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Heterotrophic microalgae production on food waste and by-products

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Abstract
The present review provides an overview of the latest research on microalgae production techniques based on carbon instead of light as energy source. The independence of light in mixotrophic and heterotrophic cultivation considerably reduces production costs and space compared to autotrophic production. Hence, this production technique may play a key role to meet future increasing food and feed demands. In order to reach this aim, it is, however, necessary to explore the possibilities of utilizing low-cost carbon sources such as molasses from industrial waste streams. This review provides an overview of worldwide potentially available low-cost carbon sources, potential microalgae species and their chemical composition, available pre-treatment methods for media sterilization and enhanced bioavailability, latest literature on growth of heterotrophic microalgae cultured on new, innovative low cost carbon sources, non-sterile culture approaches, and finally, economic considerations including a future outlook.

Keywords Heterotrophic microalgae · Aquaculture · *Chlorella* · Waste stream · By-products

Introduction

Worldwide, the requirement for proteins has been sharply increased in recent years (WHO/UNU 2007). This increasing demand is especially related to the aquaculture industry, which is the fastest growing animal producing sector (6.3% per year). To date, more than 50% of food fish for human consumption are farmed fish, and the numbers are predicted to increase further (FAO 2014). Future growth will, however, depend on the availability of alternative protein sources other than fish meal. The increasing scarcity and concomitant increase in the price of fishmeal have led the aquaculture feed industry to progressively replace the share of fishmeal with vegetable protein raw materials. Dried cells of microorganisms, also referred to as single-cell proteins (SCP), have also been considered as protein sources (Zepka et al. 2010). In contrast to higher plants, the production of microorganisms such as microalgae, bacteria, fungi, and yeasts does not require agricultural land. Microalgae, which are considered to be the most promising SCP, are traditionally produced photoautotrophically. However, the low biomass productivity makes the photoautotrophic production technically and economically challenging (Xie et al. 2012). Heterotrophic cultivation of microalgae has the potential to overcome or minimize the problems associated with autotrophic cultivation.

As heterotrophic production is done in a closed system, contamination from other microorganisms can be effectively controlled, and culture conditions can be optimized to maximize biomass yield. In addition, production can be done in production units from the brewery, the medicine, or the feed industry. Growth and biomass can be significantly higher under heterotrophic conditions than under photoautotrophic conditions, which in turn reduces the cost of down-stream processing and overall production costs. In addition, there are simple daily management and low personnel expenses (Radmer and Parker 1994; Miao and Wu 2006; Shen et al. 2010; Bumbak et al. 2011; Enzing et al. 2014). The unicellular freshwater microalgae of the genus *Chlorella* are the most promising as they are suitable for heterotrophic cultivation and are highly productive and contain high concentrations of proteins. In addition, *Chlorella* is one of a few heterotrophic microalgae that can be used in feed for both terrestrial and aquatic animals (Harel and Clayton 2004; Kotrbáček et al. 2015). However, so far algae are considered too expensive to use widely as a protein supplement in aquaculture or animal

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feed per se (Wen and Chen 2003). Therefore, decreasing media costs for microalgae production could be a step necessary to utilize microalgal biomass in animal feed in the future. One of the highest costs in heterotrophic production is the carbon source; glucose, as the most utilized carbon source, is cost intensive and can account for up to 60% of the overall production costs (Sharma et al. 2011). Another challenge related to heterotrophic production is the microalgae chemical composition. When considered for the food and feed market, protein quantity and quality is the most important parameter. It is generally considered that the protein content is lower in microalgae grown heterotrophically compared to the protein content in microalgae produced autotrophically or mixotrophically (El-Sheekh et al. 2014; Gami et al. 2014). For example, the protein content of *Chlorella vulgaris* grown mixotrophically was around 600 mg g^{-1} dry matter (dm), whereas the protein content of *C. vulgaris* grown heterotrophically was only 400 mg g^{-1} dm.

The present review summarizes the latest research results on the use of waste stream products from the agricultural and food industry for heterotrophic microalgae production.

Waste streams as carbon sources for heterotrophic microalgae cultivation

Biogenic residues from the food industry are up to 1.3 billion tonnes annually (FAO 2017). Most residues contain high levels of carbohydrates which can be converted into high-quality protein for animal feed via heterotrophic microalgae cultivation. A limited number of microalgae like some species of *Chlorella*, *Tetraselmis*, and *Nitzschia* are facultative heterotrophs (Perez-Garcia et al. 2011). These species can metabolize some organic substances like glucose, glycerol, or acetic acid under aerobic conditions (Perez-Garcia et al. 2011). The following waste products are presented because of their suitability for microalgae cultivation and availability on industrial scale.

Whey permeate

Whey permeate is a by-product from the dairy industry obtained by ultrafiltration and removal of protein from whey generated during cheese manufacturing. Whey permeate displays an overall composition of mostly lactose along with salts and non-protein nitrogen (Jelen 2009). The use of whey permeate as a direct lactose source has been neglected due to the extensive processing required for its recovery such as demineralization and dewatering (Jelen 2009). A major portion of the whey permeate produced in the world is currently being discarded as a dairy effluent. The top dairy-producing countries are the USA with 91.3×10^{12} (billion kg), India with 60.6×10^{12} (billion kg), China with 35.7×10^{12} (billion kg), Brazil with 34.3×10^{12} (billion kg), and Germany with 31.1×10^{12} (billion kg). Whey permeate represents about 85 vol% of the total milk used in the process (Panesar and Kennedy 2012). Considering these large amounts of whey permeate generated and its global production, it appears a suitable carbon source for heterotrophic microalgae production. Lactose has to be hydrolyzed into glucose and galactose prior to microalgae cultivation, leading to additional costs and process steps. Nevertheless, *Chlorella* showed biomass production of 2.8 ± 0.4 g L^{-1} on hydrolyzed galactose compared to those fed with glucose and galactose (1.5 ± 0.1 and 1.7 ± 0.4 g L^{-1}, respectively) (Espinosa-Gonzalez et al. 2014). The price of whey permeate powder of 769 US$ per tonne (Blimling and Associates 2017) would not result in reduced production costs. However, if microalgae production takes place in proximity of the dairy industry, the process of the expensive dewatering process would be unnecessary and costs could be considerably reduced.

Banana pulp

Banana pulp is a by-product of banana production and is produced mainly due to costs evolving by sorting of bananas with different ripening states. Wastes are mainly produced during harvest near the production plots. Banana plant parts are useful as insecticide, antioxidant, and color absorber, in preparation of various functional foods, wine, alcohol, biogas, and cattle feed (Mohapatra et al. 2010). Surplus and discarded bananas are a potential feed resource of great quantitative and qualitative interest in both pig and cattle production (Pérez 1997). The utilization of banana waste for microalgae cultivation may therefore compete with the food-producing industry unless a surplus is available. In 2013, 106.7 million tonnes of banana were produced worldwide, whereby the proportion of banana product to waste was 1:2 (Guerrero et al. 2016). The top banana-producing countries are India (27575000 t), China (12075238 t), the Philippines (8645749 t), Brazil (6892622 t), and Ecuador (5995527 t). In addition, there are several West and Central African (Cameroon, Côte d'Ivoire, Democratic Republic of Congo, Ghana, and Nigeria) and Eastern and Southern African countries (Burundi, Kenya, Rwanda, Tanzania, and Uganda) that produce bananas (Sheth 2017). Going back a few decades, Wai (1955) found that growth of *Chlorella* enhanced using banana extract (*Musa sapientum*). With the addition of banana extract (10 g of ripened banana fruit pulp in 100-mL boiling water to the basal culture solution after 5 days), 20 g dry matter (dm) of *Chlorella* were obtained per 20 L. A recent unpublished study conducted by the authors confirmed the enhanced growth rates of *Chlorella* grown on a media containing banana pulp and even banana peel compared to growth rates obtained for *Chlorella* grown on glucose and those grown autotrophically (unpublished data). After 7 days, final

biomass in *Chlorella* grown heterotrophically on media added with banana pulp was around 5 g L^{-1}. The good performance of *Chlorella* grown on banana could be explained by the suitable chemical composition; as part of the 12% sugar content, fully mature banana contained 48% glucose and 40% fructose (USDA 2016).

Molasses

Molasses is the by-product obtained in the preparation of sugar through repeated crystallization. The forecast of sugar production for 2017/2018 is 179 million tonnes (https://www.statista.com/statistics/249679/total-production-of-sugar-worldwide/). The top-producing countries of Molasses are Brazil (14800000 t), India (10882000 t), Thailand (4293000 t), China (3600000 t), and Pakistan (2350000 t). In addition, there are a number of African countries like Mali, Madagascar, Niger, or Somalia that produce considerable amounts of molasses. Per tonne sugar produced, about 320 kg of molasses are produced as a by-product. This corresponds to an annual worldwide molasses production of 57 million tonnes. However, the use as fermentation substrate for bioethanol and other biotechnology products as well as for animal and human consumption leads to market competition. Although global molasses production in 2016/2017 and 2017/2018 increased strongly and consequently prices have dropped in Europe, prices still range at EUR 130 (beet) and EUR 140 (cane) per tonne (Informa 2018).

Heterotrophically grown *Chlorella zofingiensis* fed pretreated cane molasses achieved a volumetric cell mass production rate of 1.79 g L^{-1} day^{-1} which is comparable to the biomass produced with glucose, and about 2- and 2.8-fold higher than values obtained with untreated cane molasses (Liu et al. 2013). Similarly, *Chlorella* sp. fed molasses showed a volumetric cell mass production rate of 1.796 g L^{-1} day^{-1} (Leesing and Kookkhunthod 2011). Furthermore, hydrolyzed molasses and glucose resulted in similar biomass after 72 h (3.5 g L^{-1}) in a study by Vidotti et al. (2014). The influence of molasses on growth depends on the molasses concentration. The highest growth response (measured optically) for *Chlorella* was obtained at a molasses concentration of 0.45% volume/volume; all lower concentrations resulted in lower optical density (El-Sheekh et al. 2014).

Protein concentration and amino acid composition

It is generally considered that the protein content is lower in microalgae grown heterotrophically compared to the protein content in microalgae produced autotrophically or mixotrophically (El-Sheekh et al. 2014; Gami et al. 2014). For example, the protein content of *C. vulgaris* grown mixotrophically was around 600 mg g^{-1} dm, whereas the protein content of *Chlorella* grown heterotrophically was only 400 mg g^{-1} dm when using high concentrations of molasses in the growth media (El-Sheekh et al. 2014). One way to overcome this problem may be over-compensation of nitrogen due to prior nitrogen starvation (Xie et al. 2017). Cellular protein content in sterile centrifugal transfer culture was improved to 53.8% in 1.50 g L^{-1} nitrate medium which was 1.43 times that of one-stage cultivation (37.5%) and higher than autotrophic microalgae cells (44.9%). The authors related the increased cellular protein content to a shift of metabolism towards protein formation, relative to carbohydrate protein synthesis (Shelly et al. 2007). In addition, the significant improvement of protein content neither had significant negative impact on microalgae growth or biomass yield nor did the amino acid composition changed. In addition, the protein content can also be increased by harvesting at the right time. For example, in heterotrophically grown *Chlorella*, the protein content of the cells in the logarithmic phase was 25–35% but increased rapidly, approaching 60%, after the glucose was consumed and the cells entered the stationary phase (Endo et al. 1974).

When heterotrophically grown *Chlorella* is evaluated as animal feed, the amino acid composition is of great importance. Deficiencies of, e.g., lysine, methionine arginine, isoleucine, leucine, threonine, or tryptophan resulted in increased lipid deposition in rainbow trout, *Oncorhynchus mykiss*. Valine deficiency resulted in reduced lipid deposition in *O. mykiss* (Rodehutscord et al. 1995a, b, 1997). Fish meal has a balanced amino acid profile and high concentration of essential amino acids such as lysine and methionine and is difficult to be replaced in animal feeds. For example, the lysine and methionine of soybean meal is about half the concentration as in fish meal (see Table 1). In contrast, *Chlorella* grown autotrophically and heterotrophically contain higher levels of lysine (see Table 1). In addition, the methionine concentration especially in heterotrophically grown *Chlorella* is considerably higher than in soybean meal nearly reaching the concentration of methionine in fish meal (Table 1).

Pre-treatment methods for media sterilization and enhanced bioavailability

Complex solid organic substrates like organic residues from food processing consist mainly of carbohydrates like starch, cellulose, and pectin; proteins and lipids need to be hydrolyzed prior to microalgae cultivation. Hydrolysis of organic residues can be performed either by acid hydrolysis or enzymatically (Fig. 1) (Pleissner and Rumpold 2018). Particularly, lignocellulosic biomass needs tougher treatments with concentrated acid or base at a high temperature in order to release sugars from cellulose and hemicellulose (Pleissner and Venus 2014).

Table 1 Amino acid (in AA in g (100 g)$^{-1}$ protein) composition of autotrophically and heterotrophically grown *Chlorella regularis* as well as fish meal and soybean meal

AA in g (100 g)$^{-1}$ protein	*Chlorella* autotrophic[a]	*Chlorella* heterotrophic[a]	Fish meal[b]	Soybean meal[c]
Isoleucine	4.21	3.35	5.23	2.63
Leucine	8.08	7.01	6.25	4.18
Lysine	7.74	9.42	6.79	3.50
Phenylalanine	5.08	3.19	3.26	2.46
Tyrosine	2.64	3.00	2.69	1.62
Cysteine	0.67	0.88	0.84	1.11
Methionine	1.25	1.83	2.50	0.99
Threonine	3.62	3.96	3.97	2.06
Tryptophan	1.52	1.40	0.84	n.a.[d]
Valine	5.94	5.05	3.93	1.94
Arginine	5.75	10.24	5.23	4.18
Histidine	1.82	2.98	1.97	1.53
Alanine	7.30	7.37	5.00	2.32
Aspartic acid	8.83	7.73	8.24	6.00
Glutamic acid	11.78	9.88	11.92	9.10
Glycine	5.42	4.91	4.40	2.01
Proline	4.32	2.16	3.26	2.20
Serine	3.03	3.35	3.95	2.54

Amino acid composition percent per $N \times 6.25$ in cell weight

[a] (Endo et al. 1974)

[b] (Øverland et al. 2013)

[c] (Winkler et al. 2011)

[d] Data not reported

In order to increase the specific surface of the substrate and facilitate enzymatic breakdown, coarse material needs mechanical pre-treatment by a homogenizer or blender prior to the hydrolysis step. The enzymatic hydrolysis can either be performed by autolysis at 30–60 °C (Zhao and Fleet 2005) via the addition of hydrolytic enzymes like cellulases, amylases, and proteases or by co-fermentation with hydrolytic fungi (Pleissner and Venus 2014). Depending on the chemical composition and digestibility, organic residues can be almost completely hydrolyzed and made available for algal cells (Pleissner and Venus 2014).

Hydrolysis converts the solid organic material into liquid which has to be diluted and sterilized prior to heterotrophic microalgae cultivation. Sterilization is either performed by heat treatment, e.g., autoclaving at 121 °C saturated steam atmosphere for 20 min. This method bears the disadvantage of the occurring Maillard reaction if proteins are present in the substrate material. The Maillard reaction results in reduced bioavailability of amino acids and sugars. The second option is the sterilization by membrane separation or sterile filtration which has the disadvantage of blocking filters and membrane fouling. Prior to the membrane separation or filtration step, a separation of coarse material has to be performed by centrifugation. Another treatment option is tyndallization. Tyndallization is a repeated heating process below

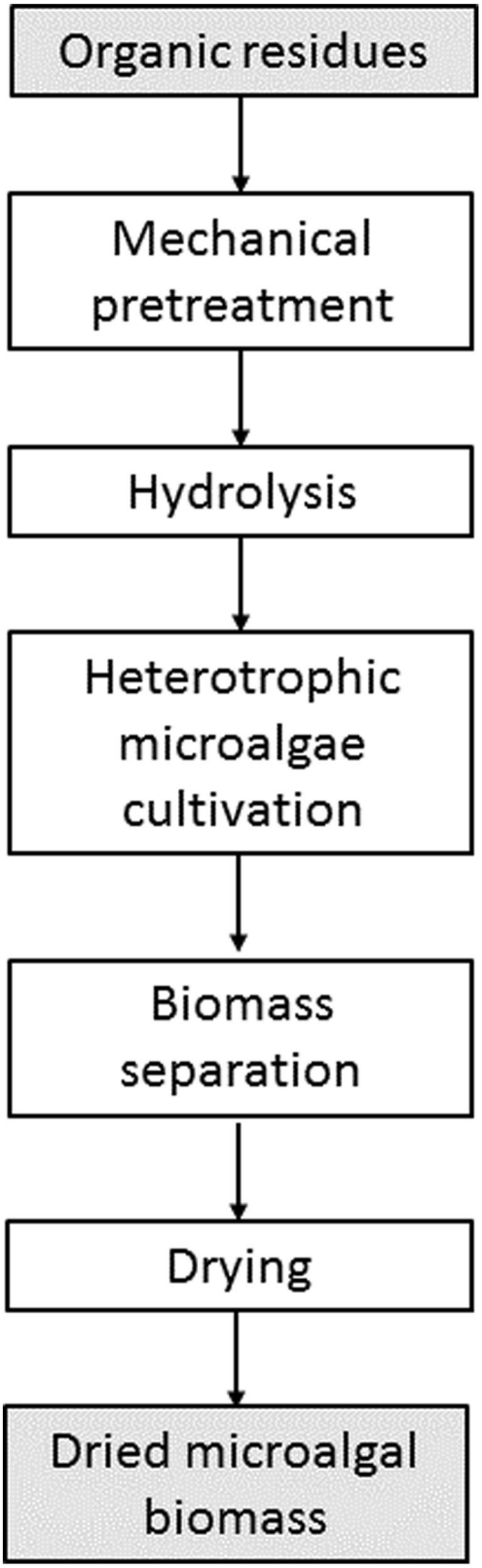

Fig. 1 Process scheme for heterotrophic microalgae cultivation

the boiling point of 100 °C, keeping the temperature for 15 min at 3 consecutive days (Heritage et al. 1997). A potential re-contamination by surviving spores is thus prevented. This technique is especially useful in countries with limited access to technical equipment like pressure resistant tanks required for autoclaving.

Since organic wastes and by-products vary in composition, the content on nutrients should be analyzed, and deficiencies in nitrogen, phosphate, or other macro- and micronutrients should be compensated prior to cultivation. Lau et al. (2014) hydrolyzed bakery and restaurant food waste using the fungi *Aspergillus awamori* and *Aspergillus oryzae* and produced a hydrolysate consisting of 17.9 g L^{-1} glucose, 0.1 g L^{-1} free amino nitrogen, 0.3 g L^{-1} phosphate, and 4.8 mg L^{-1} nitrate which could be used for the growth of *C. vulgaris* biomass without addition of further nutrients.

Non-sterile culture

To date, heterotrophic microalgae cultivation is mainly performed under strictly sterile conditions. Under non-sterile conditions, the culture is overgrown easily by bacteria, which have generally a higher growth rate than microalgae. Contamination control or strictly sterile production can amount to 20–30% of the total production costs (Hayes 2014). One possible approach is to use an extremophile microalgae that thrive at extreme pH, temperature, or salinity, which significantly reduces or prevents competitive microbial growth (Cripps and Bergheim 2000; Pulz and Gross 2004). Because bacteria and other contaminating organisms can adapt to such environments, an extremophile with more than one protective mechanism for open tank reactors is attractive. The red alga *Galdieria sulphuraria* (formerly *Cyanidium caldarium*) is such a candidate. *Galdieria sulphuraria* thrives at pH values between 0.05 and 4 and can resist temperatures of up to 56 °C as well as high salt concentrations. This is among the most extreme growth conditions for known eukaryotes. It is able to grow photoautotrophically, heterotrophically, and mixotrophically and can utilize more than 50 different carbon sources such as sugars and sugar alcohols like glycerol and amino acids (Rigano et al. 1976, 1977; Gross 1999; Oesterhelt et al. 1999). This metabolic flexibility is rarely found under eukaryotic organisms and makes it an ideal candidate for heterotrophic cultivation on organic waste hydrolysate. *Galdieria sulphuraria* replaces phospholipids by betaine lipids as an adaptation to low pH and high temperatures. In addition, the ratio of regioisomers of the polar lipids, phosphor, and glyco- and betaine lipids changes in response to culture media pH (Vítová et al. 2016). The maximum density *G. sulphuraria* grown heterotrophically at low pH of 1.8 on 25 mM glucose was about of 1.9 g L^{-1} (on dry weight basis) (Gross and Schnarrenberger 1995). This is in the range of biomass reported earlier for non-extremophile species. In addition, *Galdieria* can be grown on acid or acid-treated waste achieving a cell density of 2.5 g ash-free dry weight L^{-1} (Selvaratnam et al. 2014). However, *G. sulphuraria* is not yet used for protein production. This may be related to the relative content compared with other microalgae. The protein content of *G. sulphuraria* ranges from 265 mg g^{-1} dm in heterotrophic cultures of up to 325 mg g^{-1} dm in autotrophic cultures (Graziani et al. 2013).

Economic considerations and future outlook

The current production costs of heterotrophically grown *Chlorella* is estimated at around 2–2.6 US$ per kg (Enzing et al. 2014); hence, still there is no economical alternative to fishmeal which is currently available for 1.3 US$ per kg. However, previous cost predictions were based on an expensive carbon source (glucose). The share of glucose in the total production cost ranges from 20% up to 60% depending on the reference cited (Sharma et al. 2011). A share of 20% would equal 0.55 US$. Assuming for example that liquid whey permeate is available free of charge, a saving of around 20% can be expected. The previously reported production costs of 2–2.6 US$ could be reduced to 1.6–2.1 US$ which makes the utilization of microalgae biomass as replacement, e.g. for fish still economically challenging at current fish meal prices. If glucose, however, accounts for 60% of the total production costs as predicted for biodiesel production scenario, costs for microalgae biomass would be reduced down to 1–1.25 US$, which is within or even below the current price for fish meal.

Conclusions

Microalgae, specifically *Chlorella*, can be grown heterotrophically and of protein quantity and quality suitable for animal feeds. In addition, the use of waste streams such as whey permeates may reduce production costs to an economically viable level.

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Compliance with ethical standards

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

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