

This is the pre-peer reviewed version of the following article:

Fra-Vázquez, A. , Pedrouso, A. , Palmeiro-Sánchez, T. , Moralejo-Gárate, H. and Mosquera-Corral, A. (2018), Feasible microbial accumulation of triacylglycerides from crude glycerol. *J. Chem. Technol. Biotechnol*, 93: 2644-2651, which has been published in final form at <https://doi.org/10.1002/jctb.5618>.

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Feasible microbial accumulation of triacylglycerides from crude glycerol

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ABSTRACT

BACKGROUND: Crude glycerol, a by-product of the biodiesel production industry, was used to produce intracellular storage polymers for waste valorisation. The enrichment of a mixed microbial culture (MMC) in microorganisms with the ability to accumulate intracellular polymers was performed in a sequencing batch reactor (SBR) submitted to feast/famine conditions. The effect of different carbon sources in the accumulation of biopolymers was investigated.

RESULTS: A mixed microbial culture (MMC) enriched in yeast and bacteria was obtained using crude glycerol as feedstock. Accumulation experiments performed with crude glycerol, synthetic glycerol, and synthetic methanol, showed the feasibility of the MMC to produce different biopolymers. Triacylglycerides (TAGs) accumulation up to 46 wt.% in yeast cells was promoted by the presence of residual lipids in crude glycerol. However, bacteria from *Betaproteobacteria* class used glycerol mainly to accumulate 28 wt.% of polyglucose (PG) and methanol as carbon source for cell growth.

CONCLUSIONS: As waste valorisation, a possible advantage which comes out of the present study is the use of open, non-sterile and non-defined systems to produce TAGs. These TAGs can potentially re-enter the biodiesel production process helping on the maximisation of the feedstock used in this process.

Keywords: Bacteria, Bioprocesses, Lipids, Recovery, Waste Treatment, Yeast

INTRODUCTION

Biodiesel is a renewable fuel that emerged during the past decades as a potential alternative to petroleum. In Europe, its production capacity during 2014 was approximately 23 million tonnes (European Biodiesel Board, 2014). Biodiesel is obtained by transesterification of fats and oils, after the addition of an alcohol (methanol or ethanol) and a catalyst (like sodium hydroxide), which generates crude glycerol as a primary by-product. ¹ On weight basis, 1 kg of crude glycerol is obtained per 9 kg of biodiesel produced. This crude glycerol contains impurities such as water, methanol, inorganic salts and free fatty acids. ² Glycerol is widely used in the pharmaceutical, cosmetic and food industry. For these purposes, glycerol with high purity is required, which implies the application of several purification steps, ³ increasing its production costs. Therefore, the development of an economical, efficient and feasible utilisation of the crude glycerol is needed to increase the profitability of the biofuel production process.

The conversion of crude glycerol into added-value products represents an interesting and innovative option to improve its usefulness. ⁴⁻⁶ Besides appearing as a cheap carbon source, its high reduction degree offers the opportunity to obtain reduced chemicals at higher yields than those achieved with sugars. ⁷ In particular, some microorganisms can use crude glycerol as carbon source to produce cytoplasmic inclusions such as polyhydroxyalkanoates (PHAs) and triacylglycerides (TAGs). ⁸ PHAs, like polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV), are polyesters also known as bioplastics that may become an alternative to conventional plastics due to their properties, with the advantage of being biobased and biodegradable. ⁹ TAGs are

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3 accumulated as intracellular lipid granules, and they can be extracted and used as
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5 biodiesel feedstock, re-entering the production process and maximising the use of raw
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7 materials.¹⁰
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10 The intracellular lipid production is carried out by oleaginous microorganisms, which
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12 are defined as microorganisms with a microbial lipid excess of 20 wt.%. Most of these
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14 processes are based on the use of pure cultures and comprise microalgae, fungi, yeast
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16 and bacteria. Microalgae grow very fast, and they are very rich in oil, accumulating a
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18 TAG content up to 70 wt.%.¹¹ Prokaryotic microorganisms also accumulate TAGs,¹²
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20 but their average lipid content is lower (approximately 20-40 wt.%). Some yeast strains
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22 are also considered as oleaginous microorganisms,¹³ as they can accumulate up to 70
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24 wt.%.
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28 Technologies based on the use of mixed microbial cultures (MMC) have appeared as
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30 an alternative to pure cultures. These processes are carried out as open, non-sterile
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32 cultures, which allow the use of wastewater as feedstock.^{14, 15} In general, biopolymer
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34 production with MMC is based on a three-stage process. The first unit is an acidifying
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36 reactor to produce volatile fatty acids (VFAs) from the raw wastewater. These VFAs are
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38 used as a substrate in a second unit to select accumulating microorganisms from a
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40 MMC by imposing a feast-famine regime. This strategy consists of alternating periods
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42 with presence (feast) and absence (famine) of carbon source in the liquid medium.
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45 Finally, the maximum accumulation capacity of the enriched MMC is achieved in a
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47 third unit where nutrients (mainly nitrogen) are supplied in limiting concentrations and
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49 the organic carbon substrate is present in excess to promote the storage of polymer
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51 and prevent the biomass growth. The main advantage of using glycerol to produce
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3 biopolymers is that the acidification unit is not needed, with the consequent saving of
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5 operational costs. Most of the studies using crude glycerol as carbon source reported
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7 PHA production, mainly as the homopolymer PHB. Dobroth et al. ¹⁶ obtained a low
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9 polymer yield (0.10 g PHB/g methanol) because they enriched a MMC in
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11 methylotrophic bacteria and only the methanol fraction of crude glycerol was used to
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13 accumulate PHB. However, Moita et al. ¹⁷ observed a higher PHB content of 47 wt.%
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15 with an enriched microbial community only consuming glycerol as carbon source to
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17 produce biopolymers. Moralejo-Gárate et al. ¹⁸ also produced PHB up to 80 wt.%, but
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19 they used a MMC that was previously enriched using synthetic glycerol as substrate.
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21 In the present study, the features of the biopolymer production carried out by a mixed
22
23 microbial culture using crude glycerol (containing glycerol, methanol and residual
24
25 lipids) as substrate were assessed. Firstly, the enrichment process of a mixed microbial
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27 culture with biopolymer-accumulating capacity was researched. Then, the maximum
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29 storage capacity of the enriched MMC and differences in the obtained biopolymers
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31 were evaluated in batch experiments performed using different carbon sources.
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40 **MATERIALS AND METHODS**

41 **Sequencing batch reactor for biomass enrichment**

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43 A double jacket tubular glass reactor with a working volume of 2 L was used to select a
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45 MMC with biopolymer production capacity. The system was operated as a sequencing
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47 batch reactor (SBR) under aerobic dynamic feeding (ADF) strategy to establish the
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49 alternate feast-famine conditions. The SBR operated in 24-hour cycles according to the
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51 following scheme (in minutes): feeding (0-6), reaction (7-1,429), effluent withdrawal
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3 (1,430-1,435) and idle (1,436-1,440) periods. At the end of each cycle, half of the total
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5 volume of the reactor was discharged and the same volume of feeding of the next
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7 cycle was supplied, which resulted in hydraulic and solid retention times (HRT and SRT,
8
9 respectively) of 48 hours. The complete mixture of the reactor medium was achieved
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11 by air flow (6 L/min), which was introduced through a ceramic air diffuser located at
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13 the bottom of the reactor. The temperature was controlled at 30 °C using a water
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15 jacket connected to a thermostatic bath. The pH was maintained at 7.0 ± 0.5 with a pH
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17 controller (Crison, Germany), adding either NaOH (0.5 M) or HCl (0.5 M). The dissolved
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19 oxygen (DO) concentration was measured with an oxygen pocket meter provided with
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21 a membrane sensor (Hach-Lange, USA).
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26 Activated sludge collected from the municipal wastewater treatment plant of Calo-
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28 Milladoiro (Spain) was used as inoculum. Crude glycerol was provided by a biodiesel
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30 production facility located in Begonte, Lugo (Spain) (Table 1). Glycerol, methanol and
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32 lipids content in this waste stream amounted to 66%, 20% and 11% of the total organic
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34 carbon (TOC). The alkaline pH and salinity of the crude glycerol corresponded to the
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36 presence of NaOH, added as a catalyser for the reaction during biodiesel production.
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38 A volume of 1,000 mL of feeding was added at the beginning of each operational cycle,
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40 containing 100 mL of the carbon solution, 100 mL of nutrient solution and 800 mL of
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42 dilution water. The carbon solution contained 23 mL/L of crude glycerol. The nutrient
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44 solution was: NH_4Cl , 6 g/L; KH_2PO_4 , 7 g/L; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 2 g/L; KCl 1 g/L; and 20 mL/L of
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46 trace element solution.¹⁹ Moreover, 1.5 mL/L of a 33 g/L allylthiourea solution (ATU)
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48 were added to the nutrient solution to prevent nitrification activity.
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Batch assays to determine the maximum storage capacity

The maximum biopolymer storage capacity of the enriched MMC was evaluated in batch experiments using biomass samples collected at the end of the operational cycles of the enrichment SBR (Table 2). Batch experiments were carried out under the same conditions as those applied to the enrichment reactor. However, only crude glycerol was dosed as carbon source, and no nitrogen source was supplied to force the conversion of the substrate into biopolymer and to prevent the biomass growth. Different carbon sources were tested to evaluate their influence on the biopolymer production: crude glycerol (A1), synthetic glycerol (A2), a mixture of synthetic glycerol and methanol (75:25 ratio as Cmmol) similar to the composition of the crude glycerol (A3) and a mixture of synthetic glycerol and methanol (25:75 ratio as Cmmol) with the opposite ratio to that from the experiment A3 (A4). In all cases, approximately 500 Cmmol of substrate were added in one pulse at the beginning of the experiment.

Analytical methods

Liquid phase

Liquid samples were filtered through a mixed cellulose and ester filter of 0.45 μm of pore size (Advantec, Japan) for the quantification of glycerol, methanol, total organic carbon (TOC) and ammonium. Glycerol concentration was determined by high-performance liquid chromatography (HPLC) with HP 1100 (Hewlett Packard, USA) using an Aminex HPX-87H-87H 300 x 7.8 mm column (temperature fixed at 35 °C) coupled to a refractive index (RI) detector and H_2SO_4 (5 mM) as eluent. Methanol concentration was measured by gas chromatography (model 6850-FID, Agilent, USA) using a DB-WAX

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3 30 m x 0.25 mm x 0.25 μ m column coupled to a flame ionisation detector (FID) and
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5 nitrogen as carrier gas. TOC concentration was determined by catalytic combustion
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7 (model TOC-5000, Shimadzu, Japan) with an automatic injector (model ASI-5000-S,
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9 Shimadzu, Japan). Ammonium and suspended solids, total (TSS) and volatile (VSS),
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11 concentrations were analysed according to Standard Methods.²⁰
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14 *Solid phase*

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16 For the analysis of biopolymer content, fresh biomass samples were collected, and
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18 formaldehyde was added to stop the microbial activity. Then, samples were
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20 centrifuged, frozen and freeze-dried to obtain a solid phase. For TAGs and PHAs
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22 identification and quantification, the method proposed by Smolders et al.²¹ was
23
24 applied. Commercial standards of TAGs (palmitic acid (PO500), oleic acid (O1008) and
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26 linoleic acid (L1376)) and PHA (containing 88% of hydroxybutyrate (HB) and 12% of
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28 hydroxyvalerate (HV)) were used (Sigma-Aldrich, USA). Freeze-dried biomass samples
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30 of 5 - 15 mg and standards were weighed using an analytical balance (Mettler Toledo,
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32 USA) and transferred into borosilicate glass tubes. Volumes of 1.5 mL of
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34 dichloroethane, 1.5 mL of a solution of propanol (with 25% vol/vol of commercial
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36 hydrochloric acid) and 50 μ L of a solution of benzoic acid (1 mg of benzoic acid in 100
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38 mL of propanol) as internal standard were added. Tubes were heated for 4 hours at
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40 100 °C and then cooled at room temperature. A volume of 3 mL of water was added to
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42 each sample and two phases (aqueous and organic) were formed. One mL of the
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44 organic phase was filtered over water-free sodium sulphate into vials. The propylesters
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46 were analysed by gas chromatography (Agilent, USA) equipped with a FID, on an HP
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48 Innowax column. For the extraction of polyglucose (PG), 5 – 15 mg of freeze-dried
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3 samples was weighed using an analytical balance (Mettler Toledo, USA) and
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5 transferred into borosilicate glass tubes. A volume of 4 mL of HCl 0.6 M was added to
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7 each tube, and they were heated at 100 °C for 3 hours. Glucose was measured by the
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9 HPLC previously described above.
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11 12 13 14 **Calculations**

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16 Lipid concentration (Cmmol/L) in the liquid samples was estimated by the difference
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18 between the TOC concentration and the carbon corresponding to the addition of
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20 glycerol plus methanol concentrations in the sample. The biopolymer content of the
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22 biomass was calculated as a percentage of the measured TSS on mass basis according
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24 to equation [1]. The active biomass (X) was obtained by subtracting the quantity of the
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26 stored compounds from the VSS, following equation [2]. The maximum specific
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28 glycerol and methanol consumption rates ($-q_{gly}$, $-q_{MeOH}$) were estimated from the
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30 maximum slopes of the curves describing the evolution of the concentrations of these
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32 compounds, divided by the active biomass concentration (Cmmol/(Cmmol_X·h)). The
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34 same procedure was applied to calculate the biopolymer (TAG, PHB, PHV and PG)
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36 specific production rates (q_{TAG} , q_{PHB} , q_{PHV} , q_{PG}). The yields (Cmmol/Cmmol) for TAGs
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38 ($Y_{TAG/TOC}$), PHB ($Y_{PHB/gly}$), PHV ($Y_{PHV/gly}$), PG ($Y_{PG/gly}$), and active biomass ($Y_{X/S}$) were
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40 obtained by dividing the specific production rate of each biopolymer by the
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42 consumption rate of the substrate.
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$$48 \quad \text{Biopolymer (\%)} = \frac{g \text{ biopolymer}}{g \text{ TSS}} \cdot 100 \quad [1]$$

$$49 \quad Cmmol_X = Cmmol_{VSS} - (Cmmol_{TAG} + Cmmol_{PHB} + Cmmol_{PHV} + Cmmol_{PG}) \quad [2]$$

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3 The elemental composition of the active biomass was assumed to be $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$.
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6 7 **Microbiology**

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9 The identification of active microbial populations was performed by applying the
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11 fluorescence *in situ* hybridization (FISH) technique. Fresh biomass samples were taken
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13 and the procedure described by Amann et al.²² was followed. Bacterial cells hybridised
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15 with the following probes: EUB338I, EUB338II, EUB338III, BET42a and EUK516
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17 (Supplementary material, Table S.1). All probes were 5' labelled by fluorochromes FITC
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19 (Fluorescein-5-isocyanate) or Cy3 (Carbocyanine 3). Details on oligonucleotide probes
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21 are available at probeBase.²³ DAPI (4, 6-diamidino-2-phenylindole) was used as
22
23 universal dye for the detection of all DNA in the samples. Fluorescence signals were
24
25 captured using an acquisition system (Coolsnap, Roper Scientific Photometrics)
26
27 coupled to an epifluorescence microscope (Axioskop 2 Plus, Zeiss, Germany).
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29 Moreover, fresh samples were taken from the enrichment SBR to verify the
30
31 biopolymer accumulation by lipid staining. Sudan IV solution was applied to fresh
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33 samples for 10 min and then, after the wash-out with distilled water to eliminate the
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35 excess of dye, safranin for 15 minutes. After drying, microorganisms were observed
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37 with a binocular vision microscope Axioskop 2 Plus (Zeiss, Germany).
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46 **RESULTS AND DISCUSSION**

47 **Enrichment of a mixed microbial culture with crude glycerol**

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49 The enrichment SBR was operated for 500 days using crude glycerol as feeding. The DO
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51 concentration was measured throughout the enrichment operational cycles to
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3 evaluate the selection of accumulating microorganisms. In the present research work,
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5 the end of the feast phase was considered when both glycerol and methanol were
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7 depleted, and its length decreased with the gradual enrichment of the system. During
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9 the first operational cycles, no significant changes in the DO profile were observed.
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11 However, in few weeks the feast-famine regime appeared accompanied by the
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13 substrate consumption. After 100 days of operation, the feast lasted 6 hours which
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15 corresponded to a feast/famine ratio of 0.25. According to Dionisi et al.²⁴, values of
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17 this ratio of approximately 0.40-0.50 are associated to systems where biomass growth
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19 occurs, whereas values lower than 0.20 indicate the presence of cultures capable of
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21 biopolymer storage.
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25 The progress of the MMC enrichment was also identified by changes in biomass and
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27 ammonium concentrations at the end of the SBR cycles (Figure 1). At the beginning of
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29 the operation, the biomass concentration was 0.3 g VSS/L. Then, this value increased
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31 and remained at approximately 0.7 g VSS/L from day 100 of operation onwards. In a
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33 similar way, ammonium consumption progressively increased, and its concentration
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35 decreased from 100 mg NH₄⁺-N/L, during the first operational days, to 50 mg NH₄⁺-N/L,
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37 from day 100 onwards. This gradual decrease correlated with the increase of biomass
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39 concentration in the way that fitted with the theoretical nitrogen consumption for
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41 biomass growth. The stabilization of the value of the feast/famine ratio and the
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43 ammonium and biomass concentrations at the end of the enrichment cycles indicated
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45 that the MMC was enriched after 100 days of operation. The enrichment process was
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47 significantly longer than in previous studies using synthetic glycerol as substrate, which
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3 took 40 days.¹⁸ In the present study, the complex composition of the crude glycerol
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5 could slow down the enrichment of the microbial community.
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7 Microbial analyses were performed to the enriched biomass on day 229 of operation.
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10 Two main microbial populations were identified: *Eukarya* and *Bacteria* (from class
11 *Betaproteobacteria*). Large spherical cells corresponded to yeasts (*Eukarya*) (Figure
12 2A), whereas small cocci-shape microorganisms belonged to *Betaproteobacteria*
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14 (Figure 2B). Sudan IV staining confirmed the presence of biopolymers accumulated
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16 inside the yeasts as intracellular inclusions, which are presumably the TAGs. In some
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18 cases, they appeared as single granules and, in others, as several granules in the same
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20 cell (Supplementary material, Figure S.1).
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28 **Characterisation of an enrichment cycle**

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30 The performance of the enriched MMC was followed by measuring single operational
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32 cycles, which are represented by that of day 246 (Figure 3). Crude glycerol containing
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34 glycerol, methanol and lipids, which amounted to approximately 30 Cmmol/L in total,
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36 was added as substrate at the beginning of the cycle. After crude glycerol addition, the
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38 DO concentration rapidly decreased during the feast period. During this period, its
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40 profile was characterised by the existence of two plateaus (Figure 3A). The first one
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42 lasted approximately 2 hours and corresponded to the oxygen consumption due to
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44 lipids and glycerol uptake. Then, the second plateau was coincident with the methanol
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46 depletion, which finished after 6 hours. The estimated glycerol specific uptake rate was
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48 $0.26 \text{ Cmol}_{\text{gly}}/(\text{Cmol}_X \cdot \text{h})$, four-fold the methanol specific uptake rate (0.06
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50 $\text{Cmol}_{\text{MeOH}}/(\text{Cmol}_X \cdot \text{h})$). Lipid uptake rate was not calculated because lipids concentration
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3 was fully depleted immediately after substrate addition, presumably due to an initial
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5 adsorption phenomenon on the biomass surface. The lipids and glycerol consumption
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7 reverted in the accumulation of three different types of biopolymers during the first
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9 period of the feast: TAG, PHV and PG. The maximum accumulation percentages,
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11 reached in terms of cell dry weight, were of 25.5, 6.7 and 7.9 wt.% for TAG, PHV and
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13 PG, respectively (Figure 3B). The compositions of lipids present in the crude glycerol
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15 and the TAGs accumulated as intracellular biopolymers were very similar (20% palmitic
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17 acid, 30% oleic acid and 50% linoleic acid, wt.%). These results, combined with carbon
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19 balance calculations, indicated that the lipids from the crude glycerol were uptaken
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21 and intracellularly accumulated as TAGs. Glycerol was used for biomass growth, which
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23 correlated with the decrease of ammonium concentration during this period, but it
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25 was also accumulated as small amounts of PHV and PG. After glycerol and lipids were
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27 consumed, both methanol and biopolymer consumption started. At the same time the
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29 biomass concentration increased, associated to growth on methanol and accumulated
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31 biopolymers.
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37 Marang et al.²⁵ observed a similar two-plateau DO profile using a synthetic mixture of
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39 acetate and methanol as carbon source in an enrichment reactor. They identified in
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41 the mixed culture two bacterial species: *Plasticumulans acidovorans* as PHA-
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43 accumulating bacteria and *Methylobacillus flagellatus* as methylotroph organisms,
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45 which consumed acetate and methanol, respectively. They observed biopolymer
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47 production only when acetate was present, and simultaneous growth on methanol and
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49 PHA occurred during the second part of the feast phase. In the present research work,
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51 microbial populations from *Eukarya* and *Bacteria* were identified. It can be
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3 hypothesized that yeast were responsible for TAG accumulation, as these are
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5 recognized as the main storage component for energy in eukaryotic cells.¹³ On the
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7 other hand, bacterial cells consumed glycerol and methanol to grow and produce PHV
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9 and PG. In this way, the coexistence of both microbial populations may provide this
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11 versatile behaviour of the MMC.
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14 Few authors have previously worked on biopolymer production from crude glycerol.

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16 Moita et al.¹⁷ used crude glycerol as feedstock with a similar composition to the
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18 substrate used in the present study. They observed PHB and PG accumulation from
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20 glycerol, while methanol consumption was not complete. They obtained an enriched
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22 MMC with PHA-accumulating capacity but they did not observe TAGs production.
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25 Differences in the type of biopolymer accumulated may be due to the fact that these
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27 authors used a PHA-accumulating microbial culture previously acclimated to bio-oil as
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29 feedstock. However, in the present study activated sludge was used as inoculum for
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31 the MMC development. Dobroth et al.¹⁶ also obtained PHB as the main biopolymer
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33 but only utilising the methanol fraction of the crude glycerol as carbon source.
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37 Contrary to these results from literature, in the present study TAGs were found as the
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39 preferred storage compound when crude glycerol was supplied for the selection of a
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41 community with the ability to produce storage polymers. To the knowledge of the
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43 authors, TAG production from industrial wastes using a MMC had not been reported
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45 previously.
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50 51 **TAG-accumulating capacity using crude glycerol as substrate**

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3 The maximum accumulation capacity of the enriched MMC was evaluated using crude
4 glycerol as substrate (experiment A1, Table 2). The concentration of crude glycerol was
5 higher than that used in the enrichment SBR. Approximately 500 Cmmol were added in
6 one single pulse at the beginning of the experiment. No nitrogen source was added to
7 force the complete conversion of substrate into biopolymers and prevent the microbial
8 growth. However, nitrogen was present in the liquid media during the first hours of the
9 experiment as residual ammonium coming with the sludge collected at the end of the
10 operational cycle of the enrichment SBR.
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21 After crude glycerol addition, lipids, glycerol and methanol concentrations evolved
22 with a similar trend as in the enrichment cycle. Even though lipids were not analysed,
23 the concentration was estimated from carbon balances considering measured glycerol,
24 methanol and TOC. A sudden decrease of lipids was observed during the first 10
25 minutes of the experiment, presumably due to their adsorption on the biomass
26 surface. During the feast phase glycerol was preferred over methanol and totally
27 consumed in 7 hours after substrate dosage (Figure 4A). Only 24% of the added
28 methanol was consumed during the same period. The corresponding specific substrate
29 consumption rates were of $0.43 \text{ Cmmol}_{\text{gly}}/(\text{Cmmol}_X \cdot \text{h})$ and 0.13
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Cmmol_{MeOH}/(Cmmol_X·h), respectively (Table 3).

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The consumption of the carbon compounds present in the crude glycerol was accompanied by the production of TAG, PHV and PG (Figure 4A). TAGs were accumulated as the main storage compound up to 46.3 wt.% after 2.5 hours. Moreover, the extracted TAGs from the biomass samples composition was of 20 wt.% palmitic acid, 30 wt.% oleic acid and 50 wt.% linoleic acid, very similar to the

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3 composition of fatty acids of the crude glycerol. PG was also determined as
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5 biopolymer, and its highest amount of 28 wt.% was measured seven hours after
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7 substrate addition, which corresponded to the complete glycerol depletion. PHAs were
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9 measured as PHV and reached a low value of 7 wt.%.

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11 In the present research work, the presence of lipids in the crude glycerol presumably
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13 selected TAG-accumulating microorganisms in the enrichment SBR. Different strains of
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15 oleaginous yeasts have been previously reported as capable of the production of
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17 intracellular lipids from crude glycerol with maximum accumulations as dry weight
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19 content of 25 wt.%²⁶, 32 wt.%²⁷ and 64 wt.%.¹⁰ The maximum TAG accumulation in
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21 the present study was 46.3 wt.%, which is in the range of the obtained values in those
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23 previous studies with pure cultures.
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30 **Biopolymer production with different carbon sources**

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32 To determine the separated effects of the glycerol and methanol on the accumulating
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34 capacity of the enriched MMC, experiments with synthetic carbon sources were
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36 performed (A2-A4, Table 2). As in experiment A1 with crude glycerol, 500 Cmmol were
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38 added in one single pulse and no nitrogen source was added to force the biopolymer
39
40 accumulation.
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44 Accumulation experiments with synthetic glycerol in experiment A2 (Table 2),
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46 indicated that the complete depletion of this carbon source occurred 22 hours after
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48 substrate dosage (Figure 4B). Compared with the previous experiment with crude
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50 glycerol, the accumulation of TAGs was very low (11.4 wt.%). Moreover, with glycerol
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52 as substrate the enriched MMC could produce PG as the main accumulated
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3 biopolymer (54.3 wt.%), but also 3.6 wt.% and 14.4 wt.% of PHB and PHV, respectively.

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5 In this case, the specific substrate consumption rate was of $0.55 \text{ Cmmol}_{\text{gly}}/(\text{Cmmol}_X \cdot \text{h})$

6
7 (Table 3).

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9 In the experiment A3, the composition of the crude glycerol was mimicked with a
10
11 mixture of synthetic glycerol and methanol (75:25 ratio as Cmmol) to evaluate the
12
13 MMC accumulation behaviour (Table 2). Glycerol was consumed faster than methanol,
14
15 with specific uptake rates of $0.48 \text{ Cmmol}_{\text{gly}}/(\text{Cmmol}_X \cdot \text{h})$ and 0.09

16
17
18 $\text{Cmmol}_{\text{MeOH}}/(\text{Cmmol}_X \cdot \text{h})$, respectively (Figure 4C). These values were very similar to
19
20 those obtained in the experiment A1 with crude glycerol (Table 3). However, in this
21
22 case, only PG accumulation was observed up to approximately 40 wt.%. As it occurred
23
24 in experiment A2, TAG accumulation did not take place.

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27 A mixture of glycerol and methanol in a ratio of 25:75 as Cmmol, respectively
28
29 (experiment A4, Table 2), was used as substrate to evaluate the effects of the
30
31 methanol on the MMC. Methanol concentration at the beginning of the experiment
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33 was higher than that of glycerol, but its consumption rate was significantly lower 0.17
34
35 $\text{Cmmol}_{\text{MeOH}}/(\text{Cmmol}_X \cdot \text{h})$ compared to that of the glycerol of $0.25 \text{ Cmmol}_{\text{gly}}/(\text{Cmmol}_X \cdot \text{h})$
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37 (Figure 4D). No significant accumulation of biopolymers was obtained in this assay.

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40 Small amounts of PHB and PG were measured, reaching the maximum dry weight
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42 content of 8.6 and 8.2 wt.%, respectively, when glycerol was depleted. These results
43
44 indicated that the glycerol was the most probable substrate used as carbon source to
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46 accumulate these two polymers. However, the PG production decreased in this
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48 experiment and it was nine times lower than in A2 and five times less than in
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50 experiments A1 and A3. Moreover, contrary to the accumulation experiments with
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3 other substrates, ammonium was not completely consumed, and biomass continued
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5 growing throughout the whole cycle. These results may indicate a possible inhibitory
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7 effect of methanol, as previous studies indicated that methanol inhibits the growth of
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9 PHB accumulating bacteria at concentrations above 0.5% (vol/vol).²⁸
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11 Accumulation experiments (A1-A4, Table 2) provided different results depending on
12
13 the composition of the used carbon compounds. Glycerol uptake rate was similar in
14
15 the experiments where large glycerol concentrations were added. Glycerol was
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17 preferably intracellularly accumulated as PG, as can be proven by the results of
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19 experiments A2 (synthetic glycerol) and A3 (glycerol:methanol ratio 75:25), where
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21 percentages were higher than 40 wt.% once glycerol was consumed. These results
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23 seemed to indicate that glycerol was the main carbon source for PG accumulation.
24
25 Moralejo-Gárate et al.²⁹ reported a yield of 0.40 Cmmol_{PG}/Cmmol_{gly} using synthetic
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27 glycerol, slightly higher than the value obtained in the present study (0.33
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29 Cmmol_{PG}/Cmmol_{gly}). Both PHV and PHB were produced at low percentages, no higher
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31 than 14% and PHV as the preferred compound. However, in previous studies, PHB was
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33 obtained as the main biopolymer using both synthetic and crude glycerol. Moralejo-
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35 Gárate et al.¹⁸ obtained 80 wt.% of PHB using synthetic glycerol. This is a very high
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37 value compared to those obtained with the same substrate in the present study of 4
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39 and 15 wt.% of PHB and PHV, respectively. Dobroth et al.¹⁶ obtained 67 wt.% of PHB
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41 consuming only the methanol fraction, which corresponded to 40 wt.% of the total
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43 carbon supplied and it is higher than the methanol in the crude glycerol used in this
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45 study. With a similar crude glycerol composition, Moita et al.¹⁷ reached maximum PHB
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47 content of 47 wt.%. However, they used biomass with PHA-accumulating capacity
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3 already acclimated to bio-oil as feedstock. In all these cases, the substrate composition
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5 (synthetic) or the MMC (acclimated microorganisms) differed from the crude glycerol
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7 used in the present research work. Therefore, the selected accumulating-
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9 microorganisms in the corresponding enrichments were expected to be different. The
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11 low PHA values might be attributed to the characteristics of the enriched MMC, where
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13 TAG-accumulating microorganisms were preferably selected instead of PHA-
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15 accumulating microorganisms. Only in the experiment with crude glycerol (A1) the
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17 enriched MMC could accumulate high TAG content, indicating that the presence of
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19 lipids is required to obtain significant intracellular TAG accumulation.
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25 **CONCLUSIONS**

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27 In the present study, a mixed microbial culture with biopolymer-accumulating capacity
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29 was enriched using crude glycerol as carbon source. Microorganisms from Eukarya and
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31 Bacteria domains were selected and cohabitated in the MMC, which accumulated a
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33 mixture of biopolymers (TAG, HB, HV and PG). Yeasts were mainly responsible for the
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35 lipids storage as TAGs, while bacteria used glycerol to accumulate the remaining
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37 biopolymers and methanol for growth. Maximum TAG accumulation of 46.3 wt.% was
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39 observed only when crude glycerol containing lipids was used as substrate. When
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41 synthetic carbon sources were used, the maximum accumulation corresponded to PG,
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43 which indicated that fatty acids present in the crude glycerol promoted TAG
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45 accumulation.
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51 The conversion of crude glycerol to lipids is important for waste valorisation. A
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53 possible advantage which comes out of the present study is the use of an open, non-
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3 sterile and non-defined enrichment, which is expected to lead to a significant decrease
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5 in the production costs due to lower energy requirements and simpler operation, in
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7 comparison with the available studies that operated under sterile conditions, using
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9 pure and defined cultures.
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11 12 13 14 **ACKNOWLEDGEMENTS**

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16 This research was supported by the Spanish Government (AEI) through FISHPOL
17
18 (CTQ2014-55021-R) and GRANDSEA (CTM2014-55397-JIN) projects. The authors
19
20 belong to the Galician Competitive Research Group GRC ED431C 2017/29 and to the
21
22 CRETUS Strategic Partnership (AGRUP2015/02). All these programmes are co-funded
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24 by the FEDER (EU).
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10 **TABLE LEGENDS**
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14 **Table 1.** Crude glycerol characterisation.
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16 **Table 2.** Accumulation experiments with different carbon substrates using the
17 enriched MMC collected from the SBR.
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19 **Table 3.** Characterisation of accumulation experiments performed to test the
20 maximum biopolymer accumulation with different substrates.
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Table 1

Parameter	
pH	10
Density (g/L, 20 °C)	966.9 ± 7.2
Total Organic Carbon (TOC) (g C/L)	409.2 ± 30.3
Glycerol (g/L)	696.4 ± 54.0
Methanol (g/L)	225.4 ± 22.9
Lipids (g/L)	60.9 ± 5.8
Na ⁺ (g/L)	14.5 ± 0.3

Table 2.

Experiment	Substrate	Initial biomass (g VSS/L)	Initial substrate composition (Cmmol)		
			Glycerol	MeOH	Lipids
A1	Crude glycerol	0.52	180	120	140
A2	Synthetic glycerol	0.43	480	0	0
A3	Glycerol + MeOH (75:25 as Cmmol)	0.45	370	110	0
A4	MeOH + Glycerol (25:75 as Cmmol)	0.55	120	320	0

Table 3.

Exp	$-q_{\text{gly}}$	$-q_{\text{MeOH}}$	q_{TAG}	q_{PHB}	q_{PHV}	q_{PG}	$Y_{\text{TAG/TOC}}$	$Y_{\text{PHB/gly}}$	$Y_{\text{PHV/gly}}$	$Y_{\text{PG/gly}}$	Maximum (wt.%)			
											TAG	PHB	HPV	PG
A1	0.43	0.13	0.40	0.00	0.03	0.14	0.52	0.00	0.06	0.33	46.3	0.0	7.4	27.6
A2	0.55	---	0.05	0.02	0.04	0.18	0.08	0.02	0.06	0.31	11.4	3.6	14.4	54.3
A3	0.48	0.09	0.03	0.01	0.01	0.13	0.05	0.03	0.02	0.26	6.8	3.6	5.4	39.5
A4	0.25	0.17	0.00	0.02	0.00	0.02	0.00	0.08	0.00	0.04	3.9	8.6	2.1	8.2

- q_{gly} (Cmmol gly/Cmmol X·h); - q_{MeOH} (Cmmol MeOH/Cmmol X·h); q_{TAG} (Cmmol TAG/Cmmol X·h); q_{PHB} (Cmmol PHB/Cmmol X·h); q_{PHV} (Cmmol PHV/Cmmol X·h); q_{PG} (Cmmol PG/Cmmol X·h).

$Y_{\text{TAG/TOC}}$ (Cmmol TAG/Cmmol TOC); $Y_{\text{PHB/gly}}$ (Cmmol PHB/Cmmol gly); $Y_{\text{PHV/gly}}$ (Cmmol PHV/Cmmol gly); $Y_{\text{PG/gly}}$ (Cmmol PG/Cmmol gly).

wt.% (% g biopolymer/g cell dry weight).

FIGURE CAPTIONS

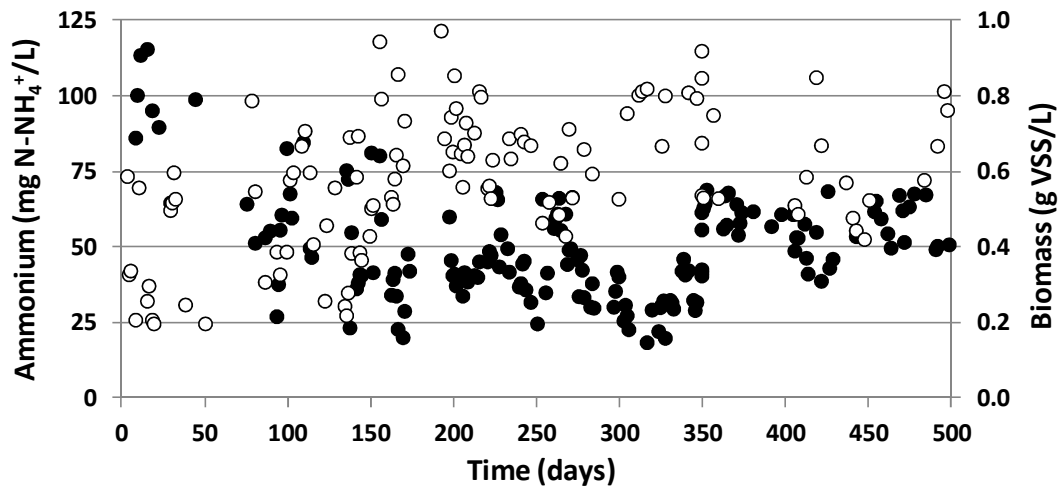
Figure 1. Evolution of ammonium (●) and biomass (○) concentration at the end of the cycles throughout the operation of the enrichment SBR.

Figure 2. FISH images of the biomass from the enrichment SBR on day 229. (A) *Eukaryota* domain (EUK516: Cy3, red) and *Bacteria* domain (EUB338mix: FITC, green). The bar represents 25 μm. (B) *Betaproteobacteria* class (BET42a: Cy3, red) and all DNA (DAPI, blue). The bar represents 10 μm.

Figure 3. Evolution of different compounds during an enrichment cycle on day 246 of operation. A) Glycerol (■), methanol (△), lipids (◇) and TOC (●), all as Cmmol, ammonium (x) as Nmmol, and DO (-) as mg O₂/L. B) TAG (◆), PHV (○), PG (□) and active biomass (- - -), all as Cmmol.

Figure 4. Evolution of different compounds throughout accumulation assays with different substrates. A) Crude glycerol-A1; B) Synthetic glycerol-A2; C) Mix of synthetic glycerol and methanol (75:25 ratio as Cmmol)-A3; D) Mix of synthetic glycerol and methanol (25:75 ratio as Cmmol)-A4. Measurements of glycerol (■), methanol (△), lipids (◇), TOC (●), TAG (◆), PHB (▲), PHV (○), PG (□), active biomass (- - -), all as Cmmol.

Figure 1



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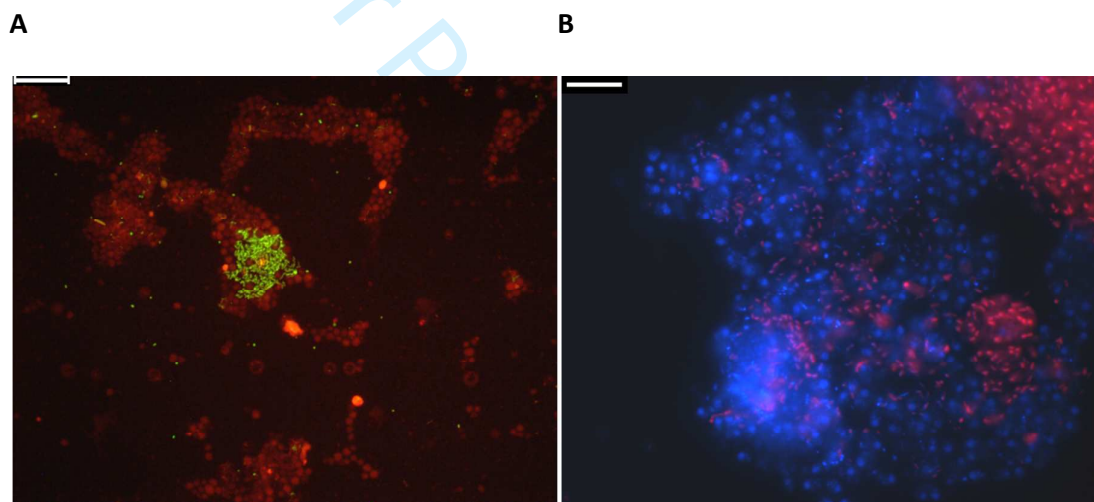
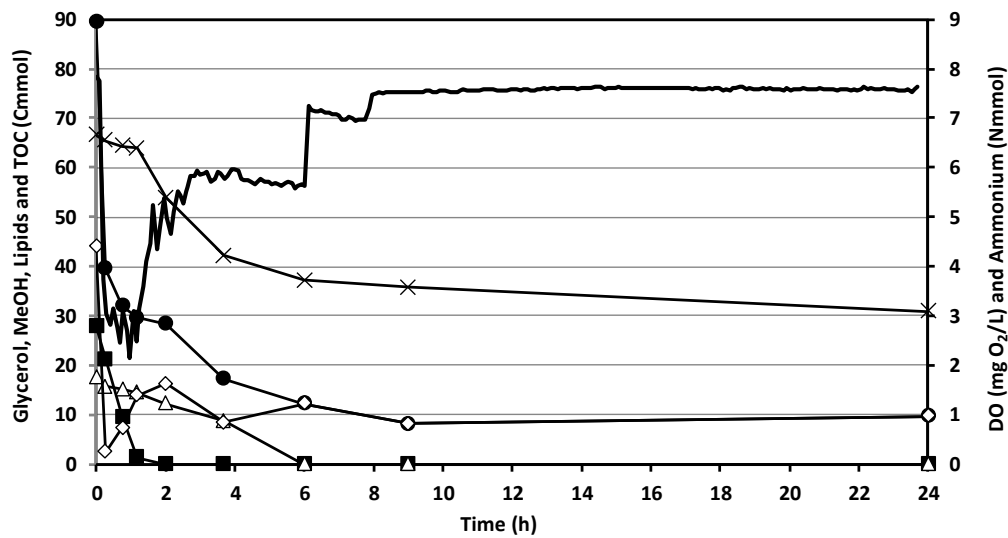


Figure 3

A



B

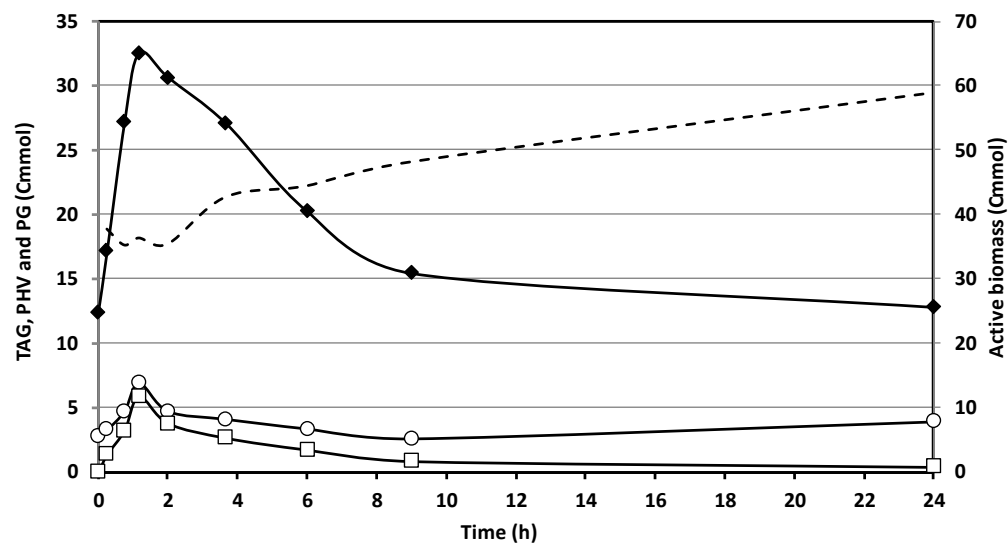
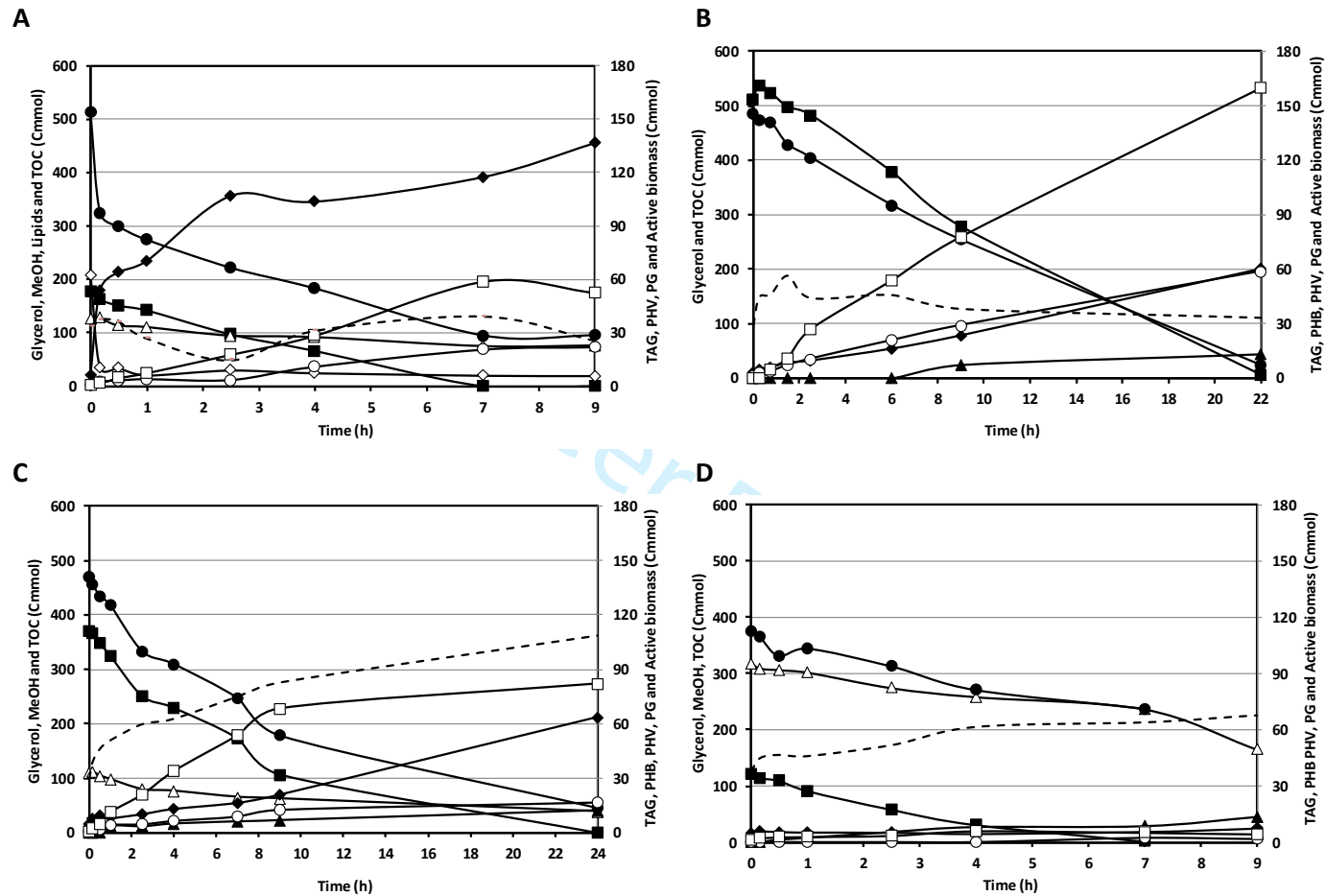


Figure 4



SUPPLEMENTARY MATERIAL

Table S1. List of oligonucleotide probes used for biomass FISH analysis, formamide percentages and identified microorganisms.

Probe	% Formamide	Target organism
EUB338I	0-50	<i>Bacteria</i> domain
EUB338II	0-50	<i>Planctomycetales</i>
EUB338III	0-50	<i>Verrucomicrobia</i>
BET42a	35	<i>Betaproteobacteria</i> class
EUK516	25	<i>Eukarya</i> domain

EUB338I, EUB338II and EUB338III were applied as an equimolar mix (EUB338mix)

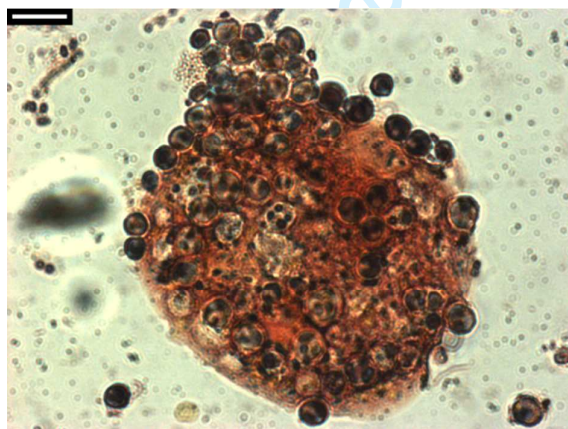


Figure S1. Biomass sample from the enrichment SBR collected during the feast phase on day 478 and treated with Sudan staining to observe the biopolymer inclusions inside the yeast cells. The bar represents 10 μm .