ARTÍCULO DE REVISIÓN

Microalgae protoplasts isolation and fusion for biotechnology research

Aislamiento y fusión de protoplastos de microalgas para aplicaciones biotecnológicas

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ABSTRACT

Protoplasts are microbial or vegetable cells lacking a cell wall. These can be obtained from microalgae by an enzymatic hydrolysis process in the presence of an osmotic stabilizer. In general, protoplasts are experimentally useful in physiological, genetic and biochemical studies, so their acquisition and fusion will continue to be an active research area in modern biotechnology. The fusion of protoplasts in microalgae constitutes a tool for strain improvement because it allows both intra and interspecific genetic recombination, resulting in organisms with new or improved characteristics of industrial interest. In this review we briefly describe the methodology for obtaining protoplasts, as well as fusion methods and the main applications of microalgal platforms.

Key words: Enzymatic hydrolysis, microalgae, cell wall, protoplast fusion, strain improvement.

RESUMEN

Los protoplastos son células microbianas o vegetales que carecen de pared celular. Estos pueden obtenerse a partir de microalgas por un proceso de hidrólisis enzimática en presencia de un estabilizador osmótico. En general, los protoplastos son experimentalmente útiles en estudios fisiológicos, genéticos y bioquímicos, por lo que su obtención y fusión continuarán siendo un área de investigación activa en la biotecnología moderna. La fusión de protoplastos en microalgas constituye una herramienta para el mejoramiento de cepas pues permite la recombinación genética intra e interespecífica, logrando así organismos con nuevas características de interés industrial. En esta revisión, describimos brevemente la metodología para obtener protoplastos, métodos de fusión y las principales aplicaciones de las plataformas basadas en microalgas.

Palabras clave: Hidrolisis enzimática, microalga, pared celular, fusión de protoplastos, mejora de la tensión.

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INTRODUCTION

The term "*protoplast*" refers to bacterial, fungal, algal or plant cells whose cell wall has been removed, either enzymatically or mechanically (Cocking, 1960; Kaladharan, 1998; Verma, Kumar, & Bansal, 2000). Experimentally it is possible to induce cell fusion by physical or chemical agents, although spontaneous protoplast fusion has also been observed in plant cells (Usui, Maeda, & Ito, 1974). Protoplast fusion enables organelle transfer between distinct species and their genetic recombination (Fowke, Gresshoff, & Marchant, 1979; Y. K. Lee & Tan, 1988). This process involves the decomposition or removal of the cell wall, purification and isolation of protoplasts, chemical fusion or electrofusion, regeneration and identification of hybrids (Tomar & Dantu, 2010). The successful fusion of two microalgal protoplasts can give a progeny that express the traits of both parental cells or a new hybrid with a different phenotype (Abomohra, El-Sheekh, & Hanelt, 2016). This technology has an immense potential for the improvement of industrially useful microorganisms, since it can give cells with improved or novel characteristics not expressed in parent strains.

Microalgae are an extensive group of photosynthetic microorganisms with a diverse phylogeny that have raised relevant interest at an industrial level due to the high efficiency of inorganic carbon conversion to various high value products including pigments (chlorophyll, carotenoids and phycobilins), fatty acids essential oils, antioxidants, protein for human and animal consumption, lipids and hydrocarbons for biofuels production and other bioactive molecules (Harun, Singh, Forde, & Danquah, 2010; Koller, Muhr, & Braunegg, 2014). However, most of algal derived bioproducts are not commercially competitive due to low productivities and production costs. In that sense, there is an important need to look for new molecular tools to increase biomass production and metabolite accumulation and harsh conditions resistance traits aiming to facilitate large-scale cultivation and management (Giraldo-Calderón *et al*., 2018). Currently obtaining and fusion of protoplasts in microalgae has been barely explored and limited to a select group of strains of commercial interest such as *Chlorella, Dunaliella, Haematococcus*, among others. However, there are still many technical gaps and there are not consensus protocols for algal cells fusion. This review focuses on the initial approaches for microalgal protoplast obtention and fusion and general methodologies from other biological systems with potential applications in microalgae.

OBTAINING PROTOPLASTS

Protoplasts from algae, bacteria, fungi and plants provide a useful cell system to support many aspects of modern biotechnology, for instance in genomics, proteomics and metabolomics (Aoyagi, 2011; Ye, Yue, Yuan, & Wang, 2013; Yokoyama, Kuki, Kuroha, & Nishitani, 2016; Yu *et al*., 2014). There are reliable procedures for protoplasts formation from different microorganisms, including both prokaryotes and eukaryotes. Isolated protoplasts are used for a wide range of experimental procedures involving membrane dynamics and function, cell structure, synthesis of metabolites, toxicological evaluations and genetic transformation (Aoyagi, 2011; Davey, Anthony, Power, & Lowe, 2005; Yang *et al*., 2015; Yu *et al*., 2014).

Methods of obtaining. Several methods for obtaining protoplasts in microbial and vegetable cells have been reported. Depending on each organism, a specific enzymatic mixture is used to remove the cell wall based on its chemical composition. In the case of plants and microalgae, fungal enzymes with cellulase, hemicellulase and pectinase activities are typically used (Baldan, Andolfo, Navazio, Tolomio, & Mariani, 2001; Juturu & Wu, 2014). Protoplast formation efficiency depends on the cell type, enzyme concentration, incubation time and other variables such as pH, temperature, osmotic pressure, among others (Peberdy, 1980). To avoid the rupture of the protoplasts, an osmotic regulator is added to the enzyme solution, being more stable if the solution is slightly hypertonic (Davey *et al*., 2005). Liu *et al*. (2006), reported that mannitol and sorbitol are excellent osmotic stabilizers to maintain cell viability and have better protoplast yields from *Chlamydomonas* sp. ICE-L when compared with glucose and sucrose.
No mechanical methods to obtain protoplasts were found for microalgae in the reviewed literature. In contrast, there are studies reporting the use of combined enzymatic and mechanical methods (sonication and microwaves) to break microalgae cell wall for applications such as pigment or lipid extraction (Al-Zuhair *et al*., 2017; Gerken, Donohoe, & Knoshaug, 2013; Sierra, Dixon, & Wilken, 2017).

Composition of the cell wall. The chemical composition of the cell wall differs between the diverse microorganism groups and determines the enzymes to be used. In bacteria, the cell wall is composed of peptidoglycan and in the case of Gram (-) there is an outer membrane composed of lipopolysaccharides and porins (Brown, Wolf, Prados-Rosales, & Casadevall, 2015; Scheffers & Pinho, 2005). The cell walls of fungi are formed by glycoproteins and polysaccharides, mainly glucan and chitin (Bowman *et al*., 2006; Erwig *et al*., 2016). In plants, cellulose acts to provide structural support to the cell wall (Domozych *et al*., 2012; Martin, De Souza, Da Silva, & Gomes, 2004; Popper *et al*., 2011). In marine algae, cellulose content is low and in some species altogether absent, while green algae have cell walls containing up to 70% of cellulose

Table 1. Composition of the cell wall of different microalgae.

Microalgae	COMPOSITION	REFERENCE
Haematococcus pluviales	Growing cells: 19% Carbohydrates- 75% Protein Stationary phase: 70% carbohydrates (66% hexoses mainly manosse), 3% cellulose and 6% proteins, 3% acetolysis-resistant material.	(Abomohra et al., 2016; Taylor et al., 2011)
Botryococcus braunni	Cellulose, algaenan, glycoprotein,	(Berrios, Zapata, & Rivas, 2015)
Chlorella sp.	25% Carbohydrates (glucose, rhamnose, arabinose, mannose, xylose, fucose, and galactose), 15-20% uronic acid, 10-15% glucosamina and 6-10 % protein.	(Gerken et al., 2013)
Scenedesmus obliquus	28% - 30 % proteins, glycoproteins, cellulose, acetolysis-resistant, biopolymers, algaenans,	(Zhang et al., 2018)

on dry weight basis. It is important to note that algal cellulose is not purely β-1,4-glucan, but also can contain other sugars like xylose and other polysaccharides such as xylans and mannans (Baldan *et al*., 2001).

There is a huge diversity of cell wall composition among different phylogenetic groups of algae. Strains like *Chlorella* sp. and *Botryoccocus braunii* have rigid cell walls, whilst and *Nannochloropsis* has thin and flexible cell walls. *Ochromonas danica* and *Isocrhysis galbana* lack this structure. In addition, apart from the cell wall, there are microalgae capable of generating an extracellular protective matrixes or structures made of polysaccharides and lipids. All these external structures must also be removed to increase the efficiency of protoplast fusion or to facilitate success in genetic transformation methods.

The composition of cell walls of microalgae is complex and poorly understood. For example, there are significant variations on cell wall structure and composition among some *Chlorella* species. Whereas some *Chlorella* species have only one layer, others have two layers, a microfibrillar layer close to the cytoplasmic membrane and a thin mono or trilaminar outer layer (Yamada & Sakaguchi, 1982). The members of the family Trebouxiophyceae and Chlorophyta have a cell wall composed mainly of cellulose, ulvans (sulfated xylorhamnoglucan), β-1,3-glucans and glycoproteins (Baldan *et al*., 2001; Domozych *et al*., 2012; Lu, Kong, & Hu, 2011; Popper *et al*., 2011). Ulvans are composed mainly of rhamnose, glucuronic acid, iduronic acid and xylose, commonly distributed in repeated units of disaccharides (Robic, Gaillard, Sassi, Leral, & Lahaye, 2009). The green alga *Haematococcus pluvialis* shows different cell wall composition depending on its life cycle stage. That is, when cells are dividing, the walls have higher content of proteins, while during stationary phase polysaccharides are more abundant on dry weight basis (Abomohra *et al*., 2016).

Other studies have indicated that only a limited number of *Chlorella* species and other green algae including *Scenedesmus*, *Pediastrum*, *Chara*, *Prototheca, Botryococcus braunii* and *Coelastrum* are able to synthesize algaenan, previously known as sporopollenin. This compound is found in the cell wall, and is a hydrolysis-resistant polymer which protects the cells from chemical and bacterial degradation and hinders the action of the enzymes during protoplast formation (Berliner, 1977; Burczyk & Czygan, 1983; He, Dai, & Wu, 2016; Ueno, 2009). Table 1 shows the composition of the cell wall for some microalgae strains.

Enzymatic hydrolysis of the cell wall: The complexity in the composition of the cell wall between organisms indicates that to remove it completely, a specific enzymatic mixture must be used, and the reaction conditions needs to be optimized for each strain. For cellulose, three enzymes working in series are needed to completely degrade this biopolymer: endoglucanaseendoglucanase, exoglucanaseexoglucanaseand β-glucosidase. As a first step of hydrolysis of cellulose, the endoglucanase breaks the glycosidic bonds present in the amorphous regions of this structure and releases oligosaccharide chains to the medium, the latter are attacked by the exoglucanase enzymes reducing them to cellobiose, which is finally hydrolyzed by the action of β-glucosidase to produce glucose monomers (Juturu & Wu, 2014). The use of pec-

tinases is indispensable to remove the wall in plant cells because the pectin is an important component in the structure of this, while in microalgae the use of cellulases is sufficient, since cellulose is the main component, especially in the divisions Cyanophyta, Chlorophyta and Xanthophyta (Baldan *et al*., 2001; Domozych *et al*., 2012).

The main factors that affect the activity of an enzyme include substrate concentration, temperature, pH, ionic strength and the nature of the salts present. Each enzyme present in the digestion mixture has optimal conditions of activity that must be guaranteed in the assay for obtaining protoplasts. Consequently, the interactions between the factors that affect the enzymatic activity will determine the efficiency in the degradation of the cell wall and finally the yield of viable protoplasts. Generally, low enzyme concentrations at low temperatures and high pH (5-8) during a short incubation period have shown to be the most suitable conditions for enzyme activity (Khentry, Paradornuvat, Tantiwiwat, Phansiri, & Thaveechai, 2006; Tomar & Dantu, 2010).

Abomohra *et al*. (2016), evaluated the effect of different enzymes such as protease K (Serine protease), lysing enzyme and mixtures of enzymes for polysaccharides (cellulase, driselase and macerozyme R-10) on the lytic activity for protoplasts formation of *Haematococcus pluvialis*, finding that the treatment with protease K at a temperature of 37°C for 90 min generates the maximum lytic activity (57%) and protoplast obtention. In this study, the fusion of protoplasts was successfully performed with *Ochromonas danica*, which, lacking cell wall, was not subjected to enzymatic pretreatment. Kusumaningrum *et al*. (2018), used only lysozymes at 2-3 g/L during 3h for the cell wall breakage of *Chlorella pyrenoidosa*, as this enzyme acts by hydrolyzing the peptidoglycans in the 1,4-beta bonds of the N-acetylmuramic acid residues and N-acetyl-D-glucosamine and in chitodextrins catalyzing the residues of N-acetyl-D-glucosamine. In previous works, the authors reported the use of this same protocol to obtain protoplasts from *Chlorella* and *Dunaliella*, however, they did not mention the efficiency of viable protoplasts formation. (H. P. Kusumaningrum & Zainuri, 2014).

Kun Lee *et al*. (1988), used cellulase and pectinase to obtain protoplasts of *Porphyridium cruentum* and only cellulase for *D. bardawil* and *D. salina*. These authors determined the percentage of protoplast formation by microscopic observation of morphological changes in *Dunalliela* cells and called protoplasts to individuals that after the enzymatic pretreatment lost mobility and changed their shape from pear to round morphology, reporting a protoplast yield of 67%. In the case of *Porphyridium cruentum*, which does not change its morphology, a different staining method with violet crystal and $CuSO_4$ was used. Normal cells retain the red coloration while protoplasts showed green color, in this case the authors obtained a protoplast production of 72%.

Few genetic studies have been carried out with *Botryococcus braunii* due to some drawbacks in transformation procedures related with cell wall thickness and cellular organization in colonies linked by an extracellular matrix of lipids and polysaccharides difficult to remove (Metzger & Largeau, 2005). Enzymatic treatments with cellulases at concentrations between 14.4 U mL and 22.4 U mL were effective for cell wall degradation in this microalga, without significantly loss of cell viability (Berrios *et al*., 2015). Cellulose rich cell walls from species such as *Chlorella*, *Coelastrum*, *Botryococcus* can be stained with calcofluor reagent, which has a high affinity for this polysaccharide. This reagent is useful to assess the effectiveness of the enzymatic treatment and the production of protoplasts by fluorescence microscopy because differentiation is determined based on the retention of the fluorescent dye by intact cells still bearing cell walls (Berrios *et al*., 2015).

Separation and purification of protoplasts

Successful cultivation of protoplasts and further fusion requires a pure, high-density population of viable and intact protoplasts. Thus, protoplasts must be separated from undigested material (debris), unviable protoplasts and enzymes (Tomar & Dantu, 2010). Enzymes are separated from the protoplast solution by centrifugation and then the cells are resuspended in an osmoregulatory solution. Then, the protoplasts and cells can be separated by centrifugation in density gradient with sucrose, percol or CsCl as used in molecular and biochemical studies (Griffith, 2010; Harms & Potrykus, 1978; Peiter, Imani, Yan, & Schubert, 2003). In this technique, continuous or stepped gradients are prepared and poured into a centrifuge tube so that the gradient has a high concentration orientation (bottom of the tube) to low (top of the tube). The biological samples are added at the top of the gradient and then centrifuged at high acceleration. Cells pass through the gradient until they reach a point where their density matches that of the surrounding medium (Eroglu *et al*., 2009; Griffith 2010).

A different separation approach is flow cytometry coupled with a classification module known as "cell sorting". This technique allows the separation of cells that differ in cell size, morphology or fluorescence derived from photosynthetic pigments (auto-fluorescence) or fluorescent markers and is widely used for cell isolation and preparation of axenic cultures (Hyka, Lickova, Pøibyl, Melzoch,

& Kovar, 2013). The heterogeneous mixtures of cells are placed in suspension and pass one by one through a laser beam by pressurization. The light signals emitted by the particles are collected and correlated with characteristics such as cell morphology, expression of intracellular proteins, gene expression and cellular physiology. The classification of the cells can be based on physicochemical, immunological and functional properties. In practice, the physicochemical characteristics are the most used for the separation or "cell sorting"; these include characteristics such as size, volume, density, light scattering properties, membrane potential, pH, electrical charge and cellular content of different compounds such as nucleic acids, enzymes and other proteins (Orfao & Ruiz-Argüelles, 1996). Based on user-defined parameters, individual cells can be diverted from the fluid stream and collected into viable homogenous fractions at exceptionally high rates and a purity approaching 100% (Ibrahim & van den Engh, 2007; Mattanovich & Borth, 2006). In most of the works carried out with microalgae, there is no detailed methodology mentioned for the purification of protoplasts and enzymatic treatment followed by centrifugation. Liu *et al*. (2006), proposed the use of low gravities and a short centrifugation time (200 g for 5 min) to eliminate the enzyme solution for protoplast formation of *Chlamydomonas* sp. ICE-L. They obtained a protoplast production of 47.8% but did not separat protoplast from whole cells.

Even though cell sorting and differential gradient centrifugation have not been used for algal protoplast separation and purification so far, those are promising alternatives to improve existing protocols, especially for lesser studied strains. Density gradient-based approaches are a simple and quick method for particle separation, however, it must be evaluated and optimized to assess at which extent it compromises protoplast viability, which is crucial for further fusion and regeneration steps. On the other hand, cell sorting is a softer and more precise technique to separate desired products as laser exposure is very short and liquid flow is laminar and non-destructive, however, the equipment required to perform this technique make it an expensive option and aggregate-forming cells require previous disaggregation steps to obtain single-cell suspensions.

FUSION OF PROTOPLASTS

The fusion of protoplasts is an effective technique in comparison with the traditional techniques of genetic modification, which has been successfully developed in different groups of organisms such as fungi, bacteria, plants and algae and does not imply a direct modification of specimen genome. (Evans, 1983; Y.-K. Lee & Tan, 1988; Peberdy, 1980; Scheinbach, 1983). One of the main advantages of this technique is that it allows a quick and economical combination of the genomes from two or more sexually incompatible species, seeking the generation of a new recombinant strain with improved characteristics of the parent strains. (Bradshaw, 2006; Peberdy, 1989). The fusion is a physical-chemical phenomenon where at least two protoplasts come into contact, either spontaneously or in the presence of fusion-inducing agents (Chawla, 2002).

Fusion methods

The fusion can be described by three consecutive phases: 1) placing the protoplasts in close contact, 2) disruption and fusion in a limited and localized place in the adjacent membranes, and 3) formation of bridges between protoplasts which allows cytoplasmic continuity between the cells (Navrátilová, 2004). The enzymatic degradation of the walls reduces constrictions in the plasmodesma in vegetative an algal tissues, as a result the plasmogamy between the neighboring protoplasts can occur spontaneously (Withers & Cocking, 1972). Normally, isolated protoplasts do not tend to fuse with each other because their surface has a negative charge (between -10 mV and -30 mV) outside the plasma membrane, which results in a strong tendency to repel each other. Based on that, fusion requires a chemical inducer agent or a system that reduces the electronegativity of isolated protoplasts and allows them to have a closer contact (Navrátilová, 2004; Verma *et al*., 2000). Chemofusion and electro-fusion are major alternatives protoplast fusion. Additionally, nuclear fusion through microinjection technique can also be explored. This last method consists in introducing the foreign material into the cell by the insertion of a glass capillary in the intracellular environment (Zhou *et al*., 2017).

Chemo-fusion. For the induction of protoplast chemical fusion between two or more viable protoplasts, it is necessary to submerge the cells in an alkaline solution (pH 9.0-10.5) in presence of a fusogen agent, an osmotic regulator and divalent cations (Muralidhar & Panda, 2000; Navrátilová, 2004). Polyethylene glycol (PEG) has been designated as a universal fusogen agent in microbiological applications, due to its excellent stability and binding action. The most commonly used divalent ions with PEG are calcium ions (Ca^{2+}). After the fusion, hybrids obtained are studied with bases on their phenotypic or genotypic characteristics (Navrátilová, 2004; Peberdy, 1980). Protoplasts can also be characterized by identifying their gene expression. The identification of new genotypes indicates that genetic recombination occurs due to cell fusion, also, the production characteristics of the hybrids can be compared in terms of enzymatic activity or production of a metabolite with the

parental strains to evaluate if there was a successful recombination (Muralidhar & Panda, 2000).

Electro-fusion. The electro-fusion technique is based on the reversible electrical rupture of cell membranes. This rupture is observed when a membrane is polarized in an electric field at voltages between 0.5-1.5 V, during a very short time interval (nanno to microseconds) (Zimmermann & Scheurich, 1981). The exposure of the protoplasts to electric fields, reversibly increases the permeability of the membrane, causing a disturbance of the local electric charge that enables the fusion between the closest protoplasts. The natural structure of the membrane is restored after few seconds or minutes depending on the experimental conditions and the properties of the membrane (Tomar & Dantu, 2010; Zimmermann & Scheurich, 1981). In comparison, electrofusion has some advantages over chemofusion because is simpler, faster and does not require chemical agents that might compromise cell viability (Navrátilová, 2004).

Fusion mechanism

The mechanism of protoplast fusion is not fully known (Peberdy 1989; Verma *et al*., 2000). Several explanations have been exposed to understand the mechanism of this phenomenon, as explained below. The first step for protoplast fusion is to guarantee physical contact between cells lacking a cell wall. However, this is not naturally common event since the surface of the plasma membrane has a negative net charge, attributed to the content of phospholipids, which generates a repulsive effect between cells (Tomar & Dantu, 2010). In the case of chemical fusion, the use of the fusogen agent helps to overcome this problem since PEG is a high molecular weight polymer that has similar polarity to phospholipid molecules and binds with protein membranes inducing cell aggregation and making a connection bridge between protoplasts. Additionally, this highly hydrophilic reagent removes water from the protoplasts, forcing their contraction and causing an increase in fluidity. The addition of divalent ions such as Ca^{2+} contributes to the reduction of the electrostatic field between protoplasts and aids in the perturbation of the for pores formation where fusion takes place (Araujo *et al*., 2016). Narayanaswamy (1994) proposed that the fusion of protoplasts occurs quickly when the PEG bound between the two protoplasts is eliminated, causing the rupture of the membranes and the transfer of the genetic material. (Narayanaswamy, 1994) In the case of electrical fusion, the approach between the protoplasts is achieved by temporarily modifying the polarity of a non-uniform electric field using the technique of dielectrophoresis and the fusion of protoplasts occurs when the cells enter the fusogenic state by the application of electrical impulses that generate a rearrangement in the lipid bilayer and the formation of water-soluble pores in the membrane where protoplast fusion occurs (Rems *et al*., 2013).

Fusion products

Despite efforts to increase the protoplast fusion frequencies, the formation of viable binucleate heterocarions is typically restricted to 1-10% of the protoplast population (Chawla, 2002). Therefore, it is necessary to select these fusion products from homokaryons, unfused parent protoplasts and/or multiple fusion products. Currently, a universal method for isolation and purification of somatic hybrids has not been developed, because each case is particular, and parental cells traits have to be considered to identify which environmental or physical parameters could serve to get the identification of these cells (Lynch, Davey, & Power, 1993).

Characterization of fusion products. The detailed characterization of the phenotype and genotype is essential to distinguish the hybrids from their respective parental cells. The fusion products obtained can be differentiated by morphological, cytological, biochemical and molecular characteristics. In several studies with microalgae, scanning electron microscopy images are used to verify the success of the fusion by identifying polynucleated cells, multi organelle and changes in cell size. (Abomohra *et al*., 2016; Gobel & Aach, 1985)

In the case of interspecies protoplast fusion, between green and red microalgae, as in the case of *Dunaliella* and *P. cruentum*, the hybrids can be identified by fluorescence microscopy. These cells can be differentiated based on to their autofluorescence signals, *Dunaliella* shows orange fluorescence, *P. cruentum* has a yellow autofluorescence and the hybrid cells have the presence of both fluorescence signals (Kun Lee & Mengtan, 1988). They can also be selected according to the complementation of the expressed characters (Chawla, 2002). In most of the works reported with microalgae the isolation of the hybrid cells is not carried out after the fusion step, therefore protoplast regeneration is carried out in normal culture media within a mixture of parental and hybrid cells. Some authors have proposed some methods to isolate fused cells. Lee and Mengtan (1988) proposed an isolation protocol for fused protoplasts of *P. cruentum and D. salina or D. bardawil*. These authors used the salinity tolerance as a parameter, taking into account that *P. cruentum* has a maximum tolerance of 1.5M whereas *Dunalliela* can tolerate concentrations of 5M NaCl, and red hybrid cells which were able to grow in solid medium with salinity above 2M were isolated and cataloged as halotolerant fused cells, with a total of 86 hybrids isolated. In another study, Abomohra *et al*. (2016), isolated hydridcells of *Ochromonas danica* and *Haematococcus pluvialis*. *Ochromonas danica* can-

not grow autotrophically in BG_{11} medium, and thus the authors grew the cells resulting from the fusion of these two microalgae in BG_{11} as selective medium and obtained growing cells after 3 weeks of incubation. Hybrid cells and *H. pluvialis* showed different coloration (Abomohra *et al*., 2016). Fatty acid profile could be useful to prove successful fusions of algal cells, since hybrids have a shared profile with the two parental cells.

Another easy and reliable method to verify hybridization and ploidy status of cells is chromosome counting. The number of chromosomes of the hybrids must be the sum of the number of chromosomes of the two parents used for the fusion (Chawla, 2002). Another perspective is the development of molecular techniques such as the analysis of restriction fragments and the hybridization of nuclear and cytoplasmic DNA, which allows a detailed analysis of the genetic constitution of hybrids. Specific DNA patterns of both mitochondria and chloroplasts confirm hybridization and elucidate the segregation of organelles and DNA recombination patterns (Chawla, 2002; Gupta, Kumari, & Reddy, 2015; Lynch *et al*., 1993). A promising alternative is the molecular karyotype by dielectrophoresis, which allows the separation of DNA molecules to the extent that in some organisms the nuclear genome can be separated into its chromosomal entities (Lynch *et al*., 1993; Martins, Horii, & Pizzirani-Kleiner, 1999; Peberdy, 1989). Pulsed field gel electrophoresis (PFGE) is a technique for the fractionation of high molecular weight DNA with an electric field that alternates in two directions. One of the most important applications of PFGE is molecular karyotyping in lower eukaryotes, whose small size of chromosomal DNA makes it susceptible to pulsed field separation (Nassonova, 2008). Molecular karyotyping allows estimating the number of chromosomes and the size of the genome, as well as the dynamics of the genome under study, in particular the chromosomal rearrangements and the resulting chromosomal polymorphism, which is common for many unicellular eukaryotes (Dzhambazov, Belkinova, & Mladenov, 2003; Higashivama & Yamada, 1991; Y. S. Lee *et al*., 2008).

For cell wall regeneration, hybrids should be incubated in hypertonic cultures. The rate of reversion of protoplasts to normal cells in bacteria can be up to 90%, although in fungi it is variable and in microalgae no information was found (Peberdy 1980).

APPLICATIONS IN MICROALGAE

Microalgae are important biological resources that have a wide range of biotechnological applications. These microorganisms have been produced for applications in different fields such as the food industry, pharmaceutical, nutraceutical, cosmetics, feed, biofertilizers and biofuels (Giraldo-Calderón *et al*., 2018). In terms of environmental biotechnology, microalgae are useful for the bioremediation of several types of wastewater (agroindustrial and domestic) (Abdel-Raouf *et al*., 2012; Cheah *et al*., 2016; Cuellar-Bermudez *et al*., 2016) and as a biological tool for the evaluation and monitoring of pollutants such as heavy metals, pesticides and pharmaceutical products (Omar, 2010, Umar *et al*., 2015; Gentili & Fick, 2016) . In recent years, microalgae have attracted a lot of interest due to their potential use as raw material in the production of biofuels (Banerjee *et al*., 2002, Chisti, 2008; Bahadar & Bilal Khan, 2013). However, some microalgae strains with potential use in the bioenergy show slow growth rates (e.g. *B. braunii)* or limited product yields (Jin *et al*., 2016). These drawbacks related with productivity can be overcome using genetic engineering tools to improve cell performance, modify the biochemical profile or increase the growth rate. In this context, protoplasts have been a useful tool in the development of algal biotechnology since they have served as a study model in physiology, biochemistry and molecular biology of these organisms (Doron, Segal, & Shapira, 2016; Inoue *et al*., 1993; Kaladharan, 1998; Reddy, Gupta, Mantri, & Jha, 2008; Yamada & Sakaguchi, 1982).

The fusion of protoplasts has an enormous potential in the improvement of microalgae of industrial interest, since super-producing strains or those resistant to adverse environmental conditions can be obtained. An interesting application was reported by Kusumaningrum and Zainuri (2014), wherein they obtained protoplasts of *D. salina* and *C. vulgaris* to fuse them with PEG and $CaCl_2$, with the goal of increasing the productivity of β-carotene for its application in sustainable aquaculture. The hybrid of *D. salina* and *C. vulgaris* maintained growth stability and increased carotenoid production for several periods of subculture. Astaxanthin is a carotenoid accumulated by the green microalga *Haematococcus pluvialis* and is a commercially important product in aquaculture as a source of pigmentation for fish. In this case, Tjahjono (1994) obtained *H. pluvialis* mutants resistant to inhibitors of carotenoid biosynthesis (such as norflurazon, fluoridone and nicotine) and carried out protoplast fusion between the mutants, finding that the hybrid strains generated had levels of ploidy two times higher and a production of carotenoids three times higher than those of parental and wild-type strains (Tjahjono, Kakizono, Hayama, Nishio, & Nagai, 1994).

As mentioned above, Lee and Tan (1988) fused protoplasts from the red microalga *Porphyridium cruentum* and *Dunaliella salina* and *Dunaliella bardawil*. The protoplasts were fused by treatment with PEG and regenerated in a selective medium and the fused strains acquired

the osmotolerance of *Dunaliella spp*. Among the hybrids, a *Dunaliella* clone was isolated with alterations in the sensitivity to antibiotics, which was the product of protoplast fusion between *P. cruentum* and *D. salina*. The acquired resistance to penicillin and erythromycin seems to be the result of a genetic transfer of *P. cruentum*. Cheng *et al*. (2012), performed a genetic transformation mediated by *Agrobacterium tumefaciens* in the marine microalga of the genus *Schizochytrium*. Transformants were successfully obtained after co-culturing *Schizochytrium* protoplasts with *A. tumefaciens*. This bacterium can transfer the geneticin resistance to the algae by means of a plasmid.

In later studies, Kusumaningrum and Zainuri (2018) modified the nutritional content of the biomass obtained from the interspecies fusion of protoplasts of *Chlorella*, obtaining an increase between 15% and 25% in the content of lipids, protein and carbohydrates compared with the wild type strain. The genetic modification in protoplasts of green microalgae has also been reported. Yang *et al*. (2015), evaluated the spectrum of sensitivity to antibiotics of *Chlorella vulgaris*, and the NPTII gene was selected as a dominant selective marker for genetic transformation. The selective marker, together with a green fluorescent protein gene was introduced into protoplasts of *C. vulgaris* mediated by PEG (Yang *et al*., 2015).

Fujita *et al*. (1990), isolated and fused protoplasts from distinct species of the red microalgae *Porphyra* through the electrofusion and PEG addition methods. These authors compared the efficiency of both methods and found that electrofusion is more efficient than the addition of PEG. The fusion products showed a mixed pattern of chromosomes and pigmentation from the parents (Fujita & Saito, 1990). Gall *et al*. (1993), also isolated protoplasts from two *Porphyra* species using enzymatic cellulase-pectinase mixtures and managed to regenerate macroalgae from isolated protoplasts (Gall, Chiang, & Kloareg, 1993).

There are other studies where optimal conditions for protoplast isolation are evaluated mainly through enzymatic treatments. Liu *et al*., (2006), developed a protocol to produce protoplasts from *Chlamydomonas sp*. ICE-L. Likewise, (Suzuki *et al*. (1997), evaluated the effect of temperature and enzyme concentration on the protoplast yield of the microalga *Prototheca zopfii*. In the biochemical context, Inoue *et al*. (1993), used *B. braunii* protoplasts to study the synthesis of farnesal and 3-hydroxy-2,3-dihydrofarnesal from farnesol as a precursor. This experiment allowed to identify the presence of the farnesal hydratase enzyme in race B of the microalga *B. braunii*.

Another very interesting application was proposed by Heller (2014), who developed a novel method of *in vitro* production of exogenous insulin. The basis of the method is the creation of a low maintenance, self-sustaining cellular hybrid that produces insulin and energy through photosynthesis. *Chlorella kessleri* and the rat insulinoma cell line RIN-5F were fused to create hybrid cells, so-called insulin-producing modified cells (MIP). Successful fusion of algae and insulinomas would lead to an efficient and inexpensive approach for the *in vitro* production of insulin through plant-animal cell hybrids that contain the biochemical properties of each cell type. The photosynthetic properties (and glucose production) of algae act as a basis on which any type of cell can remain viable and functioning, biosynthesizing a specific metabolite without the need for intensive procedures (Heller, Calabro, Queenan, & Pergolizzi, 2014). Recently, protoplast fusion between *Ochromonas danica* and *Haematococcus pluvialis* have been achieved with enzymatic lysis and treatment with PEG. The fatty acid profiles of putative fusants exhibited both *H. pluvialis* and *O. danica* fatty acids with an increase in percentage of hexadecatetraenoic acid and Tetracosanoic acid (Abomohra *et al*., 2016). All these applications of protoplasts in microalgae explain the various possibilities that exist in research around the subject. It should be noted that currently protoplasts remain a reliable tool for the improvement of industrial microorganisms and is a tool that could help solve different obstacles in the scaling of bioprocesses. This would make possible the large-scale production of different bioactive products with high added value.

CONCLUSIONS

The reports discussed in this review regarding the obtention, isolation and fusion of microalgal protoplasts, whether intra or interspecific, show that these procedures are technically feasible and can be effective tools in the way towards improvement of key traits in microalgal cells to overcome some of current bottlenecks in this biotechnological platform. However, current method reported for these processes need further improvements and new research is required to provide more information on the efficiency of protoplast formation, as well as the percentage of cell viability in each step and the effectiveness of protoplast fusion. High throughput and versatile methods for cell wall degradation need to be developed for rapid application with distinct strains, regardless the composition of the cell wall. Likewise, the need for labeling, separation and isolation of hybrids brings opportunities to apply cutting edge technologies in biotechnology into the traditional microalgae field aiming to achieve the desired metabolic responses for new sustainable biorefining process development.

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