer with the TNM- module, TOC-5000 Shimadzu, Japan).

The content of biomass on intracellular TAGs and PHAs compounds was determined in the lyophilized biomass samples according to Smolders et al. (1994) [19]. Commercial calibration standards of TAGs (palmitic-, stearic-, oleic-, and linoleic- acids) and PHAs (copolymer containing 88 % hydroxybutyrate (HB) and 12 % hydroxyvalerate (HV)) were used (Sigma Aldrich, USA). The storage compounds content of the biomass samples was calculated on a mass basis and expressed in dry weight (wt%) as a percentage of the measured VSS.

The elemental composition of the active biomass (X) was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ [9] and its mass was determined as the difference between the mass of VSS (total biomass) and that of accumulated compounds (TAGs + PHAs). Regarding kinetic parameters, specific maximum consumption (- q_N) and production (q_{TAG}, q_{PHA}, q_X) rates were estimated from the maximum slopes of the curves describing the evolution of the different parameters. q values were expressed as Cmmol/($Cmmol_X$·h) except for q_X, which was referred to the substrate (S) and defined as Cmmol/($Cmmol_S$·h). Overall production rates (Q), Cmmol/(L·h), were determined by multiplying q_{TAG} and q_{PHA} by the active biomass concentration in the system ($Cmmol_X$/L). Production yields (Y), expressed as $Cmmol/Cmmol_S$, were calculated by dividing the production rates by the carbon source consumption rates.

2.4. Microbial analysis

Samples (33 – 175 mL) from the accumulation reactors FBR-S (with salt, this study) and FBR-C (without salt, [9]) were used to perform a microbial diversity analysis. These samples were centrifuged (14,000 rpm, 1 min), supernatants were discarded, and the resulting biomass samples were frozen and kept at −20 °C. Total deoxyribonucleic acid (DNA) content was extracted using the FastDNA SPIN Kit and the FastPrep 24-Instrument (MP Biomedicals, Germany) according to the manufacturer's protocol. DNA extracts were subjected to Illumina for bacterial 16S rRNA and fungal 18S rRNA sequencing. Amplification was performed using the primers Pro341F and Pro805R [20] and FungiQuantF and FungiQuantR [21] for *Bacteria* and *Fungi*, respectively. Default settings were used for quality control, primer trimming, filtering, pre-clustering, and chimera detection through Mothur V1.44.3. Operational taxonomic units (OTUs) were assigned at the 97 % cut-off level. Singleton OTUs were removed for later analysis. Finally, taxonomic classification was made by the 16S and 18S ribosomal database from the National Center for Biotechnology Information (U.S.) using the blast tool of the Geneious Prime v.2019 software (Geneious, U. S.). The 16S rRNA and 18S rRNA sequences retrieved in this research work were deposited in GeneBank under the accession numbers PRJNA701464.

To evaluate the effect of salinity over the bacterial and fungal communities, two hierarchical clustering analyses were made considering the relative abundances (RAs) of the main bacterial and fungal OTUs (RA > 0.5 %) found in each sample. Non-parametric tests (Mann-Whitney and Kruskal Wallis) were used to analyse differences among samples ($p < 0.05$).

3. Results and discussion

3.1. Waste fish oil valorisation under saline conditions

The medium salinity (10 g NaCl/L) did not affect the hydrolysis, and subsequent metabolization of the waste fish oil fed to the enrichment reactor. Thus, the typical F/F pattern in the SBR-S became apparent after only four days from the beginning of the operation (eight enrichment cycles). DO concentration decreased after substrate feeding and it was maintained at low values during carbon source consumption (feast phase). Then it increased because of external substrate exhaustion (famine phase).

However, the culture accumulation capacity was found to be limited. Thus, the feast/cycle length ratio obtained in period I was 0.31 ± 0.01, over the value of 0.25 found as optimum [22]. This observation was confirmed by the complete characterization of an SBR-S enrichment cycle carried after reaching stable operational performance (day 22) (Fig. 1a) together with an accumulation batch assay in the FBR-S (day 29) (Fig. 1d). According to the kinetic parameters and production yields observed during the feast phase (Table 1a), the high values obtained concerning q_X, q_N and Y_X suggest that high N availability in the medium promoted extracellular carbon source consumption for growth instead of its intracellular storage. With a TAG:PHA ratio of 96:4, the culture was able to accumulate about 17 wt% at the end of the feast phase and maximum intracellular storage in the FBR-S was even lower (13.5 wt%).

At this point, the need for process optimization to increase maximum storage capacity and productivity was clear. With this objective, those selective pressures favouring preferential TAG or PHA production when using a non-pre-fermented hydrophobic substrate identified by Argiz et al. (2021) [9], were tested here under saline conditions.

3.1.1. Channelling lipids towards PHA accumulation

To promote the enrichment of the MMC in PHA-storing populations and hence maximise PHA production in the FBR-S, in period II C and N sources were added separately (DGL strategy) and N availability was limited to the minimum required for growth in the famine phase (Figure SI 2a) [9].

Results obtained in the SBR-S concerning the percentage of intracellular TAGs and PHAs accumulated at the end of the feast phase (Fig. 2) show that the DGL strategy imposed from period II, was advantageous for PHA-storing populations even in saline environments.

For example, between days 22 and 85 in SBR-S, although intracellular accumulation as a sum of TAGs and PHAs only increased from 17.0 to 17.4 wt%, in terms of biopolymer composition, the competitive advantage of PHA-accumulators was clear (the TAG:PHA ratio shifted from 96:4 to 50:50) (Table 1; Fig. 2). This enrichment of the culture was evidenced by the reduction of the feast/cycle ratio, which decreased from 0.31 ± 0.01 to 0.11 ± 0.03 after uncoupling (Figure SI 2b). N limitation during the feast phase led to insignificant biomass production (q_X) and resulted in a larger specific PHAs accumulation rate (q_{PHA}) (Table 1). The wash-out of non-storing populations that were not able to grow once imposed the DGL selection strategy caused a decrease in the VSS concentration at the end of the cycle between periods I and II (from 0.79 ± 0.12 to 0.63 ± 0.02 g VSS/L, respectively) (Figure SI 2b).

The enrichment of the culture in PHA producers improved the accumulation capacity and productivity in the FBR-S (Fig. 2). Maximum intracellular PHA accumulation and production yield increased from 0.8 wt% to 39.0 wt% and from 0.006 to 0.303 $Cmmol_{PHA}/Cmmol_S$, and the overall PHA production rate raised from 0.021 up to 1.239 $Cmmol_{PHA}$/(L·h), between days 29 (conventional ADF selection) and 85 (DGL selection), respectively.

Previous studies exhibited higher PHA production yields than the obtained in this study when working under concentrations of approximately 10 g NaCl/L (Table 2). Regarding the maximum storage capacity, differences were significant in comparison with pure strains but when considering MMCs and non-synthetic substrates, the results obtained in the present research work were maximum (Table 2). Besides, unlike previous authors reported [14,16,17], here it was not observed in the accumulation reactor (FBR-S) a negative effect over intracellular PHAs storage due to the osmotic stress caused by salinity. Thus, once reached the maximum accumulation, there was no intracellular PHA degradation (Fig. 1e) as occurred in previous research works [14,16,17]. This result might be a consequence of the origin of the inoculum and its high degree of acclimation to saline environments before the MMC

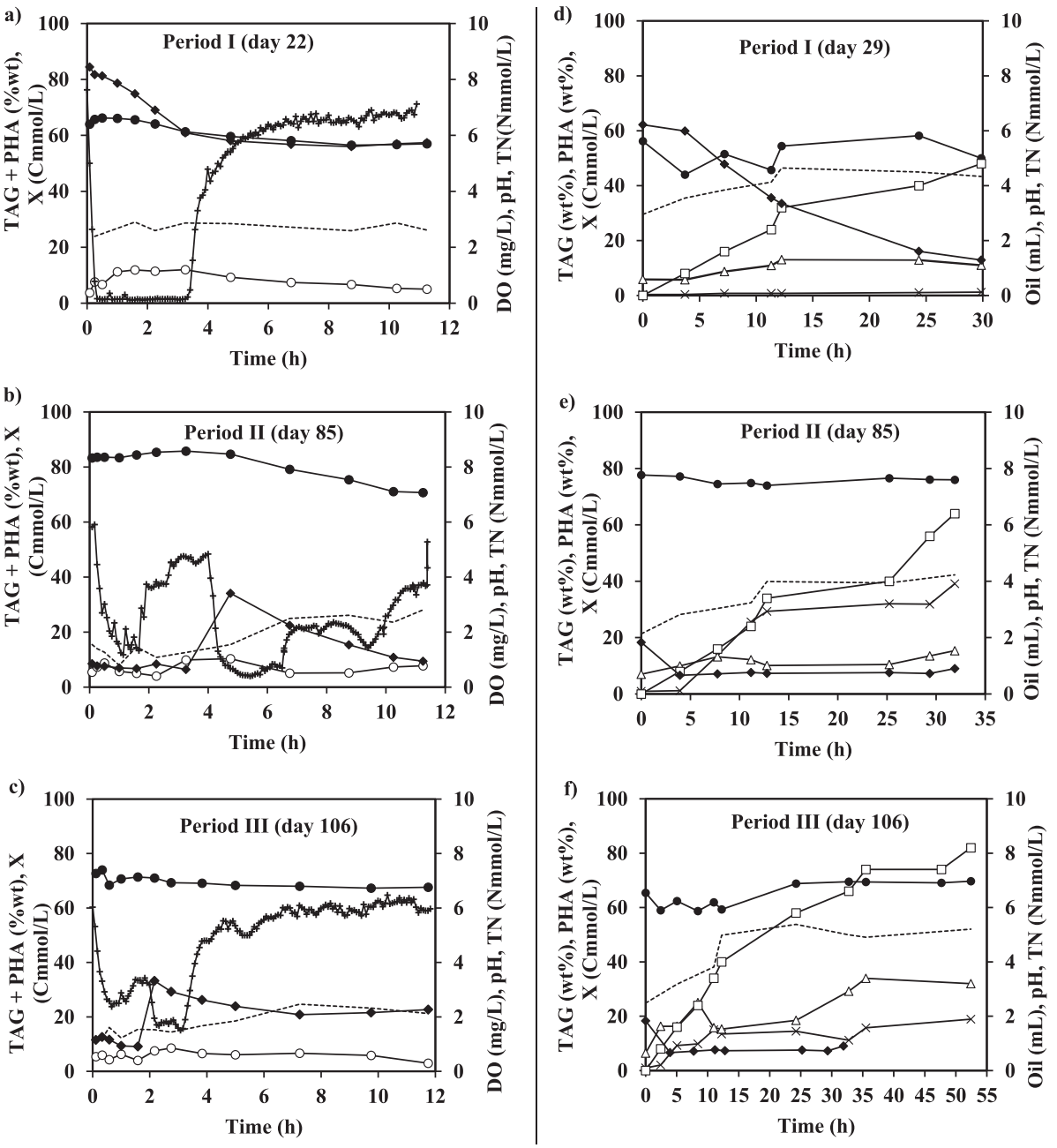

Fig. 1. Characterization of three enrichment cycles (a, b, c) and three accumulation assays (d, e, f) monitored in SBR-S and FBR-S, respectively. pH (●), DO (+), X (—), TN (◆), TAG + PHA (○), TAG (Δ), PHA (×) and cumulative waste fish oil added (□).

enrichment.

3.1.2. Channelling lipids towards TAG accumulation

TAG producers present a higher tolerance to acid environments (pH 3 – 4) than PHA ones [23]. Therefore, to select TAG producers, in period III, the amount of $NaHCO_3$ buffer added in the dilution stream was decreased (Figure SI 2c).

However, the reduction of the buffer concentration under saline conditions did not cause the expected medium acidification in the famine phase that was observed in the absence of salinity [9]. The pH at the end of the cycle decreased only 0.3 units presenting high stability during the whole cycle performance. According to Sun et al. (2016) [24], increasing salt concentrations reduce the activity coefficient of water leading to a CO_2 solubility reduction and therefore to lower acidification of the system.

However, although it was not possible to induce in the SBR-S an environment so acidic as to favour TAG-storing populations, slightly lower pH values obtained during the feast in period III, in comparison to period II, might have affected intracellular pH regulation leading to a decrease in PHA production (Fig. 2). Thus, bacteria regulate their intracellular pH (within 1 to 2 units of neutrality) by controlling the flow of protons across the membrane and when the cytoplasm becomes too acidic, protons are pumped out [25]. However, when NaCl is present in the media, Na^+ diffuses into the cells (the cell membrane is negatively charged inside and positively outside) but cells need to pump it out because it is cytotoxic [26]. Active transport of Na^+ requires proton motive force, which is generated by pumping protons inside the cytoplasm but creates an intracellular decrease of the pH that needs to be balanced. Under these conditions, the extracellular pH increases, and maintaining the intracellular pH in neutrality becomes more costly for

Table 1
Maximum intracellular accumulation (% wt max. as a sum of TAG and PHA), maximum specific production rates (q), overall production rates (Q), and production yields (Y) determined in a) enrichment cycles (SBR-S), and b) accumulation assays (FBR-S). Fed-batch reactor (FBR-S), nitrogen (N), sequential batch reactor (SBR-S), substrate (S), and active biomass (X).

a) SBR-S

Parameter	Period I (day 22)	Period II (day 85)	Period III (day 106)
% wt max.	17.0	17.4	8.5
TAG:PHA	96:4	50:50	80:20
q_{TAG} ($Cmmol_{TAG}/Cmmol_X \cdot h$)	0.063	0.017	0.028
q_{PHA} ($Cmmol_{PHA}/Cmmol_X \cdot h$)	0.002	0.042	0.020
- q_N ($Cmmol_N/Cmmol_X \cdot h$)*	0.065 – 0.000	0.004 – 0.052	0.014 – 0.020
q_X ($Cmmol_X/Cmmol_S \cdot h$) *	0.056 – 0.001	0.000 – 0.050	0.036 – 0.083
Y_{TAG} ($Cmmol_{TAG}/Cmmol_S$)	0.193	0.037	0.028
Y_{PHA} ($Cmmol_{PHA}/Cmmol_S$)	0.006	0.090	0.020
Y_X ($Cmmol_X/Cmmol_S$)*	0.181 – 0.012	0.000 – 0.363	0.098 – 0.765

b) FBR-S

Parameter	Period I (day 29)	Period II (day 85)	Period III (day 106)
%wt max.	13.5	54.2	50.9
TAG:PHA	94:6	28:72	63:37
q_{TAG} ($Cmmol_{TAG}/Cmmol_X \cdot h$)	0.009	0.015	0.026
q_{PHA} ($Cmmol_{PHA}/Cmmol_X \cdot h$)	0.001	0.035	0.010
Q_{TAG} ($Cmmol_{TAG}/(L \cdot h)$)	0.356	0.535	1.046
Q_{PHA} ($Cmmol_{PHA}/(L \cdot h)$)	0.021	1.239	0.409
- q_N ($Cmmol_N/Cmmol_X \cdot h$)	0.030	0.050	0.012
q_X ($Cmmol_X/Cmmol_S \cdot h$)	0.001	0.005	0.002
Y_{TAG} ($Cmmol_{TAG}/Cmmol_S$)	0.106	0.131	0.291
Y_{PHA} ($Cmmol_{PHA}/Cmmol_S$)	0.006	0.303	0.114
Y_X ($Cmmol_X/Cmmol_S$)	0.353	0.144	0.130

* Data referred to feast and famine phases: feast – famine.

the cell. Therefore, the intracellular pH might decrease leading to a decrease in PHA production [27]. For example, on days 85 and 106 in the SBR-S, the average pH in the feast phase was 8.4 ± 0.1 and 7.1 ± 0.2, respectively (Fig. 1b, 1c.) and while TAG storage only varied from 8.7 wt % to 6.4 wt%, intracellular PHAs decreased from 8.5 wt% to 2.1 wt%. This lower extracellular pH was also observed to affect PHAs storage in the FBR-S. Intracellular PHAs accumulation and overall PHA production rate decreased from 39.0 wt% to 18.9 wt% and from 1.239 $Cmmol_{PHA}/(L \cdot h)$ to 0.535 $Cmmol_{PHA}/(L \cdot h)$ between days 85 and 106 (average pH during the assays was 7.6 ± 0.1 and 6.5 ± 0.5, respectively). However, TAGs storage and overall TAG production rate increased from 15.2 wt% to 32.1 wt% and from 0.409 $Cmmol_{TAG}/(L \cdot h)$ to 1.046 $Cmmol_{TAG}/(L \cdot h)$ (Table 1; Fig. 3a).

Therefore, although alkalinity reduction was not enough to acidify the medium and create a clear selective advantage for TAG producers, slightly lower pH values obtained during the feast phase could have affected intracellular pH regulation and hence PHA storage.

Previous studies regarding intracellular TAG production using halotolerant strains and hydrophilic carbon sources, exhibited higher intracellular storages although lower production yields than the observed in the present research work (Table 2).

3.2. Comparison with a non-saline control system

To evaluate the effect of NaCl, results obtained in this study were compared with those reached in an analogous system fed with a non-saline medium, enrichment SBR-C, and accumulation FBR-C [9]

3.2.1. Effect of osmotic stress over TAG and PHA production

Important differences were observed between SBR-C and SBR-S regarding aeration requirements and the system buffering capacity. According to DO concentration profiles, oxygen, and hence energy consumptions appeared higher in SBR-S (Fig. 1) than in SBR-C [9]. Indeed, it has been postulated that higher NaCl:COD ratios lead to higher oxygen consumption rates due to additional cellular requirements [3]. On the other hand, the presence of NaCl appeared to bring stability to the pH of the system. $NaHCO_3$ addition did not need to be progressively adjusted in SBR-S (Figure SI 2c). The buffering capacity of the saline system remained stable and did not rise over time as happened in SBR-C, in which the amount of buffer added was diminished by > 35% during the first 50 days. This effect of NaCl over the pH could allow for a more stable and cheaper long-term operation without the need for the use of automatic control equipment. Results suggest that under high NaCl concentrations, $NaHCO_3$ buffer might not be even

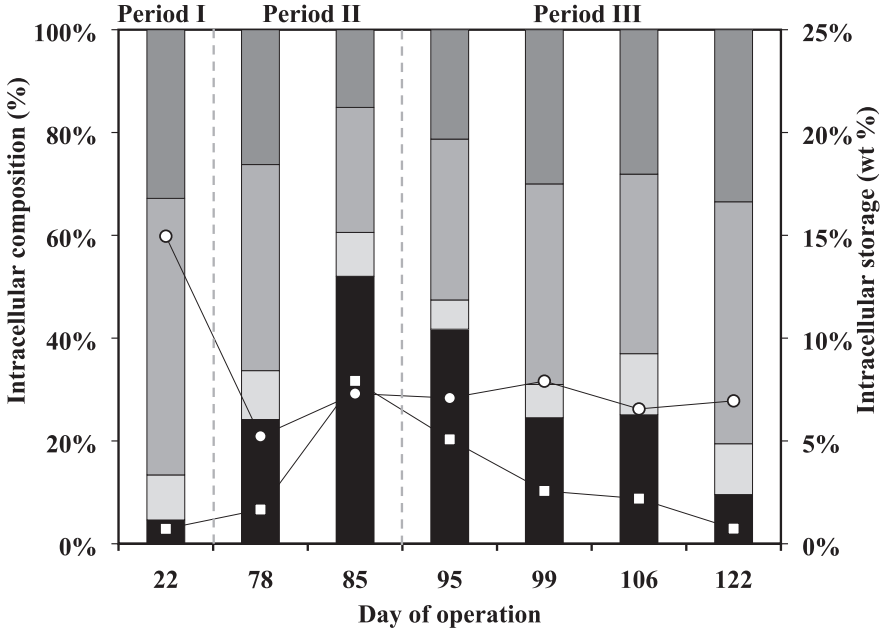

Fig. 2. Percentages of intracellular compounds accumulated (TAG (○) and PHA (■)), and composition (palmitic (□), oleic (▩), linoleic (▩), PHB (■)) measured at the end of the feast phase in different SBR-S cycles throughout periods I – III.

Table 2
Influence of increasing NaCl concentrations over TAG and PHA storage in pure and mixed microbial cultures: a comparison with literature. Production yields (Y) expressed as:(1) g/g;(2) Cmmol/Cmmol;(3) g COD/g COD. Food waste fermentation leachate (FWFL), sequencing batch reactor (SBR), wastewater treatment plant (WWTP), volatile fatty acids (VFAs), yeast-peptone-dextrose medium (YPD).

	Inoculum	Substrate	NaCl (g/L)	Y	% wt max.	Reference
PHA						
	Cupriavidus necator	Acetic acid	0	0.32(1)	61.0	[14]
			3.5	0.32	75.0	
			6.5	0.37	80.0	
			9	0.41	78.0	
			12	0.13	60.0	
			15	0.03	20.0	
	Non-acclimated activated sludge	Synthetic VFAs mixture	0	0.72(2)	53.0	[16]
			7	0.62	34.6	
			13	0.65	17.4	
			20	0.62	8.9	
	Activated sludge acclimated to salinity in the SBR	Synthetic FWFL	0	0.64(3)	60.9	[17]
			5	0.64	50.5	
			10	0.74	52.0	
			15	0.79	42.6	
		FWFL	2.5	–	33.4	
			5	–	30.9	
			10	–	30.6	
	Sludge from a SBR located at a WWTP treating saline effluents	Waste fish oil	10	0.42(1) 0.33(2) 0.28(3)	39.0	This study
TAG						
	Zygozacchorimyces rouxii	YPD	0	–	34.0	[13]
			150	–	28.3	
	Rhodosporidium toruloides	Glucose	0	0.06(1)	42.0	[28]
			10	0.08	39.0	
			50	0.15	48.0	
			100	0.13	43.0	
	Rhodosporidium toruloides	Glucose	0	–	55.1	[11]
			5	–	62.1	
			10	–	62.2	
			15	–	62.4	
			25	–	60.6	
			40	0.18(1)	71.3	
			60	–	44.4	
	Sludge from a SBR located at a WWTP treating saline effluents	Waste fish oil	10	0.29(1) 0.30(2) 0.34(3)	32.1	This study

required to maintain the pH of the system around neutrality.

The overall activity of the MMC was also affected by osmotic stress. First, its respiration capacity was altered. Concentrations of 60 – 80 mg/L of acetic acid were measured in the media at the end of the cycle in SBR-S, which seemed to increase after N addition in the uncoupled cycle (data not shown). This demonstrates that part of the acetyl-CoA produced in the β-Oxidation of the LCFAs contained in the substrate was transformed into acetic acid and brought out of the cell instead of being channelled to the Krebs Cycle for energy generation. Secondly, it affected the overall obtained ratios of TAG and PHA production, being the later the storage compound more affected. The imposition of the DGL strategy under saline conditions in SBR-S (Fig. 3) led to a lower degree of enrichment of the culture in PHA-accumulators than in SBR-C. This could be related to a possible negative effect of salinity over PHA-accumulation kinetics, which correlates with the previously reported decrease of PHA production rates with NaCl concentrations in the range of 5 – 20 g/L [16,17]. In the present study, on day 85 (period II), q_{PHA} was 0.042 $Cmmol_{PHA}/(Cmmol_X \cdot h)$ in SBR-S (10 g NaCl/L) whereas, in SBR-C (no salinity), it was 0.084 $Cmmol_{PHA}/(Cmmol_X \cdot h)$ in period C-II. Besides, in SBR-S, PHAs storage presented a lag phase at the beginning of the cycle and it did not start until 2 h after C source addition. However, in SBR-C, microorganisms were able to accumulate PHAs from the beginning of the cycle (Figure SI 3).

Because of this lower degree of enrichment in SBR-S in comparison with SBR-C after the uncoupling of C and N feedings, results obtained in the FBR-S (period III) were substantially lower than in FBR-C (period C-III). In terms of maximum biopolymer accumulation, overall production, and production yield, values of 39.0 wt% vs 82.3 wt%, 1.239 $Cmmol_{PHA}/(L \cdot h)$ vs 2.225 $Cmmol_{PHA}/(L \cdot h)$, and 0.303 $Cmmol_{PHA}/Cmmol_S$ vs 0.803 $Cmmol_{PHA}/Cmmol_S$ were obtained, respectively. Besides, unlike what happened in the non-saline system, increasing PHAs production in FBR-S after SBR-S uncoupling, did not occur at the expense of TAG production (Fig. 3a). This suggests that a higher proportion of TAG-accumulating microorganisms remained in the MMC. In fact, between days 29 and 85, TAGs production increased from 12.7 wt% (TAG:PHA ratio of 94:6) to 15.1 wt% (TAG:PHA ratio of 28:72). Overall, PHAs accumulation seemed more affected by high salinity than TAGs accumulation (Fig. 3), which correlates with previously reported literature (Table 2).

Under saline conditions, preferent TAGs or PHAs accumulation was not observed as it occurred in the control system (Fig. 3b). The fatty acid profile of the intracellular TAGs accumulated was very similar and proportional to that of the substrate (Table SI 1b; Fig. 3b). Regarding PHAs production, no PHV was detected (Fig. 3b). This was expected because no PHV was measured in the control and it was previously reported that PHV production decreases with the increase of salinity in the medium [16].

3.2.2. Effect of osmotic stress over microbial diversity

In both FBR-S and FBR-C reactors, lower diversity of *Fungi* than *Bacteria* was observed. There were 858,778 (71,565 ± 2539 per amplicon library) and 679,360 (56.613 ± 4161 per amplicon library)

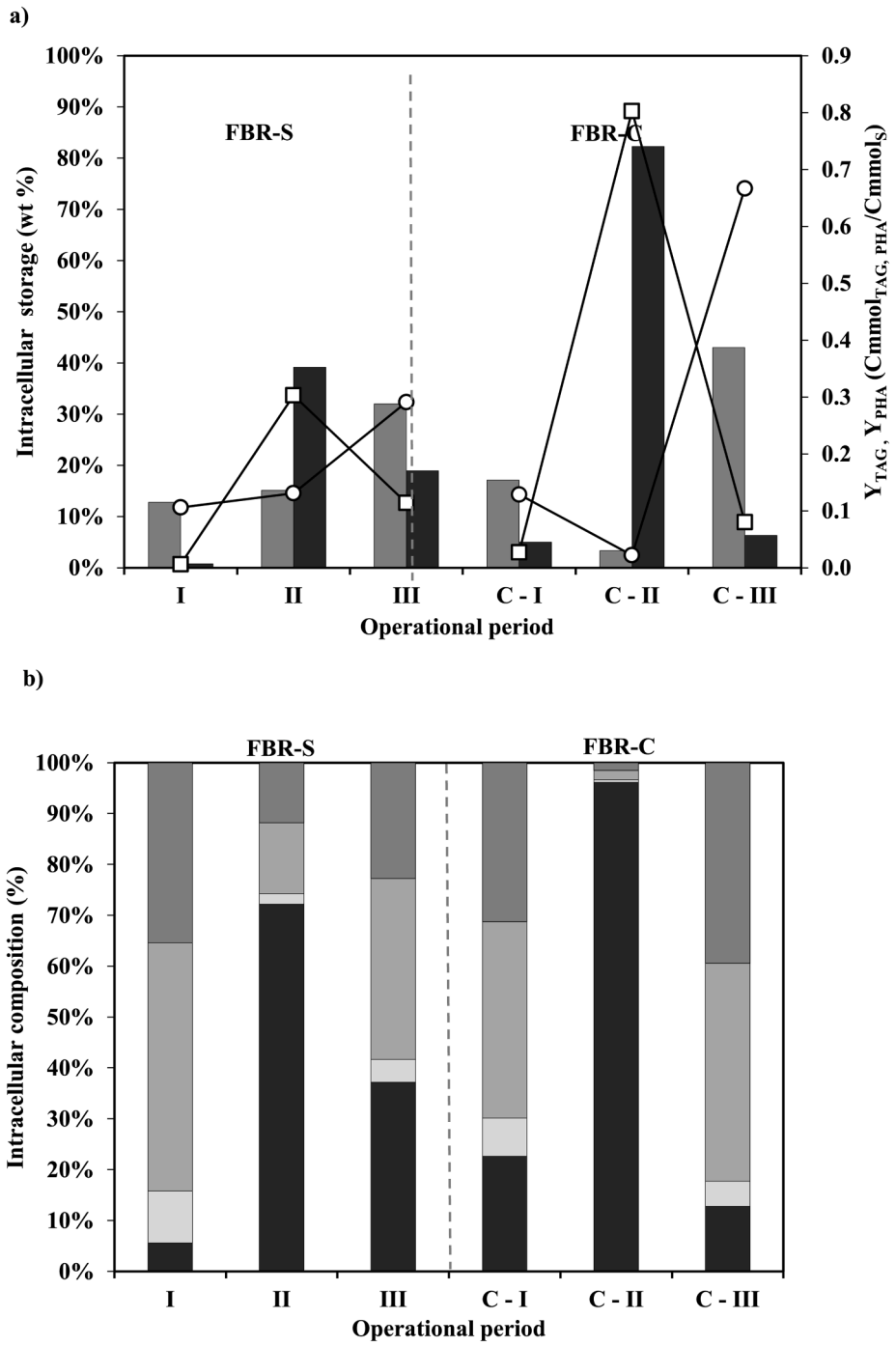

Fig. 3. Comparison between FBR-S and FBR-C considering analogous operational periods (I, II, III vs C-I, C-II, C-III). a) Maximum intracellular TAG (▒) and PHA (■) storage and production yields (Y_{TAG} (○), Y_{PHA} (■)), b) Intracellular compounds composition (palmitic (□), oleic (▒), linoleic (▒), PHB (■)).

sequences for *Fungi* and *Bacteria*, respectively, and clustering sequences into OTUs resulted in 1138 bacterial and 124 fungal OTUs.

The bacterial community comprised 15 different phyla distributed into 45 classes (Fig. 4a, Table SI 3). 24 had a relative abundance higher than 0.05 %, and 13 composed the bacterial common core (97 % of the total number of sequences). Classes *Alphaproteobacteria* and *Gammaproteobacteria* were the most abundant among the different operational periods in FBR-S. However, in FBR-C class *Gammaproteobacteria* was considerably less abundant than in the saline medium, especially in period C-II (0.30 ± 0.2 %). *Betaproteobacteria* was identified as an additional dominant class, mainly in periods C-I (33.49 ± 1.75 %) and C-III (28.48 ± 0.51 %). On the other hand, the fungal community was composed of 4 different phyla and 13 classes (Fig. 4b, Table SI 4), 8 of them with a RA higher than 0.05 %.

Saccharomycetes and *Tremellomycetes* were the main dominant classes in both reactors throughout the distinct operational periods and the only ones comprising the fungal common core (92 % of the total). According to the Kruskal-Wallis test (Conover-Iman *posthoc* test), only classes *Dothideomycetes* and *Cystobasidiomycetes* (Table SI 4) from FBR-S samples, and *Chlamydia* (Table SI 3) and *Agaricomycetes* from FBR-C samples (Table SI 4) presented statistical differences in their RAs among periods I–III and C-I – C-III, respectively. Overall, the structure of the majority of bacterial classes agreed with previous research works concerning PHA accumulation in lab-scale bioreactors inoculated with similar-origin

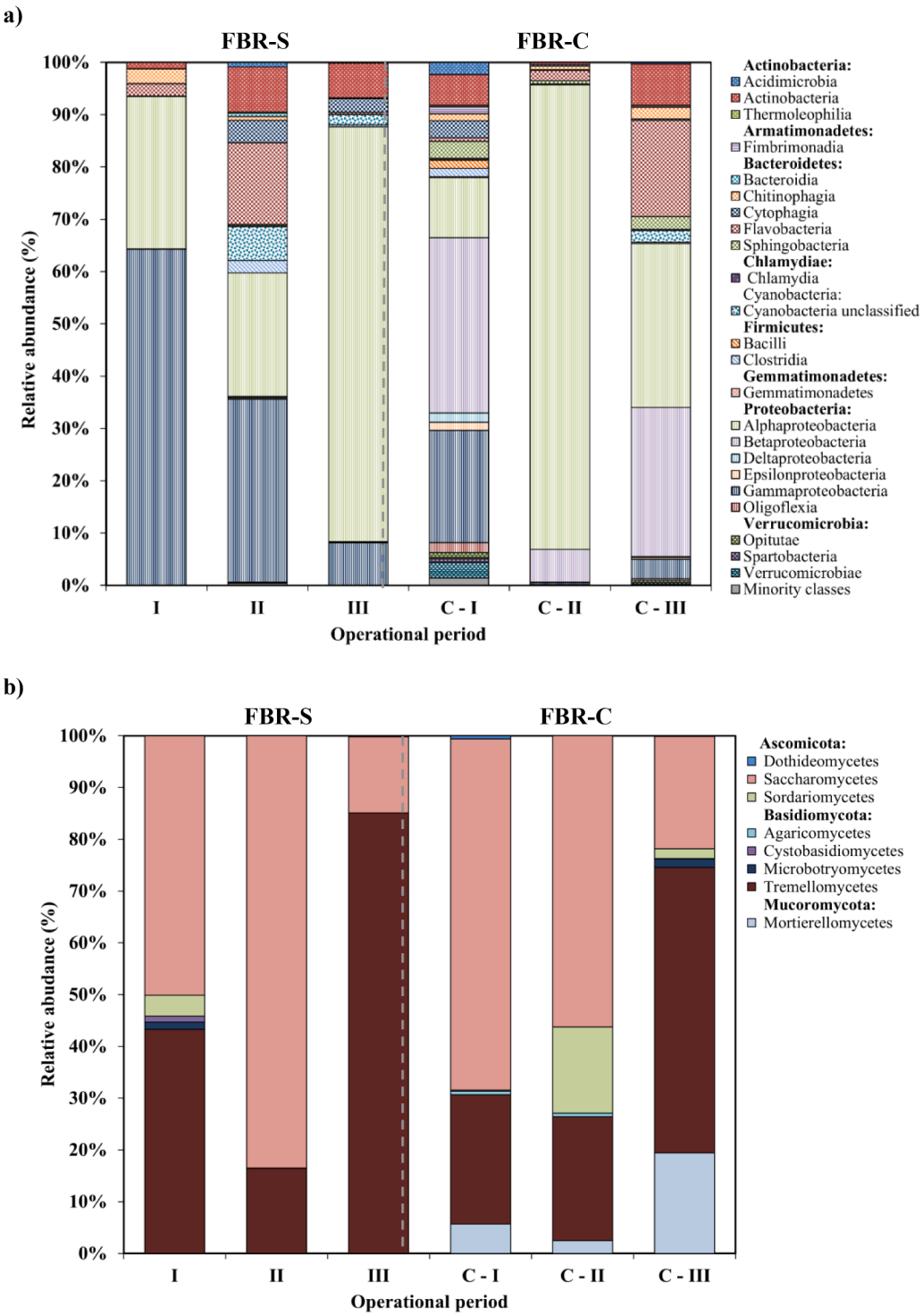

Fig. 4. Structure of main bacterial (a) and fungal (b) classes (RA > 0.5 %) identified by high-throughput Illumina sequencing in FBR-S and FBR-C biomass samples considering analogous operational periods (I, II, III vs C-I, C-II, C-III).

inocula [29]. Also, the fungal communities found here were similar to those previously described in conventional activated sludge bioreactors [30].

Concerning the OTU level, 29 of the 1138 bacterial OTUs (Fig. 5a, Table SI 5) and 7 of the 124 fungal ones were dominant (RAs > 0.5 %) (Fig. 5b, Table SI 6). Several dominant bacterial OTUs (genera *Acidovorax* (OtuB0010), *Acinetobacter* (Otus B0003, B0007 and B0008), *Comamonas* (OtuB0026), *Flavobacterium* (OtuB0013), *Lysobacter* (OtuB0022), and *Pseudomonas* (OtuB0024), *Rhizobium* (Otus B0014 and B0015)) were previously identified as able to accumulate PHAs [21,31–34]. Besides, different bacterial (genera Acinetobacter (Otus B0003, B0007 and B0008), *Gordonia* (OtuB0012) and Pseudomonas (OtuB0024)) and fungal (yeasts *Mortierella* (OtuF0007) and *Yarrowia* (OtuF0009)) ones were previously proposed as TAG-storing microorganisms [4,35–38].

Both FBR-S and FBR-C microbial communities presented important differences in each reactor between the periods studied as a consequence of the operational conditions and selective pressures implemented,

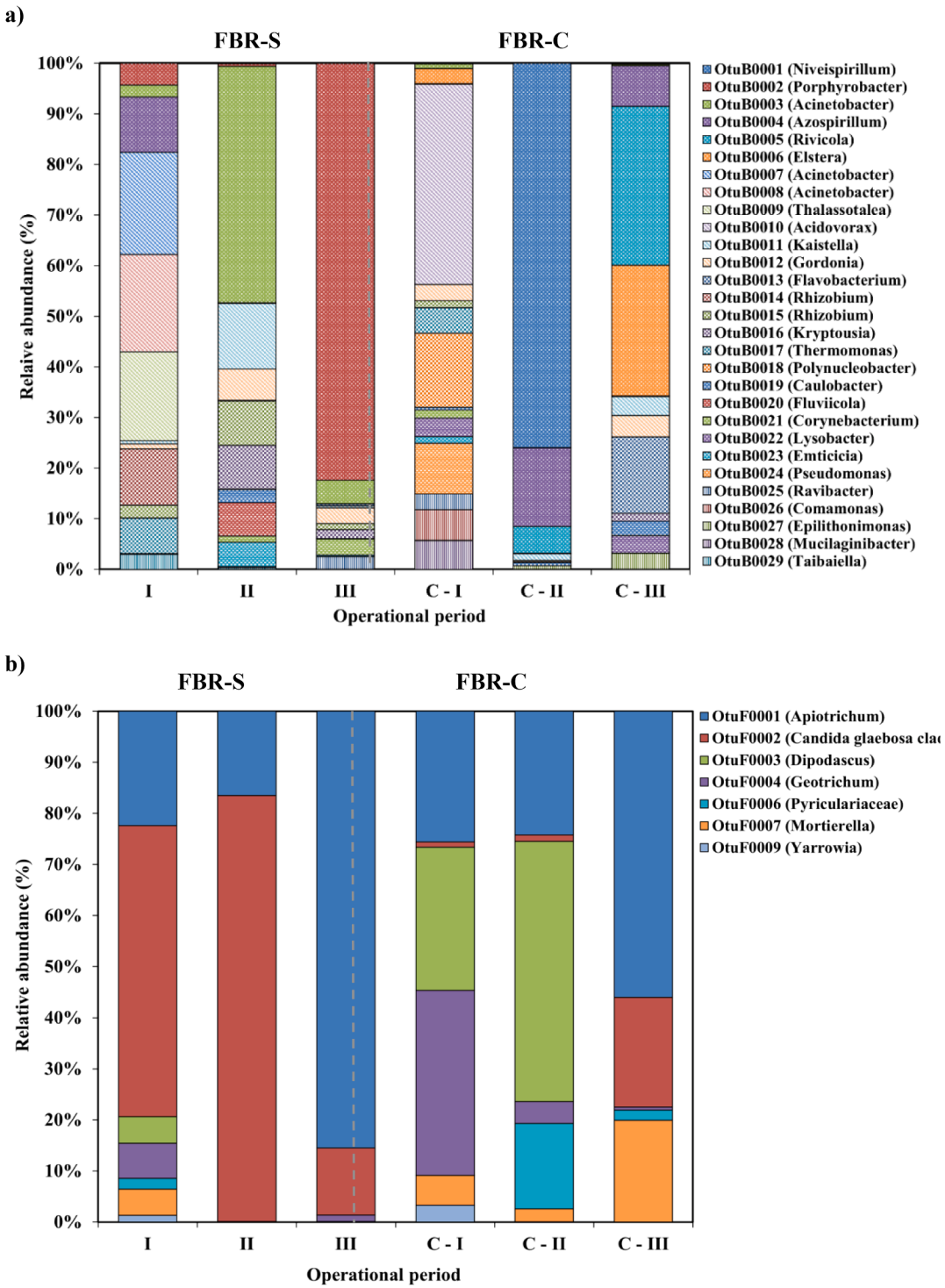

Fig. 5. Relative abundance of the main bacterial (a) and fungal (b) OTUs (RA > 0.5 %) identified by high-throughput Illumina sequencing in FBR-S and FBR-C biomass samples considering analogous operational periods (I, II, III vs C-I, C-II, C-III).

which modulated their structure. Only the bacterial groups OtuB0006 (*Elstera*), OtuB0017 (*Thermomonas*), OtuB0022 (*Lysobacter*), and OtuB0020 (*Fluviicola*) did not present statistical differences over time in both FBR-S and FBR-C (Table SI 4). Similarly, all the fungal OUT's RAs found in FBR-C were statistically different among periods C-I – C-III, and only the RAs of OtuF0003 (*Dipodascus*) did not show differences over time in FBR-S (Table SI 5).

However, the hierarchical clustering analyses performed to evaluate the global effect of salinity in the bacterial and fungal OTUs (Figure SI 4) showed that for both communities, salinity was a driver stronger than the operational period. Thus, the RAs of the dominant OTUs for FBR-S and FBR-C were separated according to their origin (except for the bacterial community of FBR-S in period III, in which RAs were separated according to the operational period) evidencing that NaCl stress specifically modulates the structure of the dominant OTUs, as previous authors observed [39].

According to the Mann-Whitney test, independently of the sampling period, high salinity negatively affected the presence of OtuB0005

(*Rivicola*), OtuB0006 (*Elstera*), OtuB0022 (*Lysobacter*) (Fig. 5a, Table SI 4), NaCl sensitive bacterial genera [40,41] that have not been identified yet as TAG or PHA-storing populations. Nonetheless, all of them reached the highest relative abundance in period C-III of FBR-C (26.21 ± 0.63 %, 21.54 ± 0.03 %, and 2.87 ± 0.08 % for *Rivicola*, *Elstera* and *Lysobacter* respectively), in which the maximum intracellular TAG accumulation was obtained (Fig. 3). Concerning the *Eukarya* domain, osmotic stress hindered the presence of OtuF0003 (*Dipodascus*) and Otu0007 (*Mortierella*) (Fig. 5b, Table SI 5). *Dipodascus* is a yeast without a TAG accumulation trait [42] which relative abundance decreased from 50.13 ± 0.21 % to non-detected between periods C-II (highest PHA accumulation) and C-III (highest TAG accumulation (Fig. 3). *Mortierella* is an oleaginous mould which accumulation capacity was reported to be negatively correlated to NaCl [43]. It presented a relative abundance of 19.41 ± 3.10 % in period C-III in FBR-C (highest TAG production, Fig. 3), whereas in FBR-S it was only detected in period II with a RA lower than 0.05 %.

On the other hand, salinity stimulated the development of OtuB0002 (*Porphyrobacter*), OtuB0003 (*Acinetobacter*), OtuB0015 (*Rhizobium*), OtuB0016 (*Kryptousia*), OtuB0021 (*Corynebacterium*), OtuB0029 (*Taibaiella*), OtuB0027 (*Epilithonimonas*), and OtuF0002 (*Candida glaebosa* clade) (Tables SI 4 and SI 5). *Porphyrobacter* was previously described as halotolerant [44] and although, until the date, it was not identified as a TAG or PHA-storing microorganism its relative abundance notably increased when the highest TAG storage was reached in FBR-S (Fig. 3, Fig. 5a). *Acinetobacter* is an osmotolerant bacterium (Hrenovic and Ivankovic, 2009) identified as capable of producing PHAs [46] and TAGs [47]. Its relative abundance notably increased in FBR-S between periods I and II (highest PHA production) whereas in period III became very scarce. *Rhizobium* was reported to survive under salt stress conditions [48] and, despite being a PHA-storing microorganism [49], its presence was only observed in period I in FBR-S (lowest intracellular PHA storage, Fig. 3). *Kryptousia* was developed after the SBR-S uncoupling and its relations to NaCl stress or biopolymer production are still unknown. *Corynebacterium*, with a scarce presence in FBR-S (highest RA of 2.88 ± 0.80 % in period III), can store amino-acids in response to osmotic stress although it is not a TAG or PHA synthesizer [50]. *Taibaiella* (highest RA of 2.67 ± 0.10 %) was previously characterized as slightly NaCl tolerant, [51] but to the best of the authoŕs knowledge, is not a TAG or PHA-storing microorganism. *Candida glaebosa* clade, affine to hypersaline environments [52] presented a high abundance in FBR-S throughout the different operational periods reaching its maximum in period II (82.92 ± 1.32). The TAG accumulation capacity in members of the current *Candida* genus has been previously characterized [53]. However, the uncertain position of the members of this clade inside the family *Debaryomycetaceae* hinders their assign within the group of oleaginous yeasts [54].

4. Conclusions

The use of saline water (10 g NaCl/L) for dilution in a two-stage process for TAGs and PHAs recovery from industrial waste fish oil appeared feasible according to the results obtained in this study. However, further optimization focused on the adaptation of the imposed selective pressures to the effect caused by salinity, would allow for the obtention of more competitive outcomes.

- When PHAs were the main storage compound (TAG:PHA = 28:72), 39.0 wt% PHA was accumulated yielding 0.303 $Cmmol_{PHA}/Cmmol_S$. On the other hand, when TAG producers dominated (TAG:PHA = 63:37), a maximum of 32.1 wt% TAG was observed yielding 0.291 $Cmmol_{TAG}/Cmmol_S$.
- The selective pressures imposed in the enrichment stage to maximize preferent TAGs or PHAs production in the accumulation reactor seemed less efficient when NaCl was present in the medium.
- PHA producers appeared more sensitive to salt and although the DGL strategy favoured their development, the culture needed to be further enriched to increase specificity. In the case of TAGs, a more severe medium acidification might be needed in saline conditions to create a clear selective advantage for TAG producers.
- Salinity was observed to be the main factor influencing the structure of both bacterial and fungal communities. Several OTUs presented a high relative abundance in the presence of salinity, some of them previously identified as TAG or PHA-storing microorganisms (*Acinetobacter*, *Rhizobium* and *Candida glaebosa* clade).

CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors declare that they have no known competing interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2021.118805.

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