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Mild cell disruption methods for bio-functional proteins recovery from microalgae—Recent developments and future perspectives

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ABSTRACT

Bio-functional proteins from microalgae have numerous biological properties with health-promoting effects. However, efficient harnessing of bio-functional proteins from microalgae is still in its infancy. One of the major obstacles that hinder the mass production of bio-functional proteins is the presence of resistant cell wall that diminishes the liberation of cell contents. As the bio-functional proteins are very sensitive to denaturation, selecting a mild disruption method to rupture the cell wall, while preserving their bioactivity and functionality, is of vital importance in downstream processing. To ensure the future development of efficient mild disruption methods for maximum recovery of bio-functional proteins from microalgae, this review provides useful information on various mild disruption approaches, current status, potential technologies that are still under development, as well as their advantages and constraints. In particular, those potential technologies that require further attention in the future (namely, explosive decompression, microfluidization, pulsed arc technology and cationic polymer coated membranes) are also discussed in this review.

1. Introduction

A biorefinery is sustainable biomass processing, aiming to maximize the efficiency of resource utilization from the biomass feedstock. The co-production of a range of end products and energy from biomass in linear production chains can be achieved through the integration of biomass conversion processes and equipment [1–3]. The biorefinery approach enables the microalgal production to be economically feasible, by allowing the optimal harnessing of all the valuable compounds present in microalgae [3–6]. The implementation of the biorefinery concept in the microalgal industry is imperative in the creation of a sustainable and more environmental friendly future. The advantages include mitigating greenhouse gas emission, reduction in fossil fuel usage and overcoming the insufficiency of future food supply [2]. Microalgae are classified as the futuristic raw material in biorefinery process, because of their relatively untapped potential to produce multiple valuable products in addition to biofuels [2,7]. Although being small in size, these photosynthetic microorganisms have the capability of accumulating different types of metabolites, which can subsequently be transformed into value-added products [2]. The growing interest and increasing commercial demand in natural and healthy products in today's market trend has forced the development of novel products with valuable functional compounds from microalgae for food, nutraceutical and pharmaceutical industries [8]. Being a natural source of highly interesting biologically active compounds with positive health effects, microalgae produce a range of functional ingredients including polyunsaturated fatty acids, polysaccharides, natural pigments, essential minerals, vitamins, proteins, essential amino acids, and enzymes, as well as bioactive molecules [2,8,9]. Most of the functional components that possess health-promoting effects are associated with proteins, protein hydrolysates or peptides. Therefore, special attention has been paid to the bio-functional proteins and peptides from microalgae with a broad spectrum of biological properties such as antioxidants, antihypertensive, anticoagulative, antitumor

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and immuno-stimulant activities [10].

The utilization of microalgae as source of high-value metabolites at commercial scale is still in its nascent form [11]. Extensive efforts have been dedicated to the development of cost-effective and feasible upstream processing particularly in the cultivation system [12], but still very limited reports on the recovery of high-value metabolites especially bio-functional proteins from microalgae are being observed. As the microalgal morphology is different from terrestrial plants, intracellular components cannot be extruded effectively from microalgae using a mechanical press, a method which is designed specifically for product extrusion from terrestrial crops, such as soy [13]. On top of that, there are many different species of microalgae grown under different cultivation conditions, varying greatly in their cell wall structure and chemical compounds concentration, making the predictions or extrapolations on disruption efficiency and the recovery of intracellular compounds impossible [1].

Biorefinery techniques, mainly focusing on downstream processes, are necessary to exploit all cell contents produced by microalgae after cultivation [3]. Downstream processing represents a major economic limitation to the mass production of high-value metabolites from microalgae at lower cost [1]. Downstream processing costs typically contribute to a big portion of the total cost, hence the development of competent and vigorous new downstream strategies is imperative to maximize product recovery from microalgae and favour the economic feasibility of the process [14,15]. One of the major problems faced in developing a suitable downstream strategy is the low recovery of high-value metabolites, limited by the rigid nature of microalgal cell wall. The structurally small microalgae are covered with multiple layers of resistant thick cellular walls that hinder the liberation of cell contents [1].

To overcome this chemical and structural barrier that limits the liberation of intracellular components, employing an appropriate cell disruption technique prior to extraction is undoubtedly one of the most crucial preliminary steps in downstream processing for the maximum recovery of cell contents from microalgae [1,16]. It is essential to ensure that the cell structure has been completely disintegrated to increase the efficiency of the extraction process [16]. However, selecting an ideal disruption method to facilitate the high-value metabolite extraction based on the biorefinery concept is quite challenging. The quantity and quality of functional compounds in the extract depends on the effectiveness of the cell disruption method [17,18]. The nature and composition of the structural cell wall has an important effect on the disruption efficiency and the extraction yield of intracellular biomolecules [18,19]. For example, lipid extraction from *Chlorella* or *Nannochloropsis* is much more difficult than *Dunaliella* due to the presence of a thick resistant cell wall [19]. Therefore, the choice of disruption technique is highly specific and strongly depends on the microalgal strain and the characteristics of the cell wall structure.

In addition to considering the structural morphology of each microalgal cell wall, the selection of a suitable cell disruption technique also largely depends upon the nature of the desired product or final product application [20]. Protein denaturation can be defined as the irreversible loss of the three dimensional structure due to the breakage of non-covalent bonds brought about by heat, alcohol, acids, bases, salts of heavy metals or other agents [21]. Most bio-functional proteins, such as enzymes with specific bioactivities, are very sensitive to denaturation; even slight changes in pH or temperature may cause inactivation and result in the loss of their biological function [21]. Therefore, achieving a good extraction yield of proteins from microalgae without degrading them is quite challenging [22]. If the target product involves the production of fragile functional compounds, the selection of a mild downstream processing without negatively affecting the activity and quality of the cell components of interest is required. Gentle breakage of the outer cell wall is necessary to promote the release of bio-functional proteins and peptides from microalgae, while preserving the functionality and biological activity of the molecules. As such, transforming biomass into a variety of high value-added products can be achieved in microalgal-based biorefinery [3].

Due to the growing significance of producing high-value compounds for food and pharmaceutical sectors based on biorefinery concept, the current research attention toward microalgae should predominantly focus on mild disruption methods. This is imperative to ensure the feasible and sustainable development in food and pharmaceutical industries. Despite the necessity, extensive and comprehensive studies on techniques to degrade cell walls of microalgae are still limited to date [1]. Hence, this review aims to provide useful information on various mild cell disruption methods, the current status, the potential innovative technologies that are still under development and require further attention in the future, as well as their benefits and constraints.

2. Microalgae

2.1. Types of microalgae

Microalgae can be grouped nutritionally on the basis of their energy sources, namely, autotrophic, heterotrophic or mixotrophic. Most microalgae are autotrophic, with the absolute requirement for light to perform photosynthesis, adequate supply of carbon dioxide and inorganic nutrients for optimal growth [23]. With the presence of these simple inorganic substances in their surroundings, autotrophs are capable of producing complex organic compounds, such as carbohydrates, fats and proteins [11]. On the other hand, some microalgae are heterotrophic. Heterotrophs are in contrast with autotrophs; they cannot fix carbon and therefore need organic carbon compounds, such as glucose, acetate, lactate and glutamate as carbon and energy source for growth. Microalgae that may have the dual capacity of both autotrophic and heterotrophic characteristics are known as mixotrophic. These phototrophic microalgae are able to adapt their metabolism to heterotrophic conditions, depending on the availability of organic compounds and light intensity [23].

2.2. Major chemical composition of microalgae

Microalgal biomass is composed of three main components: proteins, carbohydrates and lipids [15]. Studies on various microalgae demonstrated that protein is always the major constituent of the microalgae biomass (typically 25–40% of the dry weight) [23], followed by lipid and carbohydrate [24]. Microalgae have the ability to synthesise all types of amino acids, which are mostly equivalent or even better than that of other high-quality plant proteins [25]. Many metabolic studies have confirmed the capacity of microalgae as a novel source of protein in food [25] due to their abundance and complete amino acid profile.

2.3. Cell wall structure of microalgae

Microalgae are microscopic single cell microorganisms covered with a relatively recalcitrant cell wall and the intracellular compounds are mostly located in globules or bound to complex membranes, making the extraction of cell contents a great challenge [1,26]. The microalgal cell wall is a complex entity to preserve the integrity of the cell and serves as the main protective barrier against invaders and harsh environment [1,16]. Their cell envelopes are generally more rigid than the cell envelopes of other microorganisms or higher plants. It was reported that the tensile strength of the microalgal cell wall can be up to 9.5 MPa, which is about three times higher than that of carrot, *Daucus carota* [13]. These complex cell walls are typically tri-layered structures with high mechanical strength and chemical resistance, composed of: polysaccharides, such as cellulose, pectin, mannose, xylan; minerals, namely calcium or silicates; as well as proteins, such as glycoproteins [27,28]. The cell walls of most species of microalgae contain a relatively large proportion of cellulose, conferring structural stability

and rigidity to the cell [29]. An additional tri-laminar sheath (TLS) containing algaenan may also be found in certain microalgal species, making them highly resistant to degradation [30]. In addition, the cell walls of microalgae are strong, owing to the existence of a mixture of covalent bonds, hydrogen bonds and van der Waals force interaction that hold the cell wall molecules together [13].

The extracellular coverings of microalgae are diverse, complex and poorly understood. The rigidity, thickness and composition of the cell wall varies greatly within and between species and may also depend on several factors, such as growth phase, harvesting time and culture conditions [1,31]. For instance, the nascent cell wall of microalgae is in general thin and fragile, but the thickness increases gradually in the mature stage [31]. The variation in the cell wall composition and structure among *Chlorella* intraspecies can be dramatic [32]. In terms of the cell wall structure, some *Chlorella* species possess only the inner cell wall layer. On the other hand, certain *Chlorella* species such as *C. zofingiensis* and *C. homosphaera* possess both an inner cell wall layer and a trilaminar outer layer [32]. However, the cell wall sugar compositions of these two *Chlorella* species are dissimilar, but dominated mainly by glucose and mannose. Likewise, it was found that the rigid cell wall of *Scenedesmus* intraspecies is composed of glucose, mannose and galactose in different proportions [33].

3. Mild cell disruption methods

The biodegradability of microalgae is restricted by the rigid nature of the thick cell wall. To facilitate the release of the cell contents from the tiny cell, the complex structure of the microalgal cell wall must be sheared to allow complete access to the internal components, which are entrapped within the thick cell wall [34]. The internal components can then be liberated into the liquid medium, making them readily available for further separation and purification processes.

In the microalgal biorefinery, all the principle constituents from biomass, such as carbohydrates, protein and lipid, can subsequently be transformed into multiple end products [35]. Fig. 1 shows that the microalgal biorefinery consists of different procedures and involves deploying a wide range of technologies to fractionate biomass into various fractions, whereof cell disruption is the most crucial part [1]. A disruption method would have significant impact on the efficiency of the subsequent steps in the biorefinery. Cell disruption is an energy intensive process and is relatively influential on the total production cost. This process could, thereby, affect the economy and yields of bioproducts [1,13]. Recently, another team of researchers also agreed that among all the downstream processing steps, cell disruption plays a significance role in enhancing the quality and quantity of the extracted intracellular contents from microalgae [17].

However, the major bottleneck in the biorefinery is to separate the different fractions without damaging one or more of the product fractions. To ensure the successful implementation of the biorefinery concept in microalgal industry, a mild technique is necessary to enable the complete exploitation of the microalgal biomass. Therefore, the first focus in obtaining the bioproducts should be on the selection of mild disruption technique to release various types of intracellular components without degradation [3,5,6].

A variety of mild cell disruption methods are currently available to rupture the cell wall of microalgae gently. These mild techniques are divided into two main groups, namely existing technologies and potential technologies. The classification of the mild cell disruption methods is outlined in Fig. 2.

3.1. Factors affecting the selection of mild disruption methods

There are several factors that collectively determine the suitability of a cell disruption process for the release of cell contents from microalgae. In general, a good cell disruption technique should be characterized by easy handling, low energy demand, and high disruption yields, by using economic and less toxic disruption reactants [36]. The efficiency of the cell disruption method signifies the selective and efficient release of the specific intracellular content from microalgae, to obtain maximum recovery yield with minimum risk of contamination and micronization of cell debris [37].

Cell wall characteristic and microalgal strain are not the only two

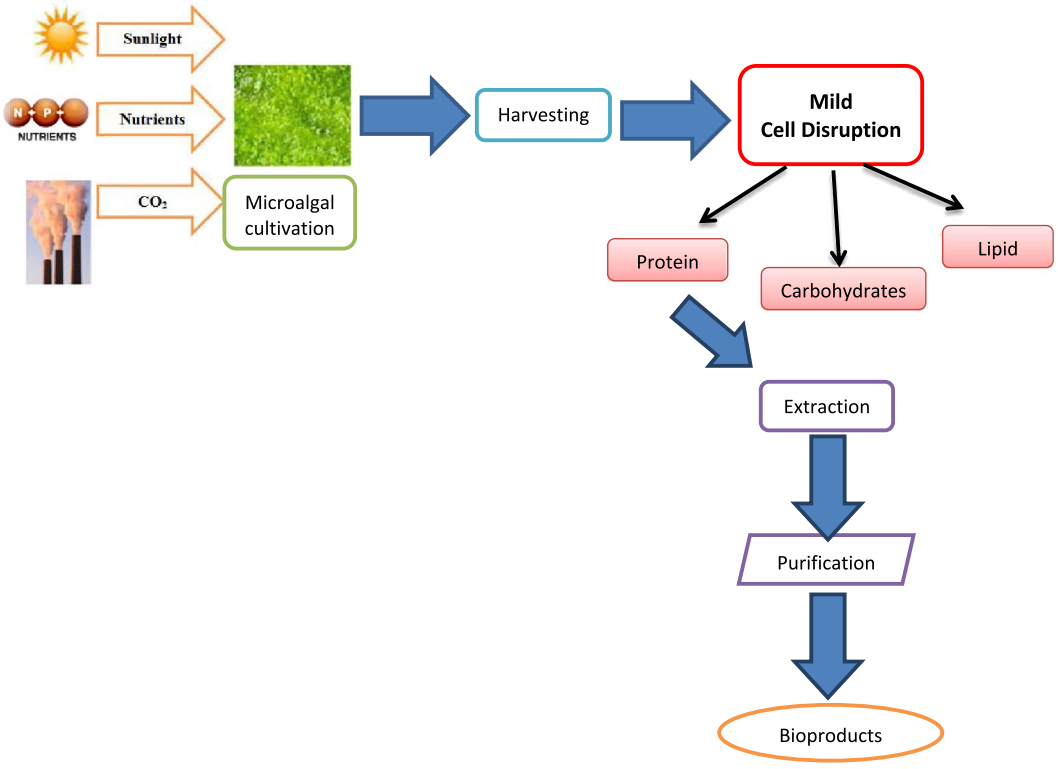

Fig. 1. Main steps involved in the operation of microalgal biorefinery.

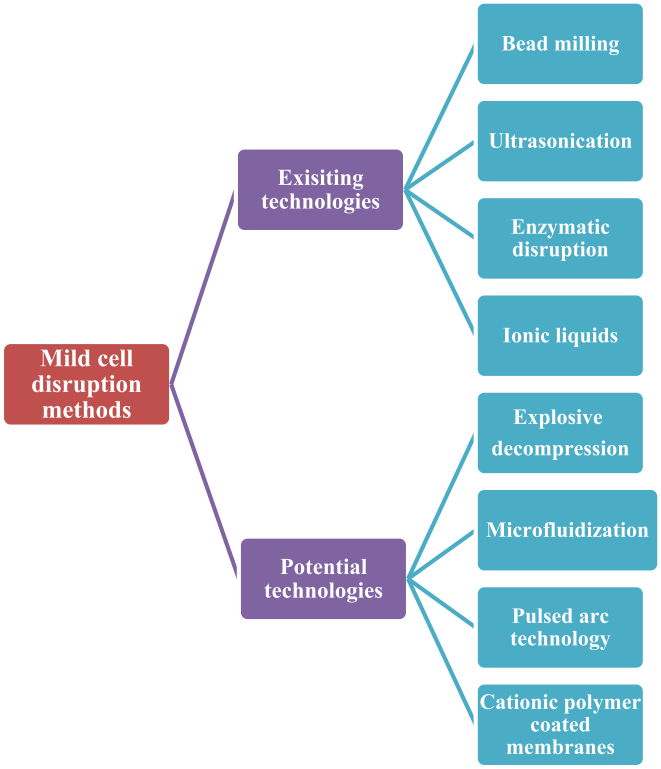

Fig. 2. Classification of mild cell disruption method.

selection criteria that determine the suitability of a cell disruption technique. The final product application should also be taken into consideration when selecting a suitable disruption technique. To extract the bio-functional proteins from microalgae while preserving their bioactivity and functionality, it is necessary to use a mild pretreatment process to facilitate the recovery of the fragile intracellular compounds [5,20]. Exposure of the microalgal cells to harsh conditions, such as high pressures, high shear levels or high temperatures that might change the structure and subsequently cause the loss of specific functionality or activity, could thus be avoided [5]. Operating parameters, such as reactant concentration, contact time, operating temperature, type of enzyme, pH, salt concentration and intracellular content, would cause significant impact on the disruption efficiency [38] and the bioactivity of the target products. For instance, the operating temperature should be kept below 35 °C during the disruption process to prevent protein aggregation [5]. Denaturation of the fragile bioactive compounds can be prevented under mild operating conditions. Hence, all these parameters should be suitably selected and adjusted to achieve maximum disruption yield, while minimizing the degradation of target biomolecules, through the use of low cost reagents and reducing the excessive production of liquid wastes [36].

An overview of the advantages and limitations of the currently employed and potential innovative mild disruption methods are provided in Table 1, while the comparison of the disruption methods in terms of their main features is summarized in Table 2.

3.2. Bead milling

Bead milling is a mechanical cell disruption method which causes direct mechanical damage to cells. Advantages such as high disruption efficiency and high biomass loading are the primary factors that make bead milling an attractive cell disintegration method [1,39]. It has been reported that the most energy-effective cell disruption can be achieved when biomass concentrations of 100–200 g/L are used [40]. An agitated bead mill is composed of a rotating agitator in a fixed vessel filled with beads, which provide high speed spinning cell breaking action [13]. The grinding effect is, thus, achieved through the physical collision of the solid beads against the cells [5,13,41]., The beads can be easily separated by gravity from the agitated solution after the cell disintegration process [13].

The energy transfer from the rotating shaft to the beads and subsequently to the cells in the chamber, as well as energy conversion into heat due to friction, would lead to undesired rise in temperature. When dealing with heat sensitive products, an effective cooling is required during the disruption process. With recent design improvements and the possibility to control the temperature of the algae suspension, bead milling can be regarded as an effective mild disintegration technique for biorefinery. Protein denaturation can be prevented, as the milling chamber is equipped with cooling jackets and the frictional heat can be removed by a cooling coil in the feed funnel [5,13].

Although high rate of cell disruption can be achieved using bead milling, this technique consumes large amounts of energy when applying at large scale. There are many parameters that affect the degree of cell disruption efficiency, energy consumption and, thus, the overall cost of disintegration process. These include the diameter, shape and composition of the beads, bead filling, agitator speed, agitator design, residence time distribution, milling chamber design, biomass concentration, as well as biomass viscosity [13]. It was reported that the optimal diameter of the beads for effective microalgal disruption is 0.5 mm [13]. Compared to glass beads with lower density, high density beads such as zirconia-silica, zirconium oxide or titanium carbide are more effective and preferred in high viscous media [1,13].

Bead milling has been found to be effective in breaking the cell wall of microalgae, but most studies mainly focus on the disruption of microalgae for lipid extraction, whereby mild operating condition is not a main concern. To date, the investigation on the mild release of fragile components from microalgae using bead milling is still limited [5]. Recently, Postma et al. demonstrated that mild disintegration of *Chlorella vulgaris* using bead milling for the release of water soluble proteins below 35 °C is possible, with yields ranging between 32% and 42%. Under this disruption process, it was estimated that the specific energy consumption can be kept below 2.5 kWh/kg dry weight [5]. Although this study did not target any specific bio-functional proteins, the possibility to disrupt the microalgal cells under mild conditions makes bead milling a suitable disintegration technology for algal biorefinery.

3.3. Ultrasonication

Mechanical methods directly destroy the cells via physical force. The main advantage of these physical pretreatment methods is that they can be universally applied to all microalgae, regardless of the species [27]. Among all the mechanical methods, ultrasonication is proposed to be able to rupture the microalgal cells mildly [3]. The effect of ultrasonication on extraction yield is attributed to micro-scale eddies and heightened mass transfer, produced by cavitation and bubble collapse that can induce stress on microalgal cells. This can be explained as ultrasonication induces cell disruption by exposing microalgae to the high intensity of ultrasonic waves, which causes tiny unsteady cavitation bubbles around cells in liquid medium. Implosion of bubbles emits shockwaves, generates chemical and mechanical energy shattering the rigid cell wall and releasing the desired intracellular compounds into the solution [34,42]. Guldhe et al. also agreed that the cavitational effect in ultrasonication enhances the extraction of chemical compounds from microalgal cells by facilitating solvent access through cell disruption and proper mass transfer [43].

As one of the most promising mechanical cellular disruption methods, ultrasonication has drawn worldwide attention in literature recently with the capability to disrupt the cell wall effectively and, thus, increasing the extraction yield of various intracellular compounds from

Table 1
Advantages and limitations of mild cell disruption methods.

Methods	Disruption mechanism	Advantages	Limitations	References
Existing technologies				
Bead milling	Physical collision of beads against cells	• High biomass loading • High disruption efficiency • Beads can be easily separated by gravity • Overheating can be prevented by cooling jacket or cooling coil	• High energy demand at large scale	[1,5,13,39]
Ultrasonication	Cavitation shear force	• Universal, less dependent on species • Can combine with other methods, thus increase efficiency, reduce energy demand and solvent consumption • Environmental friendly • Can be scaled-up • Operated continuously • Low set-up costs	• Cooling is needed to prevent overheating	[3,13,16,18,27,34,39,41,42,49,51,53]
Enzymatic disruption	Enzyme substrate interaction	• Specific • Mild operating conditions • Low energy requirements • Can combine with other disruption methods • High selectivity • Easy scale-up	• Expensive • Long process • Product inhibition • Need to know cell composition due to high substrate selectivity, species dependent	[1,3,38,54–57,60,102]
Ionic liquids	High hydrogen bond accepting ability	• Less hazardous due to low-melting point, non-volatile, non-flammable • Versatile • capable of dissolving a wide range of polar to non-polar compounds • excellent recyclability	• Expensive solvents	[56,67–71,73]
Potential technologies that are still under development				
Explosive decompression	Gaseous expansion by increased pressure	• Does not require the use of aggressive chemicals • Mild temperature • Able to disrupt high DCW of biomass • Can lower energy consumption • High extraction yields	• Require more research to obtain acceptable disruption efficiency	[1,78,79]
Microfluidization	Streams collision within chamber	• Mild temperature • Possibility of treating high DCW of biomass • No solvent usage • Scalable	• Need higher pressure more energy intensive than explosive decompression	[1,81]
Pulsed arc technology	Proliferation due to electricity	• Temperature or shear effects can be adjusted • Environmental friendly • Energy-efficient • Does not affect the quality of extracts	• Needs further development for scale-up	[49,83,84,95,96]
Cationic polymer coated membranes	Disturbance of local electrostatic equilibrium	• Very gentle • Simple • Effective	• Require more research to obtain acceptable disruption efficiency	[1,97]

microalgae, such as proteins [34], lipids [44] and pigments [45], just to name a few. A recent experiment showed that ultrasonic treatment was able to disintegrate the microalgal cells successfully. This was indicated by the increased concentrations of proteins and carbohydrates, and the highest release of both components was achieved at ultrasonic energy intensity of 0.4 kWh L^{-1} [46]. It was also reported that the application of ultrasonication has been proven to be efficient in disrupting various microalgal strains by destroying the cell walls and membranes [16]. To protect against overheating during the process, the samples are usually placed in an ice bath to absorb the ultrasonic heat [16]. Alternatively, the operating temperature can be regulated externally by circulating cold water during the disruption process [47].

Ultrasonication is generally used in conjunction with solvents in cell disruption [47,48]. Many researchers have investigated the advantages of using ultrasound-assisted extraction (UAE) compared with the other methods. UAE is an environmentally friendly and energy efficient technique [49]. It does not consume as much energy as compared to the other cell disruption techniques such as high pressure homogenization [3]. This technique induces cell damage without the addition of beads as required by bead milling [50] or offers clean extraction by avoiding the generation of fine cell debris as in high pressure homogenization, which could increase the difficulty of separation in the

Table 2
Comparison of mild disruption methods in terms of selectivity, optimum DCW concentration and energy consumption.

Disruption methods	Bead milling	Ultrasonication	Enzymatic disruption	Ionic liquid	Explosive decompression	Microfluidization	Pulsed electric field
Selective product recovery	No	No	Yes	Yes	No	No	No
Optimum DCW concentration	Concentrated	Diluted	Diluted	Diluted	Diluted/concentrated	Diluted/concentrated	Diluted
Energy consumption	High/medium	Medium/low	Low	Low	High/medium	Medium/low	Medium/low
References	[40,41]	[1,27,46]	[1,3,27]	[67,68,73]	[78,79]	[1,80–82]	[83,84,92]

subsequent purification step [1]. UAE could reduce the solvent consumption by increasing the penetration of solvent into cellular materials within short extraction times [39,51]. The synergistic disruptive effects of ultrasonic vibration and ethanol solvent on *Spirulina platensis* improved the extraction performance of phycocyanin (a pigment-protein complex) as compared to the conventional soxhlet extraction. UAE method achieved a yield of phycocyanin at 15.66% within 20 min extraction time, while the soxhlet extraction obtained a yield of 11.13% within 4 h [52]. Another researcher also agreed that ultrasound is relatively easy to use, versatile, and flexible, and requires low investment compared to other novel extraction techniques, such as supercritical fluid extraction (SFE), pressurized solvent extraction or accelerated solvent extraction (ASE) [47]. In addition, ultrasonic devices are applicable to laboratory scale use or can be scaled-up and operated continuously [53].

The effectiveness of using ultrasonication to disrupt various types of cells and the recovery of a wide range of biological compounds has been shown successfully. Nevertheless, most of these studies have concentrated on low-frequency non-focused ultrasonication, 20–40 kHz, neglecting the important role of high-intensity focused sonic energy on microalgal cell disruption [26]. Recently, Wang et al. evaluated the performances of high-frequency focused ultrasonication (operated at 3.2 MHz with input power of 40 W) and conventional low-frequency non-focused ultrasonication (operated at 20 kHz with input power of 100 W) toward lipid extraction. In the experiment, two strains of *Scenedesmus dimorphus* and *Nannochloropsis oculata* were exposed to these two treatments. It was found that for the same cell disruption efficiency, high frequency focused ultrasonication exhibited higher energy efficiency than the conventional ultrasonication in lipid recovery. In addition, the experimental results revealed that both high and low frequency techniques, when used in series could achieve a better cell disruption efficiency than single frequency treatment within the same treatment time [26].

3.4. Enzymatic disruption

Another promising mild disruption method to facilitate access to the inner cellular materials of microalgae is through enzymatic degradation. Although it is cost intensive, the use of enzymes to lyse microalgal cell walls may be advantageous as compared to the other methods and is attracting attention [54].

Enzymes, with their intrinsic catalytic activity and substrate specificity, provide a very gentle and specific means of disrupting cells to promote the release of the cell contents. Since this method can be operated under mild and gentle operating conditions, serious damage to the intracellular compounds can be avoided under the absence of toxic chemicals [55] or without going through aggressive physical conditions such as high shear stress [1,3]. Additionally, this method is effective, highly selective biologically and operates at low temperatures with low energy demand [54,56,57]. During the mild disruption process, an enzyme can selectively degrade a specific chemical linkage, without destroying particles existing in the solution or inducing non-specific reactions of the target products [27]. Moreover, enzymes are easily controlled biological materials and commercially available [27], making the scale-up of this method relatively easy [1].

There are a few variables that could affect the enzymatic activity, such as the characteristic and concentration of the enzymes, concentration of the specific reactant, intracellular composition, cell wall composition, type of microalgae and temperature [58]. Lytic enzymes, such as glycosidases, glucanases, cellulose, pectinase and lipases have been investigated for cell lysis. Researchers found that during lysis, enzymes act very specifically by binding to specific compounds on the cell wall, hydrolyse the bonds and subsequently resulting in effective cell wall degradation [55,59]. Because of the specificity of enzymatic mechanism, the type of enzymes used in the enzymatic processes is the main parameter that influences the disruption yield [60]. Enzymes are very selective in their degradation process, therefore knowing the exact composition of the cell wall is particularly important in enzymatic treatment, which enables the selection of the right type of enzymes [3]. If the type of enzyme is chosen appropriately, the enzymatic cell disruption can be very effective [27].

It was reported that enzymes can aid in the extraction of proteins, oils and other intracellular contents. For example, the use of carbohydrases to attack cell wall components allowed liberation of more proteins from rice bran and resulted in the increase of the protein yield [61]. The release of antioxidant peptides from *Navicula incerta* using enzymatic hydrolysis has been studied by evaluating the cytoprotective activity of the bioactive peptides against alcohol-induced damage in HepG2/CYP2E1 cells. It was observed that *N. incerta* hydrolysed by papain exhibited higher antioxidant activities than other enzymes, such as alcalase, α-chymotrypsin, neutrase, pepsin, pronase-E and trypsin [62]. A recent review written by Show et al. also agreed that the utilization of enzymes can help facilitate the hydrolysis of microalgal cell walls [39].

3.4.1. Mixed enzymes

A combination of cell wall-hydrolysing enzymes leads to a more effective release of intracellular contents, by allowing various types of enzymes to act simultaneously on the cellular structure of microalgae. A study demonstrated that two different types of enzymes, namely cellulases and lipases were used to breakdown cellulose and phospholipids respectively, to facilitate protein extraction [63]. The occurrence of degradation was indicated by the formation of glucose and glycerol as degradation products [54]. Another study reported that cellulase could hydrolyse the cellulosic structure, whereas lysozyme can degrade the tough polymers present on the cell envelope of microalgae, thereby facilitating the extraction of intracellular compounds [57]. It was found that the linkages within the polysaccharide matrix of oat bran were cleaved effectively after being exposed to mixed enzymatic pre-treatment, as indicated by the release of more intracellular contents [64].

3.4.2. Challenges of deploying enzymatic disruption on large scale

Despite offering several advantages, enzymatic cell wall degradation is not widely practiced in industry, at the present time [3]. Cell lysing enzymes have traditionally been cost prohibitive, making this strategy uneconomical [55]. The high cost of enzymes stems from the fact that they generally cannot be recovered and recycled after being used in production [65]. Besides that, another critical downfall that restricts the application of enzymes in biorefinery is the limited availability of suitable enzymes for microalgae disruption, due to their high substrate selectivity [1]. Additionally, low production capacity compared to mechanical or chemical disruption, because of the generally long reaction time and product inhibition, is a drawback that could affect the efficiency of an enzymatic disruption process [38].

3.4.3. Possible solution: immobilization or combination process

It was suggested that enzyme immobilization [66] could be a solution to allow the implementation of enzymatic disruption at large scale for mass production of proteins due to the fact that it could reduce the overall cost, by allowing the reuse of enzymes and thus reducing the needed amount of enzymes. Additionally, steps required to separate enzymes from the products can also be eliminated [1,55]. Due to the strong correlation between the amount of the biomass and immobilized enzymes, finding a good balance between them is also necessary for an economically attractive process [1]. It was reported that the cell walls of *Chlorella* sp. can be degraded enzymatically by immobilized cellulose on an electrospun polyacrylonitrile (PAN) nanofibrous membrane, with 62% hydrolyzing conversion. Interestingly, the immobilized cellulose still can be reused after five passes, with the hydrolysis yield remaining at 40% [66]. This finding is in agreement with another study demonstrating that the application of enzyme immobilization in degrading the cell wall of *Chlorella pyrenoidosa* was an efficient method

[27].

Since the efficiency of the specific immobilized enzymes varies among different microalgal strains and the process requires long reaction times, it was suggested that enzyme immobilization or enzymatic disruption without immobilization could potentially be upgraded by combining with other techniques [1]. The combination of enzymatic cell disruption with chemical or mechanical treatment could reduce the cost of an enzymatic process, resulting in lower overall operational costs [3,27,39]. This approach appears attractive and promising because it will not only reduce solvent usage or energy consumption but also increase the recovery efficiency of intracellular contents [56].

3.5. Ionic liquids

Ionic liquids can be an eco-friendly alternative to traditional solvents and their applications in the processing of microalgae appears to be promising [67,68]. Ionic liquids are organic salts in the liquid state, consisting of a large asymmetric organic cation and an organic or inorganic anion [68]. They are designated as “designer solvents” due to their synthetic flexibility. They can be tailored for a specific solubility, conductivity, polarity and hydrophobicity by choosing the cationic or the anionic constituents [69,70]. Furthermore, ionic liquids present a few attractive characteristics such as low-melting point, extremely low volatility under atmospheric conditions, capable of dissolving a wide range of polar to non-polar compounds, low flammability, as well as high thermal and chemical stability. All these features make them an excellent choice to conventional solvent and allow the development of safer processes at large scale [67,68,71]. Nevertheless, it should be noted that some ionic liquids are not environmentally friendly and require laborious purification process [72].

Ionic liquid extraction methods have demonstrated some unique prospects compared to traditional solvents [68] by displaying dual function, in which it can act as a cell disruption agent as well as an extraction solvent [67]. Ionic liquids have excellent properties for use in cellulosic biomass treatment due to their high hydrogen bond accepting ability, which could disrupt the extensive hydrogen bonding network of polymers, leading to the breakdown of complex networks of lignin, cellulose and hemicelluloses [67]. Ionic liquids exhibited better microalgal disruption efficiency under mild conditions compared to organic solvents [68]. *Synechocystis* sp. [73] and *Scenedesmus* sp. [74] have successfully been dissolved in ionic liquids during the extraction process. Lately, another study also verified the effectiveness of using ionic liquids in promoting the dissolution of cell walls of *C. vulgaris* [75]. Furthermore, numerous studies revealed that ionic liquids are suitable for use in the extraction of fragile and sensitive compounds from microalgae, such as phycocyanin from *Spirulina* [76] and astaxanthin from *Haematococcus pluvialis* [77].

Ionic liquids can be designed for biomass pre-treatment to suite the composition of biomass. In addition, they can also be used in combination with other pre-treatment methods for effective pre-treatment of biomass [67]. Interestingly, it was observed that recycled ionic liquids could retain their ability to dissolve microalgae *Synechocystis* sp. with no significant difference compared to the fresh ionic liquids [73]. Nonetheless, only few extraction studies have been performed on microalgae using the eco-friendly ionic liquid extraction. As such, more comprehensive investigations are still required to make the application of ionic liquids in the downstream processing of microalgal industry cost-effective at large scale [67].

4. Potential mild disruption technologies that are still under development

Due to the growing popularity of harnessing valuable intracellular contents from microalgae, the evolution of the existing approaches as well as the invention of potential innovative technologies for mild microalgal biorefinery are emerging rapidly in recent decades. These include explosive decompression, microfluidization, pulsed arc technology and disruption using cationic polymer coated membranes.

4.1. Explosive decompression

Explosive decompression has been promoted for processing a wide variety of algae in various industries. This technique can aid in extraction of desired intracellular compounds of microalgae through the disruption of cellular structure, increasing the porosity of the cell wall and, thus, causing the release of the cell contents from microalgae prior to extraction. Explosive decompression makes use of the solubility of large quantities of carbon dioxide or nitrogen in solution under pressure. In a pressure vessel, carbon dioxide or nitrogen is introduced under high pressure to treat the biomass. The sudden increase in pressure generates gas with massive volume expansion that stretches the membrane of each cell until rupture, subsequently liberating the cell contents from microalgae [78].

In an experiment conducted by Dierkes et al., explosive decompression was found to be able to rupture *Haematococcus pluvialis* efficiently, resulting in the release of cell contents. This method enabled the effective and simultaneous extraction of lipid and astaxanthin with high extraction yields, namely 72.3–92.6% of astaxanthin and 80–100% of lipid [79]. Besides being able to disrupt large amounts of biomass efficiently with high extraction yields, this energy efficient approach can be operated under mild temperature and does not require the use of aggressive chemicals during the cell disruption process. All these advantages make explosive decompression stand out among other approaches as one of the promising techniques to be applied aptly in the mild microalgal biorefinery [79].

4.2. Microfluidization

Of late, a robust but mild cell disruption treatment involving microfluidization has been proposed [80]. During the disruption process, the microfluidizer functions as a high-pressure homogenizer in which the target cells are passed through the specially designed interaction chambers at extremely high velocities. Particle size reduction can be achieved through streams of collisions within the specialized geometry of the chamber walls [81].

In general, a microfluidizer requires higher pressure to disrupt cells, thus it is more energy exhaustive compared to explosive decompression. However, microfluidization is deemed as a nascent technology for mild biorefinery, due to its ability to rupture cells mildly under mild temperature and without solvents. Besides that, it is also scalable with the possibility of treating large amounts of biomass [1].

Microfluidization with high pressure was found to effectively damage the surface of the cell wall of *Chlorella ellipsoidea*. The mean particle size and the polydispersity index of *C. ellipsoidea* were evaluated to determine the effect of microfluidization on the breakdown of the microalgal cell wall. After being pulverized by microfluidization at high pressure of 20,000 psi, the mean particle size was significantly reduced from 2463 to 361 nm. The experimental data showed that microfluidization enabled extremely effective cell disruption of *C. ellipsoidea* with improved bioaccessibility to carotenoids, such as zeaxanthin and β-carotene, compared with untreated *C. ellipsoidea* [80].

This observation is in agreement with a previous study conducted in 2011 using the rigid cell wall of *Chlorella vulgaris* as a model microalga in microfluidization processing. After the treatment, the cell surface of untreated and microfluidized *C. vulgaris* were imaged by scanning electron microscopy to evaluate the impact of microfluidization on the *C. vulgaris* cell wall. The scanning electron microscope image displayed that the treatment of microfluidization at 20000 psi was able to completely rupture the *C. vulgaris* cell wall into a nano-scale size, which was approximately 10 times smaller than untreated *C. vulgaris*.

This result demonstrated that microfluidization was effective in pulverizing the cell walls of microalgae [81].

This flexible technology is capable of rupturing a wide variety of cells efficiently by optimizing the pressure. A recent study on the optimization of intracellular product extraction from the bacteria *Neisseria denitrificans* using microfluidizer has also been conducted. At optimized conditions, the disruption rate increased two-fold. In the meantime, the yield of intracellular components such as proteins, nucleic acids, polysaccharides was increased by 26% with 1 g of cells [82].

4.3. Pulsed arc technology

Pulsed arc technology, such as pulsed electric fields (PEF) and high voltage electrical discharges (HVED) are environmental friendly and energy-efficient disruption techniques. As the temperature or shear effects can be minimized through the modification of the treatment vessel design [83], these emerging electrical techniques appear to be potentially gentle, low-temperature as well as highly effective process to be applied in the mild biorefinery [49,83]. The electrotechnologies do not introduce additional impurities during the disintegration process, so any undesirable changes of the intracellular components can be prevented. Furthermore, cell disruption using electrical treatment provides an easy approach for the subsequent downstream recovery and purification operations, because hardly any cell debris that could negatively affect the quality and purity of the extracts will be produced after the electrical disruption [84–86].

Previous studies have shown that the application of these electrical disruption techniques could enhance the extraction yields of various biomolecules from different sources, such as the extraction of oil from sesame seeds [87], nutraceuticals and antioxidant compounds from papaya peels [51], soluble phenolic bio-compounds from fermented grape pomace [49] and betanines from red beet [88]. Besides that, the effectiveness of electrical disruption techniques on microalgae has also been studied. Goettel et al. [84] studied the release of organic substances such as carbohydrates and proteins from fresh water microalgae *Auxenochlorella protothecoides* using PEF. Compared to the control samples, the total organic carbon content (TOC) of the supernatant was up to 6 times higher after PEF induced cell disintegration. Similarly, other studies also demonstrated that PEF is an effective procedure for extracting proteins from *Haematococcus pluvialis* and *C. vulgaris* [89], lutein from *C. vulgaris* [90], C-phycocyanin from *Artrosphira platensis* [86], and proteins from *Nannochloropsis* spp. [91].

Studies showed that field strength and treatment duration are decisive parameters in determining the efficacy of the PEF treatment on the extraction enhancement [86,89]. Experiments with microalgal suspensions of different biomass concentration (36–167 g dry weight per kg suspension) showed that the biomass concentration did not negatively affect the disruption efficiency. Based on this finding, it was suggested that further reduction of energy requirement by PEF is possible by increasing the biomass content of the suspensions [84]. Optimization of these processing parameters enables the electroporation of microalgae at low electric field strengths and minimal energy inputs, making PEF an economically feasible technology [86,89].

Another research team found that both PEF and HVED exhibited selective extraction of water soluble ionic components, microelements, small molecular weight compounds and proteins from *Nannochloropsis sp.* [45]. It was reported that the cell disintegration induced by both PEF and HVED methods could intensify the extraction of biological components, but HVED was generally more favourable than PEF due to its higher efficiency [92]. HVED always displayed a higher disintegration index than PEF. For instance, the HVED treatment was found to be able to extract higher amounts of proteins and polyphenols from sesame seeds [87]. Extraction assisted by HVED was demonstrated to be more effective than PEF in recovering proteins and phenolic compounds from mango peels [93]. HVED treatment was more efficient than PEF in terms of energy input and effective treatment time to extract phenolic compounds and proteins from olive kernels (*Olea europaea*) [94]. Another study also showed that HVED was able to achieve higher phenolic compounds recovery from fermented grape pomace with lower energy requirement, when compared to PEF [49]. A possible explanation for this phenomenon is that PEF treatment causes rupture of only cell membranes, whereas HVED can cause more extensive damage by acting on both cell walls and membranes of the biomass during the cell disruption process [92].

Studies found that the functionality and quality of extracted polyphenols were not affected after the electrically assisted extraction from vine shoots [95] and grape pomace [96]. Furthermore, low protein and polyphenols losses were observed during the electrical treatment of sesame seeds [87]. All these findings suggest that PEF and HVED are suitable for use in mild biorefinery to isolate fragile components, such as bio-functional proteins.

4.4. Disruption using cationic polymer coated membranes

Disruption of microalgal cells using cationic polymer coated membranes has been suggested as a nascent and novel mild cell disruption technology with high efficiency [1,97]. This gentle method was devised using a functional membrane coated with a cationic polymer, by Yoo et al. (2014), to extract the intracellular lipid directly from wet microalgae without requiring dewatering [97]. The research team used a membrane coated with positively charged tertiary-amine-containing polymer to induce the perturbation of the local electrostatic equilibrium of the amphiphilic microalgal cells with negatively charged phospholipid bilayers. As a result, the microalgae cells were attracted to a functional membrane by electrostatic interaction. The microalgal culture was simply shaken together with the membrane to promote direct interaction. In this experiment, cell bursting caused by the local rearrangement of lipid bilayers in microalgae was clearly observed. A significantly high disruption yield of 25.6 ± 2.2% was obtained from this experiment, verifying that this simple advanced technique is very efficient in facilitating the liberation of the intracellular contents entrapped in the complex cell wall of microalgae [97].

5. Expectations of mild cell disruption methods at industrial scale

The main approach to the success of microalgal-based biorefinery is the development of mild, cost effective and low in energy consumption technologies [1,3]. A feasible and practical mild cell disruption method that is suitable to be deployed on industrial scale should also possess a few characteristics. These include simplicity, minimum production of unnecessary liquid wastes and high disruption efficiency [36]. In order to generate high quality of extracted products [1], a mild cell disruption method should be able to maintain the functionality of the fragile intracellular components during the disruption process [3]. Besides that, when establishing a mild cell disruption technique for industrial-scale application, factors such as easy scalability, must also be taken into consideration [98].

Different technologies should be tailored for different products of interest. But this is quite labour-intensive and costly, if the whole production process to be developed is targeting only one or two bio-products from microalgae. To make the implementation of the biorefinery concept into microalgal industry successful, the developed techniques should be applicable to various end products with good quality at large quantity [1]. It has been suggested that the production of microalgae will become even more economically feasible with higher profitability, if more natural components can be obtained simultaneously from one microalgae production process [3,6]. The use of mild cell disruption methods should not affect the functionality of other valuable components available in the microalgae. Hence, the attempt to maximally exploit all the intracellular components produced by microalgae, to obtain a variety of high value-added end products simulta-

neously in large quantities, should not be an unattainable mission.

However, appropriate biorefinery concepts for mild extraction are relatively new [3]. Further intensive studies focusing on the use of multifunctional processes for simultaneous extraction, separation and transformation of two or more desired products are required before commercial use is possible.

6. Challenges and future perspectives

To ensure the success of microalgal biorefinery, especially for the mass production of bio-functional proteins, economic feasibility remains the most important aspect to consider when developing mild cell disruption techniques for industrial scale. The operating cost for mass production of bio-functional proteins is rather complex. The direct comparison of the energy consumption among various disruption methods is relatively perplexing, due to the absence of a common microalgal strain and disruption condition [13]. From a practical point of view, it is imperative to optimize the balance between product yields, processing cost and energy consumption, in order to maximize the efficiency and profitability [99] of bio-functional protein production at commercial level. Due to the growing interest in harnessing valuable bioactive compounds from microalgae for food and pharmaceutical industries [100], the development of cost-effective mild cell disruption for industrial scale is garnering worldwide attention. However, attempts to develop cost-effective technologies that would economically allow efficient mild disruption of microalgae, to facilitate the extraction of bio-functional proteins, is relatively new and remains an uphill battle.

As most of the current microalgal research is concentrated on lipid extraction for biodiesel production, common industrially available cell disruption methods such as microwave treatment, high speed homogenization and high pressure homogenization expose the cells to harsh conditions, with the possibility of causing denaturation to the fragile functional compounds during treatment [1,5,101]. These commonly used conventional techniques will cause complete disruption of the cells, mainly focused on obtaining one specific product while damaging the other available components [3]. Many of the other valuable ingredients, such as fragile bio-functional proteins present in the biomass, will lose their functionality if applying the same disruption technique used in lipid extraction. Therefore, all these methods are not suitable to assist the recovery of bio-functional proteins from microalgae.

Economic limitations to mass production of bio-functional proteins at lower cost are one of the main challenges encountered in developing the mild cell disruption methods, particularly in enzymatic disruption. Besides that, the high specificity and selectivity in enzymatic reaction could be the main restriction to extensive applications of enzymatic disruption for a wide spectrum of microalgae. A preliminary study on the exact composition of a target microalgal cell wall should be performed prior to treatment, when selecting the right type of enzymes for cell disruption process.

To address the challenges faced in mild disruption, continuous effort is necessary to develop a generic method that is practical to application commercially on wide range of microalgal species, regardless of their diverse cellular structure, for maximum recovery of bio-functional proteins from microalgae. On top of that, all the potential mild cell disruption methods should also be tested with different types of microalgae, to assess their general applicability to a wide variety of microalgae.

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