

Review Article

Chlorophyll Extraction from Microalgae: A Review on the Process Engineering Aspects

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Chlorophyll is an essential compound in many everyday products. It is used not only as an additive in pharmaceutical and cosmetic products but also as a natural food colouring agent. Additionally, it has antioxidant and antimutagenic properties. This review discusses the process engineering of chlorophyll extraction from microalgae. Different chlorophyll extraction methods and chlorophyll purification techniques are evaluated. Our preliminary analysis suggests supercritical fluid extraction to be superior to organic solvent extraction. When compared to spectroscopic technique, high performance liquid chromatography was shown to be more accurate and sensitive for chlorophyll analysis. Finally, through CO₂ capture and wastewater treatment, microalgae cultivation process was shown to have strong potential for mitigation of environmental impacts.

1. Background

Microalgae are microscopic unicellular organisms capable to convert solar energy to chemical energy via photosynthesis. They contain numerous bioactive compounds that can be harnessed for commercial use. Marine microalgae in particular have unique biochemical characteristics not found in higher plants [1]. Microalgae are usually cultivated in open ponds or photobioreactors. The cultivation process requires carbon dioxide, light, water and other nutrients which facilitate the photosynthetic process. Microalgal cultivation captures greenhouse gas CO₂ while simultaneously produces biomass containing high-value consumer products.

Chlorophyll is one of the valuable bioactive compounds that can be extracted from microalgal biomass. It is used as a natural food colouring agent and has antioxidant as well as antimutagenic properties. The process of extracting chlorophyll from marine microalgae begins with dewatering and desalting the highly dilute microalgal culture (biomass concentration = 0.1–1% w/v). Chlorophyll is then extracted from the dried biomass by organic solvent extraction or supercritical fluid extraction. This process is followed by a fractionation step to separate the chlorophyll pigments and derivatives. Many studies have been carried out to optimize

chlorophyll extraction and fractionation from microalgae. This review focuses on the entire chlorophyll production process beginning from microalgae cultivation to chlorophyll fractionation and purification.

1.1. Chlorophyll Classification. There are two main types of chlorophyll, chlorophyll *a* and chlorophyll *b*. However, exposure of chlorophyll molecules to weak acids, oxygen or light accelerated their oxidation and resulted in the formation of numerous degradation products [2, 4, 5]. Figures 1, 2, and 3 shows the structures of the chlorophyll compounds. The skeleton of chlorophyll molecule is the porphyrin macrocycle, which comprises of four pyrrole rings [6, 7]. An attachment of a single isocyclic ring to one of the pyrrole rings gives rise to the phorbins structure [6]. Each pyrrole ring contains four carbon atoms and one nitrogen atom. All of the nitrogen atoms face inward creating a central hole where an Mg²⁺ metal ion easily binds [7]. In chlorophyll *b*, the methyl group in ring II of chlorophyll *a* is replaced by a formyl group [5, 7]. This structural difference results in chlorophyll *a* being a blue/green pigment with maximum absorbance from 660 to 665 nm and chlorophyll *b* being a green/yellow with maximum absorbance from 642 to 652 nm [2].

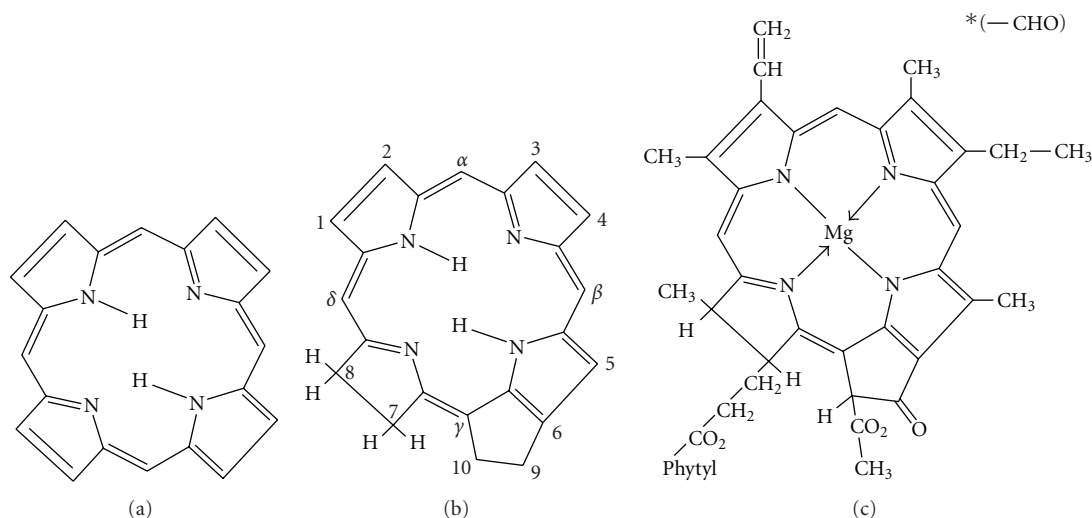


FIGURE 1: Chemical structures of chlorophyll and its constituents, extracted from [2]. (a) porphyrin macrocycle. (b) phorbins. (c) chlorophyll a, chlorophyll b is a variant with the methyl group in position 3 being replaced by a formyl group.

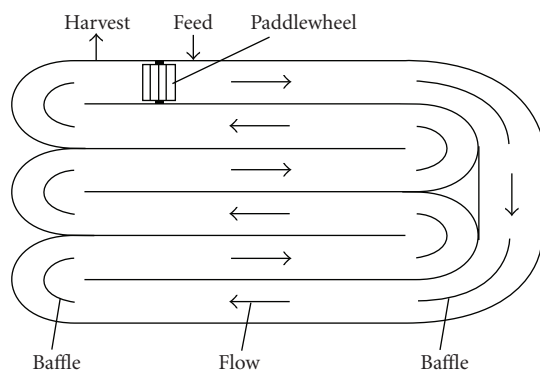


FIGURE 2: Raceway pond for open-air microalgae cultivation, extracted from [3].

1.2. Uses of Chlorophyll. Chlorophyll is present abundantly in nature and, due to its critical “light harvesting” role in photosynthesis, is vital to the survival of both the plant and animal kingdoms [6]. Chlorophyll selectively absorbs light in the red and blue regions and therefore emits a green colour. Photosynthesis is a process which uses this harvested light energy together with water and carbon dioxide to produce oxygen and carbohydrates; as such, it converts solar energy into chemical energy. The products from this chemical process reflect its significance, with carbohydrates being the primary building block for plants and oxygen being necessary for the survival of animal kingdom [6]. The importance of photosynthesis for life on earth is further highlighted by plants forming the basis of all food chains. Chlorophyll is a compound that is decomposed and reproduced continuously in significant amounts both terrestrially and in the oceans. It is estimated that 1.2 billion tons of chlorophyll are produced annually in the planet [6].

Chlorophyll is used as a colouring agent due to its selective absorbance of light of certain wavelengths and

its consequent green colour. Changes in market demands and legislation have resulted in the requirement of natural colouring agents to be used in food products in preference to artificial colourings [8]. Colouring is essential for both consumers and manufacturers, as many foods lose their original colours due to chemical processes they undergo. Consumers demand products of original appearance, while manufacturers desire uniformity for all products [8, 9]. Chlorophyll in plants is confined in chloroplasts where it is not only complexed with phospholipids, polypeptides and tocopherols but also protected by a hydrophobic membrane [6]. When chlorophyll is removed from this protective environment, its magnesium ion becomes unstable and may easily be displaced by a weak acid. In order to overcome this problem, the magnesium ion is often substituted with a copper ion to form a highly stable blue/green complex [6, 9].

Although chlorophyll is a natural food colouring agent, there are disadvantages associated with its use. Its pigment content is not precisely known and it tends to be unstable under the different pH conditions of the foods to which it is added. Additionally, it is more expensive than artificial colourings [8] and it must undergo a chemical modification by replacing the magnesium centre with a copper ion in order to improve its stability as a food colouring agent. Since the copper complex is not absorbed by the body and is removed in its entirety as an excretion product, it is considered to be safe and is permitted to be used in most countries as a food additive. However, the concentration of free ionisable copper in the colouring must be kept below 200 ppm under current regulations [2, 9].

Chlorophyll and its derivatives are also used widely in pharmaceutical products. Chlorophyll has been found to accelerate wound healing by more than 25% in some studies. Since chlorophyll stimulates tissue growth, it prevents the advancement of bacteria and speeds up the wound healing process [10, 11]. Chlorophyll is similar in chemical structure to haemoglobin and, as such, is predicted to stimulate tissue

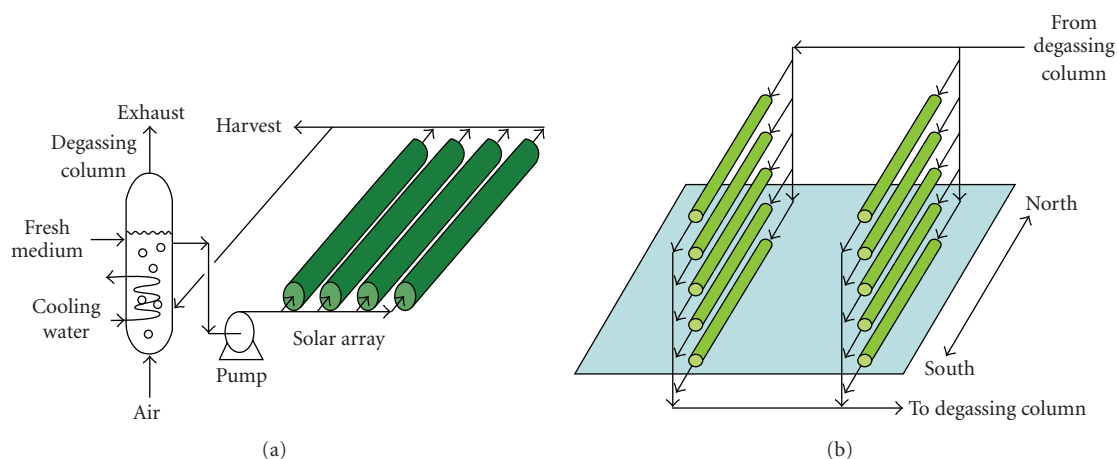


FIGURE 3: Different configurations of tubular photobioreactors, extracted from [3]. (a) parallel run horizontal tubes. (b) fence-like tubular array.

growth in a similar fashion through the facilitation of a rapid carbon dioxide and oxygen interchange [12]. Because of this property, chlorophyll is used not only in the treatment of ulcers and oral sepsis but also in proctology.

Chronic ulcer is a significant health problem in society, with lengthy periods required for its treatment [13]. Chlorophyll's ability to increase the rate of healing is a breakthrough for ulcer sufferers. The application of ointments containing chlorophyll derivatives was found not only to eliminate pain after several days but also to improve the appearance of the affected tissues [13]. The discharge from the ulcer and its characteristic odour also improved significantly after a few days of chlorophyll treatment [13].

The same properties which make chlorophyll a key compound in the treatment of ulcers also make it vital in the treatment of post-operative wounds from rectal surgery. When proctologists remove large areas of tissue, healing can be difficult and the area of the removed tissue tends to be painful [12]. The stimulation of cells in the host and the consequent acceleration in the tissue formation upon chlorophyll application increase the rate of healing in many cases by 25% [12]. Additionally, chlorophyll was found to remove odours from the wound after a few applications [12]. Its non-toxic nature, antibacterial property and deodorising function make chlorophyll a key product in the treatment of oral sepsis [14].

Chlorophyll *a* and its derivatives also have profound antioxidant properties [15]. Chlorophyll derivatives such as pheophorbide *b* and pheophytin *b* have always been known as strong antioxidants. However, these derivatives exist in very low concentrations in fruits and vegetables [15].

Fruit and vegetable consumption has been associated with decreasing the risks of cancer. Phytochemicals present in these foods, particularly chlorophyll and its derivatives, have been suggested to play a key role in cancer prevention due to their high displays of antioxidant and antimutagenic activities [15, 16]. The most significant activity of chlorophyll derivatives in the prevention of cancer is the trapping of mutagens in the gastrointestinal tract [16].

2. Capturing CO₂ with Microalgae

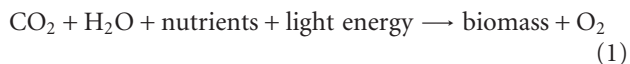
Over the past several decades, global warming has become a serious threat to both humans and nature [17]. The increase of Earth's surface temperature can bring about extreme weather occurrences, rise in sea levels, extinction of species, retreat of glaciers and many other calamities. The rise in global temperature is attributed to the high amount of carbon dioxide (CO₂) gases in the atmosphere [18, 19]. CO₂ is emitted from the burning of fossil fuels for electricity, transport, and industrial processes [17, 18, 20]. Due to the serious threat of global warming, the Kyoto Protocol in 1997 proposed a reduction of greenhouse gases by 5.2% based on the emissions in 1990.

Since then, many CO₂ mitigation options have been considered to meet the proposed target [17]. The various strategies suggested can be classified into two main categories: chemical reaction-based approaches and biological mitigation. Chemical reaction-based strategy captures CO₂ by reaction with other chemical compounds before the CO₂ is released to the atmosphere. The disadvantage of this method is that the chemical reactions are very energy-intensive and costly. Furthermore, both CO₂ and the wasted chemical compounds need to be disposed of. On the other hand, biological mitigation is deemed to be more favourable as it not only captures CO₂ but also generates energy through photosynthesis [19]. This method will be much simpler than physical CO₂ sequestration [21].

Photosynthesis is carried out by all plants and any photosynthetic microorganisms. Even though the use of plants to capture CO₂ is viable, it is by no means efficient owing to its slow growth rate. On the other hand, microalgae as photosynthetic microorganisms are able to capture solar energy and CO₂ with an efficiency of 10 to 50 times greater than that of higher plants [19]. Microalgae includes both prokaryotic cyanobacteria and eukaryotic unicellular algae [17]. The structural and functional simplicity of these microorganisms makes them a better choice for research purposes than any other terrestrial plants [22]. In addition,

microalgae have rapid growth rates and higher productivities than any other plant systems. Microalgae can also grow in variable environmental conditions [19, 20, 23].

Apart from CO₂ and sunlight, microalgae also need nutrients, trace metals and water to grow [24]. In short, biomass from microalgae cultivation is produced based on the following reaction:



see [25].

Unlike plants, microalgae can be cultivated with waste or brackish water despite its abundance of heavy metals and pathogens. The nitrogen and phosphorous in waste water can be directly used by the microalgae as nutrients. Hence, use of chemicals and freshwater needed for algal cultivation can be reduced. It also means that it is entirely feasible to incorporate wastewater treatment process with microalgal cultivation [17, 19].

There are over 30,000 species of microalgae in existence on the Earth and they contain a variety of chemicals that have commercial values [26]. Thus, the biomass produced from microalgal cultivation has numerous uses [17]: (1) a source of biofuels (biodiesel and bioethanol); (2) a nutritional supplement for humans in the form of tablets, capsules, powders and liquids; (3) a natural food colorant; (4) a natural food source for many aquacultural species; (5) a nutritional supplement for animals to improve immune response and fertility; (6) for cosmetic supplements; (7) a source of high-value functional ingredients like polyunsaturated fatty acids, ω -3 fatty acids, pigments, and stable isotope biochemicals; (8) a raw material for the formation of 'biochar' through pyrolysis that can be used as biofertiliser and for carbon sequestration [17, 27]; (9) a source of renewable hydrogen [28].

Microalgae can capture CO₂ from the atmosphere, from industrial gases (i.e., power plant flue gases) and in the form of soluble carbonates (i.e., Na₂CO₃ and NaHCO₃) [19, 20]. Generally, CO₂ concentration from power plants is higher than the atmosphere. The low CO₂ concentration in the atmosphere can slow down the growth of microalgae due to mass transfer limitation. Efficiency of CO₂ capture by microalgae increases with power plant flue gases that have up to 15% of CO₂ [19] and studies have shown that microalgal species (such as *Scenedesmus* sp. and *Chlorella* sp.) can tolerate 10% to 30% CO₂ in its gas supply [29]. Therefore microalgal cultivation can be synergistically located adjacent to a power plant where the CO₂ emitted from such a plant can be conveniently sequestered by the algae as a source of their nutrients [17].

3. Microalgal Cultivation System

There are two main microalgal large-scale cultivation systems: (1) open air-system and (2) photobioreactors [30]. The selection of a cultivation system depends on several factors: the type and the biology of the algal species, the availability of sunlight, the cost of land, the water

supply, the availability of nutrients, the desired final product, the climate conditions and the supply of CO₂ [25, 30]. The amount of nutrients and certain metals (i.e., iron and magnesium) must be optimum as they are important for the growth of microalgae and CO₂ fixation efficiency [19]. Research from an outdoor mass cultivation in New Mexico showed that with the right levels of nutrients, CO₂ fixation by microalgae could be increased [23]. A well-designed cultivation system can improve the effectiveness of CO₂ capture by microalgae [19]. Another important factor when deciding on a cultivation system is the likelihood of contamination by other microorganisms [19]. For example, algal species like *Chlorella*, *Dunaliella*, and *Spirulina* can grow only in specific environments, hence are not likely to be contaminated by other microalgae when cultivated in an open-air system. In contrast, some marine microalgae such as *Tetraselmis*, *Skeletonem*, and *Isochrysis* are susceptible to foreign invasion and must be grown in closed photobioreactors [30].

3.1. Open-Air System. There are many kinds of open-air systems with each having its own set of advantages and disadvantages. The four common types of open-air systems are shallow big ponds, tanks, circular ponds, and raceway ponds [30]. Open-air systems are less complex to construct and operate than photobioreactors [31]. They are considered as low cost methods to cultivate microalgae if compared to closed systems [19]. Nevertheless, open-air systems have many disadvantages, including diffusion of CO₂ back to the environment, requirement for a large area of land, evaporative water losses, and poor light utilization [31]. Besides that, open systems are exposed to many elements in the atmosphere. Hence, they give low biomass yields as it is difficult to control the culture conditions. The depth of the ponds also presents a problem. It needs to be shallow enough for the sunlight to reach the microalgal cells and, at the same time, deep enough for sufficient mixing. Thus, the maximum biomass that can be achieved from most microalgae is roughly between 0.1 to 0.5 g dry weight/L culture [30].

Among the many types of open-air systems, the most common design is the raceway pond as shown in Figure 2 [19]. The raceway pond is made up of a closed loop recirculation channel that includes a paddle wheel. The paddle wheel provides the circulation and the mixing, while the baffles placed in the channel guide the flow. To ensure that the light reaches the microalgal cells, the raceway pond is only about 0.3 m in depth. This design has been around since the 1950s. The raceways ponds are made from concrete or compacted earth that can be lined with plastics [3]. The continuous mixing that occurs within the pond enables high growth rate and minimises contamination risk by foreign microalgae [30].

Even though this open-air cultivation method is not ideal, there are several companies using the design, such as Aquaflow Bionomics and Live Fuels based respectively in New Zealand and the United States. Both of these companies are using alternative techniques to deal with the presence of unwanted microorganisms [32].

TABLE 1: Previous studies on organic solvent extraction of microalgal chlorophyll.

Study	Algae species	Solvent	Cell disruption	Key results
Jeffrey et al. [4]	Phytoplankton	Methanol (90%), ethanol (90%), ethanol (100%), DMF	All	DMF is superior to all the other solvents used and cell lysis improves extraction in all cases.
Macías-Sánchez et al. [33]	<i>Dunaliella Salina</i>	DMF Methanol	Ultrasound	DMF was found to be more efficient methanol.
Sartory & Grobbelaar [34]	<i>Scenedesmus quadricauda</i> , <i>Selenastrum capricornutum</i> , <i>Microcystis aeruginosa</i>	Ethanol (95%), methanol, acetone (90%)	Homogenisation, sonication, boiling	(1) Methanol and 95% ethanol were superior to 90% acetone. (2) Boiling the algae in either methanol or 95% ethanol for 5 minutes and allowing extraction for 24 hours resulted in the complete extraction of pigments without any formation of degradation products.
	<i>Stichococcus</i> , <i>Chlorella</i>	Acetone, DMF	Grinding, Ultrasound, bead beater	(1) DMF was found to be the most efficient solvent. (2) Acetone extracted 56-100% of the amount of chlorophyll <i>a</i> extracted by DMF. (3) DMF does not require cell disruption. (4) Freeze drying before analysis aids extraction
Schuman et al. [35]				
Simon & Helliwell [36]	Freshwater algae <i>Selenastrum obliquus</i>	Methanol and acetone	Probe sonication, bath sonication, tissue grinding, mortar and pestle	Under sonication, methanol removed 3x more pigment than acetone. Under tissue grinding, methanol removed 20% more than acetone.

3.2. Photobioreactor System. The photobioreactor system maximises photosynthesis by circulating the microalgal cells, nutrients, and CO₂ in a narrow enclosed transparent vessel exposed to ample illumination [32]. Its high-surface-area-to-volume ratio enables optimal light utilisation leading to higher biomass productivity. Furthermore, the photobioreactor's closed design facilitates high sterility, robust control of temperature, and good management of culture conditions. All these advantages allow the photobioreactor system to culture a variety of microalgal species without any contamination and to produce a high quality biomass. The photobioreactors are also continuously well-mixed to improve gas diffusion into the microalgal cells. Even though the photobioreactor system gives control over most cultivation parameters and achieves a higher CO₂ fixation rate than open-air system, it requires large amount of energy for its operation and construction [19, 30].

The photobioreactor system comes in several designs like flat-plate, tubular, bubble-sparged vertical column, and airlift. Of those designs, tubular photobioreactors are the most popular [19, 31]. Table 2 shows the comparison between raceway ponds and tubular photobioreactors. Tubular photobioreactors is made up of an array of clear straight tubes. These tubes are made of either glass or plastic and are usually less than 0.1 m in diameter in order to allow for light penetration. The tubes are arranged either horizontally where they are placed parallel to each other flat on the ground or vertically where they are configured to form fence-like structures. Figure 3 shows the two different configurations of tubular photobioreactors [3].

Flat-plate photobioreactors are frequently used for its large illumination surface area. Like the tubular design, flat-plate photobioreactors are also made out of transparent materials for maximum penetration of sunlight. This design can also achieve high CO₂ fixation efficiency and mass cultivation of microalgae. In fact, there is less build-up of oxygen in the flat-plate design compare to the tubular design. However, the design suffers from the following drawbacks: (1) difficulty in controlling culture temperatures; (2) scale-up requires more compartments and support materials; (3) stronger hydrodynamic stress on microalgal cultures [31].

Apart from the four primary designs, there are other types of photobioreactor systems that are currently used. The basics of the systems are the same with some modifications to maximise the efficiency. One such example is a 450 ft long by 50 ft wide photobioreactor made up of twin transparent plastic algal waterbeds patented by a company called A2BE Carbon Capture LLC. Another company, Green Shift Corporation based in New York has produced a pilot-scale photobioreactor that is incorporated with an ethanol producing facility to capture the CO₂ emitted from power plants [32].

4. Chlorophyll Extraction Methods

4.1. Organic Solvent Extraction. In order to quantify the amount of chlorophyll in a particular species, the intracellular chlorophyll must first be extracted. The traditional method that has been employed is organic solvent extraction [4, 36]. The extraction process involves the organic solvent

TABLE 2: Comparison between microalgal cultivation systems [19].

System	Raceway ponds	Tubular photobioreactors
Light efficiency	Fairly good	Excellent
Temperature control	None	Excellent
Gas transfer	Poor	Low - high
Oxygen production	Low	High
Accumulation	Low	Low - high
Hydrodynamic stress on algae	Difficult	Easy
Species control	None	Achievable
Sterility	Low	High
Cost to scale-up	Low	High
Volumetric productivity	High	Low

penetrating through the cell membrane and dissolving the lipids as well as the lipoproteins of chloroplast membranes [4]. It has been found that cell disruption, achieved through grinding, homogenisation, ultrasound or sonication, significantly improves the effectiveness of chlorophyll extraction using organic solvents [4, 33, 34, 36]. Simon and Helliwell [36] found that, without cell disruption, only a quarter of the potential chlorophyll *a* was able to be extracted by an optimal method. In addition to cell disruption, there are other parameters which affect the efficiency of organic solvent extraction, including the storage conditions of the filtered microalgae prior to the analysis, the organic solvents used, the duration of the extraction and the number of extraction steps employed in the analysis [4, 34, 35]. Since chlorophyll is highly reactive, the yield of a particular extraction procedure is also affected by the formation of degradation products. Degradation products of chlorophyll are formed when their molecules are exposed to excess light, oxygen/air, high temperatures and acidic or basic conditions [4, 5].

Table 1 shows previous studies on organic solvent extraction of microalgal chlorophyll. Simon and Helliwell [36], Sartory and Grobbelaar [34], and Jeffrey et al. [4] found methanol and ethanol to be superior extraction solvents to acetone. Simon and Helliwell [36] conducted their sonication-assisted chlorophyll extractions in an ice bath and in dark conditions to prevent degradation products from forming. They found that, with sonication, methanol removed three times more pigment than 90% acetone. Additionally, when tissue grinding was used, methanol removed 20% more pigment than 90% acetone. Sartory and Grobbelaar [34] similarly found that 90% acetone was an inefficient organic solvent compared to methanol or 95% ethanol. However, it has been shown that the use of methanol as an extraction solvent resulted in an unstable solution and lead to the formation of chlorophyll *a* degradation products [4, 37]. Although 100% acetone was found not to yield the highest amount of chlorophyll from any particular species, its use as an extracting solvent strongly inhibited the formation of degradation products [4]. In studies carried out by Jeffrey et al. [4] and Macías-Sánchez et al. [33], dimethyl formamide (DMF) was found to be a superior extraction solvent to methanol, 90% ethanol, 100% ethanol and 90% acetone.

Extraction using DMF did not require cell disruption as pigments were completely extracted after a few steps of soaking. Additionally, the pigments remained stable for up to twenty days when stored in the dark at 5°C [4, 35]. However, DMF toxic nature decreased its appeal as an efficient solvent [4]. It was also found that storage of the microalgae at low temperatures after filtration (−18°C or −20°C) assisted cell disruption and promoted the extraction of chlorophyll [4]. Schumann et al. [35] found freezing the biomass in liquid nitrogen followed by lyophilisation and then storage at −18°C to be the optimal storage procedure.

Sartory and Grobbelaar [34] found the efficiency of chlorophyll extraction from fresh water microalgae to be optimal when the filtered microalgae and solution were refluxed at the solvent's boiling point. It was shown that boiling for 3 to 5 minutes in methanol or 95% acetone prior to 24-hour extraction led to the complete isolation of chlorophyll *a* without the formation of any degradation products. However when the mixture was subjected to temperatures of 100°C, degradation products started to form [34]. Such findings are contradictory to the general assumption that chlorophyll degraded upon slight temperature elevation.

The amount of chlorophyll extracted from a particular algal species was found to be highly dependent on its growth stage. Microalgae extracted in the stationary growth phase were shown to have significantly higher amount of chlorophyll *a* compared to the same species obtained in the logarithmic phase [35].

4.2. Supercritical Fluid Extraction (SFE). The use of organic solvents for lipophilic extraction poses a serious threat to the environment. The Montreal Protocol in 1987 proposed to restrict or to eliminate the manufacture and the use of ozone depleting solvents such as chlorofluorocarbons (CFCs). From then on, the Montreal Protocol has evolved and most solvents that are damaging to the environment have been restricted. As a result, more industries are looking into a new sustainable process that does not involve environmentally damaging solvents [38]. Supercritical fluid extraction (SFE) is a popular method to replace organic solvent extraction.

Supercritical fluid extraction was first introduced as an alternative extraction method in 1879 by Hannay and

TABLE 3: Comparison between organic solvent extraction and SCCO₂ extraction, modified from [40].

No.	Organic solvent extraction	SCCO ₂ extraction
1	Solvent presence is unavoidable. The amount of solvent depends on the type of solvent used. Solvent removal requires extra unit operations and increases operational cost.	SCCO ₂ extraction produces solvent-free extracts.
2	Heavy metal and inorganic salt contents are also unavoidable. Their levels depend on the solvent, the method of solvent recycling, the source of the raw material, and the materials used to construct the contact parts of the machinery.	Extract is completely free of heavy metals and inorganic salts since they are not extractable by the SCCO ₂ even if they are present in the raw material. No heavy metals are present in the CO ₂ extractant or the SFE system.
3	Polar substances get dissolved along with the lipophilic substances from the raw material due to poor selectivity of the solvent. During solvent removal operations, these polar substances may form polymers which lead to discoloration of the extract.	Due its nonpolar nature, SCCO ₂ will not extract any polar substance.

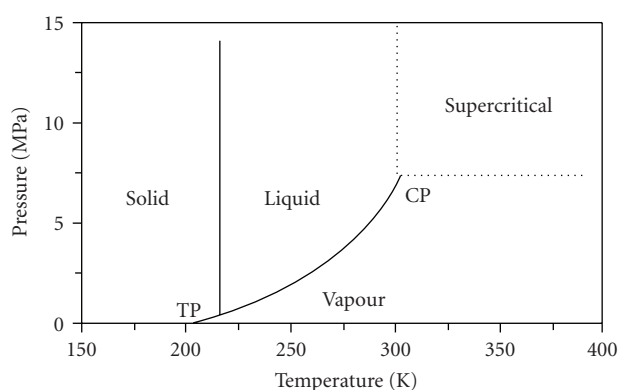
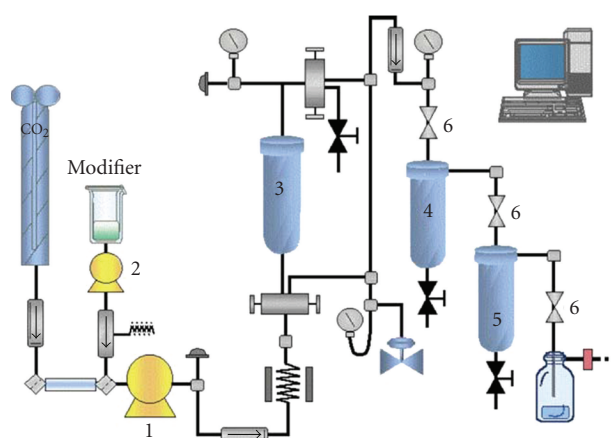


FIGURE 4: P-T phase diagram for carbon dioxide [42].

Hogarth. However, it was not until around 1960 that this extraction method started to be thoroughly investigated [39]. SFE has many advantages over organic solvent extraction. One of the major advantages is the high purity of the extract. In addition to requiring less processing steps, SFE is significantly safer than organic solvent extraction and can be operated at moderate temperatures to minimize extract degradation. Table 3 shows the comparison between the two extraction methods [40].

The supercritical state is achieved when a substance is exposed to conditions exceeding its critical temperature (T_c) and pressure (P_c). In this state, the substance has liquid-like densities with the viscosities of a gas. The solvent power of supercritical fluids is the highest for slightly polar or non-polar components and is lower for analytes with higher molecular weights. The fluid is easily removed from extract through expansion to atmospheric pressure [41].

CO₂ is the most commonly used fluid for SFE as it is cheap, non-flammable, readily available, and somewhat inert. Figure 4 shows the phase diagram for CO₂ with its supercritical region. The critical temperature and pressure of CO₂ are 304.1 K and 7.38 MPa, respectively. The moderate T_c of CO₂ yields higher quality extracts because it avoids excessive heating which often leads to degradation. This allows for supercritical carbon dioxide (SCCO₂) to be used in

FIGURE 5: Schematic diagram of supercritical fluid extraction system, extracted from [45]. (1) CO₂ pump, (2) modifier pump, (3) extraction cell, (4) fractionation cell I, (5) fractionation cell II, (6) valve.

the extraction of thermolabile compounds [42, 43]. SCCO₂ is also preferred due to its high diffusivity and the ease in manipulating its solvent strength. This can be done by adding a substance or modifier which has volatility between the supercritical fluid and the analyte that is to be extracted. For example, the addition of a polar solvent like methanol or water to supercritical CO₂ allows it to extract polar compounds [39, 42]. Studies have shown that addition of ethanol to supercritical fluid increases the yield of lipids from *Arthrospira maxima* [44].

SCCO₂ extraction is applicable in many different fields such as food and natural products extraction, environmental science and pharmaceuticals [39]. This review will focus on SCCO₂ extraction of chlorophyll from microalgae. Chlorophyll is mainly used in food technology, so there are stringent regulations regarding its quality. By using SCCO₂ extraction, a solvent-free highly pure extract can be achieved. Upon decompression, the supercritical CO₂ is removed from the extract as it evaporates to the ambient in its gaseous state [45].

TABLE 4: Previous studies on HPLC fractionation of chlorophylls extracted from phytoplanktons.

Study, Chromatography	Mobile phase	Stationary phase	Key results
Jeffrey [49], TLC	First dimension: 0.8% n-propanol in light petroleum (by volume) Second dimension: 20% chloroform in light petroleum (by volume)	Sucrose	In this two dimensional chromatography, there was complete separation of chlorophylls and carotenoids.
Jeffrey et al. [4], HPLC	90 : 10 (v/v) methanol : acetone for 8 min at a flow rate 1 mL/min Preinjection mix of sample 3 : 1 (v/v) sample: 0.5 M ammonium acetate	3 μ m C18 Pecosphere	This simple isocratic protocol was able to separate only chlorophyll <i>a</i> from other pigments and compounds.
Jeffrey et al. [4], HPLC	Solvent A is 80 : 20 (v/v) methanol : 0.5M ammonium acetate Solvent B is 90 : 10 methanol: acetone Elution order: 0–3 min: solvent A 3–17 min: solvent B flow rates: 1 mL/min Pre-injection mix of sample 3 : 1 (v/v) sample: 0.5 M ammonium acetate	3 μ m C18 Pecosphere	This step-isocratic protocol was found to successfully separate the three chlorophylls (<i>a</i> , <i>b</i> and <i>c</i>) and ten other derivative products.
Jeffrey et al. [4], HPLC	Solvent A is 80 : 20 (v/v) methanol: 0.5 M ammonium acetate Solvent B is 90 : 10 (v/v) acetonitrile : water Solvent C is ethyl acetate Elution order: 0–4 min: linear gradient from 100% A to 100% B 4–18 min: linear gradient to 20% B and 80% C 18–21 min: linear gradient to 100% B 21–24 min: linear gradient to 100% A 24–29 min: isocratic flow of 100% A	3 μ m C18 Pecosphere	This ternary gradient protocol was found to separate over 50 pigments. The resolution of this protocol is higher than that of Wright and Shearer [51]. Additionally no ion pairing reagent is required, as in Mantoura and Llewellyn [37].
Lynn Co and Schanderl [52], TLC	Three different solvent systems were experimented. Solvent system 1 (modified Bauer solvents): First dimension is benzene: petroleum ether: acetone (10 : 2.5 : 2 v/v/v) Second dimension is benzene: petroleum ether: acetone: methanol (10 : 2.5 : 1 : 0.25 v/v/v) Solvent system 2: First dimension is benzene: petroleum ether: acetone: methanol (10 : 2.5 : 1 : 0.25 v/v/v) Second dimension is petroleum ether: acetone: n-propanol (8 : 2 : 0.5 v/v/v) Solvent system 3: First dimension is benzene: petroleum ether: acetone (10 : 2.5 : 2 v/v/v) Second dimension is petroleum ether: acetone: n-propanol (8 : 2 : 0.5 v/v/v)	Silica Gel	Two dimensional chromatography was carried out on silica gel. Eight major pigments as well as eight to ten minor derivatives were successfully separated with these solvent systems.
Madgwick [50], TLC	30 mL of 1 : 1 (v/v) diethyl ether : petroleum spirit	Glucose	(1) One dimensional ascending thin layer chromatography was used. (2) Spectrophotometric analysis was found to overestimate chlorophyll <i>c</i> by up to 22% and to underestimate chlorophyll <i>b</i> by 10–20%. Chlorophyll <i>a</i> was, however, correctly quantified.

TABLE 4: Continued.

Study, Chromatography	Mobile phase	Stationary phase	Key results
Mantoura and Llewellyn [37], HPLC	Solvent P (ion pairing reagent) is made of 1.5 g tetrabutyl ammonium acetate and 7.7 g of ammonium acetate dissolved in 100 mL of water. Primary eluant is solvent P : water : methanol (10 : 10 : 80 v/v/v) Secondary eluant is 20 : 80 (v/v) acetone: methanol 0–10 min: linear gradient from primary eluant to secondary eluant 10–22 min: isocratic flow of secondary eluant	Four different columns: C3 Zorbax, C8 Zorbax, C18 Zorbax, Shandon Hypersil ODS	High resolution separation of chlorophylls and all major pigments were achieved. The method obtained a high recovery (over 90%) of pigments.
Riley and Wilson [53], TLC	Petroleum ether : ethyl acetate : diethyl amine (58 : 30 : 12 v/v/v)	Silica gel	All plant pigments were separated except for some minor components.
Sartory [48], HPLC	Solvent A is 97% methanol Solvent B is 97% acetone Elution order: 0–15 min: 100% solvent A 15–20 min: linear gradient to 77% solvent A: 23% solvent B Then isocratic flow of 77% solvent A: 23% solvent B Flow rate: 1 mL/min	Sep-Pak C18 Bondapak	(1) HPLC was found to be a rapid, sensitive, and selective method that successfully separated chlorophylls and its derivatives to high resolutions. Recovery of total pigments by HPLC is greater than 96%. (2) Spectrophotometric analysis overestimated chlorophyll <i>a</i> and underestimated chlorophyll <i>b</i> .
Wright and Shearer [51], HPLC	Linear gradient from 90% acetonitrile to ethyl acetate for 20 mins at a flow rate of 2 mL/min	C18 octadecyl silica.	Carotenes, chlorophylls, xanthophylls, and their degradation products were successfully separated with high resolutions.

4.2.1. SFE Apparatus. There are two supercritical fluid extraction systems: analytical and preparative. In the analytical system, SFE is directly combined with a chromatographic system. The preparative system is used to extract compounds from samples for example chlorophylls from microalgal biomass. SFE can process either liquid or solid samples.

Figure 5 shows a pilot-scale preparative SFE system. It is made up of a solvent (in this case the solvent is CO₂) pump, a modifier pump, an extraction cell, valves and two fractionation cells. The extraction cell is for solids whilst packed columns are used for liquids [45]. The microalgal biomass is placed in the extraction cell [42]. The extract is collected and solvent is depressurized in the fractionation cells which are equipped with temperature and pressure controllers. This configuration allows different compounds to be obtained in each fractionation cell based on their differential solubility in the supercritical fluid [45].

4.2.2. Effect of SFE Operating Conditions. The SCCO₂ extraction of chlorophyll from microalgae depends on the fluid density which is a function of operating pressure and temperature. Studies have been conducted on SCCO₂ extraction of chlorophyll *a* from at least two microalgal species *Nannochloropsis gaditana* and *Synechococcus* sp. Optimum extraction conditions were found to be 60°C and 400 bar for *Nannochloropsis gaditana* and 60°C and 500 bar for *Synechococcus* sp.

At constant temperature, an increase in pressure will increase the fluid density. Even though this will increase the

solvating power of the supercritical fluid, it will decrease its diffusion coefficient. The increase in temperature at constant pressure also has a double effect. Increasing temperature raises the vapour pressures of the pigments and hence gives better solubility. However, it will also decrease the density of the supercritical fluid which leads to a lower solvating power. Therefore, the chlorophyll yield on each operating condition will depend on which effect predominates [43, 46].

5. Chlorophyll Fractionation and Purification on Chromatographic Adsorbents

The traditional method for the quantification of chlorophyll and other pigments in a particular extract is spectrophotometric analysis [34]. The different chlorophyll compounds and other pigments are quantified by measuring the absorbance of a sample at particular wavelengths and using formulated simultaneous equations to calculate the concentrations of particular pigments. It has been found that this method of quantification is often inaccurate as absorption and emission bands of chlorophyll *b* and other pigments overlap with those of chlorophyll *a*. Chlorophyll degradation products are not detected and studies conducted by different authors provided different simultaneous equations [37, 47–50].

In order to overcome the flaws associated with the spectrophotometric analysis, chromatographic techniques are used to fractionate and to quantify chlorophyll and its derivative products. The three types of chromatography

that have been widely used are paper chromatography, thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC). Paper chromatography was mostly used in the 1950s and 1960s during the early stages of the development of chromatographic techniques. This method was found to effectively separate chlorophyll into its three fractions (*a*, *b*, and *c*), pheophytins, carotenes and other derivative products [54]. However, TLC was found to be a preferable technique to paper chromatography due to the ease in quantitatively recovering the pigments from its adsorbents. In paper chromatography, recovery was shown never to exceed 80% [49, 50, 53]. Additionally, TLC requires less sample, is less tedious, and produces chromatograms with sharper resolutions [52, 53].

Organic adsorbents such as sucrose and cellulose were found to be the most efficient stationary phases for use in two dimensional thin layer chromatography [49]. Riley and Wilson [53] and Lynn Co and Schanderl [52] utilised silica gel as their stationary phase and managed to completely separate all of the plant pigments except for some minor components. However, Jeffrey [49] found that use of silica gel promoted the formation of degradation products and resulted in multiple chlorophyll zones.

HPLC is superior to TLC because it requires even less sample for analysis, is faster and features automatic detection system [37, 51]. In addition to these, HPLC is more precise and has a higher degree of sensitivity [55]. Reverse phase HPLC is preferred to normal phase as the latter does not separate polar compounds and its polar stationary phase promotes pigment degradation [37, 51]. An additional drawback of the normal phase HPLC is that it is not compatible with aqueous samples, whereas many of the solvents used for chlorophyll extraction from microalgae are aqueous-based [37]. Several HPLC configurations have been employed, with each providing separation of pigments to varying extents and different resolutions [4]. There are different types of detectors that may be used to measure the concentrations of separated pigments as they exit the column. The most commonly used detectors rely on fluorescence and absorbance analyses. Jeffrey et al. [4] states that fluorescence detection is more sensitive and more selective than absorbance detectors when used to analyze chlorophylls amongst carotenoids. Table 4 summarizes previous studies on chromatographic fractionation of phytoplankton pigments.

6. Conclusions

This review article shows that chlorophyll has a wide range of applications due to its colouring effect, tissue growth stimulating effect, antioxidant and antimutagenic properties. Chlorophyll is best extracted from microalgae by supercritical fluid extraction method. High performance liquid chromatography was found to be the most accurate and sensitive technique to fractionate and to quantify chlorophyll along with its derivatives. Furthermore, biological CO₂ mitigation with microalgal cultivation helps to minimise the global warming impact. Microalgae seem to be a promising alternative source for chlorophyll.

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