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Rhodosporidium toruloides and *Tetradesmus obliquus* Populations Dynamics in Symbiotic Cultures, Developed in Brewery Wastewater, for Lipid Production

Carla Dias[1] · Luísa Gouveia[1,2] · José A. L. Santos[3,4] · Alberto Reis[1] · Teresa Lopes da Silva[1]

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Abstract

In this work, primary brewery wastewater (PBWW) and secondary brewery wastewater (SBWW) separately, or mixed at the ratios of 1:1 (PBWW:SBWW) and 1:7 (PBWW:SBWW), with or without supplementation with sugarcane molasses (SCM), were used as culture media for lipid production by a mixed culture of the oleaginous yeast *Rhodosporidium toruloides* NCYC 921 and the microalgae *Tetradesmus obliquus* (ACOI 204/07). Flow cytometry was used to understand the dynamics of the two micro-organisms during the mixed cultures evolution, as well as to evaluate the physiological states of each micro-organism, in order to assess the impact of the different brewery effluent media composition on the microbial consortium performance. Both brewery wastewaters (primary and secondary) without supplementation did not allow *R. toruloides* heterotrophic growth. Nevertheless, all brewery wastewater media, with and without SCM supplementation, allowed the microalgae growth, although the yeast was the dominant population. The maximum total biomass concentration of 2.17 g L^{-1} was achieved in the PBWW mixed cultivation with 10 g L^{-1} of SCM. The maximum lipid content (14.86% (w/w DCW)) was obtained for the mixed culture developed on SBWW supplemented with 10 g L^{-1} of SCM. This work demonstrated the potential of using brewery wastewater supplemented with SCM as a low-cost culture medium to grow *R. toruloides* and *T. obliquus* in a mixed culture for brewery wastewater treatment with concomitant lipid production.

Introduction

Microalgae/yeasts mixed cultures have been considered an auspicious approach for lipid and carotenoid production, presenting many benefits over single cultures, such as higher biomass, lipid and carotenoids production [1, 2]. The advantages of the symbiotic cultures result from the synergistic relationship between the two micro-organisms [1].

When grown in mixed cultures, yeast and microalgae benefit from the heterotrophic/autotrophic nutritional modes that have complementary requirements. Yeasts produce carbon dioxide during their respiration, which can be used as carbon source for the autotrophic metabolism of the microalgae, while the microalgae produce oxygen, essential for the yeast metabolism [1, 2]. In this way, it is possible to avoid carbon/oxygen limitations for yeast/microalgae populations, respectively. Furthermore, other advantages such as metabolite exchanges and medium pH auto-adjustment are observed in mixed cultures [1]. Therefore, yeast and algae when cultivated in mixed cultures produce intracellular compounds more efficiently with potential commercial interest, such as lipids and carotenoids [3, 4].

Low-cost substances, such as wastewater, have been extensively used as growth media for microbial growth, aiming at the concomitant wastewater treatment and interesting intracellular products, in order to reduce the microbial process costs [1]. This strategy can be applied to yeast and microalgae symbiotic cultures for intracellular lipid production, for biodiesel purposes. In addition, microalgae are very efficient in ammonia, phosphorus, and heavy metal removal

✉ Teresa Lopes da Silva
teresa.lopessilva@lneg.pt

1 Laboratório Nacional de Energia e Geologia, I.P., Unidade de Bioenergia e Biorrefinarias – UBB, Estrada do Paço do Lumiar 22, 1649-038 Lisboa, Portugal

2 GreenCoLab - Green Ocean Technologies and Products Collaborative Laboratory, CCMAR, Algarve University, Faro, Portugal

3 Departamento de Bioengenharia, Instituto Superior Técnico, Universidade de Lisboa, Avenida Rovisco Pais, 1049-001 Lisboa, Portugal

4 IBB, Institute for Biotechnology and Bioengineering, Avenida Rovisco Pais, 1049-001 Lisboa, Portugal

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[5], which, together with the removal of the organic carbon by heterotrophic yeast, can be a precious strategy for wastewater treatment.

The brewery industry consumes large amounts of water [8], generating between 3 and 10 L of wastewater per liter of beer [7, 8]. Usually, brewery effluents have a high level of organic pollutants (sugars, proteins, ammonia, phosphates, among others) which requires remediation, to avoid water bodies eutrophication [9–11].

To remove the organic and inorganic nutrients, there are several available approaches for brewery wastewater treatment, such as chemical, physical, or biological technologies [12]. The first treatment steps usually include mainly physical unit operations, where physical forces are applied to remove suspended solids and organic matter content in the raw wastewater, but not the dissolved pollutants [11]. After this step, the primary brewery wastewater (PBWW) is obtained.

The secondary brewery wastewater treatment step removes the biodegradable organic matter and the remaining suspended solids. Because brewery effluents often contain high organic content and microbial contaminants, biological methods are usually used for their treatment. The usual biological treatment method used is based on the activity of a wide range of micro-organisms which, through aerobic or anaerobic processes, remove the biodegradable organic pollutants in the wastewaters. After this step, the secondary brewery wastewater (SBWW) is obtained.

Although there are advantages of using brewery wastewater as a low-cost medium for microbial growth, it also contains toxic compounds, such as heavy metals, organically bound metals and metalloids, inorganic species, trace organic pollutants, polychlorinated biphenyls, pesticides, detergents, chemical carcinogens, among others [13], that may inhibit the microbial growth and affect negatively the cell metabolism [1, 14]. These toxic compounds are known to induce the impairment of photosynthetic mechanism, blockage of cell division, and inhibition of enzyme activity in eukaryotic cells [15]. Even traces of these compounds can have detrimental effect on the cell physiology and metabolism, which must be taken into consideration, especially when using raw effluents without pre-treatment, which is the case of PBWW. A possible approach to use these effluents, as culture media, consists of diluting them, to minimize the effect of the toxic compounds on the microbial cells. In addition, it is essential to have information on cell physiological status when using brewery effluents as medium culture, in order to understand the effect of the inhibitory compounds on the cell performance.

Marques (2018) [16] studied the biological treatment of a secondary effluent from brewing industry with simultaneous production of intracellular lipids, using the microalga *Scenedesmus obliquus* and the yeast *Rhodosporidium toruloides*, in order to take advantage of their nutritional requirements complementarity, to improve the effluent treatment efficiency, as well as the intracellular lipid production from these micro-organisms. The author concluded that lower intracellular lipids were produced by the pure cultures, in comparison with the mixed ones, demonstrating the advantage of the mixed cultures over pure cultures.

In this study, raw (as collected) and further diluted primary (PBWW) and secondary brewery wastewater (SBWW), with and without supplementation with sugarcane molasses (SCM), were used as culture media to develop mixed cultures of the oleaginous microalga *Tetradesmus obliquus* (ACOI 204/07) and the oleaginous yeast *Rhodosporidium toruloides* NCYC 921, for the concomitant intracellular lipid production and wastewater treatment. Flow cytometry was used to evaluate the cellular physiological status of each micro-organism, in terms of enzymatic activity and membrane integrity, in order to understand the impact of the different brewery wastewater media on the microbial populations dynamics, and how cells respond to such environments.

As far as the authors know, the strategy of using PBWW and its dilution with SBWW, with or without supplementation with SCM, has never been used as culture media to develop a mixed culture of yeasts and microalgae for concomitant wastewater treatment and lipid production.

Materials and Methods

SCM, PBWW, and SBWW

Sugarcane molasses (SCM) was kindly supplied by the sugar manufacturer Sidul Company at Alhandra, Lisbon, Portugal.

Primary Brewery Wastewater (PBWW) and Secondary Brewery Wastewater (SBWW) from beer production industry were collected from the brewer SCC—Sociedade Central de Cervejas e Bebidas, S.A at Vialonga, Lisbon, Portugal. PBWW was collected after the preliminary screening and primary sedimentation, and SBWW after the secondary treatment by anaerobic digestion. Table 1 provides the characterization of PBWW and SBWW.

Micro-Organisms

The microalga used in this work was *Tetradesmus obliquus* (ACOI 204/07) (formerly known as *Scenedesmus obliquus*) supplied by ACOI-Coimbra University Culture Collection, Portugal. *T. obliquus* was maintained as described in [17].

The yeast *Rhodosporidium toruloides* NCYC 921 (former *Rhodotorula glutinis* NRRL Y- 1091) was purchased to the National Collection of Yeast Cultures (Norwich, UK). The strain was stored on slants of Malt Extract Agar, at 4 °C.

Table 1 Characteristics of the different Primary Brewery WasteWater (PBWW) and Secondary Brewery WasteWater (SBWW) media used in this work, at inoculation time

	Test No	Culture type	COD (gO_2 L^{-1})	Ammonium N (mg L^{-1})	Sugar content (g L^{-1})			
					Sucrose	Glucose	Fructose	Total Sugars
Without supplementation	M1	PBWW	2.37 ± 0.23	23.71 ± 0.59	3.24 ± 0.04	0.15 ± 0.01	0.07 ± 0.00	3.63 ± 0.08
	M2	SBWW	0.31 ± 0.00	31.81 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
	M3	1:1 (PBWW:SBWW)	0.79 ± 0.06	37.61 ± 0.30	0.63 ± 0.04	0.00 ± 0.00	0.14 ± 0.00	0.77 ± 0.04
	M4	1:7 (PBWW:SBWW)	0.34 ± 0.02	27.63 ± 2.07	0.21 ± 0.01	0.00 ± 0.00	0.11 ± 0.00	0.31 ± 0.01
10 g L^{-1} SCM supplementation	M5	PBWW	48.00 ± 1.89	38.97 ± 0.40	9.65 ± 0.02	1.25 ± 0.03	1.12 ± 0.03	12.02 ± 0.09
	M6	SBWW	30.67 ± 0.94	36.78 ± 0.47	8.44 ± 0.64	0.90 ± 0.04	1.03 ± 0.08	10.37 ± 0.76
	M7	1:1 (PBWW:SBWW)	37.67 ± 2.36	35.08 ± 0.32	8.81 ± 0.13	0.88 ± 0.10	1.09 ± 0.03	10.77 ± 0.26
	M8	1:7 (PBWW:SBWW)	29.67 ± 2.36	41.13 ± 1.14	8.21 ± 0.13	1.48 ± 0.11	1.63 ± 0.12	10.21 ± 0.13

Growth Conditions

Inoculum

R. toruloides and *T. obliquus* inoculum growth conditions followed those described in [17].

Brewery Effluent Cultivations

In order to study the growth of *R. toruloides* and *T. obliquus* mixed cultures on brewery effluent, experiments were performed in 1 L baffled Erlenmeyers containing 200 mL of sterile medium. Eight different media, containing PBWW, SBWW, or the mixture of these two effluents at the ratio of 1:1 and 1:7 (PBWW:SBWW) with or without supplementation of SCM were tested, as described in Table 1. PBWW and SBWW were mixed at different proportions to dilute possible toxic compounds present in the PBWW. In order to promote heterotrophic growth, the effluent was supplemented with SCM. To optimize the effluent treatment efficiency, concomitantly with the maximum intracellular lipid production, different concentrations of total sugars has been studied [16], and the maximum lipid content obtained was for 10 g L^{-1} of total sugars.

Initial cell density of each micro-organism was 1.38×10^6 cells mL^{-1}, in a proportion of 1:1 (2.75×10^6 total cell mL^{-1}). Duplicates of the experiments were performed at 30 °C and 130 rpm in a shaker (Unitrom Infors, Switzerland) and continuous artificial light of two led strips with a light intensity of 3.7 µmol photons m^{-2} s^{-1}. The pH of each medium was corrected to 6.0 at the beginning of the experiments. For each condition, a parallel control experiment with the respective sterile culture media without inoculum was performed. Growth of the mixed cultures in the shake flasks was monitored for 120 h.

Biomass

Biomass growth and the determination of the dry cell weight (DCW) of each micro-organism followed the same protocol described in [17]. Briefly, the percentage of each micro-organism during the cultivation (obtained from the flow cytometry FSC/SSC density plots (Example in Fig. 1 a1 and a2) was multiplied by the total biomass concentration obtained by a correlation for each condition between the optical density (OD) and the DCW.

Kjeldahl Nitrogen, Ammoniacal Nitrogen, and Chemical Oxygen Demand (COD)

The analytical techniques were carried out according to [18].

Sugar Concentration

Total sugars, sucrose, glucose, and fructose concentrations were determined using HPLC (LaChrom Merck/Hitachi, Germany), equipped with a differential refractive index detector and a Waters SugarPak 1 column (6.5 × 300 mm, Bio-Rad Laboratories, CA, USA), operating at 75 °C with Ca-EDTA at 50 mg L^{-1} as mobile phase with a flow rate of 0.5 mL min^{-1}. The data obtained were analyzed with Chromeleon software ver. 6.40 SP6 build 783 (1994–2003, Dionex). SCM obtained by Sidul company had 754.0, 459.5, 168.3, and 126.2 g L^{-1}, respectively, of total sugars, sucrose, glucose, and fructose concentrations.

Fatty Acid Analysis

Since the yeast and microalgae saponifiable lipidic fractions are those that are used for biodiesel purposes, the yeast and microalgal lipids were converted into fatty acids

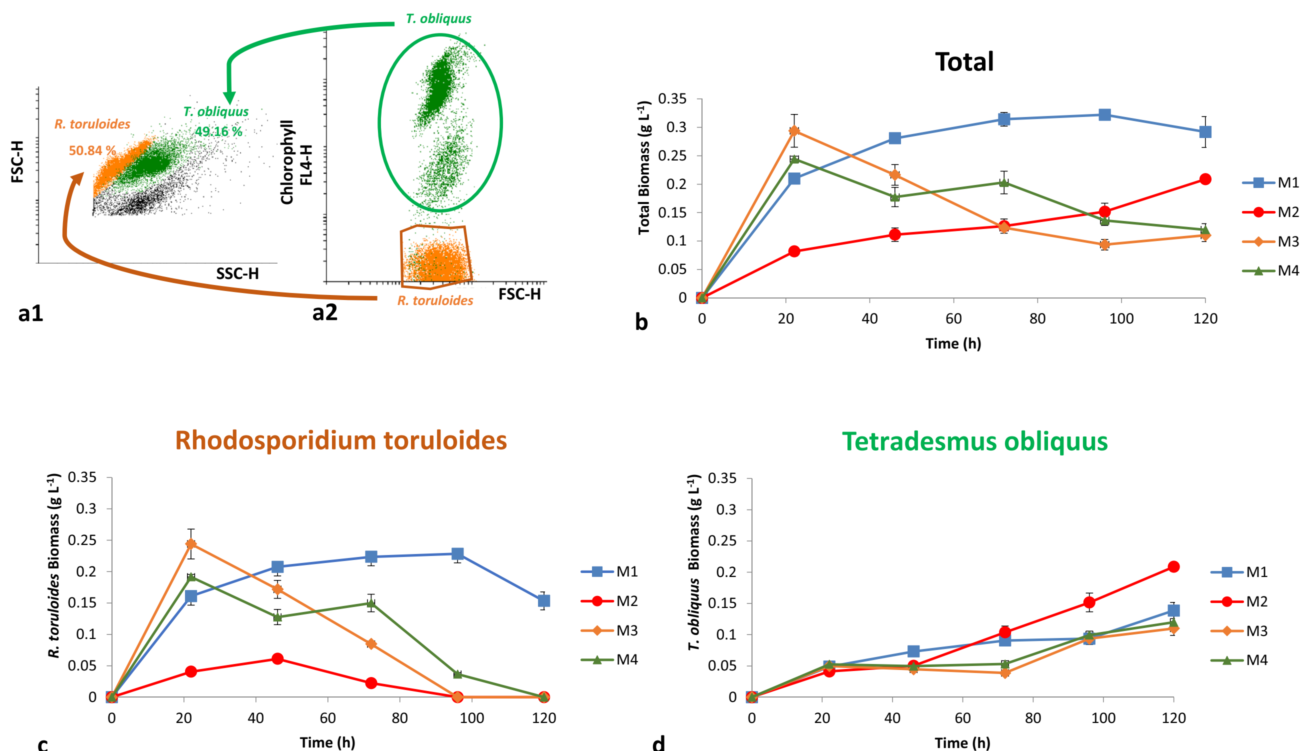

Fig. 1 **a1** FSC/SSC dot plot concerning a mixture of *Tetradesmus obliquus* cells (green) and *Rhodosporidium toruloides* cells (orange). FSC is proportional to cell-surface area or size and SSC is proportional to cell granularity or internal complexity. Based on this information, it is possible to differentiate yeast and microalgae cells as they have different sizes and levels of internal complexity. **a2** FL4/FSC dot plot concerning the mixture of *T. obliquus* (green) and *R. toruloides* (orange). Due to the chlorophyll present in microalgal cells, detected in the FL4 channel, it is possible to discriminate between microalgae cells with chlorophyll auto-fluorescence and yeast cells without chlorophyll auto-fluorescence. **b** Total, **c** *R. toruloides*, and **d** *T. obliquus* biomass concentration over time for the brewery effluent cultivations without supplementation: M1 – Primary Brewery WasteWater (PBWW) cultivation, M2 – Secondary Brewery WasteWater (SBWW) cultivation, M3 – Mixture of PBWW and SBWW at the ratio of 1:1 and M4 – Mixture of PBWW and SBWW at the ratio of 1:7. The normalized biomass in each cultivation time was calculated deducting to the DCW obtained at that time $t=0$ h. The error bars represent the standard deviation of two measurements from independent duplicates (Color figure online)

methyl esters through a transesterification reaction, and analyzed by gas–liquid chromatography, according to [19].

Flow Cytometry

Flow cytometry analysis followed the same protocol described in [17]. For cell stress detection, membrane integrity and enzymatic activity were studied. SYTOX Green 30 μM (obtained from 5 mM SYTOX Green from Invitrogen by Thermo Fisher Scientific, USA diluted in Dimethyl sulfoxide (DMSO)) was used to study the integrity of the yeast and microalgae cells. To evaluate the enzymatic activity of the microalgae and yeast cells, carboxyfluorescein diacetate (CFDA) 10 mg mL^{-1} (10 mg of CFDA (Invitrogen by Thermo Fisher Scientific, USA) diluted in 1 mL of acetone) was used to detect esterase activity.

Microscopic Observations

Samples from 120 h of cultivation were observed using an optical microscope Olympus BX60 (Tokyo, Japan) with increasing lens magnification of 100x (oil immersion lens).

Results

Brewery Effluent Cultivations

Biomass

M1, M2, M3, and M4 Figure 1 shows the normalized total (b) and individual *R. toruloides* (c) and *T. obliquus* (d) biomass concentration profiles obtained during the mixed cultures development for PBWW (M1), SBWW (M2), 1:1

Table 2 Summary of the *Rhodosporidium toruloides* and *Tetradesmus obliquus* parameters obtained in the different Brewery WasteWater (BWW) cultivations

	Test No	Maximum total biomass concentration (g L^{-1})	Total biomass productivity [a] (mg L^{-1} h^{-1})	Maximum *R. toruloides* biomass concentration (g L^{-1})	*R. toruloides* biomass productivity [a] (mg L^{-1} h^{-1})	Maximum *T. obliquus* biomass concentration (g L^{-1})	Maximum *T. obliquus* biomass productivity [a] (mg L^{-1} h^{-1})	Lipid content (% w/w DCW) (final t)	Lipid productivity [b] (mg L^{-1} h^{-1}) (final t)
Without supplementation	M1	**0.32 ± 0.01 (at 96 h)**	3.35 ± 0.08	0.23 ± 0.01 (at 96 h)	2.38 ± 0.06	0.14 ± 0.01 (at 120 h)	1.16 ± 0.11	**3.99 ± 0.00**	**0.097 ± 0.01**
	M2	0.21 ± 0.01 (at 120 h)	1.74 ± 0.06	0.06 ± 0.00 (at 46 h)	1.33 ± 0.13	**0.21 ± 0.01 (at 120 h)**	**1.74 ± 0.06**	3.55 ± 0.35	0.062 ± 0.00
	M3	0.29 ± 0.02 (at 22 h)	**13.36 ± 1.30**	**0.24 ± 0.02 (at 22 h)**	**11.09 ± 1.08**	0.11 ± 0.01 (at 120 h)	0.92 ± 0.09	3.86 ± 0.29	0.035 ± 0.00
	M4	0.24 ± 0.01 (at 22 h)	11.10 ± 0.23	0.19 ± 0.00 (at 22 h)	8.71 ± 0.18	0.12 ± 0.01 (at 120 h)	1.00 ± 0.09	3.97 ± 0.26	0.020 ± 0.00
10 g L^{-1} Sugarcane Molasses (SCM) supplementation	M5	**2.17 ± 0.11 (at 120 h)**	**18.08 ± 0.90**	**1.67 ± 0.08 (at 120 h)**	13.93 ± 0.69	**0.50 ± 0.02 (at 120 h)**	4.15 ± 0.21	4.28 ± 0.09	0.77 ± 0.04
	M6	1.13 ± 0.12 (at 72 h)	15.63 ± 1.56	0.95 ± 0.01 (at 46 h)	**20.59 ± 0.29**	0.19 ± 0.02 (at 72 h)	2.71 ± 0.27	**14.86 ± 0.86**	**1.35 ± 0.00**
	M7	2.00 ± 0.20 (at 120 h)	16.63 ± 1.66	1.66 ± 0.11 (at 96 h)	17.27 ± 1.13	0.37 ± 0.03 (at 120 h)	3.12 ± 0.31	5.00 ± 0.09	0.83 ± 0.08
	M8	1.57 ± 0.02 (at 96 h)	16.33 ± 0.23	1.37 ± 0.03 (at 120 h)	11.43 ± 0.22	0.26 ± 0.01 (at 46 h)	**5.64 ± 0.18**	9.00 ± 0.55	1.17 ± 0.02

M1 – Primary BWW (PBWW) cultivation, M2 – Secondary BWW (SBWW) cultivation, M3 – 1:1 (PBWW:SBWW) cultivation, M4 – 1:7(PBWW:SBWW) cultivation, M5 – PBWW + SCM cultivation, M6 – SBWW + SCM cultivation, M7 – 1:1(PBWW:SBWW) + SCM cultivation and M8 – 1:7(PBWW:SBWW) + SCM cultivation

[a] Biomass productivity (mg L^{-1} h^{-1}) for the maximum biomass concentration was calculated as follows: X_t—X_0/(t_t—t_0), where X_t is the biomass concentration at the instant t, X_0 is the biomass concentration at t = 0;

[b] Lipid productivity (mg L^{-1} h^{-1}) was calculated in terms of Total Fatty Acids (TFA) as follows: TFA_t—TFA_0/(t_t _ t_0), where TFA_t is the TFA concentration at the instant t, TFA_0 is the TFA at t = 0

Bold values indicate the higher values obtained for each parameter, in each group of experiments (M1-M4 and M5-M8)

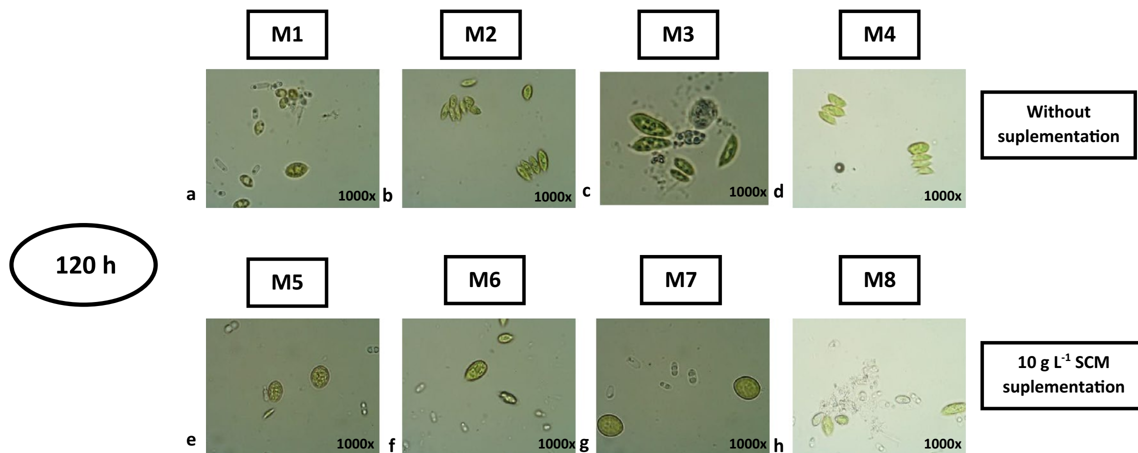

Fig. 2 Microscopic examination of *Rhodosporidium toruloides* and *Tetradesmus obliquus* cells for the different brewery effluent cultivations at *t* = 120 h: **a** M1 – Primary Brewery WasteWater cultivation (PBWW), **b** M2 – Secondary BWW cultivation (SBWW), **c** Mixture of PBWW and SBWW at the ratio of 1:1 cultivation, **d** M4 – Mixture of PBWW and SBWW at the ratio of 1:7 cultivation, **e** M5 – PBWW + Sugar Cane Molasses (SCM) cultivation, **f** M6 – SBWW + SCM cultivation, **g** M7 – 1:1(PBWW:SBWW) + SCM cultivation, and **h** M8 – 1:7(PBWW:SBWW) + SCM cultivation. Magnification 1000x

(PBWW:SBWW) (M3), and 1:7 (PBWW:SBWW) (M4) cultivations.

In M1, M3, and M4 brewery cultivations without supplementation, in the initial 22 h of cultivation, the yeasts dominated the mixed growth, being the main contributor for the total biomass concentration, attaining 0.21, 0.24, and 0.19 g L^{-1}, respectively (Fig. 1b and c, Table 2). After this time, only in the M1 cultivation was possible to observe an increase in the yeast biomass until $t=96$ h (0.32 g L^{-1}) decreasing until the end of the cultivation (0.29 g L^{-1}) (Fig. 1b and c, Fig. 2a, Table 2). In the other three cultivations (M2, M3 and M4), after the initial increase, a decrease in the yeast biomass was observed. In M2 and M3 assays, after $t=72$ h, and in the M4 cultivation, after 96 h, and until the end of the cultivations, the yeast cells could not be seen when the cultures were observed under the optical microscope (Fig. 2b-d). This could be due to possible cell lysis due to the lack of nutrients, essential for the yeast growth, as these three media contained low COD and sugar contents (Table 1).

Nevertheless, although these three media (M2, M3 and M4) contained a low organic load, an initial increase in the yeast biomass was observed, in the beginning of the cultivations, probably due to the consumption of the organic load present in the inoculum. On the contrary, in the M1 cultivation, in which PBWW was used as sole culture medium containing 2.4 gO_2 L^{-1} of COD and 3.6 g L^{-1} of total sugars (Table 1) allowed a steadily increase in *R. toruloides* biomass until $t=96$ h slightly decreasing afterward, probably as a result of nutrient depletion.

Maximum *R. toruloides* biomass productivity was obtained in the M3 cultivation (11.09 mg L^{-1} h^{-1}) despite a short timeframe interval (20 h), followed by M4 cultivation (8.71 mg L^{-1} h^{-1}), M1 cultivation (2.38 mg L^{-1} h^{-1}), and M2 cultivation (1.33 mg L^{-1} h^{-1}) (Table 2). Having in mind the total culture timeframe, the highest *R. toruloides* average biomass productivity was achieved in the M1 trial.

Relatively to *T. obliquus*, in all cultivations, there was an increase in the biomass concentration throughout the different cultivations. M2 cultivation showed the highest increase in biomass (Fig. 1d), Table 2) attaining 0.21 g L^{-1} at $t=120$ h. For the remaining assays (M1, M3 and M4), the biomass attained 0.14, 0.11, and 0.12 g L^{-1}, respectively, at the end of the assays.

T. obliquus biomass productivities followed the same pattern as the maximum biomass concentration, being the maximum obtained for M2 cultivation (1.74 mg L^{-1} h^{-1}), followed by M1 cultivation (1.16 mg L^{-1} h^{-1}), M4 cultivation (1.00 mg L^{-1} h^{-1}), and M3 cultivation (0.92 mg L^{-1} h^{-1}).

M5, M6, M7, and M8 In order to evaluate the effect of the different brewery effluents supplemented with 10 g L^{-1} of SCM on the growth of *R. toruloides* and *T. obliquus* in mixed cultures, different cultivations were performed: PBWW + SCM (M5), SBWW + SCM (M6), 1:1(PBWW:SBWW) + SCM (M7) and 1:7(PBWW:SBWW) + SCM (M8) cultivations.

In all cases, the yeast growth dominated the microalgae growth, being the total biomass concentration curves almost overlaid to the yeast biomass concentration curves. M5 culture attained the highest yeast biomass concentration (1.67 g L^{-1}) followed by M7 (1.66 g L^{-1}), M8 (1.37 g L^{-1}) and M6 (0.95 g L^{-1}) (Fig. 3 b), Table 2). At 46 h, the yeast biomass attained a plateau in M6 and M8 assays, while it stabilized at $t=96$ h for M5 and M7 assays (Fig. 3b).

In addition, the yeast biomass concentration attained higher maximum values for M5-M8 assays (1.67, 1.66, 1.37 and 0.95, respectively), than for M1-M4 assays (0.23, 0.06, 0.24 and 0.19 g L^{-1}, respectively), which was due to the SCM addition (Table 2). This was reflected in the higher biomass productivities observed for the four SCM cultivations when compared with M1-M4 assays (Table 2).

Figure 3c shows the *T. obliquus* biomass in the different mixed cultivations. In M5 and M7 cultivations there was a constant increase in the microalgae biomass during all the cultivations, attaining 0.50 and 0.37 g L^{-1} at $t=120$ h, respectively (Fig. 3c, Table 2). For M6 and M7 cultivations, there was an initial sharply increase, decreasing to 0.18 and 0.19 g L^{-1} respectively, at the end of the cultivations (Fig. 3c, Table 2).

As observed for *R. toruloides*, the two media with higher PBWW proportions (M5 and M7) favored the microalgae growth. Also, higher microalgae biomass productivities were observed for the four SCM cultivations when compared with the brewery cultivations without supplementation (Table 2).

Lipid Production and FAME Composition

M1, M2, M3 and M4 When the brewery wastewater without supplementation was used as the sole feedstock, the lipid content determined in the total biomass collected at the end of all assays was similar and low (between 3.55 and 3.99% w/w (DCW), Table 2). Such results do not make the intracellular lipid extraction and conversion into biodiesel economically viable.

In terms of lipid productivity, the pattern is different as for the lipid content, as a result of the different final biomass concentrations attained by the cultures: 0.097 mg L^{-1} h^{-1} for the M1 cultivation, 0.062 mg L^{-1} h^{-1} for the M2 cultivation, 0.035 mg L^{-1} h^{-1} for the M3 cultivation and 0.020 mg L^{-1} h^{-1} for the M4 cultivation (Table 2).

M5, M6, M7 and M8 Regarding the lipid production of the mixed culture at the end of the different brewery cultivations supplemented with 10 g L^{-1} of SCM, higher lipid content was obtained for the M6 cultivation (14.86% w/w) followed by the M8 (9.00% w/w), the M7 (5.00% w/w) and at last

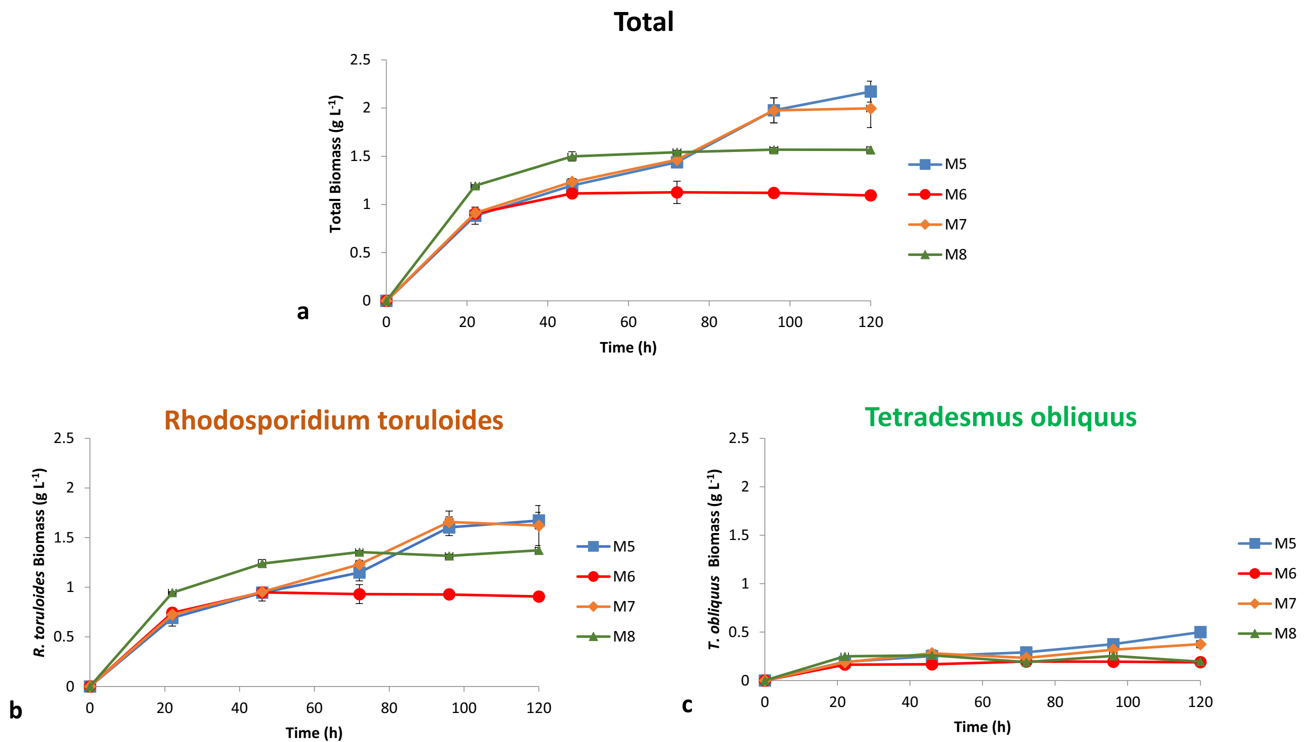

Fig. 3 **a** Total, **b** *Rhodosporidium toruloides* and **c** *Tetradesmus obliquus* biomass concentration over time for the brewery effluent cultivations supplemented with 10 g L^{-1} sugarcane molasses (SCM): M5 – Primary Brewery WasteWater (PBWW) + SCM cultivation, M6 – Secondary Brewery WasteWater (SBWW) + SCM cultivation, M7 – Mixture of PBWW and SBWW at the ratio of 1:1 + SCM cultivation and M8 – Mixture of PBWW and SBWW at the ratio of 1:7 + SCM cultivation. The normalized biomass curve in each cultivation time was calculated deducting to the dry cell weight (DCW) obtained at that time the DCW at $t = 0$ h. The error bars represent the standard deviation of two measurements from independent duplicates

Table 3 Fatty acid classes and α-linolenic acid (18:3ω3) contents at the end of the different brewery effluent cultivations with 10 g L^{-1} Sugarcane Molasses (SCM) supplementation

	Test No	SFA (% w/w TFA)	MUFA (% w/w TFA)	PUFA (% w/w TFA)	18:3ω3 (% w/w TFA)
10 g L^{-1} SCM supplementation	M5	23.28 ± 0.09	49.30 ± 0.35	27.42 ± 0.27	5.10 ± 0.04
	M6	23.01 ± 0.61	47.96 ± 0.17	29.03 ± 0.52	3.02 ± 0.04
	M7	23.42 ± 0.56	49.78 ± 0.25	26.80 ± 0.34	4.99 ± 0.08
	M8	23.10 ± 0.55	47.37 ± 0.48	29.53 ± 0.55	3.70 ± 0.11

(M5 – Primary Brewery WasteWater (PBWW) + SCM cultivation, M6 – Secondary BWW (SBWW) + SCM cultivation, M7 – 1:1(PBWW:SBWW) + SCM cultivation and M8 – 1:7(PBWW:SBWW) + SCM cultivation)

the M5 (4.28% w/w) (Table 2). In terms of lipid productivity, the pattern is the same as for the lipid content: 1.35 mg L^{-1} h^{-1} for M6, 1.17 mg L^{-1} h^{-1} for M8, 0.83 mg L^{-1} h^{-1} for M7 and 0.77 mg L^{-1} h^{-1} for the M5 cultivation (Table 2).

Table 3 shows the saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) percentages at the end of the mixed cultures cultivations supplemented with 10 g L^{-1} of SCM. The SFA proportion was the same for M5-M8 cultivations (~ 23% w/w of total fatty acids (TFA), Table 3). MUFA and PUFA proportions were similar for M5 and M7 cultivations (around 49% w/w of TFA and 27% w/w of TFA, respectively), and M6 and M8 cultivations (around 47% w/w of TFA and 29% w/w of TFA, respectively) (Table 3). The percentage of linolenic methyl ester (18:3) in all cultivations was in compliance with the European Standard EN 14,214 (describes the requirements and test methods for biodiesel purposes) which limits this percentage to 12%, being the lower value obtained for the M6 cultivation (3.02% w/w of TFA) and the higher value obtained for the M5 cultivation (5.10% w/w of TFA) (Table 3).

Table 4 Removal of organics and nutrients by *Rhodosporidium toruloides* and *Tetradesmus obliquus* mixed cultures at the end of the different Brewery WasteWater (BWW) cultivations without supplementation and with 10 g L^{-1} Sugarcane Molasses (SCM) supplementation

	Test No	Final COD (g O_2 L^{-1})	COD removal % [a]	Final ammoniacal N (mg L^{-1})	Ammoniacal N removal % [a]	Sugar consumption % [a]			
						Glucose	Fructose	Sucrose	Total sugars
Without supplementation	M1	0.15 ± 0.01	**93.38 ± 3.12**	6.37 ± 0.26	73.15 ± 1.09	100.00 ± 0.00	− 169.73 ± 1.95 [b]	96.58 ± 0.58	91.65 ± 1.17
	M2	0.12 ± 0.01	60.22 ± 4.48	8.31 ± 0.08	**73.86 ± 4.17**	–	33.10 ± 0.70	–	33.10 ± 0.70
	M3	0.22 ± 0.02	70.76 ± 3.45	15.77 ± 0.69	58.08 ± 1.84	–	40.42 ± 0.57	100.00 ± 0.00	89.11 ± 0.11
	M4	0.11 ± 0.01	68.93 ± 6.72	11.38 ± 0.64	58.83 ± 2.30	–	100.00 ± 0.00	100.00 ± 0.00	**100.00 ± 0.00**
10 g L^{-1} SCM supplementation	M5	18.0 ± 1.8	62.50 ± 4.17	27.21 ± 0.79	30.19 ± 2.02	100.00 ± 0.00	56.19 ± 4.71	67.34 ± 0.21	**69.62 ± 0.73**
	M6	20.7 ± 2.0	32.61 ± 0.51	19.88 ± 0.90	**45.95 ± 2.46**	100.00 ± 0.00	70.57 ± 2.70	37.20 ± 0.72	46.12 ± 0.53
	M7	13.0 ± 1.1	**65.50 ± 2.94**	26.17 ± 0.07	25.40 ± 0.19	100.00 ± 0.00	63.56 ± 1.11	60.57 ± 1.89	64.08 ± 1.66
	M8	11.3 ± 1.1	61.80 ± 4.49	24.10 ± 1.32	41.40 ± 3.20	100.00 ± 0.00	73.06 ± 0.85	40.55 ± 0.31	47.88 ± 0.11

M1 Primary BWW (PBWW) cultivation, *M2* Secondary BWW (SBWW) cultivation, *M3* 1:1 (PBWW:SBWW) cultivation, *M4* 1:7(PBWW:SBWW) cultivation, *M5* PBWW + SCM cultivation, *M6* SBWW + SCM cultivation, *M7* 1:1(PBWW:SBWW) + SCM cultivation and *M8* 1:7(PBWW:SBWW) + SCM cultivation)

Bold values indicate the higher values obtained for each parameter, in each group of experiments (M1-M4 and M5-M8)

[a] The removal efficiency (*ER* (%)) was calculated as follows: ((C_0-C_f)/C_0) × 100 where C_0 is the nutrient concentration at the instant 0, C_f is the nutrient concentration at the final t

[b] The negative sign indicates the accumulation of the nutrient in the medium

Wastewater Treatment

M1, M2, M3, and M4 Relatively to COD removal, the highest removal percentage was obtained for the M1 cultivation with 93.38%, followed by the M3 cultivation with 70.76%, the M4 cultivation with 68.93%, and the M2 cultivation with 60.22% (Table 4), reflecting the final biomass concentrations achieved (Fig. 1, Table 2).

The emission value limits (ELV) for wastewater discharge for COD into natural water bodies in Portugal (decree-law 236/98, Portugal [21]) is 0.15 g O_2 L^{-1}. The final COD obtained at the end of M1 cultivation was 0.15 g O_2 L^{-1} thus accomplishing the legislation (Table 4). Also, in M2 and in M4 cultivations, although the COD removal rate was lower (60.22% and 68.93%, respectively, Table 4), the COD obtained in the end of the cultivation (0.12 and 0.11 g O_2 L^{-1}) was in accordance with the ELV (Table 4). Only in the end of M3 cultivation the final COD was not in accordance with the ELV (0.22 g O_2 L^{-1}).

Regarding the ammoniacal nitrogen removal, at the end of M1 and M2 cultivations, around 73% of the ammoniacal nitrogen had been removed in both cultivations and around 58% in the M3 and M4 cultivations (Table 4). In both M1 and M2 cultivations, the final effluent was below the ELV for ammoniacal nitrogen and that limits its discharge in a maximum of 10 mg NH_4 L^{-1} (6.4 and 8.3 mg NH_4 L^{-1} for M1 and M2 cultivations, respectively, Table 4).

Relatively to the sugar consumption in the brewery effluent cultivations without supplementation, it can be seen that the mixed cultures that showed higher sugar consumption (M1, M3 and M4, with 91.65%, 89.11% and 100%, respectively) were those in which the COD removal was also higher (93.39%, 70.76% and 68.93%), which explains the yeast biomass increase in the beginning of the cultivations (Fig. 1c), revealing active heterotrophic metabolism by the yeast at that stage. After 22 h in M3 and M4 cultivations, the yeast population steadily decreased, indicating that cells were exposed to adverse conditions (other than carbon limiting conditions, since organic carbon was not completely exhausted).

As it is possible to observe in Table 4, in the case of M4 cultivation, 100% of the total sugars were removed from the medium, but the COD removal was only 69%. This indicates that not only sugars contribute for the COD present in the medium.

M5, M6, M7, and M8 The highest COD removal percentage was observed for M7 cultivation (65.50%), followed by M5 (62.50%), M8 (61.80%), and M6 (32.61%) (Table 4). The latter attained lower biomass concentration and higher lipid content.

At the end of the M5 cultivation, 30.2% of the ammoniacal nitrogen have been removed, 46.0% in M6, 25.4% in M7, and 41.4% in M8. In the cultivations with higher percentage of PBWW, the removal rates of ammoniacal nitrogen were lower (25–30% of ammoniacal N removal) comparatively with the cultivations with higher SBWW proportion where the percentage of removal of ammoniacal nitrogen was higher (41–46% of ammoniacal N removal) (Table 4).

Relatively to the sugar consumption in the brewery effluent cultivations supplemented with SCM, glucose was completely exhausted at the end of M5-M8 assays (Table 4). This demonstrates the high affinity of the yeast *R. toruloides* for glucose. In terms of total sugars, higher removal rates were obtained in the M5 cultivation (69.6%) followed by M7 (64.1%), M8 (47.9%), and M6 (46.1%) (Table 4).

In the brewery cultivations supplemented with 10 g L^{-1} of SCM, the parameters studied were not in accordance with the ELV limits of discharge. Also, in any of the brewery cultivations supplemented with 10 g L^{-1} of SCM, COD removal rates close to 100% were not observed, as the supplementation of the brewery effluent with SCM could constrain the effluent treatment, in terms of organic carbon and nitrogen [18].

Cell Stress Detection—Membrane Integrity

M1, M2, M3, and M4 All cultivations contained between 16 and 21% of *R. toruloides* cells were stained with SYTOX Green (thus SYTOX + cells) in the beginning of the assays. However, as the cultures developed, differences were observed among the four assays. The yeast cells seemed to have adapted to the culture medium, which was shown by the decrease in this proportion, in all the assays, at $t = 22$ h: 9.79% in the M1 cultivation, 10.09% in the M2 cultivation, 9.32% in the M3 cultivation, and to 7.65% in the M4 cultivation (Fig. 4a).

Thereafter, SYTOX + cells population slightly increased until the end of the experiment for M1 assay attaining 30.39%. During M2 and M3 assays, after $t = 72$ h, and in M4 cultivation, after 96 h, the yeast cells population could not be seen in the cytogram plots, neither in the cultures when observed under the optical microscope as above referred (Fig. 2b-d), which could be possibly due to cell lysis, as a result of lack of nutrients in the media, essential for the yeast growth and membrane integrity maintenance. This observation probably results from the low COD and sugar contents of the three media (M2, M3, and M4) (Table 1).

It can also be seen that the proportion of SYTOX + yeast cells was lower for the assay with higher proportion of PBWW (M1) throughout the cultivation, attaining ~ 30% at the end of the experiment. This result is in accordance with the biomass profile (Fig. 1b and c) as assay M1 attained the highest biomass concentration. Such observations suggest that PBWW contains nutrients that favor the heterotrophic yeast growth, contrarily to the assays

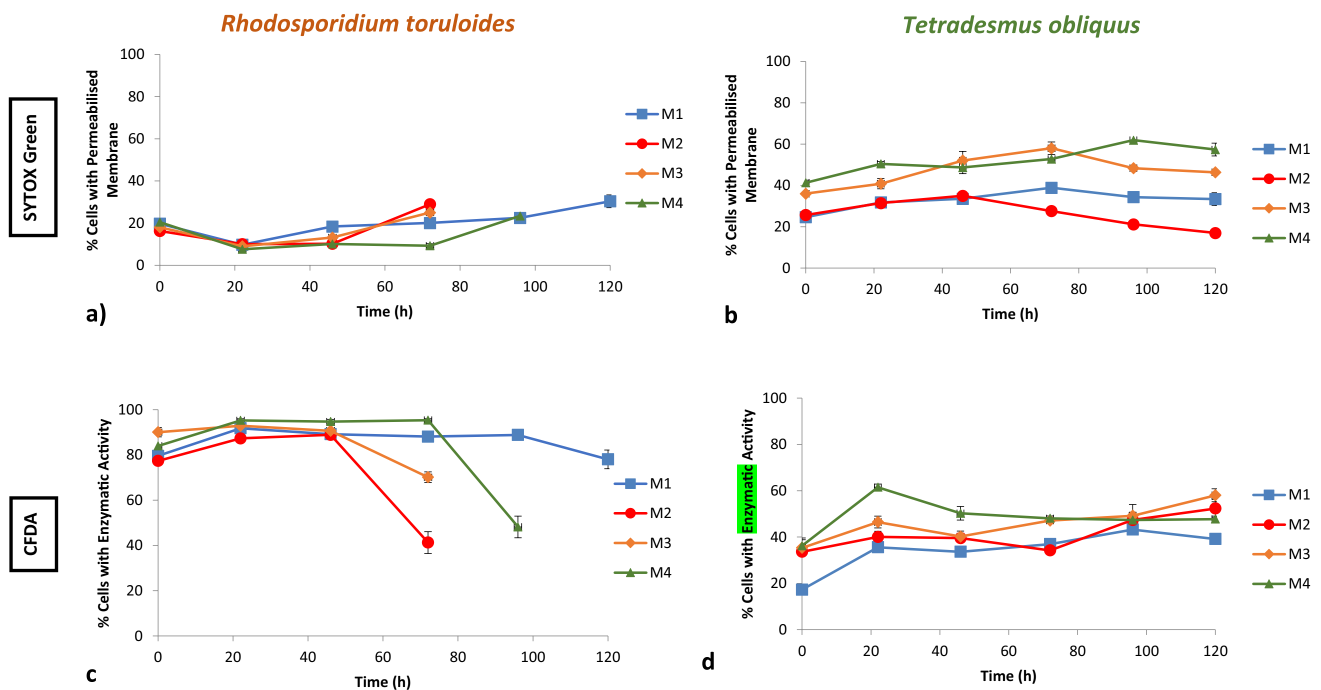

Fig. 4 **a** *Rhodosporidium toruloides* and **b** *Tetradesmus obliquus* cells with permeabilised membrane over time for the brewery effluent cultivations without supplementation: M1 – Primary Brewery WasteWater (PBWW) cultivation, M2 – Secondary Brewery WasteWater (SBWW) cultivation, M3 – Mixture of PBWW and SBWW at the ratio of 1:1 cultivation, and M4 – Mixture of PBWW and SBWW at the ratio of 1:7 cultivation. **c** *R. toruloides* and **d** *T. obliquus* cells with enzymatic activity over time for the brewery effluent cultivations without supplementation: M1 – PBWW cultivation, M2 – SBWW cultivation, M3 – 1:1(PBWW:SBWW) cultivation, and M4 – 1:7(PBWW:SBWW) cultivation. The error bars represent the standard deviation of two measurements from independent duplicates

containing higher proportions of SBWW, which probably lack a few nutrients essential for the yeast metabolism. The yeast cells behavior in the other three cultivations (M2, M3 and M4) could be due to the lower sugar content compared to M1, resulting in earlier starvation conditions, leading to eventual yeast cell lysis.

Concerning *T. obliquus* SYTOX + cells in M1/M2 assays, the proportion of SYTOX + cells was similar after the inoculation (~ 25%), slightly increasing at different times of cultivation and afterward decreasing until the end of the cultivations, reaching 33.4% and 17.0% at $t = 120$ h, respectively. The lowest SYTOX + microalgae stressed cells proportion observed during M2 cultivation is in agreement with its biomass profile, attaining the highest concentration (0.21 g L^{-1}) among all the assays (Fig. 1). For M3/M4 assays, the proportions of SYTOX + cells increased immediately after the inoculation (36.0% and 41.3%, respectively). Such results demonstrated that, in M3 and M4 assays, the microalgae cell membrane was immediately affected after the inoculation. As M3 and M4 cultures developed, the proportion of SYTOX + cells increased, attaining 46.4% in the M3 cultivation and 57.4% in the M4 cultivation, possibly due to lack of nutrients (other than ammoniacal N, since this compound was not exhausted, according to Table 4), which was intensified as the culture aged.

M5, M6, M7, and M8 After the yeast cells adaptation to the culture medium, at 22 h, the percentage of *R. toruloides* cells with permeabilised membrane decreased in all cultivations to 6.0% in M5, 3.6% in M6, 5.6% in M7, and 9.5% in M8 (Fig. 5a). After this time, and until the end of all experiments, the proportion of SYTOX + cells remained low and stable (< 10%). Comparing these results with the yeast cell membrane integrity observed during M1-M4 assays (Fig. 4a), it can be seen that the proportion of *R. toruloides* SYTOX + cells was always lower during M5-M8 cultures development, confirming that the cultures supplemented with SCM could protect the yeast cell membrane.

In the case of *T. obliquus* cells, M8 cultivation displayed the highest proportion of permeabilised cells, being always higher than 35%, and reached 49.6% at the end of the assay.

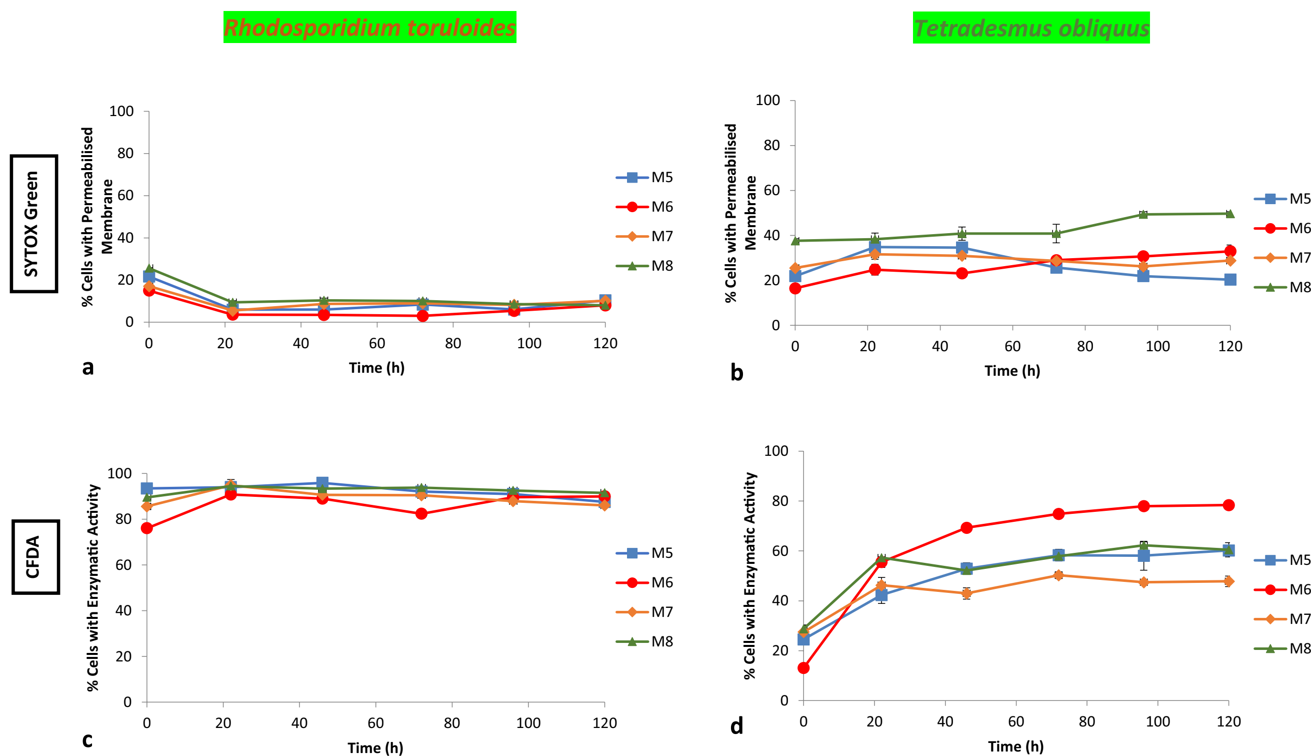

Fig. 5 **a** *Rhodosporidium toruloides* and **b** *Tetradesmus obliquus* cells with permeabilised membrane over time for the brewery effluent cultivations supplemented with sugarcane molasses (SCM): *M5* Primary Brewery WasteWater (PBWW) + SCM cultivation, *M6* Secondary Brewery WasteWater (SBWW) + SCM cultivation, *M7* Mixture of PBWW and SBWW at the ratio of 1:1 + SCM cultivation, and *M8* Mixture of PBWW and SBWW at the ratio of 1:7 + SCM cultivation. **c** *R. toruloides* and **d** *T. obliquus* cells with enzymatic activity over time for the brewery effluent cultivations supplemented with sugarcane molasses: *M5* PBWW + SCM cultivation, *M6* SBWW + SCM cultivation, *M7* 1:1(PBWW:SBWW) + SCM cultivation, and *M8* 1:7(PBWW:SBWW) + SCM cultivation. The error bars represent the standard deviation of two measurements from independent duplicates

The remaining cultures (M5, M6, and M7) displayed a lower proportion of permeabilized membrane (<35%) (Fig. 5b). Comparing the microalga cell membrane integrity in M1-M4 cultures, it can be seen that the proportion of *T. obliquus* SYTOX+cells was similar in all cultures (M1-M8), indicating that the addition of SCM did not affect the microalga cell membrane integrity during the cultures development.

Cell Stress Detection—Enzymatic Activity

M1, M2, M3, and M4 For all assays, the proportion of yeast cells with enzymatic activity (cells stained with CFDA, thus CFDA+) was always above 77% during the first 46 h of cultivation. Thereafter, this proportion abruptly decreased for assays M2 and M3, attaining 41.3% and 68.5%, respectively, at $t = 72$ h and 48.2% at $t = 96$ h for M4 assay. M1 assay was the only culture that maintained a high proportion of CFDA+cells throughout the time course of the cultivation (attaining 78.0% at $t = 120$ h) (Fig. 4c). Figure 4a supports this result, as it shows that M1 assay displayed only 30.4% of SYTOX+cells at the end of the cultivation, following the same trend displayed in Fig. 4c, which indicates that most of the yeast cells maintained the membrane intact and the esterase activity during the M1 assay. These results are also in accordance with M1 yeast biomass profile shown in Fig. 1, as the yeast biomass concentration increased until $t = 96$ h, demonstrating that the medium rich in PBWW favored the yeast growth.

The proportion of *T. obliquus* CFDA+cells are displayed in Fig. 4d. At the end of all cultivations, this proportion varied between 39.2% and 58.0% among all the assays. There was not an evident difference among all the assays throughout the cultivations, as it was observed for microalgae SYTOX+cells profiles (Fig. 4b). M2 assay displayed the lowest proportion of SYTOX+cells (17.0%) and the second highest proportion of CFDA+cells (52.3%) at the end of the cultivation, meaning that, in this assay the microalgae cells were able to maintain their integrity and their esterase activity.

M5, M6, M7, and M8 In brewery wastewater cultivations supplemented with SCM, the initial percentage of *R. toruloides* CFDA+cells was 94.1%, 76.1%, 84.6%, and 89.6% in M5, M6, M7, and M8 cultivations, respectively (Fig. 5c). After the initial time, throughout the cultivation time course, the percentage of *R. toruloides* cells with enzymatic activity was always above 82%, for the four cultivations. Comparing these results with the yeast cell enzymatic activity observed throughout the assays M1-M4 development (Fig. 4c), the proportion of CFDA+cells was always higher throughout M5-M8 assays development (Fig. 5c), confirming that the carbon addition allowed the yeast enzymatic activity maintenance, as well as the membrane integrity, as above referred.

Regarding *T. obliquus* cells, the initial percentage of microalga CFDA+cells in M5, M6, M7, and M8 cultivations was 24.5%, 13.1%, 27.5%, and 28.8%, respectively (Fig. 5d). In the four cultivations, an increase in the proportion of *T. obliquus* cells with enzymatic activity throughout the cultivation time was observed, reaching 60.2%, 78.4%, 47.8%, and 60.5% at the end of M5, M6, M7, and M8 cultivations, respectively (Fig. 5d). Comparing these results with the microalga cell enzymatic activity observed during the M1-M4 assays (Fig. 4d), the proportion of CFDA+cells was higher throughout M5-M8 assays development (Fig. 5d), indicating that the SCM addition allowed the microalga enzymatic activity maintenance, as well as the membrane integrity.

Discussion

In this work, the feasibility of using primary brewery wastewater (PBWW) and secondary brewery wastewater (SBWW) separately, or mixed at the ratios of 1:1 (PBWW:SBWW) and 1:7 (PBWW:SBWW), with or without supplementation with sugarcane molasses (SCM) as culture media for lipid production by a mixed culture of the oleaginous yeast *Rhodosporidium toruloides* and the microalgae *Tetradesmus obliquus* was studied.

When the different brewery wastewaters without supplementation were used as culture medium for the mixed culture, it was observed low biomass and lipid production because the wastewater did not contain the required nutrients for heterotrophic and autotrophic growth. This has already been described by Dias et al. [18], who concluded that secondary brewery effluent did not contain enough carbon and nitrogen source to allow *R. toruloides* growth.

In fact, industrial effluents often do not contain the required amounts of nutrients for yeast and microalgae metabolism. This situation has already been described by Xue et al. [20] and Chi et al. [22] who reported low biomass and lipid productivities when using monosodium glutamate wastewater and municipal wastewater, respectively, as verified in this work. In both works, to fulfill the micro-organisms nutritional needs, it was necessary to supplement the effluent with other effluent/waste. In this work, sugarcane molasses was used as a low-cost carbon source to supplement the brewery wastewater, as it contains high amounts of sugars (sucrose, fructose and glucose), essential for the yeast metabolism.

When 10 g L^{-1} of SCM was added to the culture medium, it was observed a predominance of the yeast population in mixed cultures. This has already been described in Zhang et al. [23] and Liu et al. [24] which used mineral mediums

with glucose as organic carbon source for mixed culture development.

For both M1-M4 and M5-M8 cultivations, the media containing the highest PBWW proportions (M1, M5 and M7) favored the yeast growth, which is an interesting result: although PBWW contains higher COD levels than SBWW (COD concentration for M5, M6, M7 and M8 assays were 48.00, 30.67, 37.67 and 29.67 g O_2 L^{-1}, respectively (Table 1), this primary effluent should contain higher proportion of toxic compounds, such as heavy metals, inorganic species, trace organic pollutants, among others, which did not affected the yeast growth in the mixed culture. Kim and Farnazo [30] studied the toxicity characteristics of sewage treatment effluents and the potential contribution of micropollutant residuals. The authors concluded that conventional wastewater treatment process reduced some organics and nutritional compounds from the wastewater, and it resulted in toxicity reduction in lethal effect and positive reproductive effect. Although these toxic compounds may have a negative impact on the microbial metabolism, microalgal cells can absorb heavy metals when present in the medium culture at low levels [25–27]; therefore, it is thought that the microalgae were able to remove the toxic components from the medium that could be affecting the yeast.

The addition of SCM also favored the microalgae growth, as the two media with higher PBWW proportions (M5 and M7) allowed higher *T. obliquus* biomass production (Fig. 3c).

For all the assays with SCM supplementation, the lipid production was higher than without supplementation since a high C/N ratio, present in these assays, is essential for lipid production. Contrary to what was observed for biomass production, lipid production increased with increasing SBWW proportions in the effluents supplemented with SCM attaining 14.86% (w/w DCW) in M6 (Table 2).

Dias et al. [18] used SBWW supplemented with 10 g L^{-1} of SCM for brewery wastewater with concomitant lipid and carotenoid production by the yeast *Rhodosporidium toruloides* NCYC 921. In the batch trials performed with the addition of a nitrogen source, the lipid contents obtained varied between 3.1 and –6.2% w/w (DCW), values considerably lower than those obtained in this work for the mixed culture, and without the nitrogen supplementation, which suggests the advantage of the mixed culture.

Other authors have used mixed effluents to supplement wastewaters that are not rich enough in nutrients to allow the growth of the micro-organisms in mixed culture (Table 5). Cheirsilp et al. [28] used a steamed fish process effluent from a seafood processing plant supplemented with sugarcane molasses from a sugarcane plant, as growth medium for the co-culture of the yeast *Rhodotorula glutinis* and the microalga *Chlorella vulgaris*. 4.63 g L^{-1} of total biomass was obtained in the mixed culture, a higher value than those obtained in this work. The authors concluded that the addition of 1% of sugarcane molasses as organic carbon source increased the total biomass production (Table 5). The authors observed 62.2% (w/w) lipid content, a higher value of any of the cultivations in this work. In addition, 79% of COD removal was obtained by the authors, higher than any of the COD removal achieved in the brewery wastewater cultivations supplemented with 10 g L^{-1} of SCM performed in this work (Table 5).

The nitrogen compounds present in the SCM do not allow the yeast growth and lipid production, being necessary the addition of assimilable nitrogen source, as referred in Dias et al. [18] and Taskin et al. [29]. The low nitrogen load in the medium might have limited *R. toruloides* and *T. obliquus* growth in the mixed culture, contributing for the low biomass concentration and low lipid contents. Ammoniacal nitrogen is consumed by heterotrophic micro-organisms as a source of nitrogen, being incorporated by the cells for protein synthesis. In the brewery cultivations supplemented with 10 g L^{-1} SCM, the ammoniacal nitrogen consumption was higher in the cultivations where the lipid production was higher.

Conclusions

This work investigated the possible use of primary and secondary brewery wastewater with and without supplementation and with 10 g L^{-1} of sugarcane molasses for the concomitant wastewater treatment and lipids production, using a mixed culture of the oleaginous yeast *Rhodosporidium toruloides* NCYC 921 and the oleaginous microalga *Tetradesmus obliquus* (ACOI 204/07). The results showed that the media with brewery wastewater without supplementation are not propitious for the yeast metabolism, not allowing the co-culture maintenance. When the brewery wastewater was supplemented with 10 g L^{-1} of sugarcane molasses, higher biomass and lipid productions were obtained.

Flow cytometry was used to differentiate the cells of both micro-organisms during the cultivations, as well as to evaluate the cell status, in order to understand the effect of the different brewery effluent media on the cells. This advanced technique also allowed understanding the yeast and microalga populations dynamics in mixed cultures, developed in brewery effluents.

To obtain higher productivities, experiments adding a nitrogen source to the culture medium are in progress.

Table 5 Co-cultivation of oleaginous yeast and microalgae using different sources of low-cost materials for wastewater treatment and lipid production

Micro-organisms		Substrates	Seed proportion Microalgae/yeast	Cultivation mode/time	Biomass production (g L^{-1})	Lipid production (g L^{-1})	Lipid content (% w/w)	Effluent treatment Removal rate (%)	Reference
Yeast	Microalgae								
Rhodotorula glutinis	*Spirulina platensis*	Monosodium glutamate wastewater	N/m [a]	250 mL flasks Batch mode 5 days	1.6 ± 0.1	0.20 ± 0.0	14.00	73% COD [b] 94% Reducing sugars 35% NH_4^+-N d	[20]
Rhodotorula glutinis	*Chlorella vulgaris*	Steamed fish process effluent from a seafood processing plant and molasses from sugarcanne plant	1:1	Shake flasks containing 50 mL medium culture Batch mode 7 days	4.63 ± 0.15	2.88 ± 0.16	62.2	79.0 ± 1.1% COD [b]	[28]
Cryptococcus curvatus and *Rhodotorula glutinis*	*Chlorella sorokiniana*	Food waste hydrolysate and municipal wastewater (1:4)	N/m	50 mL culture medium in 250 mL flasks Batch mode 12 days Two-step sequential treatment	*C. curvatus* + *Chlorella sorokiniana*: $X_{C. curvatus}$ = 7.5 $X_{C. sorokinianas}$ = 1.5 *R. glutinis* + *Chlorella sorokiniana*: $X_{R. glutinis}$ = 5.2 $X_{C. sorokiniana}$ = 0.88	*C. curvatus* + *Chlorella sorokiniana*: $L_{C. curvatus}$ = 2.14 $L_{C. sorokinianas}$ = 0.34 *R. glutinis* + *Chlorella sorokiniana*: $L_{R. glutinis}$ = 1.02 $L_{C. sorokiniana}$ = 0.18	*C. curvatus* + *Chlorella sorokiniana*: $L_{C.curvatus}$ = 28.6 $L_{C. sorokiniana}$ = 22.9 *R. glutinis* + *Chlorella sorokiniana*: $L_{R. glutinis}$ = 19.6 $L_{C. sorkiniana}$ = 20.0	In the end of the integrated process: 95.2% COD [b] 96.4% N [c]	[22]
Rhodosporidium toruloides	*Tetradesmus obliquus*	Primary (PBWW) and secondary (SBWW) brewery wastewater with or without supplementation with sugarcane molasses (SCM)	1:1	1 L baffled flasks Batch mode 120 h	PBWW [e]: 0.32 ± 0.01 PBWW [e] + 10 g L^{-1}: 2.17 ± 0.04 SBWW [f] + 10 g L^{-1}: 1.13 ± 0.12	PBWW [e]: 0.013 PBWW [e] + 10 g L^{-1}: 0.093 SBWW [f] + 10 g L^{-1}: 0.168	PBWW[e]: 3.99 ± 0.00 PBWW [e] + 10 g L^{-1}: 4.28 ± 0.09 SBWW [f] + 10 g L^{-1}: 14.86 ± 0.86	In the PBWW[e] cultivation: 93.4 ± 3.2% COD [b] 73.2 ± 1.1% NH_4^+-N [d] 91.7 ± 1.2% Total Sugars In the PBWW[e] + 10 g L^{-1} cultivation: 62.5 ± 4.2% COD [b] 30.2 ± 1.1% NH_4^+-N [d] 69.6 ± 0.7% Total Sugars	This work

[a] *N/m* Not mentioned

[b] *COD* Chemical Oxygen Demand

[c] *N* Nitrogen

[d] *NH_4^+-N* Ammonia Nitrogen

[e] *PBWW* Primary Brewery WasteWater

[f] *SBWW* Secondary Brewery WasteWater

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Authors' Contributions CD carried out the experimental work, analyzed the data and drafted the manuscript. TLS coordinated experimental work, and revised the manuscript. All authors revised and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no conficts of interest.

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