Towards a better understanding of microalgae natural flocculation mechanisms to enhance flotation harvesting efficiency

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Cécile Formosa-Dague

Irem Demir
Alexandre Besson
Pascal Guiraud IWA
Cécile Formosa-Dague (corresponding author)
TBI, Université de Toulouse, CNRS, INRAE, INSA, Toulouse,
France
and
TBI-INSA de Toulouse,
135 avenue de Rangeuil 31077 Toulouse Cedex 4,
France
E-mail: formosa@insa-toulouse.fr

ABSTRACT

In microalgae harvesting, flocculation is usually a compulsory preliminary step to further separation by sedimentation or flotation. For some microalgae species, and under certain growth conditions, flocculation can occur naturally. Natural flocculation presents many advantages as it does not require the addition of any flocculants to the culture medium and shows high efficiency rate. But because natural flocculation is so specific to the species and conditions, and thanks to the knowledge accumulated over the last years on flocculation mechanisms, researchers have developed strategies to induce this natural harvesting. In this review, we first decipher at the molecular scale the underlying mechanisms of natural flocculation and illustrate them by selected studies from the literature. Then we describe the developed strategies to induce natural flocculation that include the use of biopolymers, chemically modified or not, or involve mixed species cultures. But all these strategies need the addition of external compounds or microorganism which can present some issues. Thus alternative directions to completely eliminate the need for an external molecule, through genetic engineering of microalgae strains, are presented and discussed in the third part of this review.

Key words | flocculation, flotation, harvesting, microalgae

INTRODUCTION

Modern life is intimately linked to the availability of fossil fuels, which continue for the moment to meet the world's growing energy needs even though their use drives climate change (Georgianna & Mayfield 2012). But because of the increasing world population and energy demand, there is an urgent need for renewable sources to produce energy (Markou & Nerantzis 2013). In this context, microalgae are receiving increasing attention worldwide as an alternative and renewable source of energy because of their eminent oil producing capacity (Pragya et al. 2013). But the potential of microalgae is in fact even greater and they also represent an important source of biomass and of molecules of interest for the fields of food, feed or health. Indeed, microalgae are unique microorganisms which convert light energy, water

and inorganic nutrients into biomass resource rich in value-added products such as lipids, carbohydrates, proteins and pigments (Pragya et al. 2013; Minhas et al. 2016). Moreover, microalgae have several advantages that make them a potential new generation of feedstock for the production of biofuel and molecules of interest. First, microalgae are capable of all year round production (Brennan & Owende 2010), and they grow on aqueous media but need less water than terrestrial crops (Dismukes et al. 2008). Also, nutrients for their cultivation can be found in wastewater, and there is no need at the moment for herbicide or pesticide applications (Rodolfi et al. 2009), although this may perhaps change in the future as microalgae cultures also suffer from parasites or other unwanted algal species (Huo et al. 2017). They also have a rapid growth rate and many species have an oil content in the range of 50–70% dry weight of biomass. To give an example, compared to soybean, microalgae can produce up to 300 times more oil per area unit, considering ideal laboratory conditions (Ziolkowska 2014). Finally, as mentioned

above, they can also produce valuable co-products such as metabolites, long-chain polyunsaturated fatty acids and vitamins that are used in nutraceuticals industries as food additives (Minhas et al. 2016).

While the small-scale production of microalgae to obtain high value-added molecules is nowadays efficient, the large-scale production of molecules, substituting fossil carbon resources, from microalgae faces a number of technical challenges that have made the current growth and development of the biofuel industry economically unviable (Waltz 2009; Pragya et al. 2013). These include, among others, (i) the selection of algal species with specificities that meet the requirements for both biofuel production and the extraction of useful co-products (Rodolfi et al. 2009; Brennan & Owende 2010; Liao et al. 2016), (ii) the inexpensive production of large quantities of microalgae biomass (Chisti 2007) and (iii) the development of efficient harvesting methods (Molina Grima et al. 2003; Pragya et al. 2013; Kurniawati et al. 2014; Coward et al. 2015; Ndikubwimana et al. 2016). While recent progresses notably in synthetic biology and in culture methods provide solutions for the first two points (Jagadevan et al. 2018), the main limitation encountered by industry remains the harvesting of microalgae (Ndikubwimana et al. 2016). Harvesting consists in removing at a minimal cost the microorganisms from their aqueous culture medium where their concentration is low (Lam & Lee 2012), without destroying them so as not to lose their production in solution. This crucial step of harvesting and dewatering has been assumed to account for one third of the entire price of microalgal biomass production in industrial processes (Molina Grima et al. 2003). Several methods have been proposed for microalgae harvesting, including centrifugation, filtration, flocculation combined with settling or flotation (Garg et al. 2012). However, most of these methods are synonymous with high costs and energy consumption, often for low efficiency rates. For instance, centrifugation, the most commonly used method for harvesting, consumes a large amount of energy and can cause damage to the cells because of high shear forces (Pragya et al. 2013). Filtration involves using filtering media or membranes, which, in the case of microalgae separation, can get clogged because of the small size of the cells, resulting in high operating costs (Uduman et al. 2010). As for flocculation combined with settling, it seems to be a promising low-cost approach for large-scale harvesting of a wide variety of microalgae species (Molina Grima et al. 2003); however, contamination is a major issue in this technique, as the chemical flocculants used to induce flocculation end up in the harvested biomass,

and can interfere with the final application of the biomass (food or feed) (Vandamme et al. 2013).

In this context, flotation is believed to be a promising harvesting technique that takes advantage of algae's natural low density and self-floating tendency (Garg et al. 2012). Assisted flotation consists in air or gas being transformed into bubbles rising through a solid/liquid suspension. As a result, solid particles get attached to gas–liquid interfaces and are carried out and accumulate on the surface. Thus flotation allows for low-cost cell harvesting, without necessarily using flocculants that could damage them. In addition, it is a relatively rapid operation that needs little space, has moderate operational costs, and could thus overcome the bottleneck of feasible microalgal biofuel production. However, the problem with this technique is that the interaction between the bubbles and the cells is generally repulsive, due to the negative surface charge of the cells and the bubbles in water (Yang et al. 2001), and the low hydrophobicity of the algal cells. This results in the non-interaction of the cells with the bubbles and thus in a poor efficiency of this harvesting technique.

Among the ways to improve flotation efficiency, flocculating the cells prior to flotation is a strategy that has proven efficient. Indeed, this procedure allows aggregating the cells into large flocs that bubbles produced during the flotation process cannot avoid. This way, cells are easily removed from the water. However, in many cases, this flocculation step is performed using synthetic flocculants which, as previously stated, can contaminate the harvested biomass but also the recycled water. Therefore in many cases, natural flocculation is a preferred alternative. It is indeed possible under certain conditions to induce natural formation of algal flocs; this characteristic was first mentioned by Golueke and Oswald in 1965 who observed microalgae flocculation in cultures under optimal sunlight and heat conditions (Golueke & Oswald 1965). Among natural flocculation mechanisms, so far two types of mechanism have been identified: autoflocculation, where the flocculation is triggered by a molecule or precipitate that naturally forms in the culture medium, and bioflocculation, where a molecule produced by the cells present in the culture medium (microalgae but also other types in the case of co-cultures) is directly responsible for the flocculation. But for both auto- and bioflocculation, the mechanisms of flocculation described are the following: compression of the electric double layer, charge neutralization, bridging, patch mechanism and sweeping. Depending on several parameters such as the microalgae species used, or the conditions in which they are cultured, one or another mechanism takes place. This makes it then an important field of research to identify and understand these mechanisms for

all the different species/culturing situations, as being able to use natural flocculation, combined with flotation in harvesting processes, could be the key to reduce the costs associated with microalgae. In this review, we will focus on these natural flocculation mechanisms, and first describe and illustrate the already known mechanisms. Most studies on microalgae flocculation, and the ones that will be described in this first part of the review, propose a mechanism of flocculation. However, these studies should also be taken with caution as only a few of them also propose experiments or measurements to confirm the flocculation mechanism described or exclude alternative mechanisms. Thus in these studies, the mechanisms proposed often remain hypothetical. Then in a second part, we will detail cases where natural flocculation mechanisms are induced directly using natural molecules, and finally we will discuss what could be the future directions to further improve them.

NATURAL FLOCCULATION MECHANISMS AND KEY PARAMETERS TO CONTROL THEM

Flocculation consists in the aggregation of destabilized compounds, in our case, microalgae, to form structures of more important apparent size, called 'flocs'. In most cases, flocculation is often integrated into multi-stage harvesting processes and can for instance be used as a preliminary to sedimentation, centrifugation, flotation or filtration processes. But in all cases, the destabilization of algal suspensions by flocculation can be the result of one or more mechanisms, which are compression of the electric double layer, charge neutralization, bridging, patch mechanism or entrapment in a precipitate, also known as sweeping, presented in Figure 1. In this first part of the review, we will describe these mechanisms and, in each case, illustrate them with examples of microalgae auto- and bioflocculation where they have been identified.

Decreasing the electrostatic repulsion forces

The first flocculation mechanism described, screening, is due to the decrease of electrostatic repulsive forces via the lowering of the surface charge by pH variations, or via the well-known compression of the electric double layer (Figure 1(a)). Most microalgae have negatively charged surfaces (Molina Grima *et al.* 2003) and thus they can attract, through electrostatic interactions, positively charged ions available in the surrounding solution. While some of these
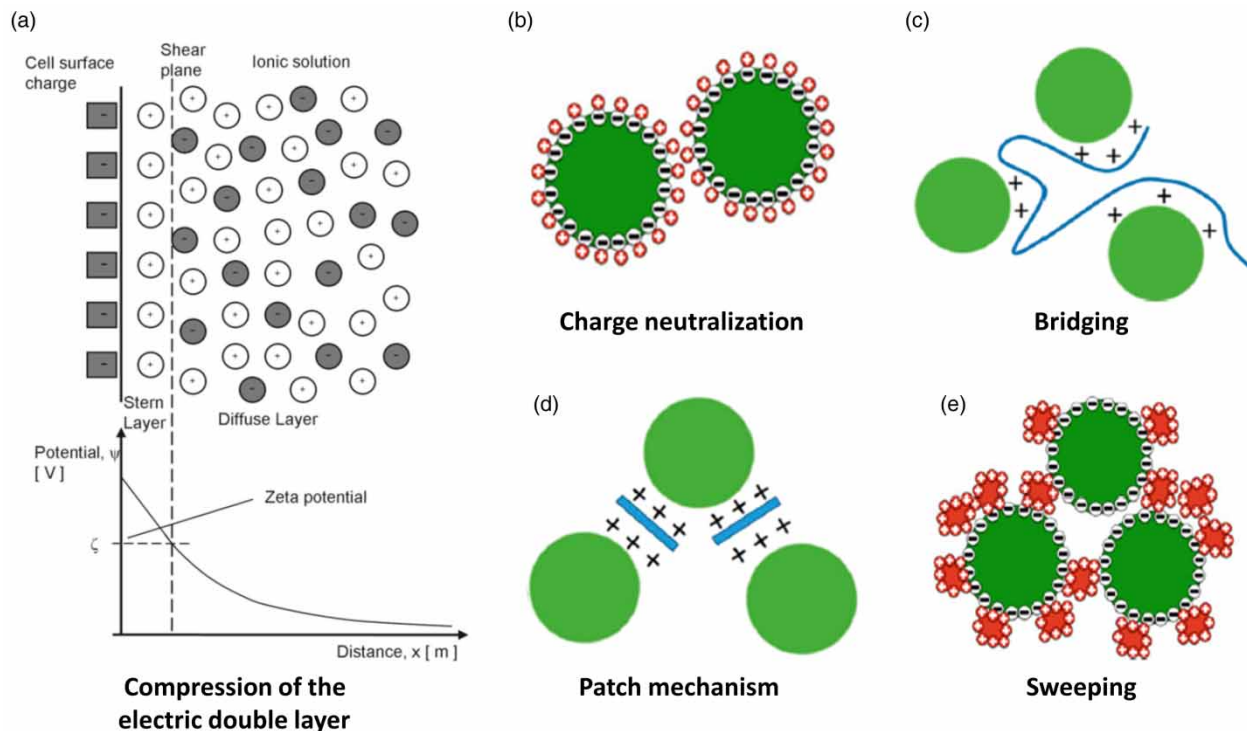

Figure 1 │ Schematic representation of natural flocculation mechanisms. (a) Compression of the electric double layer, (b) charge neutralization, (c) bridging, (d) patch mechanism, and (e) sweeping. Reprinted with permission from Pahl *et al.* (2013), Muylaert *et al.* (2015) and Blockx *et al.* (2019).

ions adsorb to the surface of the microalgae cells to form a dense layer, others remain in the solution and form what is called the diffuse layer. This two layer system is referred to as the electrical double layer, which is, according to the DLVO (Derjaguin–Landau–Verwey–Overbeek) theory, related to the ionic concentration of the solution and to the surface charge itself, the value of which depends on the pH. The interface that separates the layer bound to the cell from the unbound layer is called the shear plane, where the potential is called the zeta potential. This is depicted in Figure 1(a). The microalgae zeta potential is negative over a pH range of 4 to 10 and a point of zero charge (PZC) is regularly detected depending on the species for a pH between 3 and 4 (Phoochinda & White 2003). It is therefore possible to reduce the electrostatic repulsion forces and to promote coagulation by imposing a pH close to the PZC of microalgae. Furthermore, the more the ionic strength of a solution increases, the more the absolute value of the zeta potential of the microalgae decreases due to the compression of the unbound layer (Pahl *et al.* 2013). This decrease leads to a reduction in the electrostatic repulsion forces, which can lead to the reduction or disappearance of the energy barrier initially present and generate the agglomeration of the microalgae thanks to attractive van Der Waals' forces which in this case have become predominant. These two effects have been described in two studies. In the study by Ndikubwimana and co-workers, the authors modified the ionic strength of the culture medium of *Desmodesmus* sp. by adjusting its pH. Their results showed that this reduction of the pH induced a large decrease of the energy barrier and the further destabilization of the microalgae suspension, which flocculated with an efficiency of 78.5% (Ndikubwimana *et al.* 2015). In the study by Cui and co-authors, it was found that increasing the ionic strength of the culture medium of the microalgae species *Nannochloropsis oculata* using a higher concentration of Al^{3+} ions decreased the energy barrier of the suspension, which resulted in a higher flocculation efficiency (Cui *et al.* 2014). It must be noted that these two cases are not auto- or bioflocculation cases, as the modifications that led to the flocculation did not naturally occur.

Neutralization of negative surface charges

The second flocculation mechanism is called charge neutralization (Figure 1(b)). This mechanism also takes advantage of the negative cell surface of microalgae cells; charge neutralization then takes place when these negative charges

are decreased by a positively charged molecule that absorbs at their surface (Levy *et al.* 1992). This charge neutralization leads to the reduction of the repulsive electrostatic forces and to the predominance of van der Waals' attractive forces, which leads to the flocculation of the microalgae. In this case, the key parameter ensuring successful flocculation is thus the flocculant concentration, as it is directly proportional to the surface area that needs to be neutralized (Muylaert *et al.* 2015). In the case where the flocculant concentration is too high, the surface charge of the cells may become positive, which results in the increase of repulsive electrostatic forces and further stabilization of the suspension. In a recent work conducted in our team, it has been shown that the flocculation of *Phaeodactylum tricornutum*, a marine diatom, induced by an increase of pH in marine water, was the result of the precipitation of magnesium ions into magnesium hydroxide presenting a positively charged surface and thus flocculating the cells through a charge neutralization mechanism (Formosa-Dague *et al.* 2018). Using atomic force microscopy, it was then possible to also show the ability of the *P. tricornutum* cell wall to absorb magnesium hydroxide particles that formed a partial capsule around the cells, thus neutralizing their surface negative charges and destabilizing the suspension. In the case of this particular study, the pH was artificially increased for the flocculation experiments to mimic the natural pH increase over the course of the culture in *P. tricornutum* species. Indeed, a previous study conducted on *P. tricornutum* showed that the photosynthetic activity of this species could increase the pH of the culture medium up to 10.8 after discontinuing the CO_2 supply, and that at this pH, the cells were able to autoflocculate (Spilling *et al.* 2011). Although in this study, the flocculation mechanism is not described, it is most likely that it is also charge neutralization as described with the same species in Formosa-Dague *et al.* (2018).

Bridging the cells

A third flocculation mechanism is called bridging (Figure 1(c)). In the bridging mechanism, positively charged polymers interact with cells through electrostatic interactions and absorb at their surface. At low and intermediate concentrations, the polymers can then adsorb onto other cells if the extension of the polymer from the cell surface exceeds the distance over which the cell–cell repulsion is active, thus bridging the two. In this case the efficiency of this mechanism relies on different parameters: (i) the polymer concentration: if a higher dose is used the

cells may become completely positive, which will restabilize the suspension, or the extension of the polymer in the solution may create a steric effect preventing flocculation, (ii) its length: the polymer chains should be long enough to extend from one cell to another (Pal *et al.* 2005), although it must be taken into account that the actual size of a polymer molecule in solution is smaller than its maximum length, and depends on the concentration and the chemical conditions of the solution, (iii) its molecular weight: it is reasonable to think that length and molecular weight are directly linked (to our knowledge independent study of these two parameters has never been performed), but it has been shown that high molecular weight polymers are more efficient (Muylaert *et al.* 2015), (iv) its charge: again it has been shown that low-charged polymers are more efficient, and (v) the ionic strength of the culture medium: for instance bridging by non-ionic polymers can occur only when the adsorbed layer thickness is more than two times greater than the thickness of the electrical double layer (Bolto & Gregory 2007). While for microalgae, the bridging mechanism is mostly associated with cationic polymers interacting with the cells through electrostatic interactions, polymer adsorption onto surfaces can also take place through hydrogen bonding or through ion binding in certain conditions (Bolto & Gregory 2007). A good example of the bridging mechanism can be found in Vergnes *et al.* (2019). In this study, the authors optimized culturing conditions in which *Arthrospira platensis* cells produced exopolysaccharides (EPS). Using further atomic force microscopy imaging experiments, the authors could show that these EPS formed in a culture medium a soft and adhesive gel bridging the cells together and thus bioflocculating them. Another interesting example of bridging flocculation taking place during bioflocculation can be found in Lananan *et al.* (2016). Here the authors show that *Ankistrodesmus* sp. cells could act as a cationic flocculant bridging together cells from *Chlorella vulgaris* species. Indeed, the authors showed that *Ankistrodesmus* sp. had a positive zeta potential, thus allowing it to interact electrostatically with the negative cell surface of *C. vulgaris* at a pH between 6.10 and 7.10. Moreover, *Ankistrodesmus* sp., having an elongated shape, is able to extend from one cell to the other, and thus bridge *C. vulgaris* cells together.

Patch flocculation

The fourth flocculation mechanism that has been described is known as the patch mechanism (Figure 1(d)). This mechanism involves positively charged small polymers that adsorb at the surface of a negatively charged cell and create an irregular charge distribution at its surface, i.e. positive 'patches' or 'islands' between regions of uncoated, negatively charged surface. These positive patches can then interact with other negatively charged areas at the surface of other cells and connect them together. In this case the key parameters that will ensure successful flocculation are the length of the polymers, as only short polymers can form patches at the surface of the cells, and their charge, as highly charged polymers have been shown to be more efficient (Bolto & Gregory 2007; Muylaert *et al.* 2015). In a study by Salim *et al.* (2014), the authors identified patch mechanism as being a possible mechanism at play in a case of autoflocculation of the microalgae species *Echinocactus texensis*. Using scanning electronic microscopy (SEM) imaging, they could show that *E. texensis* cells had attached to their cell surface short EPS patches composed mainly of glycoproteins responsible for their autoflocculating behavior. Although in this study, the zeta potential measured for *E. texensis* cells was globally negative, it is not excluded that these EPS patches feature positive charges thus allowing a patch mechanism.

Sweeping flocculation

Finally, the last flocculation mechanism that we will detail here is the sweeping mechanism (Figure 1(e)). Sweeping flocculation can be described as the mechanical trapping of microalgae in the massive structure of an inorganic precipitate, resulting in their flocculation. The sweeping mechanism is often described as the result of a pH increase in the culture medium that triggers this massive precipitation; however, it should be attributed more exactly to the increase of hydroxide ions OH^- involved in the precipitation. Although this pH increase can naturally occur in microalgae cultures, as far as we know, very few examples of naturally generated sweeping flocculation have been reported (Sukenik & Shelef 1984). Natural pH variation is usually not sufficient to induce precipitation for many microalgae species, and, when flocculation is observed, often the pH was artificially increased by the addition of a base. In a first example, Besson and co-authors showed that in cultures of the hypersaline microalgae *Dunaliella salina*, the only possibility to induce flocculation was to increase the pH by addition of NaOH directly into the culture medium. The authors then showed that this increase in the pH caused the precipitation of Mg ions present in the culture medium into magnesium hydroxide, thus sweeping the cells and precipitating them (Besson & Guiraud

2013). Although magnesium hydroxide is positively charged and could also flocculate the cells through charge neutralization, this mechanism was excluded in a further work that showed using atomic force microscopy that magnesium hydroxide particles were not interacting with the surface of the cells (Besson *et al.* 2019). However, the important parameter identified to ensure the flocculation efficiency was the mixing of NaOH in the culture medium, because to achieve a high flocculation efficiency, the precipitate must be able to reach the entire volume of the suspension to entrap all the cells present. In a second example provided by Vandamme *et al.* (2015), the pH was increased in the culture medium of the diatom *P. tricornutum*, which resulted in the precipitation of both magnesium hydroxide and calcium hydroxide. Based on zeta potential analysis, the authors could conclude that while magnesium hydroxide could flocculate the cells through a charge neutralization mechanism, in the case of calcium hydroxide, the sweeping mechanism was involved. Indeed, in a culture medium lacking Mg^{2+} ions, the surface charge of the cells was not reversed to positive values at high pH, indicating that there is no adsorption of the calcium hydroxide to cells, thus excluding the charge neutralization mechanism.

Scaling-up natural flocculation for microalgae harvesting?

In natural flocculation, one or several of these flocculation mechanisms can be involved. However, as illustrated by the examples given in each case, these mechanisms are very specific to the microalgae species used and the culture conditions chosen. Indeed, for example, at high pH, magnesium hydroxide will flocculate *P. tricornutum* cells trough charge neutralization, while in the case of *D. salina*, the flocculation will occur through the sweeping mechanism. It is therefore important in each case to identify these mechanisms, in order to be able to control them and use them in larger-scale processes. For instance, to our knowledge, harvesting using natural flocculation in large-scale assays has never been reported. The only case where an attempt was made was in the study by Besson and co-workers, where flocculation of *D. salina* by sweeping was achieved in a 600 L/h continuous flocculation/flotation pilot (Besson *et al.* 2019). However, in this case, the term natural flocculation cannot be used as the pH was artificially increased in the culture medium by NaOH addition. Indeed, as it is important to understand natural flocculation mechanisms to control them, it also provides the possibility to induce them artificially, by adding ions to induce flocculation or by adjusting the ionic strength of a culture medium, as in the examples described previously, or using biomolecules to retain the sustainability aspect that is pursued in natural flocculation.

INDUCING NATURAL FLOCCULATION MECHANISMS WITH BIO-SOURCED FLOCCULANTS

As stated in the previous part of this review, natural mechanisms are difficult to implement, even at lab scales because of their specificity to each culturing situation. However, the extensive number of studies that have been dedicated to understanding these mechanisms over the last few years have allowed the research community to gain insights into these mechanisms and especially into the key parameters affecting their efficiencies. This paved the way towards new strategies where researchers started to induce these natural flocculation mechanisms, by adding biopolymers either directly extracted from other organisms, like natural polysaccharides, or modified by various means to control the functional chemical groups they present. The different bioflocculation mechanisms identified also inspired the use of mixed cultures, where one microorganism species directly flocculates the microalgae species or produces a polymer that will flocculate it. Another strategy to induce natural flocculation is to artificially increase the pH; however, this point has already been mentioned previously and described by Besson & Guiraud (2013), and will not be more detailed in this second part. The different strategies to induce natural flocculation mechanisms will be described in this second part; their advantages and drawbacks will also be discussed.

Flocculation by addition of biopolymers

Selected studies illustrating well the use of biopolymers to induce natural flocculation are compiled in Table 1. The most popular biopolymer used for microalgae flocculation is chitosan. Chitosan is a cationic polyelectrolyte obtained by deacetylation of chitin, and after cellulose, it is the second most abundant natural polymer in the world. Moreover, chitosan presents many advantages as it is non-toxic, biodegradable, biocompatible and renewable, in contrast to traditional inorganic flocculants (Renault *et al.* 2009). Finally, chitosan does not contaminate the harvested biomass as chitin-like polysaccharides are naturally present in the cell wall of many microalgae species, and thus the products extracted from the cells can then be directly used (Ahmad *et al.* 2011). So far chitosan has been successfully

Table 1 | Natural flocculation mechanisms induced by addition of biopolymers

Microalgae species	Biopolymer used	Flocculation mechanism	Harvesting efficiency	Reference
Chlorella sorokiniana	10 mg chitosan/g of algal dry weight	Charge neutralization + Patch	99%	Xu *et al.* (2013)
Chlorella vulgaris	10 mg/L chitosan	Bridging + Charge neutralization	96%	Blockx *et al.* (2018)
Nannochloropsis oculata	Up to 200 mg/L chitosan	Sweeping	90%	Blockx *et al.* (2018)
C. vulgaris	30 mg/L poly (Υ-glutamic acid)	Bridging + Charge neutralization	91%	Zheng *et al.* (2012)
Chlorella protothecoides	30 mg/L poly (Υ-glutamic acid)	Bridging + Charge neutralization	98%	Zheng *et al.* (2012)
Chlorella sp.	40 ppm cationic guar gum	Bridging	94.5%	Banerjee *et al.* (2013)
Chlamydomonas sp.	100 ppm cationic guar gum	Bridging	92.2%	Banerjee *et al.* (2013)
Chlorella sp.	35 mg/L cationic cassia	Bridging + Patch	92%	Banerjee *et al.* (2014)
Chlamydomonas sp.	80 mg/L cationic cassia	Bridging + Patch	93%	Banerjee *et al.* (2014)
Scenedesmus dimorphus	10 mg/L cationic starch	Bridging + Patch	95%	Hansel *et al.* (2014)
S. dimorphus	100 mg/L cationic starch	Bridging + Patch	70%	Hansel *et al.* (2014)
C. vulgaris	Up to 200 mg/L cationic cellulose nanocrystals	Patch	95%	Blockx *et al.* (2019)

used to harvest different microalgae species, both marine and fresh-water. For example in 2013, Xu and colleagues flocculated the fresh-water species *Chlorella sorokiniana* using an optimum dosage of chitosan with an efficiency of 99% at a pH of 6 (Xu *et al.* 2013). The pH is indeed important to control in the case of chitosan as its efficiency as a flocculant relies on the amine groups that it presents. These amino groups have a pKa value of about 6.5 (Ritthidej 2011), and thus below this pH value these groups will be protonated thus conferring a positive charge to chitosan, which allows its interaction with the negatively charged surface of microalgae cells (Bilanovic *et al.* 1988). Indeed, in the case of *C. sorokiniana*, the flocculation mechanism described is a combination of charge neutralization and patch mechanisms. While the bridging mechanism is often associated with chitosan, in this case the authors state that chitosan polymers are much smaller than the cells, thus excluding bridging (Xu *et al.* 2013). However, while chitosan is widely reported as being efficient to flocculate fresh-water microalgae species, this is not necessarily the case for marine species. Indeed, in marine water that presents high ionic strengths, it is believed that the positive charges of chitosan are screened, thus preventing the polymer to interact with the cells and further flocculate them (Bilanovic *et al.*

1988). However, it must be noted that studies have reported the successful use of other cationic polymers for flocculation of marine species ('t Lam *et al.* 2014); thus there might be other parameters perhaps influencing the efficiency of chitosan in particular. In view of this, Blockx and co-authors recently investigated the conditions under which chitosan can be used as a flocculant for marine species (Blockx *et al.* 2018). The results the authors obtained on *N. oculata* showed that in opposition to fresh-water conditions, low pH did not trigger flocculation, while a high pH (between 7.5 and 10) was efficient. This clearly indicates that chitosan-induced flocculation in marine species occurs through another mechanism than charge neutralization or patch mechanisms, as at high pH, no or very few charges are present on the chitosan polymer. But when chitosan is uncharged, its solubility decreases, which triggers its precipitation. The authors thus suggest that in the case of *N. oculata*, chitosan flocculates the cells through a sweeping mechanisms, which can only be achieved at high pH (Blockx *et al.* 2018). Thus the particular case of chitosan also illustrates well the fact that a flocculation mechanism identified in one condition cannot be extrapolated to other conditions and species, as these mechanisms are specific to the species and conditions used.

But chitosan is not the only biopolymer that can be used to induce natural flocculation in microalgae. For instance, Zheng et al. (2012) have reported the use of poly (Ɣ-glutamic acid) (Ɣ-PGA) to flocculate fresh-water species. Ɣ-PGA is a polymer of the amino acid glutamic acid produced by the bacterial species *Bacillus subtilis*. The authors showed that using this natural polymer, they could flocculate *C. vulgaris* and *Chlorella protothecoides* with efficiencies of respectively 91 and 98%. Using zeta potential measurements, they demonstrated that Ɣ-PGA could increase the potential of the microalgae cells, thus indicating that it could interact with them through a charge neutralization mechanism. Moreover, SEM imaging also revealed that this mechanism was combined with a bridging mechanism, as microalgae cells were interlaced with Ɣ-PGA directly within the flocs. In 2013 Banerjee and co-workers showed that guar gum, a natural polysaccharide extracted from plants and chemically cationized, was an efficient flocculant for both *Chlorella* and *Chlamydomonas* sp. cells. Indeed, flocculation efficiencies reached respectively 94.5 and 92.2%, and further imaging experiments confirmed that this flocculation was achieved through a bridging mechanism (Banerjee *et al.* 2013). In 2014, the same group also investigated the efficiency of another biopolymer extracted from plants, cassia, a polysaccharide that the authors also chemically cationized using a similar strategy as for guar gum. Their results showed that this biopolymer also was efficient at flocculating cells from the same species, through a bridging mechanism as well (Banerjee *et al.* 2014). In 2014, Hansel and colleagues used starch, a naturally-occurring biodegradable polysaccharide, that they modified by etherification to present positive charges, and directly used as a flocculant to harvest *Scenedesmus dimorphus* cells (Hansel *et al.* 2014). In this study, the authors found that this polymer could adsorb at the surface of several cells, thus bridging them together. The patch mechanism was also found to be at play as the adsorption of the polymer to the cell surface created localized areas of positive charges, which consequently attracted neighboring oppositely charged cells. However, it must be noted that in these three last studies, the cationic moiety used to modify guar gum, cassia and starch, CHPTAC ((2-chloro-2-hydroxypropyl)trimethylammonium chloride), presents some safety issues and may not be adapted for all applications. As a last example, in a recent study conducted by Blockx and co-workers, the authors created cellulose nanocrystals (CNCs) by acid hydrolysis of the amorphous region of cellulose, the most abundant natural polymer on earth. They then linked cationic pyridinium- and methylimidazolium-based grafts (for which the potential toxicity under this form has not yet been evaluated) to these CNCs to induce the flocculation of *C. vulgaris*. Their results showed that cationic CNCs could be used as an efficient flocculant and that, in this case, bridging was not involved, which otherwise is often the case with biopolymers. Indeed, CNCs present a rigid backbone that prevent them from coiling and bending to bridge cells together, and carry charges on both sides, which thus led the authors to suggest that the flocculation mechanism in this case was the patch mechanism (Blockx *et al.* 2019).

Flocculation by other microorganisms

But while in all these different examples described so far, the idea is to add a biopolymer, chemically modified or not to flocculate the cells, another strategy to induce natural flocculation in microalgae is to mix them with other microorganisms that will directly flocculate them. In view of this, several works have been performed involving mixed cultures of different microalgae species, or mixed cultures of microalgae with bacterial species or with fungal species. A selection of these studies are reported in Table 2. A first possibility is to mix microalgae cells with a different microorganism, a fungal species or a bacterial species. Flocculation mechanisms of the algal-bacterial and algal-fungal cultures are represented in Figure 2(a) and 2(b), respectively. Microbial flocculation was first suggested as a harvesting technique for microalgae as early as 1996 (Benemann & Oswald 1996). In most cases, the flocculation mechanism relies on the production of extracellular polymers by the bacteria or fungi, which directly flocculate the cells usually through the bridging mechanism. Then two possibilities exist; in the first one, the microorganisms are cultured separately, the bacterial/fungal species produce the bioflocculant over the culture and are then mixed with the microalgae cells to proceed to the flocculation. This is for example the case in a study by Oh *et al.* (2001), where bacterial cells from the strain *Paenibacillus* sp. AM49 were cultivated to produce a bioflocculant efficient at harvesting several species of green microalgae. Wan and co-workers also implemented this strategy in 2013 to produce a bioflocculant using the bacterial strain *Solibacillus silvetris* W01. To flocculate the marine microalgae species *Nannochloropsis oceanica*, the authors then mixed the supernatant of the bacterial culture directly with the microalgae culture and could obtain a flocculation efficiency of 90% (Wan *et al.* 2013). In both cases, there is no detailed information given on the mechanism of flocculation, as the focus of these studies was to identify and use bacteria-produced bioflocculants. Moreover, while we chose to mention these two cases as mixed cultures

Table 2 | Natural flocculation mechanisms induced by other microorganisms

Microalgae species	Micro-organism	Species	Flocculation mechanism	Efficiency	Reference
Chlorella vulgaris	Bacteria	*Paenibacillus* sp.	Extracellular secreted bioflocculant	93%	Oh *et al.* (2001)
Nannochloropsis oceanica	Bacteria	*Solibacillus silvestris*	Extracellularly secreted flocculant	90%	Wan *et al.* (2013)
Scenedesmus obliquus	Bacteria	*Solibacillus silvestris*	Extracellular secreted bioflocculant	77%	Wan *et al.* (2013)
C. vulgaris	Bacteria	*Solibacillus silvestris*	Extracellular secreted bioflocculant	51%	Wan *et al.* (2013)
Pleurochrysis carterae	Bacteria	Tap water bacterial inoculum	Increase in floc size	90%	Lee *et al.* (2009)
C. vulgaris	Fungus	*Aspergillus niger*	Fungal pelletization	98.1%	Zhang & Hu (2012)
C. vulgaris	Fungus	*Aspergillus* sp.	Fungal pelletization	89.8%	Zhou *et al.* (2012)
C. vulgaris	Fungus	*Cunninghamella echinulata*	Fungal pelletization	99%	Xie *et al.* (2013)
C. vulgaris	Fungus	*Aspergillus oryzae*	Fungal pelletization	100%	Zhou *et al.* (2013)
Chlorella sp.	Fungus	*Pleurotus ostreatus*	Fungal pelletization	64.8%	Luo *et al.* (2019)
C. vulgaris	Microalgae	*Afrocarpus falcatus*	Bridging	22%	Salim *et al.* (2011)
C. vulgaris	Microalgae	*Scenedesmus obliquus*	Patch	32%	Salim *et al.* (2011)
Neochloris oleoabundans	Microalgae	*Tetraselmis suecica*	Patch	72%	Salim *et al.* (2011)
Chlorella sp.	Microalgae	*Ankistrodesmus* sp.	Bridging	82%	Lananan *et al.* (2016)
Chlorella sp.	Microalgae	*T. suecica*	Bridging (EPS)	67.3%	Kawaroe *et al.* (2016)
Nannochloropsis sp.	Microalgae	*T. suecica*	Bridging (EPS)	42.4%	Kawaroe *et al.* (2016)
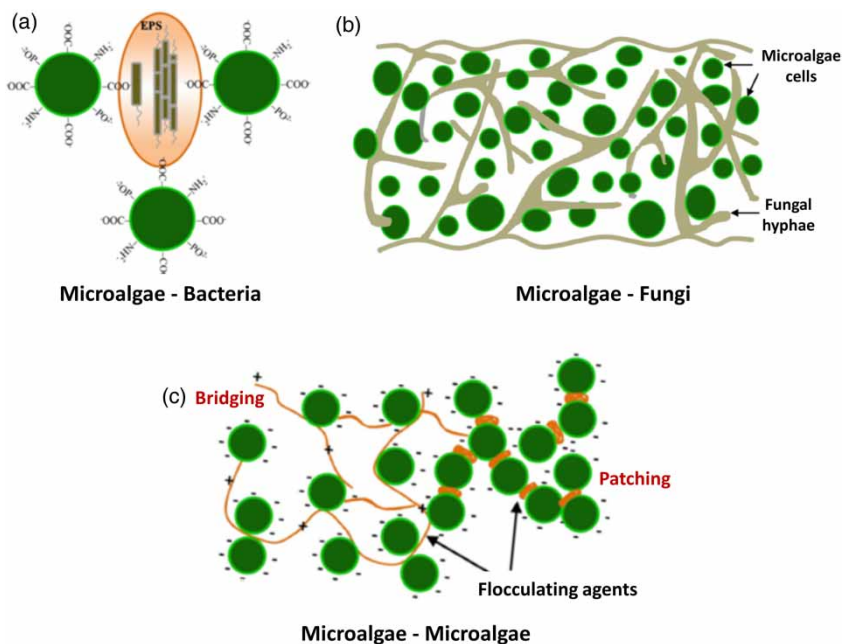
Figure 2 | Mixed species cultures mechanisms. Proposed mechanism of (a) microalgae–bacteria mixed cultures, (b) microalgae–fungi mixed cultures and (c) microalgae–microalgae mixed cultures. Reprinted and adapted with permission from Alam *et al.* (2016).

cases, one can argue that the fact that the microorganisms are not cultured together in the same broth, but separately, qualifies them more appropriately as classic bioflocculation cases.

Thus the second possibility consists in growing the different microorganisms in symbiosis, so that the microbial species directly flocculate microalgae cells *in situ*. For instance Lee and co-workers, in 2009, used directly tap water containing microbes: some heterotrophic bacteria present in the water, which therefore did not have the same nutritional requirements as microalgae cells, produced EPS under nutrient deficient conditions allowing the flocculation of the microalgae species *Pleurochrysis carterae* (Lee *et al.* 2009). Moreover, Lee *et al.* (2013) showed that in non-axenic cultures of *C. vulgaris*, the use of flocculants (CaCl$_2$, FeCl$_3$) or pH variations resulted in high flocculation efficiencies while it was not the case in axenic cultures of *C. vulgaris*. These results thus highlighted the important role of microalgae-associated bacterial species, three identified in this study, on the flocculation behavior of the microalgae cells. The suggested mechanism underlying this positive effect was that these bacterial cells and the extracellular substances they produce increased the microalgae floc size, which allowed them to be separated from the water by settling (Lee *et al.* 2013). Other studies have explored the potential of mixed cultures with filamentous fungi. In liquid cultures, filamentous fungi can either grow in filamentous form, featuring homogeneously dispersed hyphae or filaments, or in spherical pellets consisting of compact aggregated hyphal structures (Veiter *et al.* 2018). In some specific cases, these filamentous fugal strains can entrap microalgal cells and form fungi–algae pellets, thus allowing efficient algae harvesting. This technique has notably been proven efficient to harvest different microalgae species in several cases (Zhang & Hu 2012; Zhou *et al.* 2012, 2013; Xie *et al.* 2013; Luo *et al.* 2019). While the mechanism at the origin of the interaction between the fungus and the microalgae cells may be related to several possible reasons, one of these reasons may be related to surface charge. Indeed, for example in the case of co-cultures of the fungus *Aspergillus flavus* and *C. vulgaris*, it has been shown that fungal cells have a positive zeta potential, thus allowing their electrostatic interactions with negatively charged *C. vulgaris* cells (Zhang & Hu 2012). Although it is worth mentioning these examples of mixed cultures to induce flocculation of microalgae, as they represent valuable strategies to harvest cells without the addition of any flocculants or modified biopolymers, it is clear that the mechanisms of flocculation in these cases are very specific, and expand out of the 'classic' flocculation mechanisms that were described in the first part of this review.

Finally another possibility is to mix different microalgae species together, so that one will act as a flocculant for the other. This type of mixed cultures presents important advantages compared to mixing them with bacteria or fungi: it does not require different cultivation conditions, which reduces costs, and it prevents contaminations. Another advantage in this case is that both microalgae species can produce the molecule of interest in the process; thus all the biomass can then be used for downstream processes (Salim *et al.* 2011). Flocculation mechanisms of algal-algal culture are represented in Figure 2(c). An example of microalgae mixed cultures is presented in the study by Lananan and colleagues, already described earlier in this review, where *Ankistrodesmus* sp. cells, positively charged at pHs between 6.10 and 7.10, could flocculate negatively charged *C. vulgaris* cells through the bridging mechanism (Lananan *et al.* 2016). In another study performed by Kawaroe and co-workers, the marine species *Tetraselmis suecica* was directly used as a flocculant to harvest cells from the species *Chlorella* sp. and *Nannochloropsis* sp. To do so, the authors mixed *T. suecica* with the two other species, separately, at different ratio, and obtained after only 1 hour harvesting rates of 67.3% for *Chlorella* sp. and of 42.4% for *Nannochloropsis* sp. In both cases, the addition of the flocculant species in larger volume increased the flocculation efficiency. Concerning the flocculation efficiency, the fact that the two species are in competition for the nutrients as the culture goes on induces a stress on the cells, which in the case of *T. suecica* triggers the production of exopolysaccharides. These EPS are then responsible for bridging the cells together and flocculating them (Kawaroe *et al.* 2016). Other examples of successful mixed microalgae cultures can also be found in Salim *et al.* (2011), where different flocculation mechanisms are described depending on the flocculating microalgae species: bridging in the case where long EPS are partly bound to the producing cells, and patch mechanism if these EPS are short and bind completely to the producing cells, therefore creating positive patches at their surface (Salim *et al.* 2011).

Induced natural flocculation but not always so natural …

It is thus possible to artificially induce natural flocculation mechanisms in microalgae using biopolymers or mixed species cultures. As far as we know, the only biopolymer that has been successfully used to flocculate microalgae cells is chitosan, although its use should be adapted depending on the microalgae species used. Most of the

biopolymer-based strategies involve chemically modifying the natural molecules. First this involves additional costs, but also such strategies can be questionable from the toxicity point of view. So far in the examples described here no evaluation of the toxicity of the molecules was performed; thus there is for the moment no information as to whether these modified biopolymers represent a risk of biomass contamination or not. This is also a problem when using mixed cultures with different microorganisms, as fungi and bacteria also contaminate the biomass. In view of this, the ideal situation would be to be able to induce natural flocculation in microalgae species without having to add any molecules or microorganisms, but by acting directly on the microalgae itself to make it able to flocculate.

ALTERNATIVE DIRECTIONS IN NATURAL FLOCCULATION

In this idea of not to contaminate the biomass by any added molecule, an interesting strategy would be to genetically engineer the microalgae species so that they could flocculate without any modification of their culture medium. Genetic engineering consists in modifying the genome of the cells so that they express the desired molecules, or are able to use certain molecules present in the medium. While genetic engineering is widely used for bacteria, yeasts and plants (Barton & Brill 1983; Dequin 2001; Riglar & Silver 2018), its development for microalgae still remains confined. For the moment, the efforts regarding microalgae have mostly concentrated on increasing or modifying lipids or other energy storage molecules for biofuels applications (Dunahay *et al.* 1996). However, such strategies can also be used to engineer microalgae strains to enhance their flocculation capabilities, and this is what we will describe in the third part of this review. It is possible to think that such strategies could be the future directions to take in flocculation, although the use of genetically modified organisms is always controversial, and could not for instance take place in certain applications such as wastewater treatment. However, in closed photobioreactors, with no release in the environment, using genetically modified microalgae could be a possibility.

In addition to being a barrier against the environment, the algal cell wall is also an obstacle against engineering processes. Moreover, genetic manipulation of microalgae presents several other challenges, which are (i) the lack of suitable promoters and other regulatory sequences, (ii) the low efficiency and instability of transgene expression,

(iii) the fact that microalgae are a highly heterogeneous group of microorganisms and thus procedures have to be adapted in each case, (iv) the insufficient genetic data availability and (v) the lack of a standard toolbox for genetic engineering manipulations (León & Fernández 2007; Daboussi *et al.* 2014). While we will not go over the details of molecular biology strategies that are used to modify microalgae genomes as it is not the scope of the review, we will give and discuss examples where genetically modified microalgae species have been successfully used in flocculation processes. In a first example, Scholz and colleagues have tested the capacity of a mutant strain of *Chlamydomonas reinhardtii*, *cw15,* produced by Davies and Plaskitt in 1971 (Davies & Plaskitt 1971), to flocculate in the presence of the flocculant $CaCl_2$, in nitrogen-deprived conditions. The specificity of this mutant is that it lacks a cell wall and flagella, and thus has no mobility. The results obtained showed that the flocculation efficiency of this mutant strain was 83% whereas for the wild-type strain (with no genetic mutations), it was only 24% (Scholz *et al.* 2011). In the discussion the authors mention that other authors obtained similar results with another microalgae species having a cell wall (Sukenik *et al.* 1985); thus they suggest that the flocculation in this case may be due to the lack of flagella, which prevents them from moving and thus makes them more susceptible to the flocculant used. A further study by Fan *et al.* (2017) also used mutant cells of C. *reinhardtii*, deficient in a cell wall and flagella, but for the production of starch, and could also observe differences in the flocculation compared to wild-type strains, depending on the flocculant used, which they suggest can be attributed to the mutations. These examples are a good illustration that using microalgae strains where a simple genetic mutation is introduced can be a promising strategy to enhance the efficiency of flocculation and further flotation. But in this case, still a flocculant added to the culture medium is needed to achieve efficient flocculation, though it is also possible to engineer microalgae strains that self-flocculate with no addition of flocculant.

A first possibility to reach this goal has been presented by Diaz-Santos *et al.* (2016). In this study, the authors developed a strategy to express a gene from the yeast species *Saccharomyces bayanus* responsible for the flocculation, called *SbFLO5*. Flocculation in yeast is also a subject that has been extensively studied. In yeasts, a family of genes called FLO encodes specific cell surface glycoproteins, known as flocculins, which are responsible for the natural flocculating behavior of yeast cells (Stratford 1994). The mechanism by which they bind to other cells is thought to be by interacting with specific carbohydrate residues present

at the surface of adjacent cells (Miki *et al.* 1982). Later, Goossens and co-workers, among others, specified this mechanism by showing that one of these flocculins, Flo1p, could bind to the mannose residues present at the surface of yeast cells, thanks to two mannose carbohydrate binding sites present in the N-terminal region of the protein (Goossens *et al.* 2011). In a previous study in 2015, Díaz-Santos and colleagues used *S. bayanus* to produce these flocculins. The authors then extracted them from the supernatant of the yeast cultures and used them directly as flocculants in *C. reinhardtii* and in *Picochlorum* sp. cultures. Figure 3 shows the difference in the flocculation of *Picochlorum* sp. cells before and after addition of the flocculins isolated from fermentative cultures of *S. bayanus*. Their results showed that they could reach a recovery efficiency of 95% in the case of *C. reinhardtii* and of 75% in the case of *Picochlorum* sp., thus indicating that flocculins from yeasts are also able to bind to glycosidic residues present at the surface of microalgae cells, with a higher specificity in the case of *C. reinhardtii* (Díaz-Santos *et al.* 2015). It is after this study that the authors then suggested that the expression of FLO genes from *S. bayanus* to create self-flocculating *C. reinhardtii* transformants could be a promising method to enhance flocculation efficiency. After inserting the *FLO5* gene from *S. bayanus* into *C. reinhardtii*, they could show that these engineered mutant cells that express FLO

genes exhibited better self-flocculation, resulting in flocculation performance up to 3.5-fold higher compared to wild-type (Díaz-Santos *et al.* 2016). This study thus proves that self-flocculation phenotypes can be generated in microalgae through genetic engineering methods like insertion of the gene that is responsible for adhesive protein production. Then a second possibility to engineer self-flocculated microalgae strains is through the direct analysis of the microalgae genes using DNA sequencing technologies to identify flocculation genes and over-express them or transfer them from a strain to another. It has been reported that several microalgae species, such as *C. vulgaris*, *Ankistrodesmus falcatus*, *Scenedesmus obliquus* and *T. suecica*, have a higher tendency towards natural flocculation (Salim *et al.* 2011, 2012; Zhang *et al.* 2016). Such species could thus represent good candidates for DNA sequencing studies aiming at identifying which genes are responsible for their self-flocculating behavior. While over-expression or duplication of flocculating genes has not yet been performed, genetic studies have already been realized on microalgae. For instance, Blanc and co-workers sequenced the green alga *Coccomyxa subellipsoidea* C-169 genome, which was the first eukaryotic microorganism from a polar environment to have its genome sequenced. This study was conducted to analyze the mechanism of adaptation of life of this species to extreme polar environmental conditions. Their results
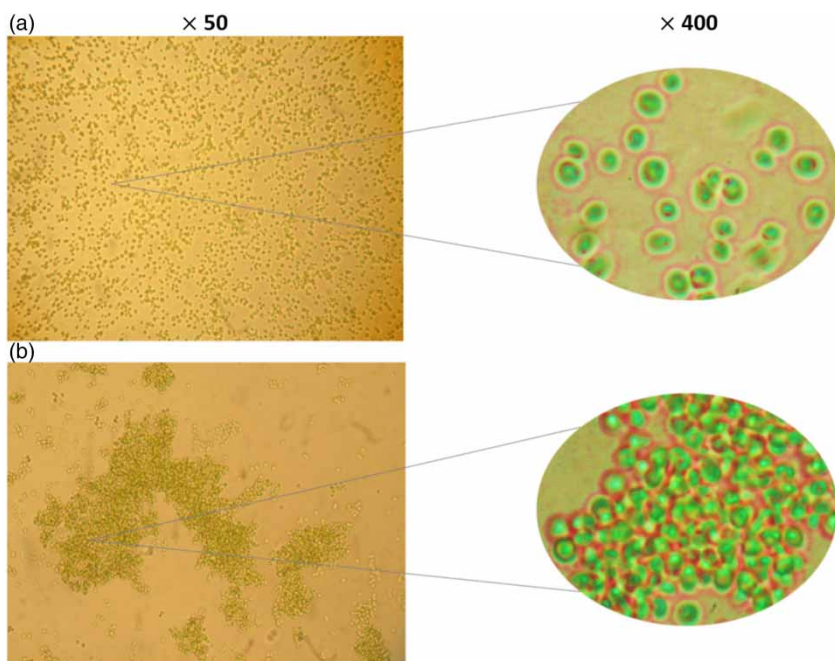

Figure 3 | Example of *S. bayanus* Flo protein induced flocculation. Microscopic analysis of *Picochlorum* sp. HM1 cells before (a) and after (b) addition of proteins isolated from fermentative cultures of *Saccharomyces bayanus* var. *uvarum*. Reprinted with permission from Díaz-Santos *et al.* (2015).

showed that this microalgae species had more enzymes engaged in the biosynthesis and lipid modification than other sequenced microalgae species. This thus implies that *C. subellipsoidea* has adapted to extreme cold conditions by making its lipid metabolism more versatile, enabling it to synthesize a wide range of cell membrane components (Blanc *et al.* 2012). However, this microalgae species is not the only species that has been sequenced. Another example is *Dunaliella tertiolecta*, whose genome has been sequenced to classify existing genes for enzyme encoding. This way corresponding lipid and starch pathways were reconstructed in order to increase the biofuel production of these species. Such results show the potential of using transcriptomic data from next-generation sequencing to identify pathways of interest and potential targets for microalgae metabolic engineering. These findings can for example be used to genetically engineer *D. tertiolecta* and this way maximize the production of commercial microalgae-based biofuels (Rismani-Yazdi *et al.* 2011). These examples demonstrate that after DNA sequencing analysis, it is possible to detect the genes that are responsible for a specific phenotype. Thus using this strategy, it could be possible to identify genes responsible for the flocculation in the case of harvesting studies.

CONCLUSIONS

While microalgae harvesting represents for the moment an economic burden slowing down the development of industrial processes to produce molecules of interest, such as biofuels, from microalgae, harvesting techniques, such as flotation, could represent a promising alternative. But flotation, at the moment, and in the specific case of microalgae, needs a flocculation step. Microalgae flocculation can be performed using chemical flocculants that in the end contaminate the harvested biomass and can interfere with downstream processes. This is why the scientific community has focused over the last few years on natural flocculation. From this review several important facts about natural flocculation can be identified. First, natural flocculation in microalgae species represents a sustainable and cost-effective alternative to the use of metal salts and other chemical flocculants. However, while the general mechanisms of flocculation (compression of the double electric layer, charge neutralization, bridging, patch mechanisms and sweeping) are well-known and described, their role in microalgae natural flocculation is specific to the microalgae species considered and to the culture conditions. Thus it is

important for each case to specifically study the mechanism underlying the observed flocculation to be able to control it and to further implement it in large-scale applications. Yet, the quite important numbers of studies produced on the subject have allowed researchers to develop strategies to artificially induce these mechanisms in microalgae, through the use of biopolymers, chemically modified or not, or through the use of mixed cultures. However, still these alternatives present some issues, as both chemically modified biopolymers and added microorganisms can be associated with biomass contamination problems. Thus new strategies need to be developed, and in this context genetic engineering can be an interesting one as it would allow creation of self-flocculating microalgae species that would require no added flocculant. And perhaps it could also be possible to simply eliminate this flocculation step, for instance in the flotation process. In view of this, an original idea is to directly functionalize the bubbles used in flotation with surfactants that would promote their adhesion to microalgae cells, with no flocculation step needed. Studies have been published on this topic, where positively charged bubbles were successfully used to harvest microalgae by flotation (Hanumanth Rao *et al.* 2018), thus demonstrating the feasibility of such an idea.

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REFERENCES

Ahmad, A. L., Mat Yasin, N. H., Derek, C. J. C. & Lim, J. K. 2011 Optimization of microalgae coagulation process using chitosan. *Chemical Engineering Journal* **173**, 879–882.

Alam, M. A., Vandamme, D., Chun, W., Zhao, X., Foubert, I., Wang, Z., Muylaert, K. & Yuan, Z. 2016 Bioflocculation as an innovative harvesting strategy for microalgae. *Reviews in Environmental Science and Bio/Technology* **15** (4), 573–583.

Banerjee, C., Ghosh, S., Sen, G., Mishra, S., Shukla, P. & Bandopadhyay, R. 2013 Study of algal biomass harvesting using cationic guar gum from the natural plant source as flocculant. *Carbohydrate Polymers* **92** (1), 675–681.

Banerjee, C., Ghosh, S., Sen, G., Mishra, S., Shukla, P. & Bandopadhyay, R. 2014 Study of algal biomass harvesting through cationic cassia gum, a natural plant based biopolymer. *Bioresource Technology* **151**, 6–11.

Barton, K. A. & Brill, W. J. 1983 Prospects in plant genetic engineering. *Science* **219** (4585), 671–682.

Benemann, J. R. & Oswald, W. J. 1996 *Systems and Economic Analysis of Microalgae Ponds for Conversion of CO_2 to Biomass*. Final report. California University, Department of Civil Engineering, Berkeley, CA, USA. https://www.osti.gov/biblio/493389 (accessed 3 December 2019).

Besson, A. & Guiraud, P. 2013 High-pH-induced flocculation-flotation of the hypersaline microalga *Dunaliella salina*. *Bioresource Technology* **147**, 464–470.

Besson, A., Formosa-Dague, C. & Guiraud, P. 2019 Flocculation-flotation harvesting mechanism of *Dunaliella salina*: from nanoscale interpretation to industrial optimization. *Water Research* **155**, 352–361.

Bilanovic, D., Shelef, G. & Sukenik, A. 1988 Flocculation of microalgae with cationic polymers – effects of medium salinity. *Biomass* **17** (1), 65–76.

Blanc, G., Agarkova, I., Grimwood, J., Kuo, A., Brueggeman, A., Dunigan, D. D., Gurnon, J., Ladunga, I., Lindquist, E., Lucas, S., Pangilinan, J., Pröschold, T., Salamov, A., Schmutz, J., Weeks, D., Yamada, T., Lomsadze, A., Borodovsky, M., Claverie, J.-M., Grigoriev, I. V. & Van Etten, J. L. 2012 The genome of the polar eukaryotic microalga *Coccomyxa subellipsoidea* reveals traits of cold adaptation. *Genome Biology* **13** (5), R39.

Blockx, J., Verfaillie, A., Thielemans, W. & Muylaert, K. 2018 Unravelling the mechanism of chitosan-driven flocculation of microalgae in seawater as a function of pH. *ACS Sustainable Chemistry & Engineering* **6** (9), 11273–11279.

Blockx, J., Verfaillie, A., Eyley, S., Deschaume, O., Bartic, C., Muylaert, K. & Thielemans, W. 2019 Cationic cellulose nanocrystals for flocculation of microalgae: effect of degree of substitution and crystallinity. *ACS Applied Nano Materials* **2** (6), 3394–3403.

Bolto, B. & Gregory, J. 2007 Organic polyelectrolytes in water treatment. *Water Research* **41** (11), 2301–2324.

Brennan, L. & Owende, P. 2010 Biofuels from microalgae – a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **14** (2), 557–577.

Chisti, Y. 2007 Biodiesel from microalgae. *Biotechnology Advances* **25** (3), 294–306.

Coward, T., Lee, J. G. M. & Caldwell, G. S. 2015 The effect of bubble size on the efficiency and economics of harvesting microalgae by foam flotation. *Journal of Applied Phycology* **27** (2), 733–742.

Cui, Y., Yuan, W. & Cheng, J. 2014 Understanding pH and ionic strength effects on aluminum sulfate-induced microalgae flocculation. *Applied Biochemistry and Biotechnology* **173** (7), 1692–1702.

Daboussi, F., Leduc, S., Maréchal, A., Dubois, G., Guyot, V., Perez-Michaut, C., Amato, A., Falciatore, A., Juillerat, A., Beurdeley, M., Voytas, D. F., Cavarec, L. & Duchateau, P. 2014 Genome engineering empowers the diatom *Phaeodactylum tricornutum* for biotechnology. *Nature Communications* **5** (1), 1–7.

Davies, D. R. & Plaskitt, A. 1971 Genetical and structural analyses of cell-wall formation in *Chlamydomonas reinhardi*. *Genetics Research* **17** (1), 33–43.

Dequin, S. 2001 The potential of genetic engineering for improving brewing, wine-making and baking yeasts. *Applied Microbiology and Biotechnology* **56** (5), 577–588.

Díaz-Santos, E., Vila, M., de la Vega, M., León, R. & Vigara, J. 2015 Study of bioflocculation induced by *Saccharomyces bayanus* var. *uvarum* and flocculating protein factors in microalgae. *Algal Research* **8**, 23–29.

Díaz-Santos, E., Vila, M., Vigara, J. & León, R. 2016 A new approach to express transgenes in microalgae and its use to increase the flocculation ability of *Chlamydomonas reinhardtii*. *Journal of Applied Phycology* **28** (3), 1611–1621.

Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M. & Posewitz, M. C. 2008 Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Current Opinion in Biotechnology* **19** (3), 235–240.

Dunahay, T. G., Jarvis, E. E., Dais, S. S. & Roessler, P. G. 1996 Manipulation of microalgal lipid production using genetic engineering. *Applied Biochemistry and Biotechnology* **57** (1), 223.

Fan, J., Zheng, L., Bai, Y., Saroussi, S. & Grossman, A. R. 2017 Flocculation of *Chlamydomonas reinhardtii* with different phenotypic traits by metal cations and high pH. *Frontiers in Plant Science* **8**. https://www.frontiersin.org/articles/10.3389/fpls.2017.01997/full (accessed 20 November 2019).

Formosa-Dague, C., Gernigon, V., Castelain, M., Daboussi, F. & Guiraud, P. 2018 Towards a better understanding of the flocculation/flotation mechanism of the marine microalgae Phaeodactylum tricornutum under increased pH using atomic force microscopy. *Algal Research* **33**, 369–378.

Garg, S., Li, Y., Wang, L. & Schenk, P. M. 2012 Flotation of marine microalgae: effect of algal hydrophobicity. *Bioresource Technology* **121**, 471–474.

Georgianna, D. R. & Mayfield, S. P. 2012 Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature* **488** (7411), 329–335.

Golueke, C. G. & Oswald, W. J. 1965 Harvesting and processing sewage-grown planktonic algae. *Journal – Water Pollution Control Federation* **37** (4), 471–498.

Goossens, K. V., Stassen, C., Stals, I., Donohue, D. S., Devreese, B., De Greve, H. & Willaert, R. G. 2011 The N-terminal domain of the Flo1 flocculation protein from *Saccharomyces cerevisiae* binds specifically to mannose carbohydrates. *Eukaryotic Cell* **10** (1), 110–117. https://ec.asm.org/content/10/1/110.short (accessed 20 March 2020).

Hansel, P. A., Guy Riefler, R. & Stuart, B. J. 2014 Efficient flocculation of microalgae for biomass production using cationic starch. *Algal Research* **5**, 133–139.

Hanumanth Rao, N. R., Yap, R., Whittaker, M., Stuetz, R. M., Jefferson, B., Peirson, W. L., Granville, A. M. & Henderson, R. K. 2018 The role of algal organic matter in the separation of algae and cyanobacteria using the novel 'Posi' – dissolved air flotation process. *Water Research* **130**, 20–30.

Huo, S., Shang, C., Wang, Z., Zhou, W., Cui, F., Zhu, F., Yuan, Z. & Dong, R. 2017 Outdoor growth characterization of an unknown microalga screened from contaminated *Chlorella* culture. *BioMed Research International*.

https://www.hindawi.com/journals/bmri/2017/5681617/ (accessed 19 March 2020).

Jagadevan, S., Banerjee, A., Banerjee, C., Guria, C., Tiwari, R., Baweja, M. & Shukla, P. 2018 Recent developments in synthetic biology and metabolic engineering in microalgae towards biofuel production. *Biotechnology for Biofuels* **11**. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6026345/ (accessed 15 October 2018).

Kawaroe, M., Prartono, T., Sunuddin, A. & Saputra, D. 2016 Marine microalgae *Tetraselmis suecica* as flocculant agent of bio-flocculation method. *HAYATI Journal of Biosciences* **23** (2), 62–66.

Kurniawati, H. A., Ismadji, S. & Liu, J. C. 2014 Microalgae harvesting by flotation using natural saponin and chitosan. *Bioresource Technology* **166**, 429–434.

Lam, M. K. & Lee, K. T. 2012 Microalgae biofuels: a critical review of issues, problems and the way forward. *Biotechnology Advances* **30** (3), 673–690.

't Lam, G. P., Vermuë, M. H., Olivieri, G., van den Broek, L. A. M., Barbosa, M. J., Eppink, M. H. M., Wijffels, R. H. & Kleinegris, D. M. M. 2014 Cationic polymers for successful flocculation of marine microalgae. *Bioresource Technology* **169**, 804–807.

Lananan, F., Mohd Yunos, F. H., Mohd Nasir, N., Abu Bakar, N. S., Lam, S. S. & Jusoh, A. 2016 Optimization of biomass harvesting of microalgae, *Chlorella* sp. utilizing auto-flocculating microalgae, *Ankistrodesmus* sp. as bio-flocculant. *International Biodeterioration & Biodegradation* **113**, 391–396.

Lee, A. K., Lewis, D. M. & Ashman, P. J. 2009 Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel. *Journal of Applied Phycology* **21** (5), 559–567.

Lee, J., Cho, D.-H., Ramanan, R., Kim, B.-H., Oh, H.-M. & Kim, H.-S. 2013 Microalgae-associated bacteria play a key role in the flocculation of *Chlorella vulgaris*. *Bioresource Technology* **131**, 195–201.

León, R. & Fernández, E. 2007 Nuclear transformation of eukaryotic microalgae. In: *Transgenic Microalgae as Green Cell Factories. Advances in Experimental Medicine and Biology* (R. León, A. Galván & E. Fernández, eds). Springer, New York, USA, pp. 1–11. https://doi.org/10.1007/978-0-387-75532-8_1 (accessed 18 March 2020).

Levy, N., Magdassi, S. & Bar-Or, Y. 1992 Physico-chemical aspects in flocculation of bentonite suspensions by a cyanobacterial bioflocculant. *Water Research* **26** (2), 249–254.

Liao, J. C., Mi, L., Pontrelli, S. & Luo, S. 2016 Fuelling the future: microbial engineering for the production of sustainable biofuels. *Nature Reviews Microbiology* **14** (5), 288–304.

Luo, S., Wu, X., Jiang, H., Yu, M., Liu, Y., Min, A., Li, W. & Ruan, R. 2019 Edible fungi-assisted harvesting system for efficient microalgae bio-flocculation. *Bioresource Technology* **282**, 325–330.

Markou, G. & Nerantzis, E. 2013 Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. *Biotechnology Advances* **31** (8), 1532–1542.

Miki, B. L., Poon, N. H., James, A. P. & Seligy, V. L. 1982 Possible mechanism for flocculation interactions governed by gene FLO1 in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **150** (2), 878–889.

Minhas, A. K., Hodgson, P., Barrow, C. J. & Adholeya, A. 2016 A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids. *Frontiers in Microbiology* **7**. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4853371/ (accessed 22 June 2016).

Molina Grima, E., Belarbi, E.-H., Acién Fernández, F. G., Robles Medina, A. & Chisti, Y. 2003 Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances* **20** (7–8), 491–515.

Muylaert, K., Vandamme, D., Foubert, I. & Brady, P. V. 2015 Harvesting of microalgae by means of flocculation. In: *Biomass and Biofuels from Microalgae: Advances in Engineering and Biology. Biofuel and Biorefinery Technologies* (N. R. Moheimani, M. P. McHenry, K. de Boer & P. A. Bahri, eds). Springer International Publishing, Cham, Switzerland, pp. 251–273. https://doi.org/10.1007/978-3-319-16640-7_12 (accessed 18 November 2019).

Ndikubwimana, T., Zeng, X., He, N., Xiao, Z., Xie, Y., Chang, J.-S., Lin, L. & Lu, Y. 2015 Microalgae biomass harvesting by bioflocculation-interpretation by classical DLVO theory. *Biochemical Engineering Journal* **101**, 160–167.

Ndikubwimana, T., Chang, J., Xiao, Z., Shao, W., Zeng, X., Ng, I.-S. & Lu, Y. 2016 Flotation: a promising microalgae harvesting and dewatering technology for biofuels production. *Biotechnology Journal* **11** (3), 315–326.

Oh, H. M., Lee, S. J., Park, M. H., Kim, H. S., Kim, H. C., Yoon, J. H., Kwon, G. S. & Yoon, B. D. 2001 Harvesting of *Chlorella vulgaris* using a bioflocculant from *Paenibacillus* sp. AM49. *Biotechnology Letters* **23** (15), 1229–1234.

Pahl, S. L., Lee, A. K., Kalaitzidis, T., Ashman, P. J., Sathe, S. & Lewis, D. M. 2013 Harvesting, thickening and dewatering microalgae biomass. In: *Algae for Biofuels and Energy. Developments in Applied Phycology* (M. A. Borowitzka & N. R. Moheimani, eds). Springer Netherlands, Dordrecht, The Netherlands, pp. 165–185. https://doi.org/10.1007/978-94-007-5479-9_10 (accessed 9 December 2019).

Pal, S., Mal, D. & Singh, R. P. 2005 Cationic starch: an effective flocculating agent. *Carbohydrate Polymers* **59** (4), 417–423.

Phoochinda, W. & White, D. A. 2003 Removal of algae using froth flotation. *Environmental Technology* **24** (1), 87–96.

Pragya, N., Pandey, K. K. & Sahoo, P. K. 2013 A review on harvesting, oil extraction and biofuels production technologies from microalgae. *Renewable and Sustainable Energy Reviews* **24**, 159–171.

Renault, F., Sancey, B., Badot, P.-M. & Crini, G. 2009 Chitosan for coagulation/flocculation processes – an eco-friendly approach. *European Polymer Journal* **45** (5), 1337–1348.

Riglar, D. T. & Silver, P. A. 2018 Engineering bacteria for diagnostic and therapeutic applications. *Nature Reviews Microbiology* **16** (4), 214–225.

Rismani-Yazdi, H., Haznedaroglu, B. Z., Bibby, K. & Peccia, J. 2011 Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: pathway description and gene discovery for production of next-generation biofuels. *BMC Genomics* **12** (1), 148.

Ritthidej, G. C. 2011 Nasal delivery of peptides and proteins with chitosan and related mucoadhesive polymers. In: *Peptide and Protein Delivery* (C. Van Der Walle, ed.). Academic Press, Boston, MA, USA, pp. 47–68. http://www.sciencedirect.com/science/article/pii/B9780123849359100033 (accessed 22 July 2019).

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G. & Tredici, M. R. 2009 Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering* **102** (1), 100–112.

Salim, S., Bosma, R., Vermuë, M. H. & Wijffels, R. H. 2011 Harvesting of microalgae by bio-flocculation. *Journal of Applied Phycology* **23** (5), 849–855.

Salim, S., Vermuë, M. H. & Wijffels, R. H. 2012 Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. *Bioresource Technology* **118**, 49–55.

Salim, S., Kosterink, N. R., Tchetkoua Wacka, N. D., Vermuë, M. H. & Wijffels, R. H. 2014 Mechanism behind autoflocculation of unicellular green microalgae *Ettlia texensis*. *Journal of Biotechnology* **174**, 34–38.

Scholz, M., Hoshino, T., Johnson, D., Riley, M. R. & Cuello, J. 2011 Flocculation of wall-deficient cells of Chlamydomonas reinhardtii mutant cw15 by calcium and methanol. *Biomass and Bioenergy* **35** (12), 4835–4840.

Spilling, K., Seppala, J. & Tamminen, T. 2011 Inducing autoflocculation in the diatom Phaeodactylum tricornutum through CO_2 regulation. *Journal of Applied Phycology* **23** (6), 959–966.

Stratford, M. 1994 Genetic aspects of yeast flocculation: in particular, the role of FLO genes in the flocculation of *Saccharomyces cerevisiae*. *Colloids and Surfaces B: Biointerfaces* **2** (1), 151–158.

Sukenik, A. & Shelef, G. 1984 Algal autoflocculation–verification and proposed mechanism. *Biotechnology and Bioengineering* **26** (2), 142–147.

Sukenik, A., Schröder, W., Lauer, J., Shelef, G. & Soeder, C. J. 1985 Coprecipitation of microalgal biomass with calcium and phosphate ions. *Water Research* **19** (1), 127–129.

Uduman, N., Qi, Y., Danquah, M. K., Forde, G. M. & Hoadley, A. 2010 Dewatering of microalgal cultures: a major bottleneck to algae-based fuels. *Journal of Renewable and Sustainable Energy* **2** (1), 012701.

Vandamme, D., Foubert, I. & Muylaert, K. 2013 Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends in Biotechnology* **31** (4), 233–239.

Vandamme, D., Pohl, P. I., Beuckels, A., Foubert, I., Brady, P. V., Hewson, J. C. & Muylaert, K. 2015 Alkaline flocculation of Phaeodactylum tricornutum induced by brucite and calcite. *Bioresource Technology* **196**, 656–661.

Veiter, L., Rajamanickam, V. & Herwig, C. 2018 The filamentous fungal pellet – relationship between morphology and productivity. *Applied Microbiology and Biotechnology* **102** (7), 2997–3006.

Vergnes, J. B., Gernigon, V., Guiraud, P. & Formosa-Dague, C. 2019 Bicarbonate concentration induces production of exopolysaccharides by *Arthrospira platensis* that mediate bioflocculation and enhance flotation harvesting efficiency. *ACS Sustainable Chemistry & Engineering* **7** (16), 13796–13804.

Waltz, E. 2009 Biotech's green gold? *Nature Biotechnology* **27** (1), 15–18.

Wan, C., Zhao, X.-Q., Guo, S.-L., Asraful Alam, M. & Bai, F.-W. 2013 Bioflocculant production from *Solibacillus silvestris* W01 and its application in cost-effective harvest of marine microalga *Nannochloropsis oceanica* by flocculation. *Bioresource Technology* **135**, 207–212.

Xie, S., Sun, S., Dai, S. Y. & Yuan, J. 2013 Efficient coagulation of microalgae in cultures with filamentous fungi. *Algal Research* **2** (1), 28–33.

Xu, Y., Purton, S. & Baganz, F. 2013 Chitosan flocculation to aid the harvesting of the microalga *Chlorella sorokiniana*. *Bioresource Technology* **129**, 296–301.

Yang, C., Dabros, T., Li, D., Czarnecki, J. & Masliyah, J. H. 2001 Measurement of the zeta potential of gas bubbles in aqueous solutions by microelectrophoresis method. *Journal of Colloid and Interface Science* **243** (1), 128–135.

Zhang, J. & Hu, B. 2012 A novel method to harvest microalgae via co-culture of filamentous fungi to form cell pellets. *Bioresource Technology* **114**, 529–535.

Zhang, X., Zhao, X., Wan, C., Chen, B. & Bai, F. 2016 Efficient biosorption of cadmium by the self-flocculating microalga *Scenedesmus obliquus* AS-6-1. *Algal Research* **16**, 427–433.

Zheng, H., Gao, Z., Yin, J., Tang, X., Ji, X. & Huang, H. 2012 Harvesting of microalgae by flocculation with poly (γ-glutamic acid). *Bioresource Technology* **112**, 212–220.

Zhou, W., Cheng, Y., Li, Y., Wan, Y., Liu, Y., Lin, X. & Ruan, R. 2012 Novel fungal pelletization-assisted technology for algae harvesting and wastewater treatment. *Applied Biochemistry and Biotechnology* **167** (2), 214–228.

Zhou, W., Min, M., Hu, B., Ma, X., Liu, Y., Wang, Q., Shi, J., Chen, P. & Ruan, R. 2013 Filamentous fungi assisted bio-flocculation: a novel alternative technique for harvesting heterotrophic and autotrophic microalgal cells. *Separation and Purification Technology* **107**, 158–165.

Ziolkowska, J. R. 2014 Prospective technologies, feedstocks and market innovations for ethanol and biodiesel production in the US. *Biotechnology Reports* **4**, 94–98.