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Review

The Role of Heterotrophic Microalgae in Waste Conversion to Biofuels and Bioproducts

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Abstract: In the last few decades, microalgae have attracted attention from the scientific community worldwide, being considered a promising feedstock for renewable energy production, as well as for a wide range of high value-added products such as pigments and poly-unsaturated fatty acids for pharmaceutical, nutraceutical, food, and cosmetic markets. Despite the investments in microalgae biotechnology to date, the major obstacle to its wide commercialization is the high cost of microalgal biomass production and expensive product extraction steps. One way to reduce the microalgae production costs is the use of low-cost feedstock for microalgae production. Some wastes contain organic and inorganic components that may serve as nutrients for algal growth, decreasing the culture media cost and, thus, the overall process costs. Most of the research studies on microalgae waste treatment use autotrophic and mixotrophic microalgae growth. Research on heterotrophic microalgae to treat wastes is still scarce, although this cultivation mode shows several benefits over the others, such as higher organic carbon load tolerance, intracellular products production, and stability in production all year round, regardless of the location and climate. In this review article, the use of heterotrophic microalgae to simultaneously treat wastes and produce high value-added bioproducts and biofuels will be discussed, critically analyzing the most recent research done in this area so far and envisioning the use of this approach to a commercial scale in the near future.

Keywords: wastes treatment; heterotrophic microalgae; high value-added products

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1. Introduction

Each year, the European Union generates 2.5 billion tons of waste, or five ton per person. The waste may contain large volumes of valuable materials for the EU industry, such as nutrients, paper, wood, industrial minerals, and metals. Some of these can be recycled close to 100% with no losses in quantity and quality [1]. The EU waste management policies are aimed at the reduction of the environmental and health impacts of waste and improving Europe's resource efficiency.

The long-term goal is to turn Europe into a full recycling society, avoiding waste and using unavoidable waste as a resource, wherever possible. The release of waste streams into the environment and natural waterbodies affects ecosystems, which raises serious problems for human society in terms of sustainability. Waste stream treatment aims to significantly reduce the quantity of pollutants, namely carbonaceous organic, nitrogen (N) and phosphorus (P) compounds, and recalcitrant compounds, prior to being discharged into the environment [2,3] as these materials, in large concentrations, may affect the equilibrium of ecosystems, fauna, and flora, and threaten human health [4].

Microalgae are considered robust and versatile microorganisms, as they can have autotrophic, mixotrophic, or heterotrophic metabolisms. Therefore, these microorganisms can be used to treat different types of waste streams, simultaneously producing valuable

products (i.e., polyunsaturated fatty acids, pigments, proteins, animal feed, biofertilizers, and biofuels) that may be used in diverse industries, such as food, feed, pharmaceutical, nutraceutical, cosmetic, and chemical, as real biorefinery alternatives. Furthermore, this approach takes advantage of the whole or part of the microalgal biomass and the various products synthesized by the cells, therefore maximizing the value derived from the whole process, with environmental benefits [5].

Although conventional wastewater treatment systems are currently used worldwide, their application is considered an environmental problem due to the high amounts of waste sludge produced [6]. In order to reduce the environmental impact of wastewater treatment, it is therefore necessary to use processes with a substantial reduction in energy consumption and sludge production [7].

The major advantages of microalgal-based waste stream treatment are that additional pollution is not generated when the biomass is harvested, allowing efficient nutrient recycling and interesting product production [5,8–12].

Most of the published works describing biological waste treatment use autotrophic microalgae, which require light as a source of energy and carbon dioxide (CO_2), as a carbon source. Little work on using heterotrophic microalgae to treat real waste streams has been done; despite the higher biomass concentrations, growth rates and lipid productivities can be obtained, compared to autotrophic microalgae growth, because the former is grown in conventional bioreactors that are easily operated, controlled, and maintained. As a result, higher cell masses are obtained due to the higher carbon source energy density when compared to CO_2. In addition, fed-batch culture strategies can be implemented to increase the microalgal biomass and products even more, resulting in the biomass harvesting costs reduction [13]. Direct air can be used, instead of previously treated flue gas, because oxygen (O_2) is consumed and CO_2 generated. All these benefits lead to efficient waste treatment by heterotrophic microalgae; thus, knowledge in this field is needed. In addition, most of the published works reporting heterotrophic microalgae grown on organic substrates are limited to pure glucose, glycerol, and acetate [14]. However, heterotrophic microalgae growth on these substrates is costly. According to Wang et al. [6], the total feedstock costs can achieve up to 80% of the total process costs when using glucose as a carbon source. In this way, zero or low-cost substrates such as wastes (including wastewater and industrial byproducts and residues) should be used as a source of nutrients for heterotrophic microalgae growth, not only to reduce the overall process costs [15] but also to accomplish the EU Circular Economy Action Plan 2020, known as The European Green Deal, which is aimed at boosting the efficient use of resources by moving to a clean, circular economy, restoring biodiversity and cutting pollution [15].

This review article will focus on the use of heterotrophic microalgae to treat wastes, specifically food wastes, municipal/domestic wastewater, and glycerol derived from the biodiesel industry because these streams are suitable for heterotrophic metabolism due to their high organic carbon content. The most recent research done in this area will be critically analyzed, and the use of this approach to a commercial scale in the near future is envisioned.

2. Autotrophic Versus Heterotrophic Mode for Microalgal Waste Stream Treatment

Autotrophic microalgae convert solar energy into valuable biomass, incorporating nitrogen and phosphorous [16]. Due to their capacity to fix carbon dioxide (CO_2), using this compound as a carbon source, and light as the source of energy, these microorganisms contribute to greenhouse gas effect mitigation (Table 1). The use of autotrophic microalgae to treat waste streams shows other benefits, such as the consumption or biotransformation of pollutants. Organic matter degradation can be enhanced in the presence of heterotrophic bacteria because the microalgae supply additional oxygen from photosynthesis to the bacteria populations, improving their growth and metabolism, and thus COD removal from the waste stream. This approach reduces the total energy costs of direct (gassing performance) or indirect (stirring performance) oxygen supply [17].

Table 1. Microalgal autotrophic/heterotrophic growths.

	Autotrophic Cultures	Heterotrophic Cultures
Advantages	• CO_2 fixation ⇒ GHG mitigation • Efficient N and P removal	• Non-dependence of light and season • Efficient organic carbon removal • Simpler and cheaper conventional bioreactors than photobioreactors • High cell-density cultures ⇒ high intracellular product productivities
Drawbacks	• Light and season dependence • Low cell-density cultures due to light shadow • Photobioreactors are expensive and difficult to scale-up	• CO_2 emission • High contamination risk by other heterotrophic microorganisms • Sterilization requirement is an expensive energetic step

However, autotrophic microalgae for waste treatment show a few bottlenecks: (i) it is only possible if CO_2 is available; (ii) the microalgae growth is light-depending, requiring expensive and specific equipment design; (iii) for an efficient conversion, the waste streams are usually treated in large volumes, in photobioreactors, in which light penetration into the dense cultures is hindered due to the self-shading effect; (iv) as a result, the microalgae cell concentration in the culture is usually low, due to the inefficient light penetration aggravated by the light shading effect. Furthermore, when the wastes contain particles (which often occurs), the cells do not receive adequate light due to the high turbidity that hinders adequate light penetration. These limitations are particularly evident when autotrophic microalgae are used for primary wastewater treatments [13]. In addition, microalgae grown under autotrophic conditions usually produce low amounts of intracellular products, such as lipids and pigments, due to the low biomass concentrations and productivities. Moreover, autotrophic microalgae growth is affected by temperature and light availability; hence, this technology is not suitable in areas of high latitude, where most seasons have low temperature and fewer daylight hours.

Most of the works reporting microalgae waste treatment use a symbiotic biological system between autotrophic/mixotrophic microalgae and bacteria populations because autotrophic microalgae are inefficient in removing high COD loads. Mohsenpour et al. [7] stated that wastewater treated by algal-bacterial co-cultures efficiently removes either inorganic nitrogen or phosphorous, requiring only a single-step treatment stage, which reduces the complexity and energy of the whole treatment process. In fact, phosphorous and nitrogen are efficiently removed from the waste by autotrophic and mixotrophic microalgae, but these microorganisms are unable to remove high COD loads from high polluting wastes, such as dairy or food effluents. In such systems, heterotrophic bacteria present in the waste are essential for removing the COD and supply the CO_2 required by the photoautotrophic/mixotrophic microalgae metabolism. This approach may be efficient for treating effluents and waste but usually does not allow the sustainable production of valuable microbial products because the bacteria presence reduces the final concentrations of microalgal biomass and related microalgal-based bioproducts. Generally, bacteria do not produce valuable products [18]. Therefore, the consortia autotrophic microalgae/heterotrophic bacteria, although efficient in treating effluents, is unable to treat wastes with concomitant high value-added product production.

Heterotrophic microalgae use organic compounds as carbon and energy sources to grow and do not use light as an energy source (Figure 1, Table 1).

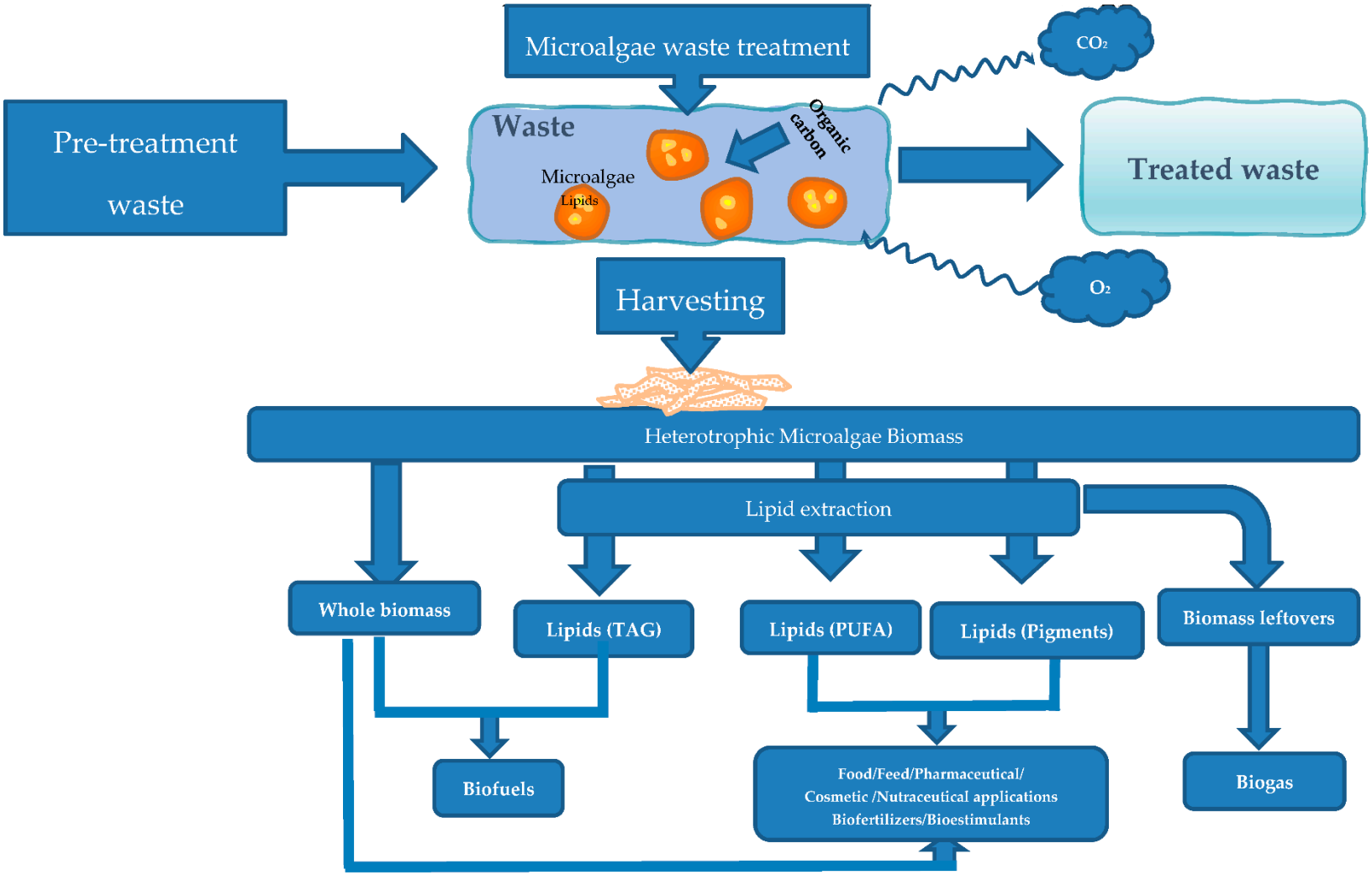

Figure 1. Heterotrophic metabolism and microalgal biomass applications.

Indeed, these microorganisms show several benefits over the autotrophic microalgae to treat waste streams, such as: (a) they can grow in cheaper bioreactors, requiring less sophisticated equipment, and are therefore easily scaled-up; (b) they do not require light to grow, which reduces the equipment requirements and costs; (c) the cultures attain higher, denser cell concentrations and intracellular product productivities than autotrophic cultures; (d) the algal biomass composition can be tailored by changing the type of organic substrate in the medium; (e) heterotrophic microalgae can remove organic carbonaceous, nitrogen, and phosphorus compounds from the wastes more efficiently than autotrophic growth [19–21].

According to Morales-Sánchez et al. [20], cell densities in the order of 100 g/L can be easily achieved in heterotrophic microalgal cultivations, which simplifies the biomass harvesting step. Xu et al. [22] reported an increase in lipid content of 40% in a *Chlorella protothecoides* culture after the cultivation mode was changed from photoautotrophic to heterotrophic. In contrast, under autotrophic conditions, the maximum cell density of microalgae that can be achieved in photobioreactors is around 5 g/L, while in outdoor open-pond or raceway-pond cultures, the cell concentration is usually lower than 0.5 g/L, which significantly increases the energy consumption of the biomass harvesting step, and thus the cost of biomass production [23]. In addition, as above referred, the heterotrophic cultivation can be carried out in conventional industrial-scale fermenters, which ensures better control over process parameters such as pH, temperature, oxygen levels, and carbon source [13].

Substrate inhibition due to very high initial substrate concentration can be overcome by process strategies, such as fed-batch and continuous regimes; even at very high cell densities, the cell growth is not limited by self-shading of the light supply, which usually limits the photoautotrophic cultures [24].

3. Heterotrophic Microalgae Waste Treatment

3.1. Heterotrophic Metabolism

Heterotrophic microalgae use respiration to produce energy by organic substrate oxidization. The most-used carbon sources to grow heterotrophic microalgae are glucose, glycerol, and acetate (Figure 1). Glucose has been the most used organic carbon source for microalgae cultivation because it produces more energy per mole than other substrates. This monosaccharide is also abundant in sugarcane molasses and syrups that result from vegetable pulp extraction, such as carob pulp, which has been used in media formulation for heterotrophic microalgae growth [25,26]. Glucose oxidative assimilation involves two metabolic routes: Embden–Meyerhof (EM) and Pentose Phosphate (PP) pathways, as shown in Figure 1.

Acetate (or acetic acid) is another common carbon source used to grow heterotrophic microalgae [25,27]. Once inside the microalgae cells, in the cytoplasm, acetate is metabolized by acetylation of coenzyme A by acetyl-CoA synthetase (EC 6.2.1.1) in a single-step catalyzed reaction, using a single ATP molecule, to form acetyl coenzyme A (acetyl-CoA). Acetate (carried by coenzyme A) is generally metabolized through two pathways: (i) the glyoxylate cycle, to form malate through the glyoxylate cycle, and (ii) through the Tricarboxylic Acid Cycle (TCA) to form citrate in the mitochondria, which provides carbon skeletons, energy source as ATP, and energy for reduction (NADH) (Figure 2). However, acetate can be toxic for many microorganisms at high concentrations, inhibiting growth. Therefore, it is commonly used for buffering high pH levels in bioreactors, keeping the acetate concentration at low levels in fed-batch configurations or pH-auxostat systems, in which pH is maintained constant [28].

Glycerol is another carbon source that has been used as a carbon source for heterotrophic microalgae growth [25,29,30]. It is a by-product from the biodiesel industry, being considered a waste product because of the associated disposal cost, and if left unattended, poses an environmental threat. Previously, only pure glycerol was used as a carbon source in microbial media formulations because the impurities present in crude glycerol

(methanol, ethanol, salts, metals, and soaps) could inhibit microbial growth, hindering the biological conversion of crude glycerol [31,32].

However, purification of crude glycerol is expensive and burdensome; hence, the conversion of glycerol through biological routes using microorganisms is a viable way to enhance the economy of the process. When inside the cells, glycerol is firstly phosphorylated to glycerol phosphate, using ATP, and is eventually oxidized to triose phosphate. Enzymes glycerol kinase (EC 2.7.1.30), sn-glycerol-3-phosphate NAD oxidoreductase (EC 1.1.1.8), and triose-phosphate (EC: 5.3.1.1) are involved in the conversion of glycerol into glyceraldehyde-3-phosphate and glycerate, which are intermediates involved in the EMP pathway of glycolysis, to form pyruvate that enters the TCA cycle [13,21] (Figure 2).

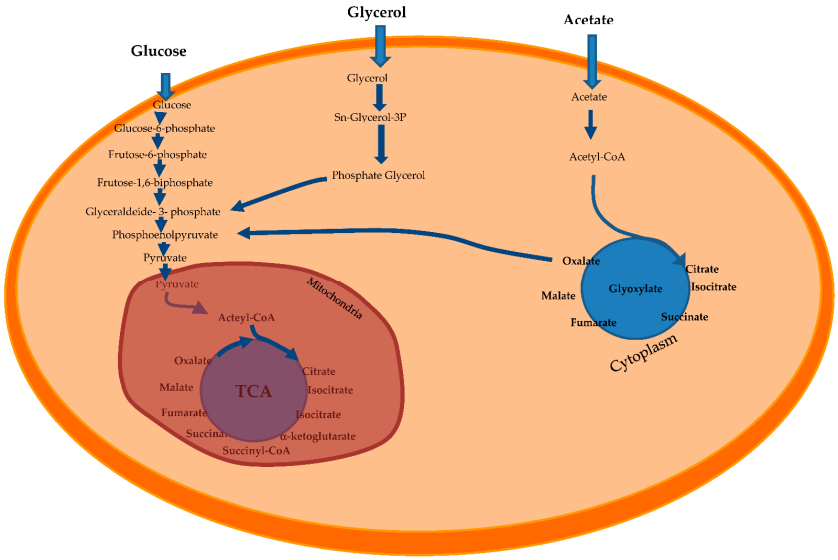

Figure 2. Carbon metabolism by heterotrophic microalgae.

3.2. Waste Pre-Treatment Step

Different types of waste can be converted into biofuels and high value-added products by heterotrophic microalgae. As shown in Table 2, wastes from the food industry are the most used for this purpose because they contain appreciable organic carbon amounts. Domestic, municipal, and aquaculture wastewaters have also been used. Glycerol was also used as a carbon source to grow *Crypthecodinium cohnii* microalga, previously distilled to remove methanol [26].

The organic carbon present in wastes is generally heterogeneous and is often in the form of compounds ranging from simple molecules, such as short-chain organic acids and peptides, to more complex compounds, such as polycyclic aromatic hydrocarbons, synthetic polymers, polysaccharides, polyphenols, proteins, lipids, large fatty acids, detergents, antibiotics, and chemical derivatives [7]. However, as unicellular organisms, microalgae can only use relatively simple molecules, such as nitrogen compounds, sugars, organic and amino acids, and several aromatic compounds [13]. Therefore, a previous waste pretreatment step is usually needed to remove particles and recover or extract the soluble nutrients (e.g., C, N, P) required for the microalgal heterotrophic metabolism, which are unavailable in the untreated waste.

Centrifugation [33,34], filtration [14,35], and decantation [36] have been used to remove solid particles from the media (Table 2). After this step, enzymatic or chemical hydrolysis is frequently used. Enzymatic hydrolysis using a commercial amylolytic enzyme was used to obtain hydrolysates rich in sugars and amino acids/peptides from restaurants and bakery food waste [37].

Table 2. Heterotrophic microalgae that have been reported to treat wastes.

	Waste	Pre-Treatment	System/Duration of the Experiment	Nutrients Removal	Products	Observations	Reference
Chrypthecodinium cohnii CCMP 316	Carob pulp syrup	Carob pulp residues were mixed with distilled water (1:2 *w/w*) for the syrup extraction, carried out at 150 rev/min for 6 h at 50 °C. Thereafter, the syrup was pressed and squeezed, and the supernatant was filtered and centrifuged. The liquid fraction was acidified to pH 2, in order to promote sucrose hydrolysis, and stored at −18 °C.	2 L bioreactor, fed-batch,		Lipids: 9.2% *w/w* DHA: 1.99 g /L; 45.2 mg/g DCW DHA/TFA: 48% (*w/w*)		[38]
Schizochytrium limacinum SR 21	Cull potato	Cull potato was boiled and minced and mixed with water in a 5-L tank with agitation. α-amylase and glucoamylase were used to hydrolyze the potato starch into glucose.	150 mL Erlenmeyer/6 days		DHA: 5.35 g/L	50% of hydrolyzed potato broth in the culture medium was used with 20 g/L glucose supplementation	[39]
Scenedesmus sp. and *Chlorella* sp.	Acid rich effluent collected from a bench scale anaerobic sequencing batch reactor (AnSBR) operated with composite food waste		250 mL conical flasks with 180 mL liquid medium/11 days	COD: 91.4 ± 0.6%	Biomass: 1.42 g/L Lipids: 26.4 (*w/w*)	Microalgae cultures flasks were grown at a photo period of 12 h sunlight and 12 h dark	[40]

Table 2. *Cont.*

	Waste	Pre-Treatment	System/Duration of the Experiment	Nutrients Removal	Products	Observations	Reference
Chlorella sp.	Lipid extracted microalgae biomass residues (LMBs) + sugarcane molasses Mixture ratio: 1/4 (*v/v*)	LMBRs: 50 g lyophilized LMBRs dissolved in distilled water were hydrolyzed using cellulase, neutrase, and alcalase; Crude molasses: Dilution with distilled water (1:9); followed by hydrolysis with neutrase and alcalase, followed by the acidic hydrolysis (addition of H_2SO_4 5 M, adjusting pH to 3.5, at 60 °C for 1 h).	500 mL Flasks with 300 mL liquid medium, 150 rpm, 25 °C/7 days		Biomass: 5.6 g/L Lipids: 43% (*w/w*)		[41]
Schizochytrium mangrovei	Food waste (rice, noodles, meat, vegetables collected from canteens)	Food waste hydrolysis using *Aspergillus awamori* and *Aspergillus oryzae* fungal glucoamylases, proteases, and phosphatases.	2 L bioreactor batch mode/7 days		Lipids: 3.3 g/L; 16.5% (*w/w*); DHA: 85.5 ± 11.2 mg/g		[42]
Chlorella pyrenoidosa					Lipids: 1.050 g/L; 20.99%(*w/w*) DHA: 0		
Scenedesmus sp. ZTY2, *Scenedesmus* sp. ZTY3 *Chlorella* sp. ZTY4	Domestic wastewater	Centrifugation followed by sterilization	500 mL shake flasks /11 days		Lipids *Scenedesmus* sp. ZTY2: 69.1% (*w/w*) *Scenedesmus* sp. ZTY3: 52.9% *w/w* *Chlorella* sp. ZTY4: 64.4% (*w/w*) Biomass, respectively: 0.04, 0.045, 0.054 g/L		[43]

Table 2. *Cont.*

	Waste	Pre-Treatment	System/Duration of the Experiment	Nutrients Removal	Products	Observations	Reference
Chlorococcum sp. RAP-13	Dairy effluent supplemented with 6% biodiesel industry waste glycerol	Stored at 4 °C in sterilized containers before use	500 mL with 200 mL untreated effluent /15 days	COD: 93% BOD: 82%	Biomass: 1.96 g/L Lipids: 42% *w/w*		[29]
Chrypthecodinium cohnii ATCC 30772	Rapeseed meal hydrolysate + crude waste molasses	Rapeseed meal: solid-state fermentation using *Aspergillus oryzae* LZ01, *Penicillium oxalicum* J1, and *Neurospora crassa* J2 fungal strains; Crude waste molasses: diluted with distilled water and acidified to pH 3.0, heated to 100 °C for 1 h, followed by centrifugation	500 mL-Erlenmeyers, batch, 7 days		Lipids: 27.3% (*w/w*) (*w/w* DCW); 26.9 g/L DHA: 8.7 mg /L; DHA/TFA: 22–34% *w/w*		[44]
Scenedesmus sp., *Chlamydomonas* sp., *Chlorococcum humicola*, *Botryococcus braunii* *Chlorella* sp., Chlorella sp.	Surfactant mediated municipal wastewater (SMMW)	Stored at 4 °C to minimize substrate decomposition, followed by mixing for 5 min, followed by a 30 min settling, to allow the settlement of excess colloidal particles		COD > 98% PO_4-P > 98% NO_3-N > 99% NH_4-N ~ 100%	Biomass: 0.41–0.50 g/L Lipids: 21.5–42.0% (*w/w*)	Previous facultative heterotrophic strains screening for the capacity to grow in SMMW	[36]
Chlorella pyrenoidosa (NCIM 2738)	Treated cane molasses	Dilution 1:1 with distilled water followed by centrifugation at 2000 rpm, 30 min. The clarified liquid was passed through a strong acidic cation exchange resin for the removal of metal ions.	250 mL flasks containing 100 mL liquid medium	Total sugars: 92.2%	Biomass: 1.2 g/L Lipids: 66% (*w/w*)		[34]

Table 2. *Cont.*

	Waste	Pre-Treatment	System/Duration of the Experiment	Nutrients Removal	Products	Observations	Reference
Scenedesmus obliquus, *Chlorella protothecoides*	Cheese whey permeate	Filtration (0.2 µm)	Shake flasks; sequential cultivation of *S. obliquus* and *C. protothe-coides*/34 days	Total lactose: 62%	Biomass *Scenedesmus obliquus*: 8 ± 0.2 g L^{-1} *Chlorella protothecoides*: 6.2 ± 0.4 g L^{-1}	*S. obliquus* and *C. protothecoides* sequential cultivation	[14]
Chlorella sorokiniana	Aquaculture wastewater supplemented with 400 mg/L $NaNO_3$	Filtration using glass fiber filter papers	1 L Shake flasks containing 500 mL/7 days	COD: 71.9% PO_4-P: 73.6% NO_3 –N: 84.5% NH_4-N: 75.6%	Biomass: 3.5 g/L Lipids: 30.2% (*w/w*) Carbohydrates: 34.7% (*w/w*) Proteins: 28.4% (*w/w*)		[35]
Crypthecodinium cohnii CCMP 316	Cheese Whey + Corn Steep Liquor		250 mL-Erlenmeyers,		Lipids: 28.7% (*w/w* DCW) DHA/TFA: 8.5–27% *w/w*		[30]
Galdieria sulphuraria 074G	Food waste from restaurants and bakeries	Autoclaving for 30 min at 121 °C, followed by enzymatic hydrolysis for 24 h at 50 °C and pH 4.5 to produce a hydrolysate rich in sugars and amino acids/peptides	500 mL conical flasks containing 150 mL of liquid medium		Phycocyanin: 20–22 mg/g		[37]
Schizochytrium sp. BCRC 33482	Sugarcane bagasse with 40 g/L glucose	Alkali followed by phosphoric acid treatment, followed by enzymatic hydrolysis (cellulase)	250 mL Flasks with 50 mL liquid medium/ 72 h for the pre-treatment step: 120 h for the microalgae cultivation		Biomass: 10. 5 g/L Lipids: 45.2% (*w/w*)		[45]

Table 2. *Cont.*

	Waste	Pre-Treatment	System/Duration of the Experiment	Nutrients Removal	Products	Observations	Reference
Crypthecodinium cohnii ATCC 30772	Sugarcane molasses and distilled crude glycerol	Sugarcane molasses: hydrolysis by pH drop to 3 with HCl; storage at 50 °C for 24 h; thereafter, the pH was raised to 6.5 using NaOH 50% (*w*/*w*) Crude glycerol previously distilled to remove methanol	500 mL shake flasks with 200 mL liquid medium/192 h	Molasses: Glucose: 100% Fructose: 0% Crude Glycerol: 100%	Sugarcane molasses: Lipids: 11.2% (*w*/*w*) DHA 5.5 mg/g DCW DHA/TFA: 49.6% (*w*/*w*) Crude Glycerol: Lipids: 14.7% (*w*/*w*) DHA: 6.6 mg/g DCW DHA/TFA: 44.7% (*w*/*w*)		[25]

Cellulase, neutralase, and alcalase enzymes were used to promote de-oiled *Chlorella* biomass residue hydrolysis; the resulting hydrolysate, mixed with hydrolyzed molasses, was used as a substrate for *Cholorella* heterotrophic microalgae growth for lipid production [41]. Pleissner et al. [42] used the inocula of *Aspergillus awamori* and *Aspergillus oryzae* as a source of fungal glucoamylases, proteases, and phosphatases to hydrolysate food wastes, reporting 85%, 40%, and 100% of carbohydrates, total nitrogen, and phosphorous yield recovery, respectively. Fungal strains (*Aspergillus oryzae*, LZ01; *Penicillium oxalicum*, J1; and *Neurospora crassa*, J2) were also used as a source of enzymes to pre-treat rapeseed meal (RSM) (Table 2). In fact, according to these authors, fungal pre-treatment is preferable to other treatments such as chemical or commercial enzymes because the former not only increased the release of nitrogen but also promoted the degradation of toxicants in meal, preventing further microalgae growth inhibition.

Nguyen et al. [46] dried and ground sugarcane bagasse to small particles (<0.25 mm) to ensure that the substrate had a high surface area before the hydrolysis step. An alkali followed by phosphoric acid treatment was used to remove lignin and enhance xylose release because delignification enhances the pore size of the lignocellulosic biomass due to the removal of lignin, hemicellulose, and unknown substances, thus increasing the digestibility of the cellulosic substrate for the subsequent enzymatic hydrolysis. An enzymatic hydrolysis with cellulose was used to produce a glucose and xylose rich sugarcane bagasse hydrolysate, with 54.7% and 12.5% recovery yield, respectively (Table 2).

Other wastes require the extraction of nutrients. The carob tree (*Ceratonia siliqua* L.) is a leguminous plant that has been widely cultivated in Mediterranean countries for years. The seeds of carob (10% of the fruit dry weight) produce 12% of the world market of gum and are widely used in the food industry for candies and cakes. The pulp, which consists of 90% of the fruit dry weight, is a food industry by-product, which contains a high content of sugar (sucrose, glucose, and fructose) and tannins, and has a low content of protein and fat, which limits its application as animal feed. The sugars were extracted by Mendes et al. [39] for docosahexaenoic acid production (DHA, a compound that has many applications in the pharmaceutical, nutraceutical, and food industries due to its well-known benefits on human health) (Table 2), using the marine heterotrophic microalgae *Chrypthecodinium cohniii*. An additional step to hydrolyze sucrose to glucose and fructose was carried out by acidifying the carob pulp syrup to pH 2.

Sugarcane molasses is an important by-product from sugarcane refining factories, being used as a source of readily fermentable sugars. It contains approximately 50% (*w/w*) of total sugars (mainly sucrose, glucose, and fructose) and water, with a low concentration of crude protein and fat, heavy metals, vitamins, and other nutrients.

Gong et al. [44] used crude waste molasses to produce DHA, using the microalga strain *C. cohnii*, ATCC 30772. The waste molasses was previously acidified to pH 3 and heated to 100 ºC, followed by centrifugation to remove particles. Taborda et al. [25] also used sugarcane molasses to produce DHA from *C. cohnii*, ATCC 30772, having previously hydrolyzed the molasses by acidification, followed by storage at 50 °C for 24 h.

Other procedures are also required when wastes contain toxic materials, such as recalcitrant compounds and heavy metals, which may inhibit the microalgae growth. The simplest and cheapest way to reduce the toxic concentrations in the wastes consists of diluting them, as Chi et al. [40] have reported, using 50% diluted hydrolyzed potato broth (HPB) in the medium to grow *Schizochytrium limacinum*, SR 21, for DHA production. However, some nutrients, essential to microalgae heterotrophic metabolism, are also diluted, decreasing their availability in the culture medium, thus reducing the process yield. In these cases, it may be necessary to supplement the medium with the missing nutrients, as Chi et al. [39] have done, by adding 20 g/L glucose to the 50% diluted HPB.

Other approaches can be used to remove toxic compounds from the wastes or reduce their concentrations. Gaurav et al. [34] have used a strong acidic cation exchange resin to remove metal ions from treated cane molasses, before using it to grow *Chlorella pyrenoidosa*, NCIM 2738, for lipid production. Taborda et al. [25] have used, for the first time, crude

glycerol from the biodiesel industry, previously distilled, in order to remove the methanol, a transesterification reaction by-product resulting from the biodiesel production process, which is known to inhibit microbial growth (Table 2).

The use of these pre-treatments to make nutrients available for heterotrophic microalgae metabolism, or to remove toxic from waste, increases the overall process costs. In this way, the overall process is only economically sustainable if other profits are obtained, such as high value-added microalgal products produced together with the waste treatment, which should be evaluated before the process scale-up.

3.3. Strains

The selection of heterotrophic microalgae strains for waste treatment should consider a few criteria, such as: (i) robustness to the adverse stress conditions that exists in the wastes; (ii) versatility and capacity to grow on different types of wastes; (iii) capacity to produce high cell dense suspensions, simultaneously producing valuable intracellular compounds, in order to improve the process profit; (iv) the cell walls must resist hydrodynamic and mechanical shear, which are present in the large bioreactors used to grow heterotrophic microorganisms.

Only a few microalgae strains are able to grow under heterotrophic conditions. According to Table 2, the most used genera that can grow heterotrophically on wastes are *Chlorella* and *Scenedesmus*, being the microalgae considered the most versatile and robust to the adverse conditions that exist in a wide range of wastes, being able to produce significant amounts of intracellular lipids during the growth on the wastes.

The obligatory heterotrophic microalgae *Chrypthecodinium cohnii*, *Schizochytrium limacinum*, and *Schizochytrium mangrovei* can grow on different types of wastes containing high COD contents, producing not only appreciable amounts of lipids but also valuable polyunsaturated fatty acids, such as DHA. *Galdieria sulphuraria* was used to treat food waste from restaurants and bakeries to produce phycocyanin, a valuable pigment widely used as a food colorant [38].

3.4. Media Supplementation

Wastes are usually composed of different types of complex materials [46], which often do not fulfil the required nutrients that allow heterotrophic microalgae growth. For instance, after microbial secondary treatment, most municipal and domestic wastewater still contains large amounts of organic carbon, which is suitable for bacterial growth but not for microalgae [13] because the former can degrade these compounds but the latter cannot. In other cases, after the secondary treatment, the effluents contain low amounts of dissolved organic carbon that do not allow massive heterotrophic growth.

As mentioned above, microalgae can only uptake relatively simple molecules, such as nitrogen compounds, sugars, organic and amino acids, and several aromatic compounds. Therefore, nutritional supplements required for heterotrophic microalgae metabolism must be added to the effluents and wastes used as media culture. Table 2 shows that different organic carbon sources have been added to different types of wastes. Glucose (20 g/L) has been added as organic carbon to cull potato wastes [39] and sugarcane bagasse (40 g/L) [45]. Different types of wastes have been mixed to grow heterotrophic microalgae. Sabeela and Sukumaran (2015) [29] supplemented dairy effluent with 6% biodiesel industry waste glycerol to grow *Chlorococcum* sp., RAP-13, for lipid production, and Gong et al. [44] mixed diluted rapeseed meal hydrolysate (7% *v/v*) with 9% crude waste molasses to grow *Chrypthecdnium cohnii*, ATCC 30772, for DHA production. According to the authors, this approach allowed the conversion of two or more wastes with low market value into valuable microbial products, with obvious environmental and economic benefits. The addition of sodium nitrate (400 mg/L) improved *Chlorella sorokiniana* biomass growth on aquaculture wastewater as well as the productivities of lipid, carbohydrate, and protein [35].

The addition of supplements may also enhance the nutrient/toxics removal efficiency. Perez-Garcia et al. [13] reported that *C. vulgaris* heterotrophic growth on secondary wastewater required the addition of an organic carbon source. Several carbon sources were tested and allowed *C. vulgaris* heterotrophic growth, but acetate and glucose were the most efficient supplements that led to the highest ammonium heterotrophic removal rate. The authors concluded that heterotrophic microalgae growth has a higher potential than autotrophic growth to produce denser microalgal cultures because the *C. vulgaris* population densities, under heterotrophic conditions, were one order of magnitude higher than those under autotrophic culturing. Moreover, ammonium removal efficiency under heterotrophic culturing was similar to autotrophic cultivation.

3.5. Microalgae Heterotrophic Wastewater Treatment Efficiency

From Table 2, it can be seen that high COD removal percentages (70–99%) have been reported by several authors using heterotrophic microalgae to treat different types of wastes.

Even a waste containing high COD loads, such as dairy effluents, as reported by Sabeela and Sukumaran [29], was successfully treated by the microalga *Chlorococcum* sp. under heterotrophic conditions, with 93% COD and 82% BOD removals. A removal of 62% of lactose was observed by Girard et al. [14] after a sequential cultivation of the microalgae *Scenedesmus obliquus* and *Chlorella protothecoides* grown on whey permeate (obtained during the whey protein isolation, after an ultrafiltration step). The authors reported that glucose and galactose resulting from the previous extracellular lactose hydrolysis step performed by *S. obliquus* were completely consumed by *C. protothecoides*.

Wastes containing potential inhibitors were successfully treated by heterotrophic microalgae. *Scenedesmus* sp. and *Chlorella* sp. were able to remove 91.4% of COD from an acid-rich effluent collected from an anaerobic sequential batch reactor [24]. Hena et al. [36] reported 98%, 98%, 99%, and 100% COD, PO4-P, NO3-N, and NH4-N removal, respectively, when several microalgae species were grown on surfactant mediated wastewater.

Crude glycerol was completely consumed by *C. cohnii*, ATCC 30772, when used as the organic carbon source in a synthetic medium [25]. However, when hydrolyzed sugarcane molasses were used as the carbon source, glucose was entirely consumed by this microalga, but fructose was not. The same results were reported by Mendes et al. [26], using the strain *C. cohnnii*, CCMP31, grown on carob pulp syrup.

As already stated, some wastes do not contain the adequate nutrient proportion to allow heterotrophic microalgae growth, and thus do not provide an efficient nutrient removal treatment. As mentioned above, Perez-Garcia et al. [13] reported that the ammonium heterotrophic uptake by *Chlorella vulgaris* grown on municipal wastewater was enhanced by adding sodium acetate or glucose.

4. Microalgal Products Obtained from Heterotrophic Growth

4.1. Products Extraction

So far, the most common products extracted from heterotrophic microalgal biomass are lipids because these microorganisms usually contain high amounts of intracellular oils with high proportions of high value-added lipids, such as poly-unsaturated fatty acids (PUFA), but low protein and carbohydrates contents [47].

After the microalgae fermentation, the biomass must be separated from the liquid culture by filtration, centrifugation, or by using rotary vacuum filtration. The supernatant can be used to produce biogas, avoiding its discharge into water bodies. Before the product extraction, the microalgae cells must be dried to obtain a free water microalgal biomass, which can be stored for long time periods, without degradation, using spray drying or a freeze dryer. In this step, care must be taken because the microalgal biomass should not be exposed to temperatures higher than 50 °C and intense light, as the intracellular lipids, particularly PUFA, are heat and light sensitive, being readily oxidized due to the presence of double bonds in the fatty acid chains when exposed to such conditions [5].

Afterwards, the microalgae cells must be disrupted to facilitate the next extraction step. A variety of methods can be used to disrupt the microalgae cells, such as solvent extraction, ionic liquids, direct saponification, high-pressure homogenization, hydrodynamic cavitation, ultrasound/microwave/pulsed electronic field and ozone treatments, and hydrolytic enzymes, followed by extraction with solvent [48]. Nevertheless, solvent extraction is the most used, with the mixtures chloroform–methanol, hexane, and hexane–isopropanol being the most used solvents [5].

The heterotrophic microalgal oil can be further fractioned into different fractions with different usages. PUFA can be separated from saturated/monounsaturated fatty acids using winterization and/or urea complexation techniques. In this way, PUFA enriched fraction can be used for pharmaceutical/food/feed purposes, while the remained fraction, composed of saturated/monounsaturated fatty acids, can be further converted into biodiesel. In this way, all the microalgal oil fractions are valorized [5,26,48].

Additional purification steps are needed if the microalgal extracted lipids are used for pharmaceutical/food/feed purposes. Supercritical fluid extraction has been used to concentrate docosahexaenoic acid (DHA, 22:6ω3) in *C. cohnii* oil [49]. PUFA enriched fractions can also be obtained using lipase enzymatic reactions, producing different forms and compositions of PUFAs in triglycerides, phospholipids, other fatty acid esters, and free fatty acids [50].

4.2. Microalgal Products

Research to improve heterotrophic microalgae species to grow in high-density cultures, producing target products in significant amounts, has been carried out over the last few decades [51], the fed-batch heterotrophic microalgae cultures being those that achieved higher biomass and lipid productivities, because high carbon amounts can be added to the culture, without substrate inhibition [27,50–54]. According to Lowrey et al. [51], a key aspect to reduce the heterotrophic microalgae cultivation process costs is the search for alternative low-cost carbon sources that allow high biomass and product productivities, replacing expensive organic carbon sources such as glucose.

According to Table 2, various heterotrophic microalgae species that can grow on wastes are potential producers of commercially attractive products. These include lipids that may have different applications to PUFA and pigments (phycocyanin), both with applications in pharmaceutical, nutraceutical, cosmetic, and food industries. Moreover, the saponifiable lipid fraction (triacylglycerols) can be converted into biodiesel, as mentioned above (Figure 1).

From the references shown in Table 2, the heterotrophic microalgal lipid content varied between 9.2% and 69.1% (*w/w*), depending on the species, strain, culture conditions, and media formulation. The highest lipid content (79.2%) was observed for *Chlorella* sp., ZTY4, heterotrophic growth on domestic wastewater [43].

The heterotrophic microalgae *Schizochytrium mangrovei*, *Schizochytrium limacinum*, and *Crypthecodinium cohnii* can use wastes to produce considerable amounts of DHA (Table 2). This compound is a long-chain fatty acid with well-known benefits for human health in the treatment of many diseases such as cancer, atherosclerosis, rheumatoid arthritis, Alzheimer's, and psoriasis. DHA is an essential ω-3 PUFA of the human brain and nervous system, playing a crucial role in infant brain development. Several reports claim that many commercial formula-fed infants contain lower levels of DHA and arachidonic acid (ARA, 20:4ω6) compared to breast-fed infants, thus they require the addition of DHA. As a result, in the last few decades, the global microalgae-based DHA market has increased, due to increasing public awareness about healthcare and chronic diseases and the public preference for natural sources, such as microalgae. DSM enterprise, a major worldwide DHA producer, commercializes microalgal oil rich in DHA obtained from the heterotrophic microalga *Schizochytrium* sp., sold as Life'sTM OMEGA, Life's DHATM products. DHASCO, and oil rich in DHA, used in the food industry is also produced by DSM, and is obtained from *C. cohnii* microalga. Solazyme Bunge Renewable Oils

(SB oils), based in Brazil, uses sugarcane to produce *Schizochytrium* microalgae, which is commercialized as a whole algal biomass and used in the aquaculture feed industry, such as in the AlgaPrime DHA product. The facility uses sugarcane waste as an energy supply for the process [5].

According to Oliver et al. [55], glucose is the most used carbon source for ω-3 PUFA production, contributing to around 80% of total cultivation cost, which is a major drawback of DHA heterotrophic production from an economic point of view. Although DHA heterotrophic production is being carried out at a commercial scale by several companies, the high economic cost and environmental impact of glucose usage as a carbon source imply that ω-3 PUFA high-quality production should be carried out in cheaper ways, using cheaper carbon sources. According to Table 1, *C. cohnii* can produce considerable amounts of DHA when grown on wastes such as carob pulp syrup (45.2 mg/g) [38], rapeseed meal + crude waste molasses [44], cheese whey + corn steep liquor (5 mg/g) [30], and sugarcane molasses and crude glycerol (5.5 mg/g and 6.6 mg/g, respectively) [24]. The microalga *Schizochytrium* has also been grown on food waste to produce 85.5 mg DHA /g [42].

The pigment phycocyanin has been used as a fluorescent marker in clinical diagnostic and as food and cosmetic dye commercially produced by autotrophic microalgae. Under these conditions, it may be difficult to achieve high cell densities due to the shelf-shading effect, particularly at a larger scale, because surface area and culture volume ratio decreases, resulting in longer light paths inside the culture and darkness, which both hinder cell growth. Sloth et al. [37] have used bakery and restaurant food waste to produce phycocyanin using the microalga *Galdieria sulphuraria* under heterotrophic and mixotrophic conditions and reported that this microalga accumulated 10–30 mg/g DCW of phycocyanin under carbon limiting conditions.

The whole biomass can be used as feed, as mentioned above, if its composition fits this application. Alternatively, it may be used as biofertilizers or biostimulants (Figure 1). Hydrothermal liquefaction (HTL) allows the thermochemical conversion of wet whole microalgal biomass into a liquid energy carrier called 'bio-oil' or 'biocrude', which may be used as fuel. The microalgal biomass leftovers can be used as a substrate to produce biogas in an anaerobic digester [56] (Figure 1).

5. Heterotrophic Microalgae Waste Treatment Process Monitoring

Studies reporting heterotrophic microalgae growing on wastes to produce biofuels and high value-added products, with the simultaneous waste treatment, have been published (Table 2). Most of these works used conventional microbiological methods to monitor the microalgal cultivations (growth and intracellular products), such as optical density, which only provide average data, not giving any information on individual cell status. Other methods for cell growth detection, such as dry cell weight or serial dilution methods, present a few limitations, as the results are usually only available a period after the sample is taken, frequently when the process is over, too late to change the process control strategy [57].

However, despite several wastes being used as culture media as they contain nutrients that allow microbial growth, they may also contain inhibitor compounds, as mentioned above, such as antibiotics, chemicals, and heavy metals that are toxic for the microalgae cells and affect their metabolic activity. In particular, contaminants of emerging concern (CECs) such as pharmaceuticals, personal care products (PCPs), nanomaterials, and perfluorinated compounds have raised increasing public concerns because they are harmful to human health and ecosystems, causing endocrine disruption, chronic eco-toxicity, encouragement of antibiotic resistance, and uptake into the food chain [58]. Many CECs (especially personal care products) enter wastewater through domestic use and discharge and, for many pharmaceutical compounds, via feces and urine, after medicinal use. Therefore, wastewater is a major release point of CECs into the environment. These compounds may affect the microorganisms, specifically heterotrophic microalgae, used to treat these effluents.

Lopes da Silva and Reis [57] described the inhibitor compounds present in potential wastes that may be used for heterotrophic growth. As previously referred, some wastes require a pre-treatment step before being used as culture media for microbial growth in order to release monomeric sugars that will be used by the microorganisms [37,42]. However, the pre-treatment step usually releases inhibitors that may affect the cell metabolism, thus reducing the process performance and yield.

In order to understand the effect of all these inhibitors on microbial cell physiology, it is essential to monitor, near real-time, the cell physiological status during the waste treatment process development. Moreover, a few heterotrophic microalgae, particularly dinoflagellates (such as *Crypthecodinium cohnii*), are negatively affected by the shear stress present in turbulent environments. Under high shear stress levels, the microalgae flagella were damaged [59], and the cell cycle was arrested at the G1 phase [60].

Multi-parameter flow cytometry (FC) is an advanced technique for bioprocess monitoring that gives near real-time (at-line) information on several cell functions and compartments at the individual cell level. This technique is ideal for assessing the microalgae cell stress response to adverse environmental conditions, such as those present in the wastes and mechanical bioreactors. An understanding of the microalgal cell response will allow the development of more tolerant microalgal strains to these environments, as well as more efficient bioprocess control strategies. As at-line information is available, this technique also allows changing the control strategy during the time course of the bioprocess by changing the operating conditions to achieve the highest product yields and optimal process performance.

In addition, FC also allows microalgal intracellular products at-line quantification, such as lipids and carotenoids. The addition of specific fluorescent dyes, such as Nile Red and BODYPI, to microalgae cells, in association with flow cytometric analysis, allows for at-line microalgal intracellular lipid quantification, avoiding the time-consuming gravimetric techniques for lipid quantification, which involves high toxic organic solvent volumes. The use of flow cytometry for at-line carotenoid content evaluation in autotrophic microalgae cells based on the autofluorescence cells has also been reported [61], using the same protocols for heterotrophic microalgae, with adjustments if necessary. Therefore, the at-line intracellular product content information allows the microalgal biomass harvesting at the highest product productivities.

Despite the benefits, the application of FC to bioprocesses involving low-cost waste conversion by heterotrophic microalgae is still rare because the equipment is expensive and requires specialized training. Lopes da Silva and Reis [57] published a detailed description of the effects of the most known inhibitors present in low-cost feedstock on the microorganisms, highlighting the benefits of using FC to monitor such processes.

6. Drawbacks and Bottlenecks

The production of carbon dioxide from the respiration of organic carbon during heterotrophic growth is a drawback of the heterotrophic microalgae metabolism, contrarily to the autotrophic microalgae metabolism, which indubitably contributes to greenhouse effect mitigation. However, symbiotic heterotrophic/autotrophic microalgae consortia may be used to treat effluents. When heterotrophic and autotrophic microalgae grow in mixed cultures, the complementary nutritional requirements of each microorganism may reduce the CO_2 produced by the heterotrophic microalgae because they consume O_2 and produce CO_2, which in turn is consumed by autotrophic microalgae that consume CO_2 and produce O_2, up-taken by the heterotrophic microalgae [62].

The presence of high organic compound loads in the wastes used as culture media to grow heterotrophic microalgae increases the risk for contamination by competitive heterotrophic bacteria and fungi, compromising the quality of the process and products if good laboratory practice is not followed [63]. For this reason, heterotrophic microalgae growth requires media sterilization, or, at least, sanitation, an energetic requirement that can account for 20–30% of the total production process costs. This cost may be compensated

if high value-added products are produced by the heterotrophic microalgae due to their high market price.

One possible approach to overcome the sterilization step consists of using extremophile heterotrophic microalgae that can grow at extreme pH, temperature, or salinity, significantly reducing or preventing competitive microbial growth [21]. The red extremophile alga *Galdieria sulphuraria* can grow photoautotrophically, heterotrophically, and mixotrophically and can utilize more than 50 different carbon sources such as sugars and sugar alcohols such as glycerol and amino acids. This versatility makes this microalga ideal for heterotrophic cultivation on non-sterile organic waste hydrolysate. Another possibility of reducing the heterotrophic microalgae cultivation contamination risk consists of adding antibiotics such as chloramphenicol, penicillin, and streptomycin to the media.

7. Heterotrophic/Autotrophic Microalgae Waste Treatment Economic Evaluation

So far, there are no industrial plants to treat waste streams using heterotrophic microalgae. The published studies using this approach were carried out at bench-scale. Nevertheless, according to Yang et al. [64], heterotrophic cultivation can be much more beneficial than photoautotrophic cultivation from an economic perspective. The input energy to ATP conversion ratio is higher for heterotrophic cultivation (18% of the energy obtained can be converted to ATP, while only 10% is converted under photoautotrophic conditions). Similar observations were made by Behrens [65], who calculated the conversion efficiency of input energy in the form of electricity to ATP and NADPH and concluded that heterotrophic cultivation is economically more advantageous than photoautotrophic cultivation; the cost per kg of dry biomass for heterotrophic cultivation was calculated as US$2, while for photoautotrophic cultivation it was about US$11 [65]. Compared to microalgae autotrophic cultivation, which depends on light intensity, heterotrophic cultivation could decrease the land area needed and water evaporation significantly. In addition, Orfield et al. [66] demonstrated that the heterotrophic microalgae cultivation mode attained the highest net energy ratio (NER) among a study that compared the three microalgae cultivation modes autotrophic, heterotrophic, and mixotrophic. Because heterotrophic microalgae cultivations attain higher cell densities that are obtained under axenic conditions, they are adequate for producing high-quality bioactive compounds such as pharmaceuticals [67].

Techno-economic assessment (TEA) is the most recognized methodology to analyze a certain system cost and has embedded economic indicators such as CAPital EXpenditures (CAPEX), OPErational EXpenditures (OPEX), Net Present Value (NPV), and Internal Rate of Return (IRR). Regarding the TEA of heterotrophic microalgae use to treat waste streams, the query ALL (waste AND heterotrophic AND microalgae) and entitle ("economic assessment"), excluding patents and citations, retrieved 21 publications on the Google Scholar platform, and 4 publications in the Scopus database, as at 20.03.2021.

Within this universe, the authors identified a total of 8 publications, of which, 1 focussed on TEA uncertainty systematization needs in heterotrophic systems [68]; 1 focussed on the model for TEA in VFA-volatile fatty acids used as a low-cost carbon source to obtain biodiesel replicable from heterotrophic microalgae [69]; 1 focussed on nutrient recycling on heterotrophic systems [51], 1 focussed on liquid waste streams (Table 3) as a carbon source for heterotrophic microalgae; and 3 focussed on organic solid waste streams as a carbon source of heterotrophic microalgae (Table 3). The bioreactor is identified as the most costly equipment of scaled-up facilities. In terms of materials consumed, the carbon source is usually the bottleneck, as well as some input materials. The most expensive carbon source is glucose. Its cost in international market price is around $500/ton, as of 2010 [69]. The price of food wastes and other agricultural wastes (rice straw) in Korea ranges from $50 to $130/ton, so it is considered a potential low-cost carbon source.

Table 3. Summary of TEA studies focused on heterotrophic cultures.

Carbon Source	System	TEA Method	Conclusions	Reference
Food waste	Batch; *Chlorella protothecoides* in heterotrophic cultures (max 48.7% lipid content; max 0.187 g/g yield) Product: biodiesel	OPEX w/carbon source cost (30$/ton), NH4Cl $150/ton, utilities cost of $0.035/kg (0.1 $/kWh^{-1}); labor cost of $0.056/kg; general works $0.029/kg including maintenance, supervision, administration, property taxes, and insurance. CAPEX not included. w/o co-product valuation	cost of biodiesel $0.6/L compared with glucose-based biodiesel $3.79/L	[70]
Molasses from sugar cane processing	Scale-up simulation on SuperPro Designer™ Auxenochlorella protothecoides (50% lipid content; lipid 0.25 g/g yield) Product: Biodiesel and animal feed	Lipid productivity 8.2 gL^{-1} d^{-1}, 330 working days, 10 ML bioreactors CAPEX 130 M$-160 M$ (function of equipment cost covering equipment Installation, pipping, electrical, buildings, design and engineering, Contractor's fee and contingency) OPEX 50–60 M$/year including materials costs + utilities costs ($ 0.058/kWh) + waste treatment costs ($1.5 × 10^{-3} kg^{-1}) + labor costs ($11.50 per work hour) + facility related costs 30-year net present value (NPV) and internal rate of return (IRR) Co-product animal feed ($ 0.45/kg)	Biodiesel price range Minimum 2.6–3.0 $/liter for IRR 12%	[71]
Food waste	Simulation of process scale-up 56.3 t of wet food waste per day 7.14 t dried biomass per day *Chlorella pyrenoidosa* to food and feed	300 working days/year CAPEX (equipment) 3.3 M€ OPEX (materials, utilities, labor 8 €/h) 31.9 k€/day Dry biomass revenue 36 k€/ton	20 years NPV w/ 5% discount rate 948 M€ In comparison with autotrophic microalgae for food/feed applications, heterotrophic microalgae cultvation operational cost is up to 50% lower	[72]
Agroindustrial wastewater (poultry and swine slaughterhouse wastewater)	Up-scale wastewater flow rate 16,000 m^3/day from bench-scale facility data Phormidium	Working 24 h/d and 336 days/year CAPEX 71 M$ (equipment, installation, deployment, instrumentation, piping) OPEX 14 M$/year (raw materials and supplies, utilities, labor costs ($8.50/h), supervision, payroll charges, maintenance, operating supplies, general plant overheads, tax, and contingency)	10-year lifetime facility $2.66/m^3 ($ 0.70/m^3 considering only operational costs) Potential revenue dry biomass production cost $ 0.03/kg (much lower than conventional heterotephic fermenters and autotrophic photobioreactors, and below theoretical target of 0.55 $/kg) Return on investment in the first year of operation	[73]

The research presented in Table 3 is supported by bench-scale experiments, scaling-up. Therefore, the scale limitations of these economic estimates emphasize the importance of field data coming from pilot plants of suitable size to finally reach an industrial scale. Nevertheless, these exploratory results showed that heterotrophic microalgae have the potential to be used in both food waste and wastewater streams. The products may be food, feed, or biodiesel, or simply clean water, as already mentioned (Figure 1). What is noticeable is that, even with lower-cost carbon sources and accounting for the CAPEX and OPEX costs, the minimum biodiesel cost is still not competitive with traditional biodiesel from 1st generation plants (Figure 3).

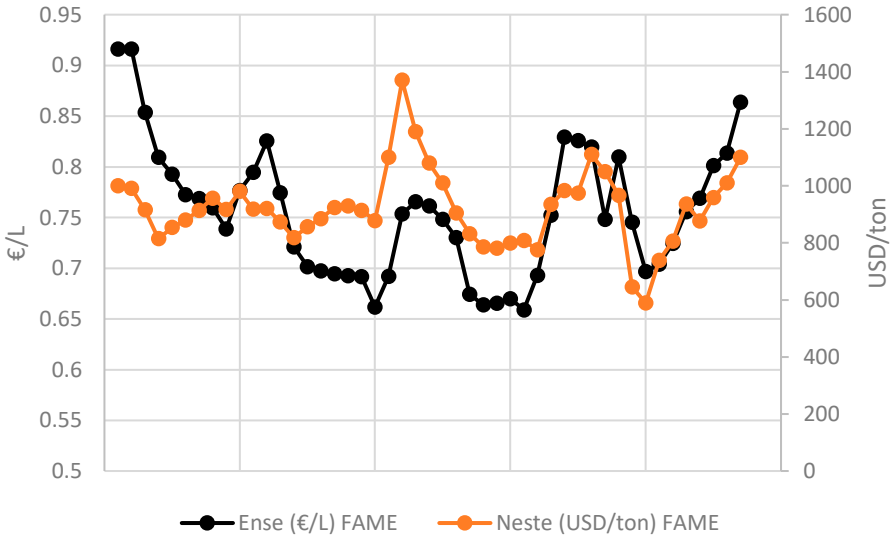

Figure 3. Market price of biodiesel (source: Ense and Neste [74]). Monthly prices since January 2017 until December 2020.

The market prices of biodiesel taken from Neste company (in $/ton) and assuming a biodiesel density of 890 kg/m^3 would range within 0.45–1.25 $/L.

The Renewable Energy Directive (2018/2001), together with the waste directive 2008/98/EC (on waste destination hierarchy), could be a game changer in the third-generation market from waste feedstocks. Advanced biofuels are defined as liquid or gaseous biofuels made from materials listed in Part A of the REDII Annex. They have a specific sub-target starting at 0.2% in 2022, at least 1% in 2025, and increasing to at least 3.5% in 2030. In addition, process optimizations such as nutrient and media recycling (lowering materials input operational costs) were never considered in the marketed studies presented in Table 3, but are being evaluated by others [51].

Typical wastewater treatment costs for agro-industrial wastes range between 1.06 and 2.58 $/m^3, covering only operational costs [75,76]. The system developed with a heterotrophic bioreactor replacing typical secondary and tertiary treatments presented in Table 3 showed the potential usefulness and role of heterotrophic microalgae in this context.

One of the reviewed studies [77] focused on protein concentrate for food and feed (protein powder). The study does not perform a TEA but points out directions of cost savings because it estimates the CO_2eq emissions (directly related to utilities, heat, electricity, and material carbon sources, and others) of three microalgae species and autotrophic and heterotrophic conditions. The latter achieved the lower impacts (for heterotrophic fermenter cultivation of *C. vulgaris* with glycerol as a carbon source and *C. pyrenoidosa* with food waste as a carbon source, less than 3–14.7 kg CO_2eq/kg protein) and was considered better than most traditional food and feed protein sources (egg concentrate, 23.4 kg CO_2eq/kg protein; spirulina, 78–196 kg CO_2 eq/kg protein).

8. Conclusions

Heterotrophic microalgae show many advantages over autotrophic microalgae to treat food wastes and municipal/domestic wastewater, as the former has the ability to grow on wastes with high COD loads, simultaneously producing microalgal biomass, biofuels, and high value-added products such as lipids, PUFA, and carotenoids, with different commercial applications.

In addition, heterotrophic growth is non-dependent on the light season and climate, being able to grow at latitudes far from the Equator, contrarily to the autotrophic microalgae.

The main disadvantages are the need for media sterilization, the need for an organic carbon source, and the biogenic release of CO_2. These drawbacks can be overcome if high value-added products are produced and if symbiotic heterotrophic-autotrophic microalgae systems are used when treating wastes.

Coupling waste management with concomitant heterotrophic microalgae production may reduce the overall waste treatment process costs, with simultaneous environmental benefits, as this strategy is based on circular economy principles.

However, further research is needed to improve heterotrophic microalgal biomass and high value-added product productivities when using wastes as substrates. The literature review recognizes the need for field data from pilot plants of suitable size to finally reach an industrial scale.

The role of heterotrophic microalgae in treating food waste and wastewater streams is being explored, but pilot scale data are needed to reduce TEA uncertainty at a future industrial level.

Up-scales based on bench-scale experiments project heterotrophic microalgae as competitive and commercially attractive. The target cost to produce 1 kg dry microalgae should be 0.55$ to compensate for downstream processing into biofuels and bioproducts.

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