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REVIEW

The Use of Oleaginous Yeasts and Microalgae Grown in Brewery Wastewater for Lipid Production and Nutrient Removal: A Review

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Abstract

Brewery wastewater has been proposed as an attractive low-cost substrate for microbial lipid production for oleaginous yeast and microalga with promising results. For each liter of beer produced, from 3 to 10 L of wastewater are generated which can be used as culture medium for autotrophic or heterotrophic metabolism. This strategy allows reducing the culture medium cost, as well as obtaining high lipid contents and other high value compounds which can make the process profitable. Additionally, the use of industrial effluents/wastes as substrates for microbial growth can be a strategy to treat them based on the circular economy rules. This review presents the different brewery wastewater treatment strategies using oleaginous yeast and microalga pure and mixed cultures for the concomitant wastewater treatment and lipids/carotenoids production so far reported, highlighting the benefits/disadvantages of such strategies and comparing their performance in terms of wastewater treatment, lipids and carotenoids production between pure and mixed cultures performance.

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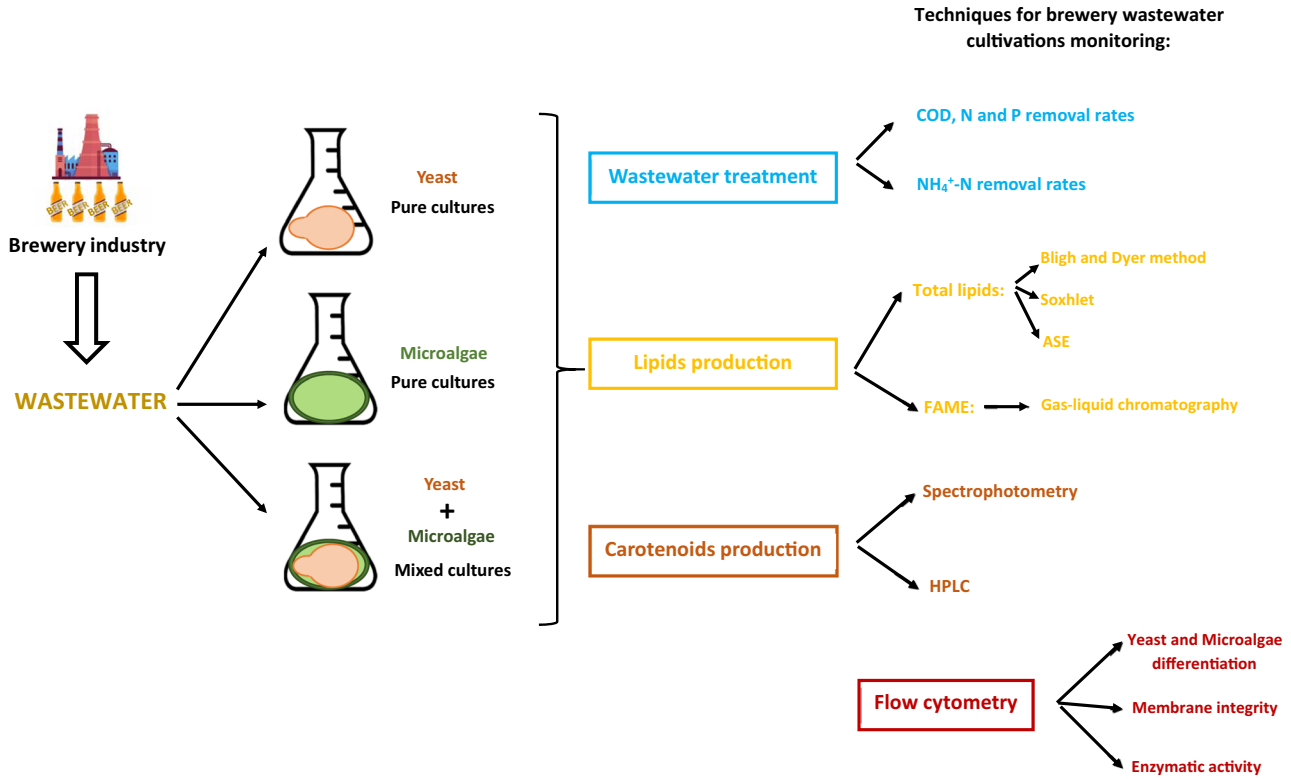
Graphical Abstract



Keywords Oleaginous microorganisms · Yeast · Microalgae · Brewery wastewater · Effluent treatment · Lipids

Abbreviations

ABWW	Anaerobically treated brewery wastewater
BWW	Brewery WASTEWATER
C/N	Carbon/nitrogen ratio
CFDA	Carboxyfluorescein diacetate
COD	Chemical oxygen demand
DCW	Dry cell weight
ELV	Emission limit values
FC	Flow cytometry
FSC	Forward scatter
GHG	Greenhouse gas
HAc	Acetic acid
HBut	Butyric acid
HProp	Propionic acid
N	Nitrogen
NAA	1-Naphthaleneacetic acid
OrgAc	Organic acid
P	Phosphorus
PBWW	Primary brewery wastewater
SBWW	Secondary brewery wastewater
SCM	Sugarcane molasses
SSC	Side scatter

Statement of Novelty

Due to the high consumption of beer worldwide, the amount of brewery wastewater that is produced is massive as 3 to 10 L of wastewater are produced for each liter of beer made. Therefore, the waste generated is enormous as well as the costs to treat it, for the brewery companies. Despite that, the current brewery wastewater treatment processes are still the conventional which generate huge amounts of sludge and use excessive quantities of chemicals, which can cause environmental concerns. The use of oleaginous microorganisms in the brewery wastewater treatment with concomitant lipids and carotenoids production, in particular using symbiotic cultures, has been described by several authors as a potential way to reduce the brewery wastewater treatment costs attaining high biomass, lipid and carotenoid productions. This review pretends to demonstrate how Science can have

an important role evolving and optimizing bioprocesses, such as the brewery wastewater treatment, to take the most advantage of all the steps of the process, interfering, as little as possible, with the surrounding ecosystems.

Introduction

In the last decades, the world energy consumption has increased considerably [1]. To obtain the necessary energy for the world's population and economy, fossil fuels, especially petroleum, coal and natural gas, have been indiscriminately exploited. The use of fossil fuels has raised serious environmental concerns such as greenhouse gas (GHG) emission, which is pointed as the main responsible for the climate change [2]. Carbon dioxide is one of the GHG that contributes for global warming, causing the rise of the Earth temperature, with detrimental effects for living life on Earth [2, 3]. In 2018, 89% of the global CO_2 emissions were from the fossil fuels usage and industry [4]. To reduce the effects of this severe energy crisis, countries have taken actions: in November 2021, the Glasgow Climate Pact was signed by 197 world leaders to cut down global GHG emissions. Also, in the transports sector, the European Union (EU) have established a goal of 14% of renewable energies until 2030 (https://www.eea.europa.eu/ims/use-of-renewable-energy-for, accessed on 25.07.2022).

Biofuels such as biodiesel are renewable sources of energy which are less pollutant alternatives to conventional diesel [2, 5]. Nowadays, the majority of the biodiesel produced is derived from vegetable oils such as palm or soybean, which has raised a public controversy: on one hand, the biodiesel derived from food crops competes with food production for farmland; on the other hand, the constant increase in the raw material prices rises, constantly, the biodiesel price [6, 7].

Oleaginous microorganisms such as yeasts and microalgae can be used as biodiesel feedstocks, as they are capable of accumulating between 20 to 80% lipids of their dry cell weight (DCW) and have many advantages when compared to oil plants: higher growth rate and oil productivities, less arable land and water are needed, as well as no fertilization requirements, and does not depend on location, climate or season [8–10]. Moreover, both microorganisms are able to produce carotenoids with commercial interest. However, the cost of the biodiesel produced from oleaginous microorganisms is still not economically sustainable since production costs are still high [2, 11]. To reduce the overall cost of the process, it is essential to develop new strategies to produce low-cost biofuels from oleaginous microorganisms.

A possible strategy consists of using low-cost substrates such as lignocellulosic biomass, effluents, wastes and by-products from industries which usually have high organic and inorganic loads that can be used as feedstock for heterotrophic or autotrophic oleaginous microorganisms, respectively. Also, if these wastes could be used as resources, the treatment cost would be reduced [7]. An example is the brewery wastewater (BWW): since 3 to 10 L of BWW are created for 1 L of beer produced, the amount of waste created is enormous, as well as the costs to treat it for the breweries [12]. The usage of BWW as feedstock for yeast and microalga has been successfully described by several authors with encouraging results [1, 12, 13]. However, the use of industrial effluents has some disadvantages: it has been reported that brewery wastewater present organic acids that can inhibit the microorganisms growth [14].

Moreover, it is possible to improve the BWW process efficiency selecting, for instance, yeast and microalga mixed cultures as an alternative to pure cultures. Higher biomass and lipid productivities have been described when compared with the individual pure cultures, as well as higher nutrient removal rates from wastes [5, 7].

This review will analyze the different brewery wastewater treatment strategies using oleaginous yeast and microalgae pure and mixed cultures for the concomitant wastewater treatment and lipids/carotenoids production so far reported, highlighting the benefits/disadvantages of such strategies and comparing their performance in terms of wastewater treatment, lipids and carotenoids production between pure and mixed cultures performance.

The Brewery Industry

Overview

Beer is the fifth most consumed beverage in the world, with a medium consume of 9.6 L per capita (value considering only people with more than 15 years old) [15]. It is produced throughout alcoholic fermentation using selected yeast from the *Saccharomyces* genera and wort prepared with malt cereals, to which were added hop flowers, or their derivatives, and adequate water [16]. Beer production process can be divided in 3 parts: wort manufacturing, fermentation and filling. A schematic scheme from the technologic process of beer production is presented in Fig. 1.

For beer production, several chemical and biochemical reactions (mashing, boiling, fermentation and maturation) and several solid–liquid separations (wort separation, wort clarification and rough beer clarification) are necessary to perform [17]. Due to this, a large amount and different varieties of wastes are produced: water, spent grains, spent hops, surplus yeast, trub, caustic and acid cleaners, waste beer and waste label [16].

Water is used in almost every step of beer production, being very important in all the process. Water consumption depends on the beer type and volume, the existence of bottles washing machines, the type of packing and

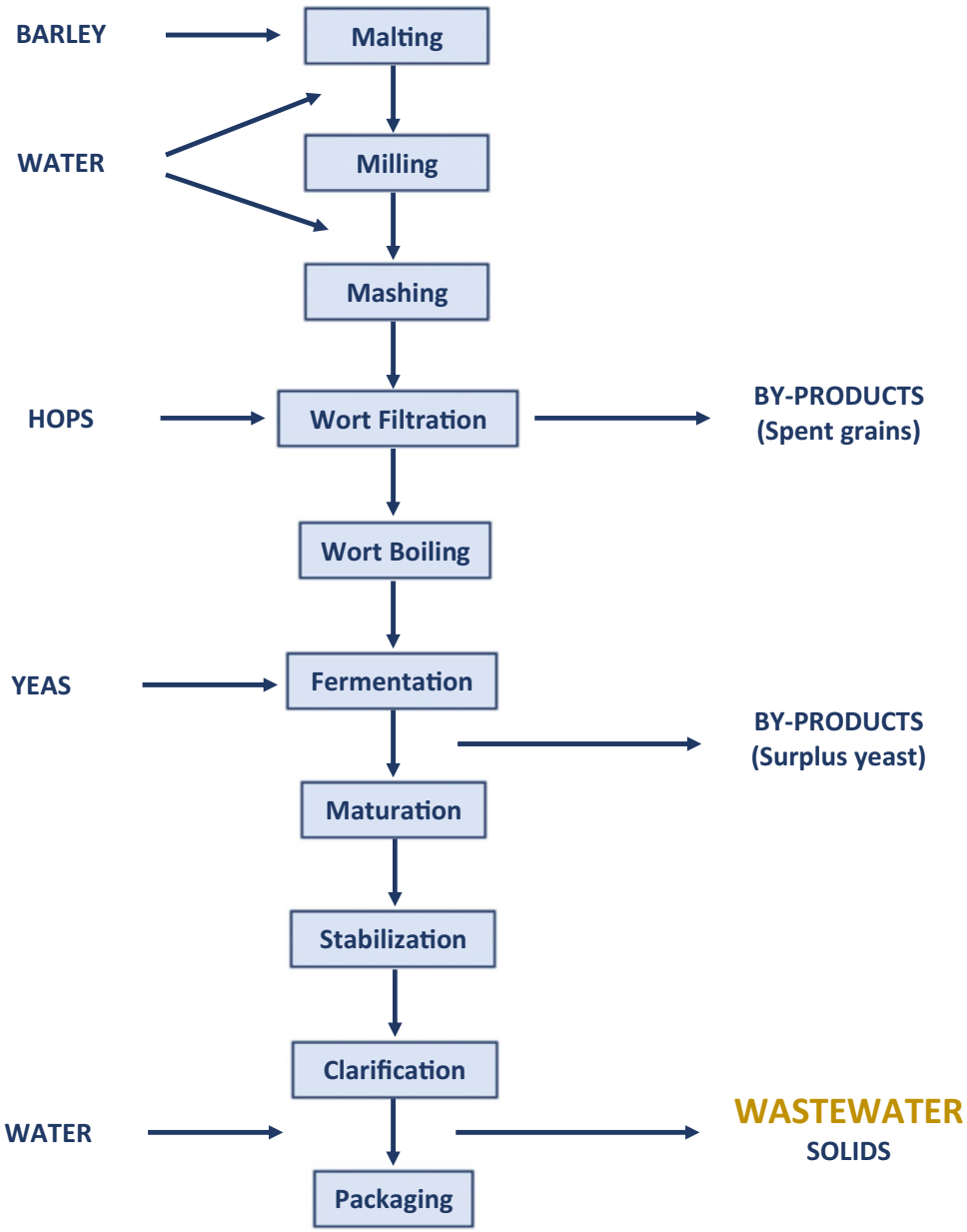


Fig. 1 Schematic scheme from the technologic process of beer production

Table 1 Characteristic of the brewery effluent [19]

Parameter	Range of values
BOD (mg L^{-1})	1200–3600
COD (mg L^{-1})	2000–6000
Total suspended solids (TSS) (mg L^{-1})	200–1000
Temperature (°C)	18–40
pH	4.5–12
Nitrogen (mg L^{-1})	25–80
Phosphorus (mg L^{-1})	10–50
Heavy metals (mg L^{-1})	Very low

pasteurization, the cleaning system used and the type and age of the equipment, and it can vary between 0.4 and 1 m^3 L^{-1} h^{-1} of beer produced [15]. Due to this, 3 to 10 L of brewery wastewater are produced for each liter of beer produced [15, 18], which makes this a major problem for breweries, since the wastewater produced is massive representing a tremendous cost to treat it for the breweries.

Characteristics of the Brewery Effluent

BWW has been described by several authors to have a high chemical oxygen demand (COD) from the organic

components present (sugars, soluble starch, ethanol, volatile fatty acids, among others) which are easily biodegradable [19, 20]. Usually, it does not contain significant quantities of heavy metals and it is not toxic. However, the presence of organic acids such as acetic, propionic and butyric acids has been already reported [14, 21]. Nitrogen (N) and phosphorus (P) levels can vary depending on the handling of the raw material and the yeast leftover present in the BWW after the beer production. The BWW temperature can range from 25 to 40 °C, sporadically reaching temperatures higher than 80 °C [22]. pH levels are very depending on the cleaning and sanitizing chemicals used and can range from 4.5 and 12 [19]. Table 1 summarizes the most common composition of the brewery effluents described in literature.

Brewery Wastewater Treatment with Concomitant Lipid and Carotenoids Production by Oleaginous Microorganisms

The potential of using oleaginous microorganisms as a renewable and environmentally friendly resource, in alternative to the traditional biofuels feedstocks such as oil crops, waste cooking oil and animal fat, is gathering continuous attention [23, 24]. The energy crisis related to the reduction of the main fossil fuels, as well as the increase in the CO_2 levels in the atmosphere, has led to a conscientious search for alternative systems for biofuels production [2, 24].

Oleaginous yeasts and microalgae microorganisms can be used as biodiesel feedstocks, as they are capable of accumulating between 20 to 80% lipids of their DCW. There are many benefits of using oleaginous microorganisms when compared to oleaginous plants: higher growth rate and oil productivities, less arable land and water are needed for their growth, as well as no fertilization requirements, and their cultivation does not depend on location, climate or season [9–11]. Moreover, when compared to traditional wastewater treatment methods, the use of oleaginous microorganisms to treat wastewater has several advantages: it can reduce energy consumption as well as the formation of dangerous sludge and the costs to treat them [25]. Low initial investment is required as this technology can be performed in basic bioreactors/fermenters and is able to treat efficiently, in a brief period of time, the effluents while producing high value-added products with great commercial value [26].

However, as above mentioned, the biodiesel produced from oleaginous microorganisms is still not a sustainable process [2, 11]. To decrease the overall cost of the process, it is crucial to investigate new strategies to produce low-cost biofuels from oleaginous microorganisms. Beyond the use of low-cost substrates already mentioned, to decrease the overall cost of the process, the use of oleaginous microorganisms that not only accumulate high lipid contents, but also produce high value-added products such as carotenoids with a commercial interest, may improve the economics of the whole process, as the high value-added biocompounds production may sustain the microbial biofuel production [2, 27, 28].

In fact, most of the research works reporting concomitant microbial lipids/carotenoids production while performing wastewater treatment has used single yeast or microalga cultures [1, 12, 13, 29, 30]. However, recent studies have highlighted the advantages of the use of yeast and microalga mixed cultures instead of the pure cultures [14, 31–33].

The next sections will address the several strategies for oleaginous yeast and microalgae pure and mixed cultures for BWW treatment with concomitant lipids/carotenoids production presented in the literature.

Oleaginous Yeast Pure Cultures

Selection of Suitable Yeast Strain

As referred before, oleaginous microorganisms are able to accumulate between 20 to 80% (w/w DCW) of lipids under specific conditions. Yeast species such as *Yarrowia*, *Rhodosporidium*, *Rhodotorula*, *Candida*, *Cryptococcus*, *Lipomyces*, among others, have been widely described as being able to accumulate considerable proportions of lipids using industrial effluents and residues as low-cost substrates for lipid production [7].

In addition, there are a few oleaginous yeast species such as *Rhodotorula* or *Rhodosporidium* that are able to produce pigments with commercial interest (β-carotene, torulene and torularhodin) [12, 27].

The study of brewery wastewater treatment by oleaginous yeast pure cultures with concomitant lipids and carotenoids production is still in an incipient step, which can be corroborated by the utilization of only two yeast strains for this end: *Rhodotorula glutinis* and *Rhodosporidium toruloides*, as observed in Table 2. Nevertheless, these strains have been widely used for effluent treatment with lipid production of different effluents such as cassava, distillery or sewage wastewaters with promising results [34–36].

Substrates/Supplementation Used

Heterotrophic organisms, as yeast are, need to consume organic compounds, such as sugars, to obtain energy and to synthetize biomolecules essentials to their metabolism (Fig. 2). Although brewery wastewater has been described, as referred in section 2.2, to contain a high nutrient and carbon load, which could serve as an adequate substrate for yeast growth, Dias et al. [12] and Dias et al. [14] reported

Table 2 Yeast oleaginous species cultivated on brewery effluents for biomass, lipids and carotenoids production with concomitant brewery wastewater treatment

Type of cultivation	Microorganism Yeast	Substrate	Total sugars concentration ($g\ L^{-1}$)	C/N ratio	Cultivation mode	Biomass production ($g\ L^{-1}$)	Lipid content (% w/w)	Carotenoid production ($mg\ L^{-1}$)	COD removal (%)	Sugar consumption (%)[a]	Total N consumption (%)[a]	Reference
Pure	*Rhodosporidium toruloides* NCYC 921	PBWW	–	–	Batch/ Shake flasks	0.18	19.4	–	–	–	− 34.7	[14]
	Rhodosporidium toruloides NCYC 921	SBWW	–	–	Batch/ Shake flasks	1.29	–	–	–	–	–	[12]
	Rhodotorula glutinis (CGMCC No. 2258)	Industrial Brewery effluent	32.04	13	Batch/ Shake flasks	5.22	~10.0	0.6	low	~22.0	–	[1]
	Rhodotorula glutinis (CGMCC No. 2258)	Industrial Brewery effluent with glucose supplementation as carbon source	41.42	33	Batch/ Shake flasks	7.82	~7.1	1.2	low	~34.8	–	[1]
	Rhodosporidium toruloides NCYC 921	PBWW supplemented with SCM as carbon source	10	80	Batch/ Shake flasks	0.23	15.9	–	–	-8.6	-6.0	[14]
	Rhodosporidium toruloides NCYC 921	SBWW supplemented with SCM as carbon source	10	–	Batch/ Shake flasks	2.20	–	–	–	–	–	[12]
	Rhodosporidium toruloides NCYC 921	PBWW supplemented with SCM as carbon source and Urea as nitrogen source	10	5.7	Batch/ Shake flasks	0.84	12.7	–	–	-2.1	-0.9	[14]
	Rhodosporidium toruloides NCYC 921	SBWW supplemented with SCM as carbon source and Urea as nitrogen source	10	5.7	Fed-batch/ 7 L bioreactor	42.5	29.9	11.4	81.7 (after batch phase)	100 (after batch phase)	45.8 (after batch phase)	[12]

PBWW primary brewery wastewater; *SBWW* secondary brewery wastewater; *SCM* sugarcane molasses

[a]Negative sign indicates the accumulation of the nutrient in the medium

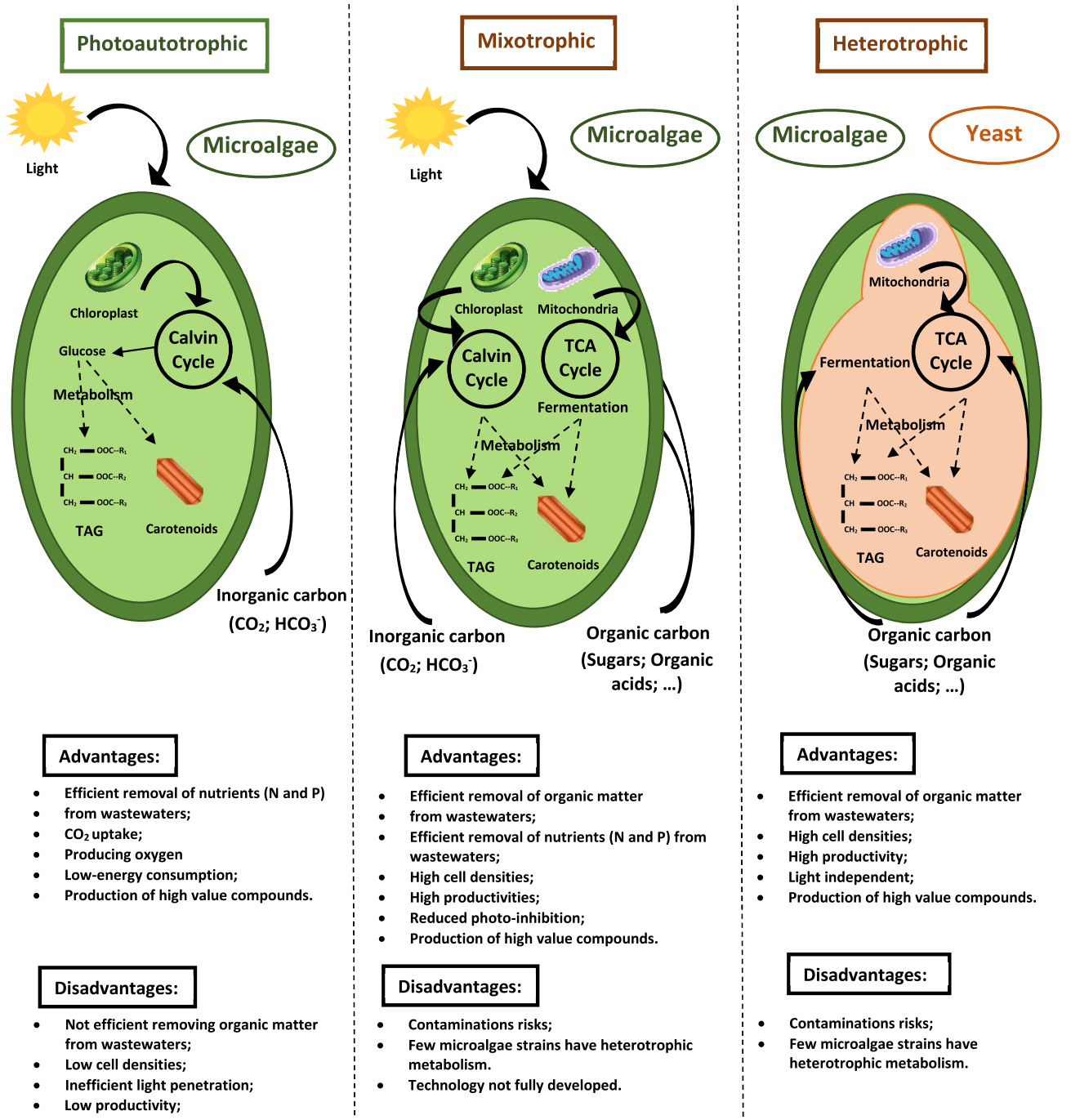


Fig. 2 Advantages and disadvantages of photoautotrophic, mixotrophic and heterotrophic metabolism cultivations for brewery wastewater treatment and lipids and carotenoids production by oleaginous microalgae and yeasts

that Primary BWW (PBWW, collected after the preliminary screening and primary sedimentation) and Secondary BWW (SBWW, collected after the secondary treatment by anaerobic digestion) without any supplementation could not allow *Rhodosporidium toruloides* growth in a pure culture: only 0.18 and 1.29 g L^{-1} of biomass production was observed for the PBWW and SBWW cultivations without supplementation (Table 2). When the authors supplemented the BWW with sugarcane molasses (SCM) as carbon source, growth limitations were also observed (0.23 and 2.20 g L^{-1} of biomass production was observed for the PBWW and SBWW cultivations supplemented with 10 g L^{-1} of SCM, respectively, Table 2), due to low nitrogen concentrations in the effluents [12, 14]. The authors supplemented the effluents

with an external nitrogen source (urea), which improved the biomass production (0.84 and 42.5 g L^{-1} for the PBWW and SBWW cultivations supplemented with 10 g L^{-1} of SCM and 2 g L^{-1} of urea, respectively, Table 2).

In the work of Schneider et al. [1], the brewery effluent used was loaded with several sugars (sucrose, maltose, glucose and fructose) which made it suitable for yeast growth. Nevertheless, sucrose was not significantly reduced by *Rhodotorula glutinis* throughout the cultivation, as well as maltose, which was the main sugar present in the effluent, which limited the yeast growth.

As already mentioned, the brewery wastewater collected from different breweries present different characteristics, in particular in terms of sugar content and COD values. This might be a limitation in the treatment process as, if the selected yeast cannot use all the sugars present in the effluent or the total organic load, COD will not be reduced sufficiently, and a clean effluent will not be obtained in the end of the treatment.

To achieve the highest performance in the BWW treatment process, it is crucial to first study the characteristics of the collected BWW. Afterwards, it is important to select an oleaginous yeast that is, not only resistant to industrial wastes and effluents, but also can fulfill its nutritional requirements with the nutrients present in the BWW. In none of the works presented in Table 2 this requirement was observed: Schneider et al. [1] had to supplement the BWW with glucose, which is an expensive carbon source, enhancing substantially the BWW treatment process; In the other two works presented in Table 2 [12, 14], the authors supplemented the BWW with SCM as carbon source, since it is a low-cost sub-product of the sugar industry (about 50€/ton) and urea as nitrogen source [12]. However, as referred by the authors, SCM contains organic compounds, including nitrogenous organic compounds such as melanoidins and other aromatic compounds that are not assimilated by the yeast *R. toruloides* which limited the BWW treatment in terms of nitrogen and organic carbon.

Cultivation Mode

Most of the works presented in Table 2 for brewery wastewater treatment by oleaginous pure yeast cultures are developed in shake flasks in batch mode, which demonstrates the incipient development of this technology. As observed in Table 2, only one of the presented works uses a fed-batch strategy developed in a 7 L bioreactor.

Dias et al. [12] developed a fed-batch cultivation where it was possible to expand the microorganisms exponential and stationary growth phases, in order to increase the biomass production and activate secondary metabolic pathways. When optimizing the lipid production by yeast cultures, the fermentation process should be carried out in two stages: during the microorganisms active growth phase, the biomass concentration increases and structural intracellular lipids are produced, since they are growth-associated compounds [37]. In this phase, the authors fed the culture with a concentrated BWW solution supplemented with SCM and urea, which promotes cell proliferation, since carbon is directed towards cell division, resulting in low lipid production. However, when the cell proliferation stops by the lack of a nutrient, usually nitrogen, the supplied fed solution is replaced by a carbon rich solution. As a result, the DNA, RNA and protein synthesis are halted, and the production of storage lipids by the cells is promoted. These compounds are usually synthesized under carbon excess conditions, as a cellular survival strategy, being used when the cells are exposed to starvation and/or other adverse conditions. This is the reason why, during the lipid production phase, it is important to maintain carbon excess conditions in the broth. Such conditions not only promote the lipid synthesis, but also avoid that cells consume the internal storage lipids.

Synthesized Products

Regarding the lipid production, a high Carbon/Nitrogen (C/N) ratio is considered benefic for lipid accumulation by oleaginous yeast, since the carbon in excess triggers intracellular lipid accumulation, as cells uses carbon for storage materials synthesis, instead for division, as referred above [14]. In the work of Schneider et al. [1], although a C/N ratio of 50 was used, not all the sugars present in the brewery effluent were utilized by *R. glutinis* as mentioned before, which resulted in an early carbon limitation in the BWW medium, not allowing *R. glutinis* to achieve the lipid accumulation phase. The authors report a lipid content of 10.0% (w/w DCW) when the raw BWW was used as culture medium and of 7.1% w/w in the glucose supplemented BWW medium, which are considerable low values for the lipid production (Table 2).

In the case of *R. toruloides* grown in PBWW, 49.1, 52.0 and 670.4 mg L^{-1} of total nitrogen were present in the PBWW, PBWW supplemented with 10 g L^{-1} of SCM (PBWW + 10SCM) and PBWW supplemented with 10 g L^{-1} of SCM and 2 g L^{-1} of urea media (PBWW + 10SCM + 2U), respectively [14]. As the level of nitrogen increased in the media, the lipid content of *R. toruloides* pure cultures decreased as expected (Table 2, 19.4, 15.9 and 12.7% (w/w DCW) of lipid content in *R. toruloides* grown in PBWW, PBWW + 10SCM, PWWW + 10SCM + 2U, respectively, Table 2). However, despite the high lipid contents observed for *R. toruloides* pure cultures, the lipid production was not viable, since the biomass production was considerably low as referred before (0.18, 0.23 and 0.84 g L^{-1} for PBWW, PBWW + 10SCM, PWWW + 10SCM + 2U cultivations, respectively, Table 2). Dias et al. [12] reported

29.9% (w/w DCW) of lipid content when SBWW was supplemented with 10 g L^{-1} of SCM and 2 g L^{-1} of urea (SBWW + 10SCM + 2U) as culture medium (Table 2). However, the authors performed this cultivation in a fed-batch regime using a 7 L bioreactor which allows better mass transfer conditions than in shake flasks, which is essential for an efficient lipid production.

Regarding the carotenoid production, usually, contrarily to intracellular lipids that are growth-associated compounds, the former are mixed compounds and are produced in both exponential and stationary phases [38]. However, in the work published by Schneider et al. [1], carotenoids were synthesized once the cell growth reached the stationary phase, which indicates that carotenoid synthesis was not related to cell growth. As for lipid production, the lack of an available carbon source inhibits carotenoid synthesis, which might have been the reason for the low carotenoid production obtained by the authors (0.6 mg L^{-1}, Table 2). Dias et al. [12] obtained a considerably higher carotenoid production (11.4 mg L^{-1}, Table 2). These authors performed a dual-stage pH control fed-batch cultivation in order to increase *R. toruloides* lipids and carotenoids production: pH 4 was used during the active yeast growth and pH 5 during the lipid accumulation phase. This strategy was based on the work of Dias et al. [39] who concluded that the medium pH strongly influences *R. toruloides* growth and lipids and carotenoids production (biomass and lipid production are maxima at pH 4.0; maximum carotenoid content was obtained for pH 5.0; [39]).

Brewery Wastewater Treatment Efficiency

Relatively to the brewery wastewater treatment, the COD removal reported by Schneider et al. [1] was low (Table 2), as well as the sugar consumption (32.5%, Table 2), probably due to the constraints previously referred of the yeast *R. glutinis* to consume the sugars sucrose and maltose present in the brewery effluent used. However, Dias et al. [12] described that, after the batch phase, 81.7% of COD removal, 100% of sugar consumption and 45.8% of total nitrogen removal were observed (Table 2). Although 100% of sugar consumption was observed by the authors, 81.7% of the COD was consumed. Also, as referred before, the use of SCM as carbon source conditioned the organic carbon and nitrogen removal rates. To allow better effluent treatment rates to comply with the emission limit values (ELV) for wastewater discharge, other low-cost carbon sources should be considered to supplement the brewery effluents, as referred before.

Oleaginous Microalgae Pure Cultures

Selection of Suitable Microalgae Strain

The potential of using oleaginous microalgae to produce high value products with the concomitant BWW treatment is considered an appealing option, since it presents several advantages compared to the traditional treatments—oleaginous microalgae are able to grow in wastewaters, removing undesired compounds, simultaneously producing biomass, carotenoids and lipids suitable for biofuels [20, 40] (Fig. 2). Moreover, microalgae can efficiently and simultaneously remove total nitrogen and phosphorus [24, 41], which is difficult to obtain with conventional treatment methods, while uptaking CO_2 as carbon source throughout photosynthesis process, and convert it into organic molecules such as lipids, sugars and proteins [5, 25] (Fig. 2). However, there are several limitations in the BWW treatment by microalgae: relatively low growth rate, low cell densities, mostly due to inefficient light penetration; long cultivation time to achieve high biomass and lipid concentrations; inefficiency in removing organic matter (COD) from the wastewaters; limitation in the salts removal, odor and color from the effluents, which requires a combination with other wastewater treatment methods; optimum light and temperature conditions requirements; contaminations risks [10, 20] (Fig. 2).

Nevertheless, depending on the light conditions and the carbon presence/absence, microalgae can assume different types of metabolism: photoautotrophic, heterotrophic (or photoheterotrophic) and mixotrophic [10, 30] (Fig. 2). In all of these metabolisms, microalgae can produce several value-added products such as lipids and carotenoids (Fig. 2). Moreover, when grown in heterotrophic or mixotrophic metabolisms, microalgae can also remove organic carbon from the effluents to obtain energy and to synthetize biomolecules essentials to their metabolism [30]. If the microalgae cells can shift from an exclusively autotrophic mode, where inorganic carbon and light are used as carbon and energy sources, to heterotrophic or mixotrophic modes, where organic matter is used, photo-inhibition will be reduced and cell density and microalgae productivity will be increased [42–44] (Fig. 2).

Regardless the type of metabolism that the microalgae cells use, they always produce value compounds which can be used for the biofuels production such as biodiesel, bioethanol, biohydrogen and biogas as alternative energy sources or carotenoids that have a high commercial value [13, 45, 46] (Fig. 2). The lipid content of microalgae usually ranges from 20–50% (w/w DCW) but can achieve 80% (w/w DCW) under certain conditions [47].

Table 3 summarizes the published studies using oleaginous microalgae pure cultures for BWW treatment with

Table 3 Microalgae oleaginous species cultivated on brewery effluents for biomass and lipids production with concomitant brewery wastewater treatment

Type of cultivation	Microorganism Microalga	Type of metabolism	Substrate	Total sugars concentration (g L^{-1})	C/N ratio	Cultivation mode	Biomass production (g L^{-1})	Lipid content (% w/w)	COD removal (%)	Sugar consumption (%)	Total N consumption (%)	References
Pure	*Chlorella vulgaris*	Photoautotrophic	Industrial BWW	-	-	Batch/ 15L plastic bags	N/m	5.0	15	–	85–90	[29]
	Chlorella protothecoides (UTEX-1806)		Anaerobically treated BWW	-	-	Batch/ Shake flasks	1.88	36.4	74	–	96	[46]
	Scenedesmus dimorphus		BWW supplemented with BG11 medium	-	-	Batch/ Bubble column tube reactor	6.82	33.0	63	–	100	[24]
	Scenedesmus obliquus (ACOI 204/07)		SBWW	-	-	Batch/ Bubble column photobioreactor	0.93	7.2	70	–	75	[13]
	Scenedesmus obliquus (ACOI 204/07)		SBWW	-	-	Continuous/ Bubble column photobioreactor	0.86	7.3	74	–	76	[13]
	Coccomyxa subellipsoidea C-169		Industrial BWW supplemented with NAA phytohormones	-	-	Batch/ Shake flasks	5.30	~43.0	100	–	100	[41]
	Tetradesmus obliquus ACOI204/07		PBWW	-	-	Batch/ Shake flasks	1.33	16.3	–	–	29	[14]
	Chlorella vulgaris (UTEX-265)	Two-stage photoautotrophic-mixotrophic	Industrial BWW supplemented with glucose as carbon source	3	N/m	Batch/ Shake flasks	2.80	28.0	1st stage: > 80	–	1st stage: > 80	[30]
	Chlorella vulgaris (UTEX-265)	Two-stage photoautotrophic-photoheterotrophic	Industrial BWW supplemented with glucose as carbon source	10	N/m	Batch/ Shake flasks	3.20	42.0	1st stage: > 80 2nd stage: > 70	–	1st stage: > 80 2nd stage: > 70	[30]
	Tetradesmus obliquus ACOI204/07	Mixotrophic	PBWW supplemented with SCM as carbon source	10	80	Batch/ Shake flasks	2.26	17.2	–	44	37	[14]
	Tetradesmus obliquus ACOI204/07		PBWW supplemented with SCM as carbon source and Urea as nitrogen source	10	5.7	Batch/ Shake flasks	4.10	4.0	–	98	46	[14]

BWW Brewery Wastewater; *NAA* Synthetic phytohormones 1-Naphthaleneacetic acid; *PBWW* Primary Brewery Wastewater; *SBWW* Secondary Brewery Wastewater; *SCM* Sugarcane Molasses; *N/m* Not mentioned

simultaneously organic carbon and inorganic nutrients removal, while producing lipids for biodiesel. Microalgae strains from the genera *Scenedesmus*, *Tetradesmus*, *Coccomyxa* and *Chlorella* have already been used for this purpose, which are microalgae species that have been intensively used in effluents treatment processes due to their resistance to harsh wastewater conditions [7]. However, most of the works were performed under photoautotrophic mode (Table 3). Only the microalgae *Chlorella vulgaris* and *Tetradesmus obliquus* were used under mixotrophic or photoheterotrophic modes.

Substrates/Supplementation Used

As referred in the previous section, most of the studies that use microalgae grown in BWW were performed using photoautotrophic conditions, where no organic carbon source was added to the culture medium. However, as observed for the yeast pure cultures developed in BWW, several authors also reported that the BWW used had low concentrations of macronutrients such as nitrogen and phosphorus, which are essential for the microalgae growth [24, 30, 41]. Unlike standard culture medium that have a balanced composition in terms of macronutrients, some BWW have a low content of N and P, which, without any supplementation, leads to the microalgae growth and biomass production slowdown. An adequate balance N/P ratio is critical to obtain higher algal growth, lipid productivity and nutrient-removal efficiency [46]. Due to this, some of the authors presented in Table 3 supplemented the BWW with nutrients or other additives and compared the results obtained with BWW cultivations without any supplementation. For instance, Lutzu et al. (2016) reported that *Scenedesmus dimorphus* grown on BWW supplemented with some nutrients (nitrogen and phosphorus) led to an increase in biomass and lipid accumulation. Liu et al. [41] supplemented BWW with synthetic 1-naphthaleneacetic acid (NAA) phytohormones, which enhanced the algal biomass and lipid productivity of *Coccomyxa subellipsoidea*. Although the works of Lutzu et al. [24] and Liu et al. [41] reported 6.82 and 5.30 g L^{-1} of biomass production, respectively, and 36.4% w/w and 43% w/w of lipid content, respectively, which are the highest values for biomass production and lipid content in the photoautotrophic works presented in Table 3, these results were obtained after BWW supplementation with expensive external nutrient sources (BG11 medium and NAA phytohormones, Table 3) that increase the BWW treatment cost (Table 3). Moreover, when no supplementation was added to the BWW, biomass production obtained ranged between 0.86 g L^{-1} and 1.88 g L^{-1}, and low lipid contents were obtained (0.5% w/w and 7.3% w/w) (Table 3).

Dias et al. [14] used mixotrophic conditions to grow *Tetradesmus obliquus* cells in PBWW + 10SCM and in PBWW + 10SCM + 2U (Table 3). The authors attained 4.10 g L^{-1} of biomass production in PBWW supplemented with SCM and urea cultivation (Table 3). This cultivation was performed in only 72 h, indicating that this microalga, when grown under a mixotrophic mode, requires lower cultivation time to achieve higher biomass concentrations [14]. However, low lipid content was observed (4.0% (w/w DCW), Table 3).

Cultivation Mode

As for the yeast pure cultures, most works presented in Table 3 use shake flasks under batch cultivation mode to perform the effluent treatment. However, Marchão et al. [13] grew the microalga *Scenedesmus obliquus* on BWW using a 5 L Bubble Column photobioreactor operated in batch and in continuous regimes, without any previous sterilization step prior to the cultivation, which is an advantage in the treatment process, since the energy costs associated to this step were saved [13]. Although only 7.3% of lipid content (w/w DCW) was obtained in the continuous mode, 50% (w/w DCW) of protein content was obtained, which can be a way to valorize the biomass as animal feed. Also, high nutrient removal rates were obtained, which makes this a promising method to efficiently treat the BWW [13].

To improve the overall process efficiency, it is possible to use other strategies: the use of sequential growth under different metabolism modes can maximize microalgae biomass and lipid productivity [48]. Farooq et al. [30], developed two cultivation systems in a two-stage photoautotrophic–photoheterotrophic or photoautotrophic–mixotrophic modes in order to maximize lipid production by the microalgae *Chlorella vulgaris* grown in BWW (Table 3). During the two-stage photoautotrophic–mixotrophic cultivation, *C. vulgaris* was first grown under photoautotrophic conditions for seven days and, in the late-exponential stage, the conditions were changed to induce mixotrophic metabolism during five days, adding 3 g L^{-1} of glucose as organic carbon source (Table 3). The authors obtained a biomass production of 2.80 g L^{-1} and a lipid content of 28% (w/w DCW) (Table 3). When the two-stage photoautotrophic–photoheterotrophic growth mode was performed, *C. vulgaris* cells were grown for eight days in the photoautotrophic mode, followed by the settlement of the culture for 12 h, which was afterwards collected and grown for five days in the photoheterotrophic mode, with 10 g L^{-1} of glucose as carbon source (Table 3). In this mode, higher biomass production was obtained (3.2 g L^{-1}, Table 3) and maximum lipid content was achieved: 42% (w/w DCW) (Table 3).

Synthesized Products

Contrary to what was observed in the oleaginous yeast pure cultures section, in which, after brewery wastewater treatment, it was possible to obtain microbial oil and carotenoids, the usual product obtained from the microalga biomass after BWW treatment is only lipids. Only two of the works presented in Table 3 obtained protein contents after the BWW treatment [13, 42] and none of the works obtained carotenoids.

However, the lipid content presented in several works that use oleaginous pure microalgae cultures for BWW treatment in Table 3 are considerably higher than those presented in Table 2 for the oleaginous pure yeast cultures, reaching, for example, approximately 43% (w/w DCW) in the work of Liu et al. [41]. Farooq et al. [30] also obtained a lipid content of 42% (w/w DCW) using a two-stage photoautotrophic-photoheterotrophic strategy and supplemented the BWW with glucose as carbon source.

Nevertheless, under autotrophic conditions, and without any BWW supplementation, the results of lipid content obtained were considerably lower, attaining 5.0%, 7.2–7.3% and 16.3% (w/w DCW) in the works of Raposo et al. [29], Marchão et al. [13] and Dias et al. [14], respectively (Table 3). However, in the work of Darpito et al. [46], 36.4% (w/w DCW) of lipid content were obtained when using anaerobically treated BWW (ABWW) without any supplementation under photoautotrophic metabolism (Table 3). The authors concluded that the higher amount of lipid produced by the *Chlorella protothecoides* (UTEX-1806) cells might have been due to the nitrogen deprivation and available organic compounds in the ABWW.

In the *Tetradesmus obliquus* ACOI204/07 mixotrophic cultivations performed by Dias et al. [14] presented in Table 3, a lipid content of 17.2% (w/w DCW) was obtained when the BWW was supplemented with 10 g L^{-1} of SCM, which allowed obtaining a C/N ratio of 80. However, when the same medium was supplemented with 2 g L^{-1} of urea, the C/N ratio reduced to 5.7, and the lipid content dropped significantly to 4.0% (w/w DCW) (Table 3) which indicates, as expected, that the increase in the nitrogen level, in the medium, did not favor the lipid production.

Brewery Wastewater Treatment Efficiency

In terms of wastewater treatment, the results of COD, sugar and total N removal percentages presented in Table 3 for individual microalgae cultures are considerably higher than the ones presented for oleaginous yeast pure cultures in Table 2, which evidence the higher efficiency of microalgae to remove nutrients from the wastewaters.

In terms of COD removal, except for the work of Raposo et al. [29] in which only 15% of COD removal was observed, in all the other works presented in Table 3, which analyzed the COD removal, more than 60% removal was observed, attaining 100%, in the work reported by Liu et al. [41], (Table 3). The authors observed that the supplementation of the BWW with NAA facilitated the COD removal, when compared to the BWW cultivations without NAA supplementation. Moreover, the authors also observed complete removal of total N and total P, which was not observed in the BWW cultivations without NAA supplementation (Table 3). Nevertheless, except in the work of Dias et al. [14], in which the total N removal was lower (29%, 37% and 46% in the PBWW cultivations without supplementation, with 10 g L^{-1} of SCM supplementation and 10 g L^{-1} of SCM and 2 g L^{-1} of urea supplementation, respectively, Table 3), all the other works presented in Table 3 present total N removals higher than 70%.

Oleaginous Yeast and Microalga Mixed Cultures

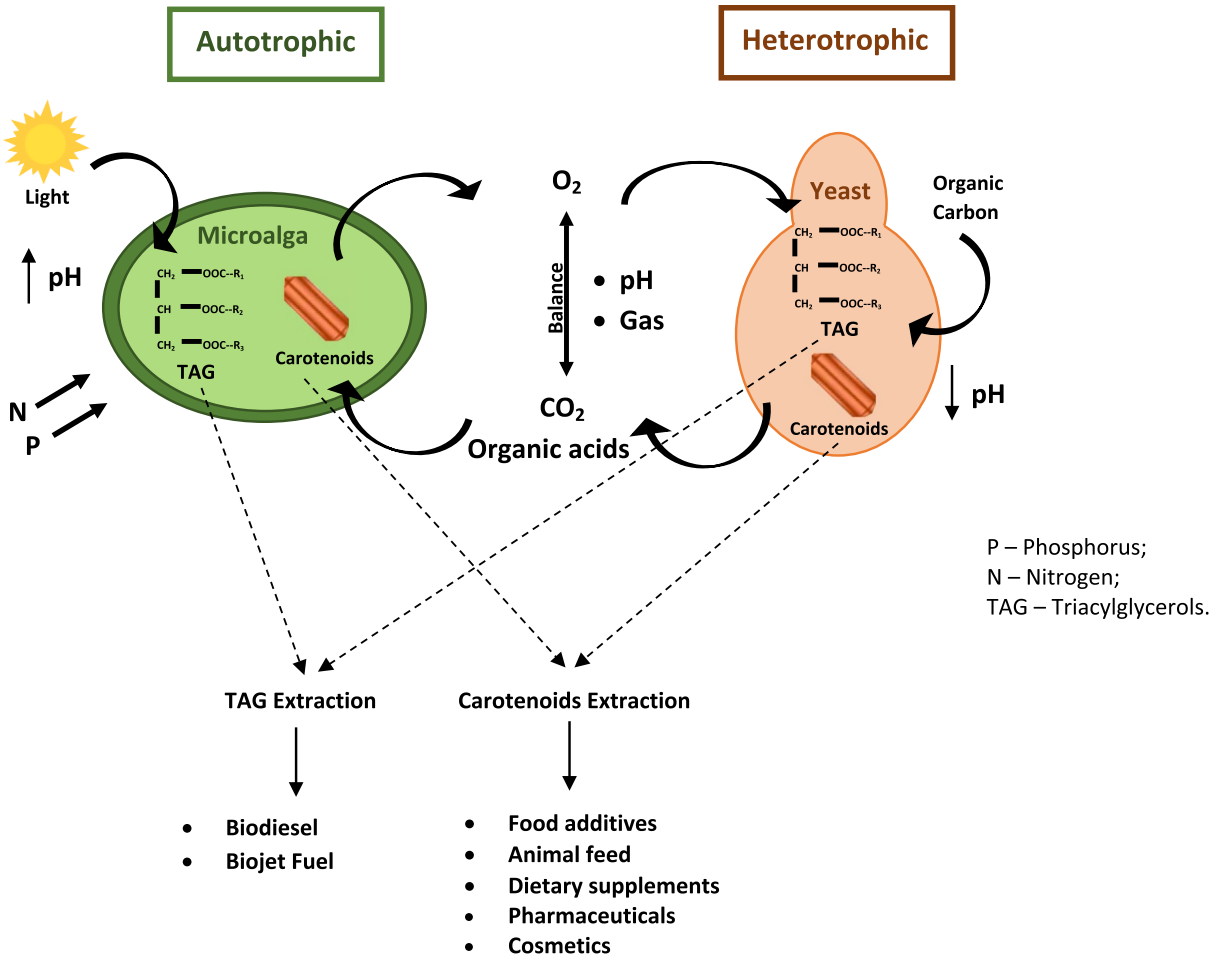
Recently, microalgae and yeast symbiotic cultures have been considered an attractive option: comparatively to individual pure cultures, higher biomass and lipid productivities, as well as higher nutrient removal rates have been described [5, 7]. Yeast and microalga mixed cultures take advantage not only from the complementary metabolisms of the two microorganisms (heterotrophic and autotrophic metabolisms, respectively), but also from gas and metabolite exchanges and medium pH auto-adjustments, which provide a more suitable environment for the production of intracellular compounds with commercial interest, such as lipids and carotenoids, by both of the microorganisms [7, 49] (Fig. 3).

Yeast and microalga mixed culture works using brewery effluents as feedstock are still limited in literature. However, recent studies presented in Table 4 have highlighted the several advantages of using yeast and microalgae mixed cultures over pure cultures.

Microbial Consortia

In the previous sections, several oleaginous yeast and microalgae species that have been used, individually, to perform BWW treatment with concomitant lipids and/or carotenoid production have been presented. However, presently, only one yeast and microalga microbial consortium has been used for BWW treatment: the yeast *R. toruloides* NCYC 921 with the microalga *T. obliquus* ACOI 204/07 (Table 4). Nevertheless, the use of this consortia in other contexts had already been described by other authors [50].

An important parameter that affects the biomass, lipids and carotenoids production of the mixed culture is the



Advantages:

- High cell densities;
- Higher productivities than pure cultures;
- Higher production of high value compounds than pure cultures;
- Complementary metabolisms;
- O_2/CO_2 balance;
- Efficient removal of organic matter from wastewaters;
- Efficient removal of nutrients (N and P) from wastewaters;
- Substance exchange (e.g: organic acids);
- Autoadjustment of the médium pH;
- Cell stress reduced.

Disadvantages:

- Contaminations risks;
- High energy and cost demanding sterilization step;
- BWW not rich enough in carbon and nutrients to allow autotrophic and/or heterotrophic metabolism;
- Necessity of supplementing the effluent with a carbon and/or nitrogen source;
- Inefficient light penetration if high concentration of yeast cells.

Fig. 3 Advantages and disadvantages of symbiotic oleaginous yeasts and microalgae cultures for brewery wastewater treatment with concomitant lipid and carotenoid production

proportion of each microbial population, throughout the culture development. As previously observed for the pure cultures, the nutrient supplementation of brewery effluents, in particular with a source of organic carbon, for microbial co-cultures development is a requirement. However, this can lead to a predominance of the yeast population in the mixed cultures, reaching proportions higher than 99% of the total mixed culture cells [14, 32]. Since *R. toruloides* is a heterotrophic organism, it will use the carbon compounds present in the culture medium to produce biomass and other valuable biocompounds such as lipids and carotenoids (Fig. 2). Contrarily, as referred before, *T. obliquus* is able to develop under photoautotrophic, heterotrophic or mixotrophic conditions, depending on the environmental

Table 4 Microalga and yeast mixed cultures developed on brewery effluents for biomass, lipids and carotenoids production with concomitant brewery wastewater treatment

Type of cultivation	Microorganisms		Substrate	Carbon concentration (g L^{-1})	Cultivation mode	Biomass production (g L^{-1})	Lipid content (% w/w)	Carotenoid production (mg L^{-1})	COD removal (%)	Ammoniacal N removal (%)	Sugar consumption (%)	Total N consumption (%)	References
	Yeast	Microalga											
Mixed	*Rhodosporidium toruloides* NCYC 921	*Tetradesmus obliquus* ACOI 204/07	SBWW	–	Batch/ Shake flasks	0.32	3.55	–	60.22	73.86	33.10	–	[32]
	Rhodosporidium toruloides NCYC 921	*Tetradesmus obliquus* ACOI 204/07	PBWW	–	Batch/ Shake flasks	0.21	3.99	–	93.38	73.15	91.65	–	[32]
	Rhodosporidium toruloides NCYC 921	*Tetradesmus obliquus* ACOI 204/07	SBWW supplemented with SCM as carbon source	10	Batch/ Shake flasks	1.13	14.86	–	32.61	45.95	46.12	–	[32]
	Rhodosporidium toruloides NCYC 921	*Tetradesmus obliquus* ACOI 204/07	PBWW supplemented with SCM as carbon source	10	Batch/ Shake flasks	2.17	4.28	–	62.50	30.19	69.62	–	[32]
	Rhodosporidium toruloides NCYC 921	*Tetradesmus obliquus* ACOI 204/07	PBWW supplemented with SCM as carbon source and Urea as nitrogen source	10	Batch/ Shake flasks	3.73	4.80	–	–	–	87.40	34.00	[14]
	Rhodosporidium toruloides NCYC 921	*Tetradesmus obliquus* ACOI 204/07	PBWW supplemented with SCM as carbon source and Urea as nitrogen source	100	Batch/ Shake flasks	30.60	26.20	–	–	–	100.00	71.60	[14]
	Rhodosporidium toruloides NCYC 921	*Tetradesmus obliquus* ACOI 204/07	PBWW supplemented with SCM as carbon source and Urea as nitrogen source	100	Fed-batch/ 7 L bioreactor	58.60	31.20	2.8	38.80 (after batch phase)	–	44.10	67.60 (after batch phase)	[33]

SCM sugarcane molasses; *SBWW* secondary brewery wastewater; *PBWW* primary brewery wastewater

conditions (Fig. 2). Since, usually, yeasts grow faster than microalgae, consuming quicker the organic carbon in the medium, the heterotrophic/mixotrophic microalga growth is hampered. Moreover, since in some mixed cultivations the yeast biomass concentration attains 30 g L^{-1} or even more (Table 4), the light penetration into the culture medium is insufficient to allow the microalga autotrophic metabolism. Nevertheless, Dias et al. [14] describes the beneficial effect of the microalga presence on the yeast metabolism detected by FC, even when the microalga cell population was present in a low proportion during the evolution of the mixed culture. Also, the microalga can have an essential role in the consumption of the potential toxic compounds for the yeast (HAc, HProp and HBut) present in the brewery effluents that can negatively affect the yeast growth, as described by Dias et al. [21]. Therefore, the presence of a few microalgae cells in mixed cultures can alleviate the stressed environment due to the toxic compound presence, favoring the yeast metabolism [21, 32].

Effect of Medium Composition in the Biomass, Lipids and Carotenoids Production

Dias et al. [32] studied *R. toruloides* and *T. obliquus* populations dynamics in symbiotic cultures, developed in BWW, for lipid production. The authors studied both PBWW and SBWW separately, or mixed, at the ratio of 1:1 (PBWW:SBWW) and 1:7 (PBWW:SBWW), with or without supplementation with SCM as culture medium. The dilution of the PBWW was performed with the intention of dilute possible inhibitors present in the raw effluent, which could have toxic effects on the microbial cells [32]. Low biomass and lipid production were observed for the mixed cultures developed without any supplementation as described in Table 4: 0.21 and 0.32 g L^{-1} of total biomass concentration and 3.99 and 3.55% (w/w DCW) of lipid production were reported for the PBWW and SBWW cultivations without supplementation, respectively. These results demonstrate that neither the SBWW and the PBWW without supplementation is suitable for the development of the mixed culture since they do not contain the necessary nutrients for heterotrophic and autotrophic metabolisms, as previously observed for the pure yeast and microalga cultures developed in PBWW and SBWW brewery effluents. To fulfill the microorganisms nutritional needs, the brewery effluents were supplemented with SCM [14]. When 10 g L^{-1} of SCM was added to the medium, 1.13 and 2.17 g L^{-1} of total biomass concentration and 14.86 and 4.28% (w/w DCW) of lipid production were reported for the PBWW + 10SCM and SBWW + 10SCM cultivations, respectively [14] (Table 4). Although higher biomass production was observed for the PBWW + 10SCM cultivation, low lipids production was observed (Table 4).

Dias et al. [14] also performed a *R. toruloides* and *T. obliquus* mixed culture using PBWW supplemented with 10 g L^{-1} of SCM, and the authors observed that although the sugars were not depleted in the medium culture, the yeast growth ceased soon in the cultivation, which indicated that the yeast might have been limited by another nutrient other than carbon, such as nitrogen.

In the other works presented in Table 4, urea was added at a concentration of 2 g L^{-1} to the mixed culture medium as nitrogen source. With the urea addition, the total mixed culture biomass concentration increased to 3.73 g L^{-1} and the lipid production to 4.80% (w/w DCW) [32] (Table 4). To increase the lipid production, the strategy developed by the authors was to increase the sugars concentration in the culture medium. When 100 g L^{-1} of SCM was added as carbon source to the culture medium, 30.60 g L^{-1} of biomass concentration and a 26.20% (w/w DCW) of lipid content was obtained [32] (Table 4). When the same medium was used in a 7 L bioreactor to grow the mixed culture in a fed-batch cultivation, 58.60 g L^{-1} of total biomass, 31.20% (w/w) DCW of lipid content and 2.8 mg L^{-1} of carotenoid production was obtained [33] (Table 4), which are the highest values for biomass concentration and lipid production presented in all the brewery wastewater works in this review. The authors were able to prove that BWW, supplemented with an external source of carbon and nitrogen, can be used as a low-cost culture medium to obtain lipids and carotenoids from a *R. toruloides* and *T. obliquus* mixed culture, which can be a strategy to decrease substantially the price of the biofuels obtained.

Cultivation Mode

Most of the brewery wastewater treatment works using yeast and microalgae co-cultures presented in Table 4 use shake flasks developed in batch mode, which shows the initial stage of development of this technology. Nevertheless, Dias et al. [33] used a fed-batch strategy with promising results, as referred before.

Brewery Wastewater Treatment Efficiency

In terms of wastewater treatment, as referred before, the utilization of BWW supplemented with SCM limits the effluent treatment, in terms of organic carbon and nitrogen removal. Higher COD and ammoniacal N removal rates were observed for the mixed cultures performed using PBWW and SBWW without supplementation (93.38 and 60.22% of COD removal and 73.15 and 73.86% of ammoniacal N

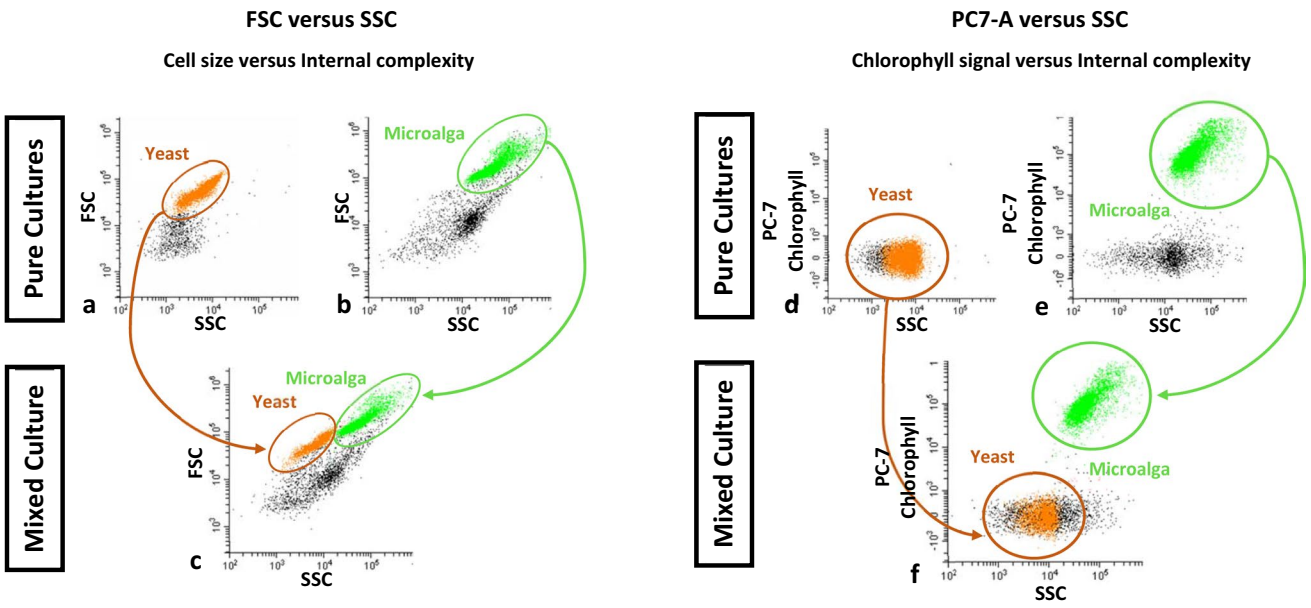


Fig. 4 **a**, **b** and **c** FSC/SSC dot plots concerning a pure yeast (orange), a pure microalga (green) and a mixture of yeast and microalga cells (orange and green), respectively. FSC is proportional to cell-surface area or size and SSC is proportional to cell granularity or internal complexity. Based on this information, it is possible to differentiate yeast and microalga cells as they have different sizes and levels of internal complexity. **c**, **d** and **e** PC-7/SSC dot plots concerning a pure yeast (orange), a pure microalga (green) and a mixture of yeast and microalga cells (orange and green), respectively. Due to the chlorophyll present in microalgal cells, it is possible to discriminate between microalga cells with chlorophyll autofluorescence and yeast cells without chlorophyll auto-fluorescence

removal for the PBWW and SBWW media without supplementation, respectively, Table 4).

Techniques for Brewery Wastewater Cultivations Monitoring

During any bioprocess development, it is necessary to assess several parameters in order to monitor the culture performance. The first parameter that is generally used to evaluate the culture development is the biomass concentration, which gives the information on cell growth. When pure yeast or microalgae cultures are developed, it is possible to monitor the individual growth of the microorganisms through optical density readings at 600 nm and 680 nm, which are proportional to yeast and microalga biomass concentrations, respectively [7]. Also, DCW protocols are usually used to assess the biomass produced by a microorganism. However, in a symbiotic mixed culture, these methods only allow to obtain the average biomass of the whole mixed culture, being impossible to discriminate the biomass production by each microorganism. Although individual cell counts, using a hemocytometer under an optical microscope, allows counting individual microalga and yeast cells in a mixed culture, it is a sluggish and time-consuming method. For the first time, Dias et al. [31] used FC to count the cells from each microbial population.

FC is based on light scattering and fluorescence detection, which occurs when light from a light source (commonly a laser beam) strikes moving particles. Light is deflected around the edges of the cell, after the laser strikes it, also called light scattering. Two types of light scattering occur named as forward scatter (FSC) and side scatter (SSC): FSC is proportional to cell-surface area or size, and SSC is proportional to cell granularity or internal complexity (Fig. 4). Based on this information, it is possible to differentiate yeast and microalga cells, as they have different sizes and levels of internal complexity, as shown in Fig. 4. Moreover, since autotrophic/mixotrophic microalga cells produce chlorophyll, which emits fluorescence at 683 nm, it is possible, using the PC-7 detector of the flow cytometer, to detect its fluorescence. This allows discriminating microalga cells with chlorophyll autofluorescence from yeast cells without chlorophyll auto-fluorescence (Fig. 4). Analyzing the FSC/SSC dot plots, it is possible to obtain the percentage of each cell population, during the development of the mixed culture. Additionally, the authors were successful in obtaining the individual DCW for each microorganism in the mixed culture, multiplying the proportion of each cell population by the total DCW, throughout the mixed culture. To achieve this, it was previously necessary to determine the average

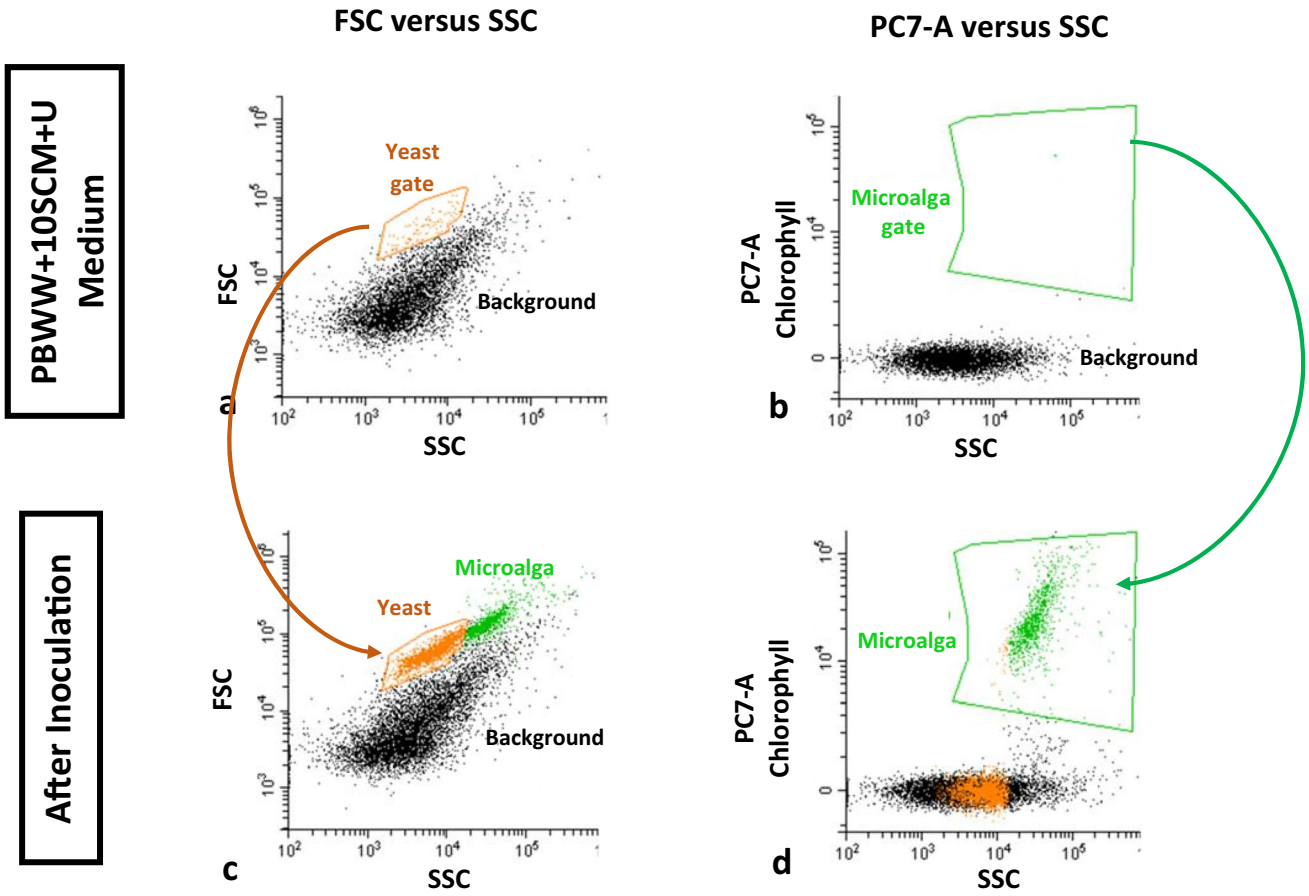


Fig. 5 **a** and **b** FSC/SSC and PC7-A/SSC dot plots concerning the noise generated by the primary brewery wastewater (PBWW) medium supplemented with 10 g L^{-1} of sugarcane molasses (SCM) and 2 g L^{-1} of urea (U); **c** FSC/SSC dot plot concerning the mixture of *Rhodosporidium toruloides* (orange) and *Tetradesmus obliquus* (green) cells after inoculation. **d** PC7-A/SSC dot plot concerning the mixture of *R. toruloides* (orange) and *T. obliquus* (green) cells after inoculation. In both FSC/SSC and PC7-A/SSC dot plots it is possible to differentiate between the yeast and the microalga cells and the background

cell mass of each of microorganism, which was determined to be roughly the same [31].

However, BWW contain large amounts of particles. Moreover, when the wastewater is supplemented with other low-cost substrates such as SCM, the number of waste particles increases, being important to assure the differentiation between the microbial cells from the background containing dust particles and cell debris (Fig. 5).

In the oleaginous yeast and microalga mixed cultures section, it was referred that the adjustment of the proportion of each cell population (yeast and microalgae) affects the biomass, lipids and carotenoids production of the mixed culture, as well as the removal efficiency of the microorganisms. Due to this, the control of the cells populations proportion ratio throughout the mixed culture is essential, for the optimization of the bioprocess. Schlembach et al. [51] reviewed different measurement techniques to resolve and control the population dynamics of mixed-culture processes. The authors provide an outlook on the possible implementation of external feedback control strategies, which could be enabled by the availability of online monitoring methods to precisely sense the population composition or differential performance parameters as control input. The authors state that external control of parameters such as pH, oxygen availability and temperature can be performed experimentally and can be a strategy to dynamically control the populations in a mixed culture [51, 52]. However, up to now, such external control has been rarely realized experimentally [51].

As referred before, even though many benefits of using BWW as a low-cost medium for microbial growth have been described, BWW can contain toxic compounds that may inhibit the microbial growth and affect negatively the cell metabolism [32]. As observed by Dias et al. [14], even relatively low concentrations of HAc, HProp and HBut in the BWW supplemented with SCM had a detrimental effect on the cell physiology and metabolism in *R. toruloides* cells.

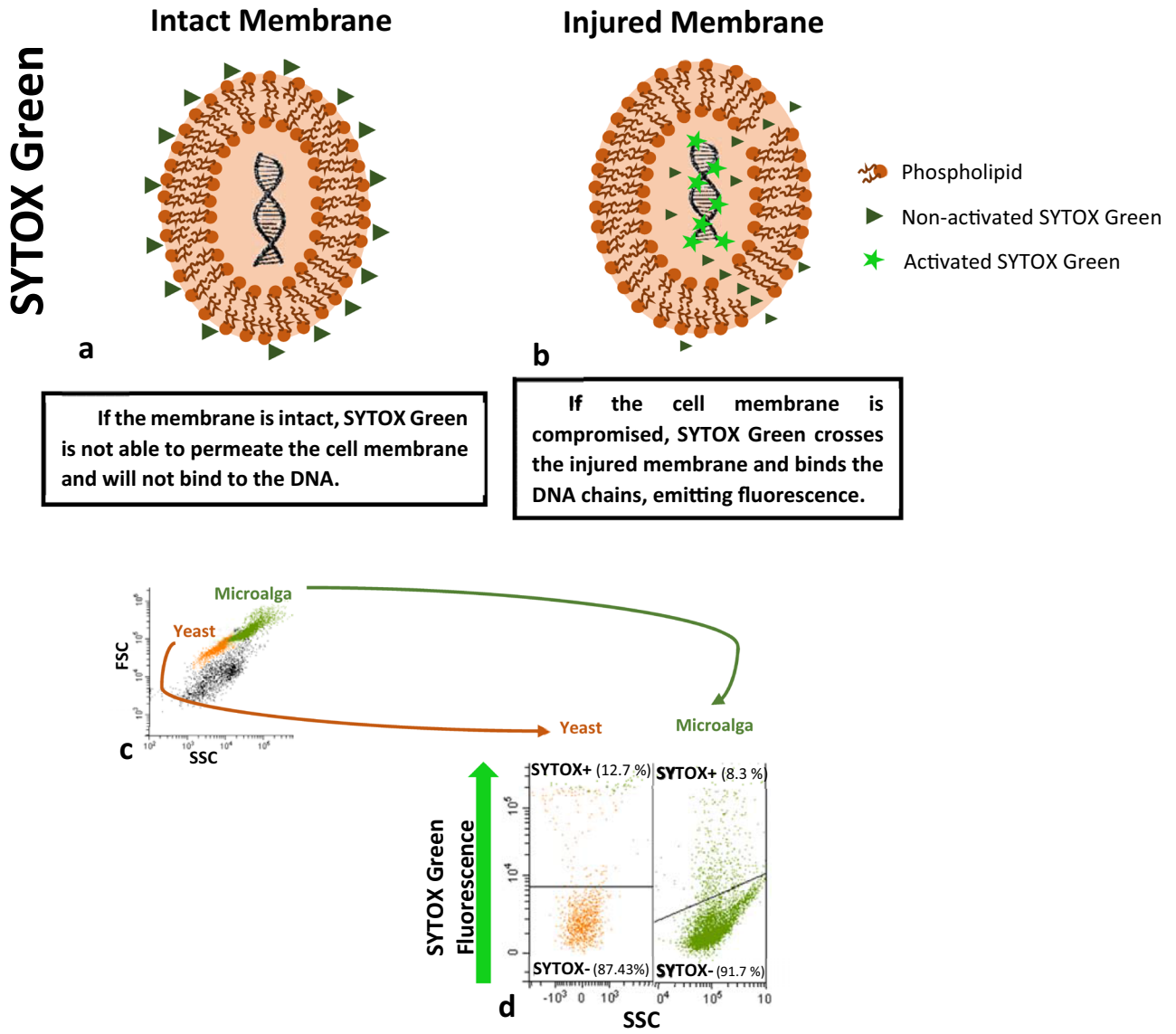


Fig. 6 Schematic diagram of SYTOX Green mechanism of action to study cells membrane integrity using flow cytometry (FC). **a** SYTOX Green is an unsymmetrical cyanine dye with three positive charges, that only permeate the cell membrane if it is compromised. **b** When SYTOX Green is inside the cell, it binds to nucleic acids and become fluorescent. **c** and **d** When SYTOX Green binds to the DNA chains, the fluorescence emitted can be detected by FC. In a yeast and microalga mixed culture, it is possible to discriminate between yeast cells with damage membrane (SYTOX+, orange), yeast cells without damage membrane (SYTOX−, orange), microalga cells with damage membrane (SYTOX+, green) and microalga cells without damage membrane (SYTOX−, green)

When using BWW as culture medium, it is crucial to determine the cell physiological status, to evaluate the impact of the inhibitory compounds on the cells performance. Multiparametric FC analysis coupled with fluorescent dyes provides detailed information on cell targets. Relative changes in cell physiological status can be detected during a cultivation, since the cells are exposed to different environments, as the growth conditions changed throughout the culture development [53]. As referred before, Dias et al. [31] developed a simple and easily implementing method to monitor the individual stress response of yeast and microalgae cells grown in a mixed culture, using FC coupled with the fluorescent dyes SYTOX Green and carboxyfluorescein diacetate (CFDA). SYTOX Green was used to study the yeast and microalgae cells membrane integrity. SYTOX Green is a three positive charged dye which only permeates the cell membrane if it is compromised. If the cell membrane is defective, SYTOX Green enters the cells, and binds to the nucleic acid chains, increasing their fluorescence [54] (Fig. 6). For esterase activity detection, CFDA was the

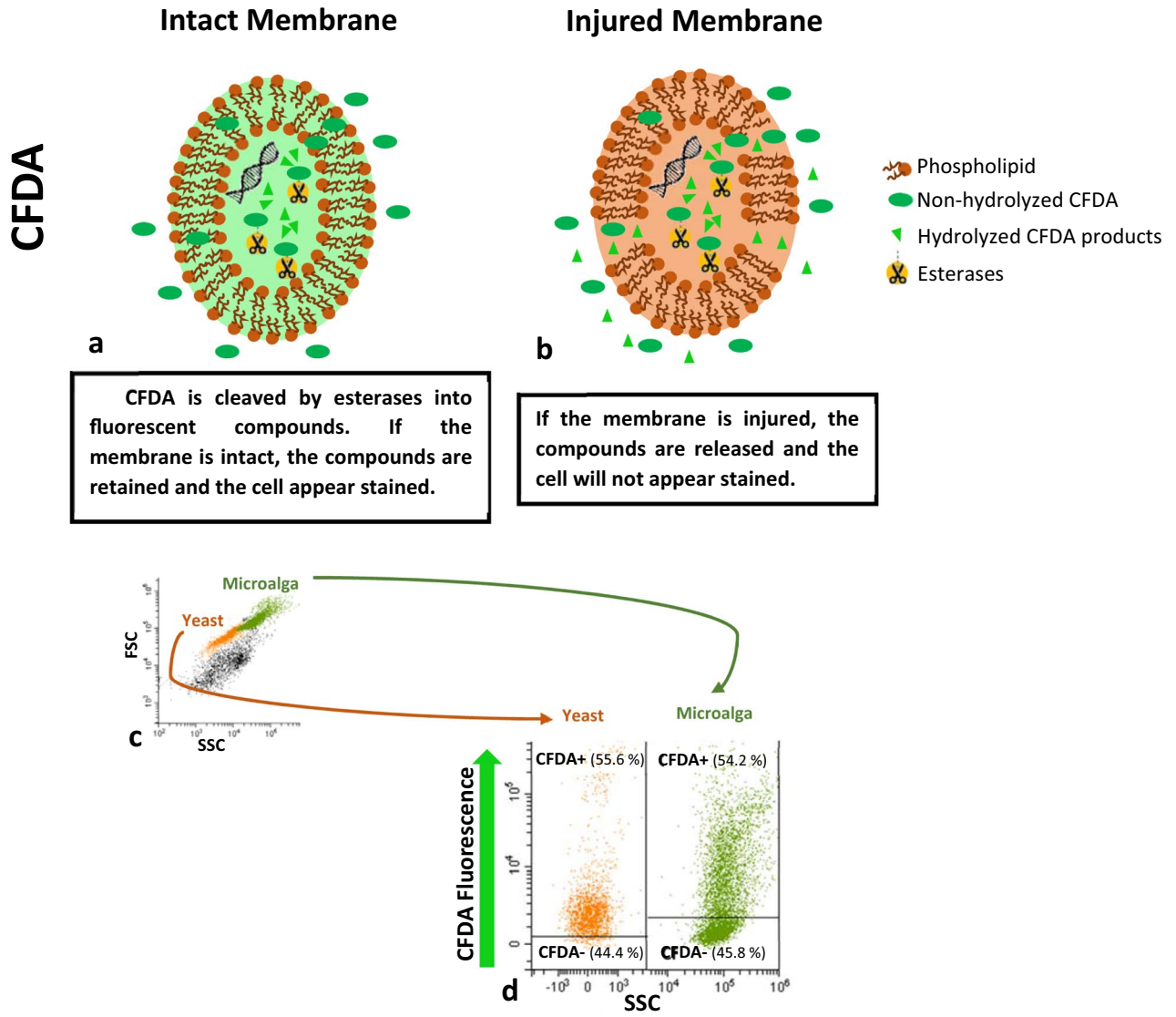


Fig. 7 Schematic diagram of carboxyfluorescein diacetate (CFDA) mechanism of action to study esterase activity using flow cytometry (FC). **a** CFDA is not a fluorescent compound. Being neutral, CFDA permeates the cell membrane by diffusion. Once inside the cell, it is cleaved by esterases into fluorescent products that are retained by the cells if the membrane is intact. **b** If the cytoplasmic membrane is not intact, both non-hydrolyzed CFDA and products are released and thus the cells will not appear to be stained. **c** and **d** The produced fluorescent by-products can be detected by FC. In a yeast and microalga mixed culture, it is possible to discriminate between yeast cells with enzymatic activity (CFDA+, orange), yeast cells without enzymatic activity (CFDA-, orange), microalga cells with enzymatic activity (CFDA+, green) and microalga cells without enzymatic activity (CFDA-, green)

compound used [55]. On the contrary to SYTOX Green, CFDA is not a fluorescent compound: it permeates the cell membrane by passive diffusion. When CFDA is inside the cell, active esterases cleave it, producing a fluorescent compound that is retained inside the cell, if its membrane is intact. However, if the cytoplasmic membrane is compromised, both non-hydrolyzed substrate and products will be released and the cell will not be stained [55] (Fig. 7).

The use of FC in a bioprocess development and monitoring, as well as for the optimization and scale-up process, is of an extreme relevant as it provides near real time information on the intrinsic heterogeneity of a microbial population, that cannot be assessed with the conventional methods. Nonetheless, it is still not often used in such processes.

Other parameters should be assessed during the development of pure and mixed cultures of oleaginous microorganisms in BWW. As described before, the selection of the culture conditions, such as the medium pH, is essential for high bioprocess performance. pH monitoring should be performed throughout all the cultivations. The

quantification of inhibitory compounds, as well as extracellular products production resulting from the microbial metabolism, which can be detected by high-performance liquid chromatography (HPLC) is also important, as these compounds affect the cells performance, thus the process efficiency.

The production of lipids and high-value added compounds such as carotenoids produced by the oleaginous yeast and microalgae in pure and mixed cultures should also be determined. For the intracellular lipid production, the Bligh and Dyer method for total lipid extraction is the most common method used at laboratorial scale [1, 24]. However, it is not viable at industrial scale, where the most common methods used are Soxhlet or Accelerated Solvent Extraction (ASE). The fatty acid profile is usually generally performed by gas–liquid chromatography [12, 13, 30, 41, 46]. For carotenoid quantification, Schneider et al. [1], Dias et al. [12] and Dias et al. [33] performed the carotenoids extraction using different solvents, which are sequentially analyzed by spectrophotometry to obtain the concentration of the total carotenoids, or by HPLC, to identify and quantify the major extracted carotenoids.

To understand the nutrient removal efficiency of the BWW treatment process, nutrients concentrations of COD, N and P in the beginning, and in the end of the cultivations should be analyzed. Most of the BWW works presented in Tables 2, 3 and 4 used HACH and Standards Methods protocols to determine these concentrations [1, 12–14, 24, 32]. NH_4^+-N is also quantified by several authors throughout the BWW cultivations [12, 13, 24, 32].

Challenges and Perspectives for the Near Future

As above referred, the use of oleaginous microorganisms in BWW treatment can be an alternative to conventional BWW treatment processes, enhancing the bioprocess with the production of useful products such as lipids and carotenoids, reducing the treatment costs. However, such methodology is still in an early step presenting several challenges that must be effectively overcame before the application of this technology at an industrial scale.

Effluents, such as the BWW, present indigenous microorganisms which will, inevitably, reduce the process efficiency since, usually, these microbial contaminants are not able to produce lipids or other high value compounds and, when present, will reduce the lipid production. In addition, if the indigenous microorganisms are predators, they will eat the inoculated oleaginous microalgae and/or yeasts, and spoil the cultivations [7, 37]. Indeed, these is a major concern in this scale-up process, since, as referred before, for each liter of beer produced, between 3 to 10 L of BWW is generated [12]. Such quantities of effluents may be impossible to sterilize at an industrial scale since the sterilization step is highly energy and cost demanding. A solution can be the use of inexpensive sterilization methods such as sanitation/disinfestation protocols in the cultivations systems and in the BWW, that use chemical compounds such as detergents, phenols, or sodium hypochlorite, to reduce or prevents the contamination [7, 37]. However, on one hand, the efficiency of such methods is not guaranteed, since there are many microorganisms and predators that are resistant to these compounds; on the other hand, these compounds can also affect the inoculated microalgae and yeasts, which will reduce the process efficiency. Investigation to identify low-cost sterilization methods at large scale is necessary, in order to reduce the BWW treatment costs.

Another important issue is that, usually, the BWW without supplementation does not contain enough organic carbon load to allow heterotrophic metabolism, or does not contain the necessary amounts of nutrients for autotrophic metabolism. Frequently, it is necessary to supplement the BWW with an organic carbon stream to be used as the major carbon source for the process. To reduce the costs of the process, it is necessary to use low-cost substrates as carbon sources. The BWW will provide not only several nutrients that the microorganisms need for their metabolism, as well as can be used as water source which would allow saving precious clean water and also to reduce the production costs. However, it is important to assure that the chosen low-cost substrate, together with BWW, contain the necessary nutrients to fulfill the nutritional requirements of the oleaginous microorganisms that will be used to grow in that mixture.

Although most of the BWW treatment works published with concomitant lipid and carotenoids production by oleaginous microorganisms are developed at lab scale and a few at pilot scale, so far this process is not viable at the industrial scale, being necessary to scale up the process using large fermenters and raceways. However, several parameters must be optimized to assure the highest process productivity: Efficient mixing and aeration is a very important issue regarding heterotrophic cultures, as inefficient mixing of the culture leads to nutrients concentrations, pH and temperature gradients, which will significantly reduce the process yield and induce cellular stress [7, 37]; Moreover, O_2 and CO_2 sufficient conditions for oleaginous yeast and microalgae cultures, respectively, in mixed cultures, are of extremely important for the highest cultures performance. Yeast cells require high aeration and stirring rates which can cause microalgae shear stress and cell damage, with detrimental effects on the microalgae cells, and, consequently, decreasing the process productivity. It is important to optimize the aeration and stirring rates to achieve the highest yeast and microalgae productivities, without causing cell damage [7, 37]; Furthermore, when operating the culture under

autotrophic conditions, it is essential to supply the adequate light intensity to the microalgae cells. In addition, since the yeast cells usually grow faster than the microalgae cells, the proportion of yeast and microalgae cells can be disproportionated in the mixed culture, resulting in low light diffusion in the culture medium for the microalgae autotrophic metabolism, which will be hindered by self-shading and light absorption. It is important to use efficient light systems inside and/or around the bioreactor vessel in order to supply the necessary light to the microalgae cells [7].

For biodiesel production from oleaginous microorganisms, several steps must be performed: biomass production, cell disruption and lipid extraction, followed by a transesterification reaction [56]. From all these processes, the lipid extraction step is reported to be the most expensive [56]. To decrease the process price, it is important to develop more efficient lipid extraction methods to produce low-cost biodiesel from oleaginous microorganisms. One of the biggest challenges to overcome when developing microorganisms in aqueous culture media is the water removal [11], since most of the lipid extraction protocols uses dry biomass. Indeed, the water presence hinders the efficient organic solvent penetration in the cell membranes. However, the microbial biomass drying step (trough freeze-drying or oven) is highly energy demanding and involves high costs. Indeed, harvesting and dewatering techniques such as centrifugation not only represent high energy costs, as well as only can remove efficiently the moisture to a level of 90% (w/w) [57], being necessary to use drying techniques such as freeze-drying among others, which are extremely energy demanding and costly [58]. Cell disruption methods such as ultrasound, milling and high pressure homogenization (HPH) have been used to improve lipids and carotenoids extraction from the cells, since this step enhances the intracellular lipids extraction [59]. Afterwards, the lipids and carotenoids can be extracted using different solvents that can access easily to the cell content [57]. It is important to investigate and to develop new protocols combining cell disruption techniques and solvent extractions to co-extract lipids and carotenoids from the oleaginous microorganisms biomass, directly from the broth culture, without using any previous either harvesting or dewatering steps, in order to reduce the lipid and carotenoids production costs from microalgae and yeasts cultures.

Conclusions

Beer is, indisputably, one of the most beloved drinks in the world. However, the amount of brewery wastewater produced in its manufacturing is enormous, as well as the cost to treat it by the brewery companies. This review pretended to demonstrate how Science, in particular Biotechnology, can have an important role evolving and optimizing bioprocesses, such as the brewery wastewater treatment, taking advantages of all process steps, interfering, as little as possible, with the surrounding ecosystems. The use of oleaginous microorganisms in the brewery wastewater treatment with concomitant lipids and carotenoids production, in particular in symbiotic cultures, is a potential way to reduce the brewery wastewater treatment costs. Moreover, the use of sophisticated tools and methodologies such as the multiparametric flow cytometry allows understanding, under a short timeframe, the microorganisms stress responses to eventual adverse conditions, which allows a fast optimization of the bioprocess.

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Declarations

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