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Potential of *Chlorella* Species as Feedstock for Bioenergy Production: A Review

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Abstract **– Selection of appropriate microalgae strain for cultivation is essential for overall success of large-scale biomass production under particular environmental and climate conditions. In addition to fast growth rate and biomass productivity, the species ability to grow in wastewater must also be considered to increase the economic feasibility of microalgae for bioenergy purposes. Furthermore, the content of bioactive compounds in a strain must be taken into account to further increase the viability by integration of biorefinery concept. *Chlorella* spp. are among the most studied microalgal species. The present review attempts to unfold the potential of species of the genus *Chlorella* for bioenergy production integrating applicability for wastewater treatment and production of high added-value compounds. Several key features potentially make *Chlorella* spp. highly beneficial for bioenergy production. Fast growth rate, low nutritional requirements, low sensitivity to contamination, adaptation to fluctuating environments, ability to grow in photoautotrophic, heterotrophic and mixotrophic conditions make *Chlorella* spp. highly useful for outdoor cultivation coupled with wastewater treatment. *Chlorella* is a source of multiple bioactive compounds. Most promising high-value products are chlorophylls, lutein, β-carotene and lipids. Here we demonstrate that although many *Chlorella* spp. show similar characteristics, some substantial differences in growth and response to environmental factors exist.**

Keywords **– Biomass; biorefinery; microalgae; wastewater treatment**

1. INTRODUCTION

Microalgae are regarded as a promising sustainable energy source due to their fast growth rate, high productivity and ability to accumulate large quantities of lipids [1]. Microalgae biomass has vast applicability, it can be converted to various types of renewable bioenergy, e.g. biogas, biodiesel, biomethane, biohydrogen, bioethanol. Moreover, microalgae biomass and high added-value compounds extracted from the biomass can be used in food industry, medicine, textile industry, feed, aquaculture, agriculture and cosmetology [2]. Several studies have been conducted on the potential and economic feasibility of large-scale microalgae cultivation for bioenergy production [3]–[5]. However, most studies have concluded that economic viability of bioenergy production from microalgae biomass is still an ambitious goal and vast improvements must be implemented before the stage of a commercial low-cost microalgae biomass production. Currently large-scale biomass production is not viable mainly due to high production costs and low productivity of microalgae strains. Lately studies have been focusing on possible solutions to decrease production costs at the same time increasing the efficiency of biomass yield. Optimisation of cultivation conditions must be attained to increase the productivity of microalgae

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mass cultures. Furthermore, effective and low-cost cultivation technology is essential to increase overall biomass productivity. Open ponds and various types of closed photobioreactors have been utilized for microalgae cultivation with certain advantages and disadvantages. Although no consensus has been reached on the most suitable type of cultivation vessel for large-scale cultures, some researchers have proposed that open pond cultivation is more commercially viable for bioenergy production [2], [3], [6], [7].

Lately several novel strategies have been proposed to increase the efficiency and eliminate the costs of microalgae cultivation. Use of wastewater as a nutrient source for microalgae growth and biorefinery are two of the most promising strategies suggested [8], [9]. Many studies have been conducted recently to test microalgae growth in various wastewaters in search for low-cost nutrients. Microalgae cultivation in wastewater offers the possibility of low-cost biomass production at the same time treating wastewater. Furthermore, integrated extraction of high added-value co-products from microalgae biomass is a more sustainable and economical approach to microalgae biomass utilization. Additionally, integration of a cultivation system close to the combustion power plant can benefit from the use of flue gas as a source of CO_2 for increased microalgae growth.

Selection of an appropriate microalgae strain is a crucial factor for high productivity under the selected environment and for the overall success of large-scale biomass production. There are over 70 000 species of microalgae, many of them have not been characterized [10]. Moreover, only a very small fraction of all species has been used in studies of biomass and bioenergy production. The ideal strain for large-scale outdoor biomass production must have the following characteristics: fast growth rate, wide temperature tolerance, high competitiveness, limited nutrient requirements, high CO_2 uptake, tolerance to shear force and to various contaminants in flue gas (e.g. NO_x, SO_x) and wastewater (e.g. heavy metals, ammonium), adaptation ability to fluctuating environmental conditions (light, pH, etc.) and source high-value co-products [1], [11]. Extensive research has been carried out focusing on selection of microalgal strains that can be cultivated for large-scale biomass yield. Among microalgal strains, various *Chlorella* species have been studied extensively. Green microalga *Chlorella vulgaris* has received much attention and is probably the most studied microalga.

This review attempts to investigate the potential of *Chlorella* species for large-scale microalgae cultivation for bioenergy production, with an emphasis on investigation of the capacity for biorefinery and the use of wastewater streams for cultivation of potential species to increase the economic feasibility of microalgal biofuels.

2. CHARACTERIZATION OF GENUS *CHLORELLA*

2.1. General description of Chlorella

Chlorella is a genus of small, single-celled green algae belonging to the division *Chlorophyta. Chlorella* cells are non-motile without flagella in a size of 2 to 10 µm in diameter [12]. *Chlorella* spp. have been widely studied since early 1950ies when the first large-scale cultivation was set up in USA for biodiesel production; however, commercial cultivation started in 1961. in Japan, where *Chlorella* was grown as a source of protein for food and feed [13]. *Chlorella* spp. are microalgae with high commercial importance [14] and *C. vulgaris* is one of a few microalgae cultivated commercially for food and feed [13]. *Chlorella* is a cosmopolitan genus with its species found in diverse water habitats including freshwater, marine and wastewater [13], [15]. Species can grow well in a wide temperature range that makes them particularly useful for various applications in outdoor conditions.

Research shows that *Chlorella* spp. can withstand temperatures from 5° C to 42° C [16], [17]. Key characteristics of selected *Chlorella* spp. are shown in Table A1 in the Annex.

Although *Chlorella* has low nutritional requirements [13], the species can withstand high nutrient concentration [18] that may be advantageous for cultivation in high strength wastewater thereby increasing its competitiveness over other species of microorganisms particularly in outdoor cultivation. Other characteristics such as fast growth rate, low sensitivity to contamination and unfavourable environments [19] also make *Chlorella* favourable for wastewater treatment and cultivation in open ponds under fluctuating environmental conditions.

Genus *Chlorella* has a simple life cycle. Reproduction is exclusively asexual by cell division, most often producing four to eight daughter cells [20]. Cells have thick resistant walls with glucosamine as a main wall component [20], [21]. It has its advantages and disadvantages. Robust, non-flagellated cells make *Chlorella* shear resistant beneficial for cultivation in bioreactors where cells are less likely to be damaged by mixing. In contrast, large flagellated cells like those of *Dunaliella* spp. are shear sensitive and are more prone to damage during mixing and pumping in bioreactors [18]. However, looking from a biorefinery point of view, the hard resistant cell walls of *Chlorella* are a major drawback as they require pre-treatment for efficient extraction of bio-compounds increasing the extraction time and costs.

2.2. Taxonomy

Classification of *Chlorella* is not straightforward and species cannot be identified based on morphological features alone [13]. More than 100 microalgae from various habitats have been historically assigned to genus *Chlorella* [22]. Classification of *Chlorella* remains a challenge even today. Although, the genus *Chlorella* has undergone extensive changes in recent years, reorganization of the genus is not complete, several new suggestions for rearrangements of the genus have been proposed [14], [20], [23]. More powerful methods than morphological features are required for the identification of species. Novel, more sensitive identification techniques, such as molecular phylogeny and bioinformatics have been introduced making classification more reliable. Use of molecular markers has revealed that many species formerly assigned to *Chlorella* in fact belong to different lineages of green microalgae [24]. Based on biochemical, physiological, ultrastructural characters and molecular tools, Huss suggested only four species to be kept in the genus *Chlorella*, namely, *C. vulgaris*, *C. lobophora*, *C. sorokiniana* and *C. kessleri* [20]. However, another research on taxonomy of *Chlorella* suggests that five "true" *Chlorella* species exist, namely *C. vulgaris*, *C. lobophora*, *C. sorokiniana*, *C. heiozoae* and *C. variabilis* [23]. *Chlorella kessleri* has been reclassified as *Parachlorella kessleri* [22]. Most strains formerly identified as *C. pyrenoidosa* have been reclassified as other strains of the genus *Chlorella* and other taxa [14]. In the study of Kessler and Huss, several strains of *C. pyrenoidosa* from UTEX collection have been tested with biochemical and physiological markers and it was found that most of the strains belong to different strains of *Chlorella* such as *C. vulgaris*, *C. sorokiniana* and *C. fusca* var. *vacuolate* [25]. *C. pyrenoidosa* is no longer a valid species and most of the strains formerly assigned as *C. pyrenoidosa* have now been reclassified. Champenois suggested that *Coelastrella vacuolate* is the current valid name for this *C. pyrenoidosa* [14]. Just a few studies are available on *C. lobophora* [26], [27], therefore, more research is required to assess the potential of *C. lobophora* for bioenergy production.

In the current review we focus on the most frequently studied *Chlorella* species, although some species have been suggested for reclassification into different genus such as *Parachlorella* and *Auxenochlorella*.

2.3. High-Value Products

Lately, microalgal biorefinery is receiving increasing interest. A commercial potential of microalgae biomass is still an untapped resource. Microalgae are a source of bioproducts such as pigments, proteins, lipids, carbohydrates, vitamins and antioxidants with high commercial value. The extraction of these co-products is essential to improving the economic feasibility of microalgal bioenergy. Microalgae biorefinery concept is a new approach for better utilization of biomass potential, achieve higher viability and sustainability of bioenergy and move towards "zero waste" production in a circular economy framework. Biorefinery results in a cost-effective simultaneous production of bioenergy and various valuable bioproducts. Moreover, besides economic benefit biorefinery also minimizes the environmental impact with the more efficient use of resources and reduction of greenhouse gas emissions.

Studies indicate that *Chlorella* biomass has a wide range of potential applications in pharmaceutical, nutraceutical, agricultural and cosmetics industries [28], [29]. *Chlorella* is a source of many high value compounds nevertheless; it has most often been exploited as a protein source. Depending on the culture conditions, *C. vulgaris* contains up to 58 % proteins and *C. pyrenoidosa* 57 % that are between the highest rates of green microalgae [30]. Due to its high protein content *C. vulgaris* is used in health food industry and aquaculture [31]. It has been reported that *C. vulgaris* contains 37 % starch [32] that can be useful for bio-ethanol production. Sulphur deficiency has been shown to increase starch content in cells that is followed by lipid accumulation in *Chlorella* species [33]. Many high value products are secondary metabolites that are biochemical compounds involved in adaptation of microalgae to changing environmental conditions. Examples of secondary metabolites are carotenoids, phycobiliproteins, phenolic compounds, alkaloids and lignin [34]. Synthesis of some secondary metabolites increases under stress conditions such as oxidative, osmotic or nutrient stress. Therefore, stress conditions must be induced to increase the production of these biochemicals.

Chlorella is a source of high value pigments such as lutein, astaxanthin and β-carotene; vitamins, especially vitamin B complex, ascorbic acid, α-tocopherol and several other bioactive compounds [28]. Othman tested 6 green freshwater microalgae and found that *C. vulgaris* had the highest total carotenoid and β-carotene content, 81 µg/g DW and 18 µg/g DW, respectively [35]. Moreover, highest lutein content (69 µg/g DW) was found in *Chlorella fusca*. Although lutein traditionally is extracted from marigold flowers, the production of lutein is hampered by seasonal availability of marigold flowers. Microalgae can contain a considerable amount of lutein [36]–[38] and can be harvested all year round. *Chlorella* has shown good potential as a lutein source. However, lutein content is dependent on cultivation conditions, therefore, lutein extraction rate reported is highly variable. D'Este was able to extract 0.69 mg/g DW lutein from *C. vulgaris* [37]. Wei achieved 1.98 mg/g DW lutein content in *C. protothecoides* [39], but Chen reached 5.88 mg/g DW lutein with two-stage heterotrophic culture of *C. sorokiniana* [38]. Lutein content in *C. minutissima* reached 8.24 mg/g DW in Dineshkumar's study [40], but the highest extraction reported was 10.4 ± 5.5 mg/g DW in *C. vulgaris* in McClure's study using photoautotrophic cultivation mode [41]. In contrast, very low lutein was reported in Othman's study. *C. vulgaris* was found to produce 63 µg/g DW lutein but *C. fusca* 69 µg/g [35]. Although sustainable and economically viable lutein production still needs extensive research, lutein production rate of microalgae is reported to be 3 to 6 times higher than that of marigold flowers [42] and the results achieved so far are promising.

Recently, emphasis has been placed for the search of new bioactive compounds in microalgae with antibacterial, antifungal and anticancer activities. *Chlorella* spp. contain valuable bioactive peptides with antioxidant, anti-inflammatory and anticancer properties [43]. For example, *C. pyrenoidosa* contains polypeptide that exhibits antitumor activity [44]. This field is very promising, but requires more studies. Another example of bioactive compound with potentially high commercial interest is β-1,3-glucan – a polysaccharide best known for its immunostimulatory activity [28].

In agriculture *Chlorella* biomass has been applied as a bio-fertilizer and as a feedstock for animals. Algae biomass have been incorporated as a dietary supplement in farm animal, fish and poultry feed. For example, the addition of *Chlorella* biomass to poultry feed has showed improved growth, immune response and gut microflora [28].

2.4. Wastewater Treatment

Large-scale microalgae cultivation requires considerable amount of water and nutrients that makes up a large part of the cultivation costs. On the other hand, large volumes of wastewater in food and processing industries are generated containing valuable micro- and macro elements that can be used for microalgae cultivation. The use of wastewater as a low-cost nutrient source is one of the strategies proposed to reduce biomass production costs and increase the feasibility of low-cost bioenergy [45]. Wastewaters are complex mixtures with a variable composition depending on their origin. Generally, wastewater streams contain organic, inorganic and man-made compounds [46]. Microalgae are known to remove nutrients and heavy metals from wastewaters to the level that meets the requirements for discharge.

Wastewater use has multiple advantages on microalgae cultivation: (1) it is a source of nutrients for microalgae growth, (2) it provides a sustainable water source, and (3) it is a source of organic carbon for heterotrophic and mixotrophic growth [47]. The main environmental issue of microalgae cultivation – the need for enormous amounts of freshwater thus could be mitigated, moreover, it reduces expenses of nutrients required for microalgae cultivation.

Simultaneous nutrient removal and biomass production requires microalgae species to survive in specific conditions and reach high biomass yield. Species for wastewater treatment must exhibit good pollutant removal capacity mainly ammonium, nitrogen, phosphorus and heavy metals under specific environmental conditions. Due to large quantities of organic carbon in wastewaters, microalgae with heterotrophic metabolism are beneficial. It has been demonstrated that *Chlorella* spp. are capable to grow in autotrophic, heterotrophic and mixotrophic conditions [48]. In contrast to photoautotrophy that use solar energy and carbon dioxide, in heterotrophic metabolism microorganisms can utilize organic compounds from the environment as a source both for energy and carbon [47]. Simultaneous use of carbon dioxide and organic carbon, known as mixotrophy, can more efficiently utilize the available light and organic nutrients form wastewater thus potentially enhancing microalgae growth. Recently many studies have been aiming at optimizing heterotrophic and mixotrophic cultivation to overcome the limitations of autotrophic growth such as light deficiency. Several studies have shown higher efficiency in nutrient removal and biomass production in mixotrophic and heterotrophic cultivation mode compared to photoautotrophic conditions [49]–[51]. When cultivated in wastewater *Chlorella* is able to switch from phototrophic to heterotrophic or mixotrophic growth. Glucose is found to be the preferred source of carbon for *Chlorella* species [52]. Mixotrophy with glucose has resulted in a higher growth rate than autotrophic or heterotrophic cultivation [50]. Some studies have shown that mixotrophic cultivation is the most efficient [51] while others demonstrated better growth in heterotrophic cultivation [49].

Species competitiveness is another important consideration for assessment of species suitability for cultivation in wastewater. Wastewater contains biological contaminants such as bacteria and protozoa, therefore robust and fast-growing microalgae that can outcompete other species are crucial for cultivation in wastewater. Wastewater treatment requires fast and efficient pollutant removal in a possibly shortest period of time therefore, in addition to fast growth rate the potential algal strain must also be tolerant to weather fluctuations and high nutrient concentrations. *Chlorella* spp. are natural inhabitants of wastewater ponds [15], [53] and can survive in various wastewater streams showing great potential to adapt to various environmental conditions [15], [54]–[56]. Oberholster demonstrated that a combination of *C. vulgaris* and *C. protothecoides* is effective in nutrient removal from wastewater stabilization ponds (75 % total phosphorus and 43 % total nitrogen removal) and *Chlorella* spp. stayed dominant after inoculation of ponds, moreover other microalgae species coexisted with *Chlorella* spp. in treatment ponds [57].

Chlorella spp. are found to be between predominant strains in wastewater ponds. Exploring waste stabilization ponds Palmer found that the most abundant and frequent genera were *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium* and *Golenkinia* [58]. Furthermore, Palmer published another study with the results of an extensive research covering 165 studies and reported that *Chlorella* is between the top eight pollutant-tolerant genera [53]. Moreover, screening top 17 strains with the best performance in wastewaters collected locally from natural freshwater habitats and wastewater, Zhou found that 60 % belongs to *Chlorella* spp. [15] demonstrating superiority of *Chlorella* over other microalgae strains and indicating its potential for wastewater treatment. Ayre studied microalgal consortium in anaerobic digestate of piggery effluent with high ammonia content and found that *Chlorella* was dominant species at all ammonium concentrations [59]. Moreover, the consortium was able to grow in 800 and 1600 mg NH_4^+-N L^{-1} showing superior resistance to high ammonium concentrations than other microalgae species. *Chlorella* spp. have been used in numerous studies and have shown good nitrogen and phosphorus removal rates. Generally, *Chlorella* spp. can remove 23 %–100 % nitrogen while phosphorus removal efficiency is 20 %–100 % [45]. However, not all microalgae strains can grow in wastewater. Caporngo observed lower growth and nutrient removal level of *Nannochloropsis ocultata* compared to *C. vulgaris* and *C. kessleri* [55]. Alvarez-Diaz found that *Neochloris oleoabundans* did not grow in wastewater [60]. According to Caporngo freshwater microalgae are preferable to wastewater cultivation than marine algae [55]. However, Chinnasamy observed that also marine algal species exhibit good growth in some wastewater (e.g. carpet mill effluent) without salt addition [61].

2.5. Biomass Yield and Lipid Production

Chlorella spp. are among the fastest growing microalgae, often reported being superior to other species [1], [62]. However, because growth rate is highly dependent on cultivation system and growth conditions, reported values are very wide making comparison between studies difficult. Growth rates and biomass productivity of *Chlorella* spp. are summarized in Table A2 in the Annex. Li compared biomass productivity of various *Chlorella* species and observed the highest productivity for *C. kessleri* UTEX 398 (2.01 g TVSS/L), followed by *C. protothecoides* strains UTEX 25 and UTEX 256 [48]. The lowest productivity (0.38 g TVSS/L) was observed for *Chlorella fusca* var. *vacuolata*, the species that is no longer assigned to genus *Chlorella* and reclassified as *Coelastrella vacuolate*. In the same study two strains of *C. sorokiniana* (UTEX 1230 and UTEX 2805) exhibited biomass productivity of 0.70 and 0.76, respectively.

Chlorella species are capable of accumulating significant amounts of lipids, generally under stress conditions; furthermore, several strains are producing a fatty acid profile suitable for biodiesel production [2], [63], [64]. Lipid content in *Chlorella* spp. under normal growth conditions is generally around 20 %, higher lipid content has been reported in *C. minutissima* (31 %) but lower in *C. protothecoides* (11 %) [65]. However, by adjusting the growth conditions lipid content can reach >50 % [2], [65]. Nitrogen limitation is an effective strategy to increase lipid content in all *Chlorella* strains [65]. Reported lipid concentration in *C. vulgaris* ranges from 5 % to 58 % (DW) and lipid productivity from 11 to 40 mg L d^{-1} [34]. Such a wide range of values could be explained with various growth conditions used in different studies.

Illman compared the growth and lipid production of five strains of *Chlorella*, *C. vulgaris*, *C. emersonii*, *C. protothecoides*, *C. sorokiniana* and marine strain *C. minutissima* [65]. Under nitrogen deficiency conditions the growth rate decreased in all strains except *C. minutissima* which remained in the same level. Highest lipid content was achieved in *C. emersonii* (63 %), *C. minutissima* (57 %) and *C. vulgaris* (40 %). Microalgae *C. vulgaris* and *C. emersonii* are promising species because of high growth rates that stay relatively high also under N limitation condition coupled with good lipid productivity. *C. minutissima* showed no decrease in growth rate under N limitation conditions and high lipid content. *C. emersonii* and *C. minutissima* show high lipid content in optimal growth conditions, 29 ± 2.5 % and 31 ± 3.2 %, respectively. Although, reported lipid productivity of *Chlorella* spp. is variable, high lipid content achieved in some studies are suggesting that high lipid concentration in *Chlorella* can be reached, however, optimization is required.

3. CHARACTERIZATION OF *CHLORELLA* SPECIES

3.1. Chlorella vulgaris

C. vulgaris is a type species of genus *Chlorella* and the most widely studied algae of the genus. *C. vulgaris* has spherical, non-motile single cells with a cell size from 2 to 10 µm in diameter [2]. *C. vulgaris* is a freshwater species and is known as one of the fastest growing microalgae strains with a doubling time of 16 h in photoautotrophic conditions [65]. *C. vulgaris* has rigid cell wall mainly composed of a chitosan-like layer, cellulose, hemicellulose, proteins, lipids and minerals [66]. Cells have a single chloroplast. *C. vulgaris* can accumulate starch or lipids under unfavourable conditions stored in cytoplasm or chloroplast [2], [65]. Reproduction is asexual by autosporulation. Most commonly four daughter cells are formed. *C. vulgaris* has a remarkable ability to withstand a wide range of temperatures, especially low temperatures. It has been demonstrated that *C. vulgaris* can withstand 5 °C and still do slow but continuous growth [16]. However, cells are not resistant to high temperature, already at 30 °C considerable decrease in cell viability has been observed [67]. Optimal temperature of the species is between 25 °C and 28 °C [67], [68].

Although a more alkaline medium is generally thought to be optimal for *C. vulgaris* growth [69], other studies have found that neutral pH (pH 7) leads to a higher growth rate [70]. While a vast number of studies on *C. vulgaris* have been performed, data reported can significantly vary. For instance, it has been reported that *C. vulgaris* contains 42 %–58 % total proteins, but lipid content under optimal growth conditions varies between 5 % and 40 % of dry weight (DW) [2]. The observed wide range of values reported most likely originates from various growth conditions applied in different studies. Lipid content under normal growth conditions of *C. vulgaris* is around 20 % [65]; however, during stress conditions, normal biochemical composition of cells changes, and an increase in lipids and decrease in proteins is often observed. Application of stress such as nitrogen starvation, can increase lipid content up to 58 % [2] and lipids are mainly in the form of triacylglycerols (TAG). For example, dos Santos observed total lipids 19.6 % DW and 27.7 % TAG under optimal growth conditions [66]. Lipid content increased to 25.4 % DW after nitrogen starvation was applied, moreover, TAG content increased to 41.3 %. Lower percentage of PUFAs was also observed under nitrogen starvation mode compared to optimal growth conditions being more suitable for biodiesel production [66].

C. vulgaris is a source of bioactive compounds with commercial value that could be used for a biorefinery approach. *C. vulgaris* is rich in proteins, carbohydrates, lipids, pigments, minerals and vitamins [2]. Therefore, it has vast applicability in various fields such as human food, animal feed, cosmetology and medicine. Cells contain significant amounts of chlorophyll. Their content in *C. vulgaris* cells can reach up to 1–2 % DW [2]. Cells also contain significant amounts of carotenoids, such as β-carotene, astaxanthin and lutein that have multiple therapeutic properties. Lately many studies have focused on optimization of pigment extraction and increase in pigment content [37], [41]. *C. vulgaris* biomass has been used as a biofertilizer with good results [28].

C. vulgaris has demonstrated high potential for wastewater treatment. Rapid growth and high nutrient removal have been shown in various wastewater streams such as urban [55], industrial [61] and agricultural wastewater [56], [71]. *C. vulgaris* has shown some remarkably high ammonia nitrogen and total nitrogen removal rates over 96 % and total phosphorus removal 69 to 98 % in various wastewaters [55], [56], [71], [72]. Efficiency of heavy metal removal depends on the species, *C. vulgaris* has shown good removal of cadmium, copper and zinc [73].

3.2. Chlorella sorokiniana

C. sorokiniana is the most heat and high light resistant species in the genus *Chlorella* [74]. Species can tolerate temperatures up to 42 °C [17], [75]; however, optimal growth temperatures seem to depend on a combination of several biotic and abiotic factors, as reported optimal temperatures vary across studies and are in range from 30 °C to 40 °C [74], [76], [77]. Still, the most frequently reported optimal temperatures are 36 °C–38 °C [78]–[80]. The performance under extreme environmental conditions was demonstrated by Morita et al., who observed good photosynthetic productivity even at 46.5 °C that was coupled with high light intensity [77]. The common growth temperature which is optimal for some other microalgae strains is not suitable for *C. sorokiniana*. Cuaresma Franko found that temperature below 20 °C had an inhibitory effect on microalga growth [79]. *C. sorokiniana* can withstand not only high temperatures but also high intensity light up to 2500 μmol m^{-2} s^{-1}[74]. Testing five different light intensities of 100, 200, 400, 600 and 750 μmol m^{-2} s^{-1}, Tan found that at 750 μmol m^{-2} s^{-1} resulted in the best growth, indicating higher light requirements than other common microalgae species [31].

Considering tolerance to high temperature and light intensity *C. sorokiniana* can be a good candidate strain for biomass production in outdoor cultivation systems in regions with high insolation. Open ponds tend to reach high temperatures and light intensity especially during mid-day [81], often exceeding the optimum temperature of the strain particularly in the upper layer of the water. Therefore, a heat resistant strain is preferred in these conditions. Temperature has an impact also on lipid productivity. Li observed that highest lipid content of *C. sorokiniana* was reached at 30 °C (37 %), however the highest lipid yield at 37 °C [80].

C. sorokiniana has substantial tolerance to high nutrient concentrations in wastewater and is able to remove up to 99 % of nitrogen and phosphorus depending on the initial concentration [82]. Microalga has exhibited good capability of ammonium removal under extreme temperature and light conditions [74]. Kim [49] found that *C. sorokiniana* exhibited the best growth rate and nutrient removal while cultivated under heterotrophic conditions with glucose as a carbon source compared to autotrophic and mixotrophic conditions. Moreover, the growth rate was more than two-fold higher for heterotrophic cultures than autotrophic [49]. However, Li found that mixotrophy resulted in considerably higher biomass concentration, growth rate and lipid productivity than either heterotrophic or mixotrophic cultivation [83]. Rosenberg reported a rapid nine-hour heterotrophic doubling time [84], while Rai found a remarkable doubling time of 2 h 9 min under mixotrophic conditions [70].

3.3. Chlorella protothecoides (Auxenochlorella protothecoides)

C. protothecoides is a robust, fast growing species able to grow in various wastewaters [57], [72]. The species has received most attention regarding biomass production under various cultivation modes, specifically mixotrophic and heterotrophic conditions to increase lipid production. Furthermore, extraction of value-added compounds, mainly pigments, has been a focus of *C. protothecoides* cultivation [85], [86]. This species is a valuable source of bioactive compounds. Particular attention has been paid to extraction of pigments. High concentrations of carotenoids and chlorophylls have been found under phototrophic and mixotrophic growth modes. Higher cellular accumulation of pigments has been observed at phototrophic mode, however concentration per unit volume was higher under mixotrophic growth [85]. Salt and light stress are known to induce the carotenogenesis process in *C. protothecoides* allowing accumulation of astaxanthin and lutein/zeaxanthin [85]. *C. protothecoides* CS41 had the highest biomass yield and lutein content when seven Chlorella strains were compared (3 strains of C. pyrenoidosa, three strains of C. vulgaris and one strain of *C. protothecoides*) under heterotrophic conditions using glucose as a carbon source [87].

Li was able to achieve 48.7 % lipid content in heterotrophic conditions in 750 L bioreactor [88]. In the same study, successful scale-up was demonstrated, heterotrophic culture density reached 15.5 g L^{-1} in 5 L, 12.8 g L^{-1} in 750 L, and 14.2 g L^{-1} in 11 000 L bioreactors. Shi succeeded to reach remarkable 48 g L^{-1} biomass yield in a 3.7 L fermenter and 45.8 g L^{-1} in upscaled 30 L fermenter [89]. Moreover, lipid content reached 57.8 % in batch and 55.2 % in fed-batch culture of heterotrophic *C. protothecoides* grown on glucose [63].

Studies indicate that *C. protothecoides* can grow in different wastewaters with similar performance and is resistant to high chemical content. Microalga showed good performance in raw, untreated urban wastewaters exhibiting high growth rate and efficient removal of N and P [90]. Results showed that endogenous bacterial contamination did not limit algal growth rate. *C. protothecoides* demonstrates a high growth rate and efficient removal of NH4+-N also from various anaerobic digestion effluents [72]. An additional benefit of this species is the significantly faster settling of cells compared to *C. vulgaris* that is particularly important for biomass harvesting [72].

3.4. Chlorella kessleri (Parachlorella kessleri)

C. kessleri cells are larger than *C. vulgaris* [55] that might be advantage for biomass harvesting. *C. kessleri* has been studied for its potential for biodiesel production and extraction of high value products. TAG accumulation in *C. kessleri* is induced by high light intensity, hyperosmosis and nutrient limitation. Hayashi showed that low temperature could also stimulate TAG accumulation but only for a limited time [91]. Moreover, the same study demonstrated very high TAG accumulation up to 48.5 % in *C. kessleri* cells due to synergetic effects of hyperosmosis, nutrient-limitation, increased light intensity and low temperature. A notable 54.7 % total fatty acid content was achieved in mixotrophic cultivation with 300 mmol L^{-1} glucose under nitrate depletion conditions that was about 5-hold increase compared to autotrophic cultures [92]. Some high value bioactive compounds have been extracted from *C. kessleri* biomass. Although lutein content in *C. kessleri* cells is not significant, the strain is a natural source of astaxanthin. Soares reported nearly 23 mg g^{-1} astaxanthin in photoautotrophic cultivation conditions [93]. Encouraging results have been achieve for its application in wastewater treatment. Biomass production of *C. kessleri* in wastewater is reported to be comparable to that of *C. vulgaris* [55]. *C. kessleri* has showed more tolerance to some pollutants, like chromium, copper and herbicide than other microalgae species [92]. It was capable to remove 94 % of chemical oxygen demand and 96 % of NH4+-N and P from aquaculture wastewater just in 3 days and was superior to Scenedesmus spp. and *C. vulgaris* [94]. Likewise, *C. kessleri* has demonstrated high uptake of N and P also in urban wastewater showing more than 96 % and 99 % removal, respectively [55].

3.5. Chlorella minutissima

C. minutissima is a high CO_2-tolerant microalga with easy cultivation and fast growth [64]. It has small unicellular spherical cells from 2 µm to 4 µm in diameter when grown in synthetic medium and larger cells, from 2 µm to 8 µm in medium with organic carbon [95]. *C. minutissima* is tolerant to pollution and fluctuating environmental conditions [95]. It can grow at exceptionally wide pH range from 4 to 10, although growth at pH 4–5 is strictly constrained. The optimum growth has been observed at pH 7 [95]. Tolerance to a wide pH range is especially valuable in open raceway pond cultivation where control of environmental parameters is not always straightforward. Moreover, another advantage for outdoor cultivation is dominance over other microorganisms diminishing the risk of contamination with fungi, bacteria and other algae [95].

It has been noted that *C. minutissima* has a fatty acid profile desirable for biodiesel production [64]. Moreover, nitrogen starvation is an effective method for enhancement of total lipid and TAG content in *C. minutissima* [64]. Tang found that neither light source nor intensity or photoperiod had a significant effect on fatty acid methyl esters (FAME) content [64]. Lipid content can reach 57 % when cultivated in low nitrogen medium [65].

C. minutissima can grow in photoautotrophic, heterotrophic and mixotrophic conditions, however, Bhatnagar found that growth in heterotrophic conditions was significantly lower than that of autotrophic whatever the carbon source was used [95]. *C. minutissima* can utilize several carbon sources, such as glycerol, glucose, succinate, molasses and press mud [96]. According to Bhatnagar, glucose is the preferred carbon source for mixotrophic growth and resulted in synergistic growth in the presence of light [95]. Other study proposed that glycerin is the optimal carbon source however, glucose was not tested in this study [97]. *C. minutissima* shows halotolerance up to 3 % NaCl suggesting potential application in treating municipal wastewaters that are often characterized by high sodium content [95]. Bhatnagar demonstrated that *C. minutissima* exhibits better growth on diluted wastewater (up to 75 %) compared to synthetic BG-11 medium [95]. Moreover, 50 % wastewater supported 146 % better growth than BG-11 medium indicating high potential of this microalga for wastewater treatment

4. Conclusion

Data on the growth rate and productivity of microalgae reported in the literature varies extensively. The observed dispersion of data is mainly due to cultivation conditions of the microalgae. Growth rate, biomass productivity and lipid content of a microalgal species are parameters particularly difficult to compare across studies as they depend highly on cultivation conditions such as light intensity, temperature, photoperiod, cultivation mode (batch, semi-batch, continuous), metabolic conditions (phototrophic, heterotrophic or mixotrophic growth), scale of the cultivation, growth media and nutrients used (synthetic growth media, wastewater etc.). All these parameters make comparison of various experiments and microalgae strains difficult. Every experiment is carried out in unique conditions and are generally not comparable across studies. Thus, it is of great importance to compare different microalgal strains in one study under the same culturing conditions. There are not enough studies comparing several productive microalgae strains simultaneously to get comprehensive comparable results for the selection of the most promising strains. Another aspect to consider is the degree of variation between strains of the same species. Specific strains have been isolated from different habitats under various environmental conditions and can therefore exhibit different responses to various conditions.

Cosmopolitan species of the genus *Chlorella* can be found in diverse habitats throughout the world. A number of key features potentially make *Chlorella spp.* highly beneficial for large-scale biomass production. Fast growth rate, low nutritional requirements, low sensitivity to contamination and flexibility to fluctuating environments, ability to grow in autotrophic, heterotrophic and mixotrophic conditions make *Chlorella spp.* highly useful for outdoor cultivation coupled with wastewater treatment. Results of the present study demonstrate that *Chlorella spp.* are suitable feedstock for bioenergy production. Some studies have reported very high growth rates of various Chlorella species supporting the goal of high biomass yield. *C. vulgaris* is often receiving the highest rating among microalgae strains in terms of growth rate, resistance to pollution and lipid productivity, moreover it can withstand wide temperature range showing its usefulness in outdoor conditions. Results indicate that *C. vulgaris* is not only a widespread model organism but holds real potential for bioenergy production. *C. sorokiniana* shows potential at locations with warmer climates and high insolation due to its resistance to high temperature and light intensity. Several studies have shown that *Chlorella* species are natural inhabitants of wastewater ponds indicating their potential in wastewater treatment. Indeed, all *Chlorella* species studied are suitable for cultivation in various wastewater streams showing high nutrient removal rates and resistance to contaminants. However, the suitable dilution rate of a stronger wastewater must be obtained to exclude the inhibitory effect of excessive ammonium level. Furthermore, *Chlorella* is a source of many bioactive compounds with commercial value that can be co-extracted to further increase the viability of microalgal bioenergy. The most promising value-added products are chlorophylls, lutein, β-carotene and lipids. The drawback for biorefinery is a thick resistant cell wall that makes downstream processing of algal biomass difficult. On the other hand, a thick cell wall makes *Chlorella* shear resistant and is an advantage for cultivation in bioreactors. Numerous studies show that *Chlorella* species hold great potential for a large-scale biomass production, however optimization of cultivation conditions is of primary importance to achieve high biomass yield and increase the content of high value compounds

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FLPP FUNDAMENTAL AND APPLIED RESEARCH PROJECTS

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ANNEX

TABLE A1. KEY PARAMETERS OF SELECTED *CHLORELLA* SPECIES

Species	Growth conditions					Optimum CO_2 absorption	Cultivation type	Application in wastewater treatment	High-value compounds	Reference
	Temp. opt.	**Temp. min.**	**Temp. max.**	**pH opt.**	**Light, opt. µmol /m²/s**					
C. vulgaris	25–28	5	28–30	7–10	150–750	4–15 %	Phototrophic Heterotrophic Mixotrophic	Urban Industrial Agricultural Municipal	Chlorophyll Lutein, β-1,3-glucan	[12], [14], [15], [47], [48], [56], [68], [1], [17], [41], [55], [57], [61], [69]–[71]
C. sorokiniana	28–40	20	38–42	6–7.5	100–2500	5 %	Phototrophic Heterotrophic Mixotrophic	Synthetic Municipal Agricultural	Chlorophyll Lutein	[31], [60], [74], [76], [78]–[80], [100], [101], [77], [82], [102]–[104]
C. protothecoides	25–30	NA	28 - 32	NA	30–150	NA	Phototrophic Heterotrophic Mixotrophic	Industrial Brewery waste Municipal	Chlorophyll Astaxanthin β-carotene Lutein	[39], [57], [61], [85], [86], [89], [105], [106]
C. kessleri	26–30	NA	34–36	NA	70–150	18 %	Phototrophic Heterotrophic Mixotrophic	Urban Aquaculture	Astaxanthin	[1], [20], [55], [91]–[94]
C. minutissima	25–30	NA	32	7	350	NA	Phototrophic heterotrophic Mixotrophic	Municipal	Lipids	[17], [64], [95]–[97] [107]

TABLE A2. GROWTH RATE, BIOMASS YIELD AND LIPID CONTENT OF *CHLORELLA* SPECIES

Species	**Max specific growth rate, μ_{max}, d^{-1}**	**Biomass yield, g L^{-1}**	**Biomass productivity, g L^{-1} d^{-1}**			**Lipid content, %/ Lipid productivity mg L^{-1} d^{-1}**	**Reference**
			Phototrophic	**Heterotrophic**	**Mixotrophic**		
C. vulgaris	0.293–1.457	0.4–20	0.02–4.64	0.105	2–5	5–58 %/7.5–132.4	[1], [15], [31], [62], [65], [66], [108]–[110]
C. sorokiniana	0.397–1.60	25–37.6	0.18–4.35	0.7–12.2	0.7–1.98	24–31.5 %/49.4–94.8	[15], [31], [38], [48], [80], [111]
C. kessleri	1.27	4.46–13	NA	NA	2.01	48.5–54.67 %/3.3–7110	[48], [91], [92], [112]
C. protothecoides	0.33–0.92	12.73–51.2	0.27	0.88–6.6	1.2–1.31	44.3–57.8 %/77.7–2120	[15], [48], [63], [65], [86]–[89]
C. minutissima	0.43	NA	0.143	0.76–1.78	0.76	5–15 %	[48], [64], [65], [95], [97]

NA – data not available.