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ARTÍCULO DE REVISIÓN

Algal enzymes, biotechnological potential uses: A review

Enzimas algales, usos biotecnológicos potenciales: Una revisión

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ABSTRACT

Enzymes are considered the most proficient catalysts for industrial processes. Nowadays, a few bacterial or fungal enzymes dominate the enzymatic global market (e.g. cellulases, amylases, proteases); however, algae have interesting metabolic routes related to nitrogen, phosphorous and carbon biogeochemical cycles, which could serve as enzyme sources with industrial, biomonitoring and bioremediation applications. Algae are a polyphyletic group of organisms that live in different, and some cases extreme, environments. These characteristics and their metabolic adaptations, make them ideal sources for novel enzymes. The aim of this review was to summarize the available knowledge in the field of algae-based enzymes production, which have potential uses in different fields, ranging from human health applications, carbon sequestration, mining effluents treatments and water bodies monitoring, among others. In addition, seven enzymatic algal complexes are described: carbonic anhydrase, hydrogenase, lipoxygenase, nitrilase, nitrogenase, phosphatase, and thiolase; also, we propose to consider the algal-derived enzymes in the list of added value byproducts of these organisms, mainly when harnessed under a biorefinery scheme.

Keywords: *carbonic anhydrase, hydrogenase, lipoxygenase, nitrogenase, phosphatase*

RESUMEN

Las enzimas se consideran los catalizadores más adecuados para los procesos industriales. Hoy en día, unas pocas enzimas bacterianas o fúngicas dominan el mercado enzimático global (e.g. celulasas, amilasas, proteasas); sin embargo, las algas tienen interesantes rutas metabólicas relacionadas con los ciclos biogeoquímicos de nitrógeno, fósforo y carbono, que podrían servir como fuentes de enzimas con aplicaciones industriales, de biomonitoreo y biorremediación. Las algas son un grupo polifilético de organismos que viven en ambientes diferentes, y en algunos casos extremos. Estas características y sus adaptaciones metabólicas las convierten en fuentes ideales de nuevas enzimas. El objetivo de esta revisión fue resumir los conocimientos disponibles en el campo de la producción de enzimas basadas en algas, las cuales tienen usos potenciales en diferentes campos, desde aplicaciones de salud humana, secuestro de carbono, tratamientos de efluentes mineros y monitoreo de cuerpos de agua, entre otros. Además se describen siete complejos enzimáticos de algas: anhidrasa carbónica, hidrogenasa, lipoxigenasa, nitrilasa, nitrogenasa, fosfatasa y tiolasa. Se propone también considerar las enzimas derivadas de algas en la lista de subproductos de valor agregado de estos organismos, principalmente cuando se aprovechan bajo un esquema de bio-refinería.

Palabras clave: *Anhidrasa carbónica, deshidrogenasa, lipoxigenasa, nitrogenasa, fosfatasa*

INTRODUCTION

Algae are a polyphyletic group of organisms which exhibit a wide diversity of forms and sizes (Hoek *et al.* 1995). They inhabit in countless environmental conditions, like fresh, salt and marine waters and in the surface of moist soil or rocks (Singh & Singh 2015). There are many algae groups, and it is estimated that exist 72 500 species (De Clerk *et al.* 2013), many of them can express high metabolic growth and high biomass production, when suitable environmental conditions are fit: high luminosity, enough CO_2 concentration, and nutriments availability (Graham *et al.* 2009; Singh & Singh 2014).

This characteristic has been exploited to produce biomass-derivate products, like biodiesel (Speranza *et al.* 2015), bioethanol (Fasahati *et al.* 2015), biohydrogen (Bedoya *et al.* 2008) and biogas (Dębowski *et al.* 2013). Although algae are more productive per area of cultivation compared to first-generation biofuel feedstocks, its production may not be economically sustainable without high-value co-products (Rothlisberger-Lewis *et al.* 2016). Many authors support that, to increase the industrial processes sustainability, biomass needs to be harnessed properly, under a biorefinery scheme (Cheali *et al.* 2015).

In addition to serve as energetic feedstocks, many non-energetic byproducts can be obtained from algae (Trivedi *et al.* 2015) as phycocolloids (Zajic 1970) and pigments (Muñoz-Crego & López-Cruz 1992), many of them are known to have applications in cosmetic, pharmaceutical and food industries (Hallmann 2007). Of the added-value products from biomass, enzymes highlights. Actually, different kinds of enzymes are required in many industrial processes, because enzymes are the most proficient catalysts, offering much more competitive processes compared to chemical catalysts (Choi *et al.* 2015).

Nowadays, microbial and fungi enzymes are utilized at large scale in food industry (i.e. amylase, protease, lipase, gluco-oxidase, pectinase and tannase), due to their high specificity and least by-products (Saxena *et al.* 2001). Despite the global enzyme market is dominated by few microbial enzymes, the potential of utilizing novel enzymes as biocatalysts for many processes is unmeasurable. As is stated above, algae are important biomass factories which can growth in many environments, and have enzymes mediated metabolisms, that turns them into good candidates for industrial enzymes production (Table 1). Thus, this review aimed to summarize the "state of the art" of some algae-based enzymes production, focused in the enzymes with industrial or environmental applications.

ENZYMATIC REGULATION IN ALGAE

Algae, like any other organism, regulate their metabolism according to different environmental changes. This metabolic modification affects the type and the amount of the enzymes produced, and in turn, changes in the enzymatic pool modify the metabolic pathways, leading to the accumulation or depletion of different cell compounds. Understanding the metabolic response of algae to variation in the environmental factors, is crucial to produce any metabolite. Variations in nutrient availability (e.g. nitrogen, phosphorous, sulfur), pH, salinity, light quality, temperature, and the presence of xenobiotics have been associated with changes in the metabolism of algae, which modifies their enzymatic production.

In this way when light quality or CO_2 concentration changes, we observe variations in enzymes related to photosynthesis. For example, when *Chlamydomonas reinhardtii* Dangeard is exposed to low CO_2 concentrations, the pyrenoid structures development starts, and with it, the accumulation of high amounts of 1,5 biphosphate carboxylase/oxygenase (Kuchitsu *et al.* 1991) for CO_2 fixation. Light quality affects the algal fitness as well; it is known that blue light (LED) is a better stimulus than white light for the production of biomass and lipids in *Chlorella vulgaris* Beijerinck (Atta *et al.* 2013), and an excess of luminous energy causes the production of protective proteins, like the antenna complex LHCSR (in *C. reinhardtii*; Peers *et al.* 2009). Protective proteins balance the light absorption capacity from photosystem II and photosystem I, acting by the mediation of thylakoid-associated Ser-Thr kinase Stt7 (Bellafiore *et al.* 2005).

Understanding the regulation of the photosynthetic enzymes in algae, is very important not only for biomass development, but also because these enzymes (fructose 1,6 bisphosphatase, NADP glyceraldehyde 3 phosphate dehydrogenase, and ribulose 5 phosphate kinase) from *Euglena*, *Chlamydomonas*, *Synechococcus* and *Synechocystis*, have insusceptibility to H_2O_2 and hydroxylamine (Takeda *et al.* 1995), which could have biotechnological applications, contrary with enzymes from other sources (Kaiser 1976).

Nutrient availability/starvation is other of the main factors that drive changes in the enzymatic-algal pool. It is known that nitrogen and phosphorous starvation lead the lipid accumulation; the same effect is observed in conditions of high salinity. Even when this is important to produce lipids for biodiesel, the phosphorous starvation can negatively affect the production of enzymes. In algae, enzymes are produced by phosphorous–rich ribosomes

(Ågren 2004); thus, phosphate limitation have a severe impact on enzyme production (Courchesne *et al.* 2009). On the other hand, nitrogen starvation can cause the induction of some proteases (Berges & Falkowski 1998), and the induction of nitrogenases (with or without heterocyst) for N fixation and hydrogen production, in the genus *Anabaena* (Masukawa *et al.* 2014). Proteases production is related to self-protein digestion to serve as carbon/energy supplies for triacylglycerols anabolism; in addition, genes codifying for diacylglycerol:acyl CoA acyltranferases increase their transcript abundance under nitrogen depletion (Msanne *et al.* 2012).

Sulfur is other important nutriment for algae. The metabolism of sulfur in algae, mainly marine phytoplankton, is highly important because the major input of dimethylsulfide (DMS) into the atmosphere is mediated in a great manner by phytoplankton (Simó 2001); this process involves the action of several enzymes, like ATP sulphurylase and APS sulphotransferase (Stefels 2000). In chlorophytes, when sulfur deprivation and anoxic conditions are fit, algae switch their metabolism towards fermentation. Under these conditions, [Fe]-hydrogenases are synthetized to serve as energy donors for anaerobic metabolism (Winkler *et al.* 2002).

Some algal enzymes are activated for the metabolism of xenobiotics. Algae can endure and degrade some herbicides, as metflurazon, thanks to the enzyme cytochrome P450 monooxygenase, which exhibit a N demethylase activity in *Chlorella* (Thies *et al.* 1996). Other herbicides like atrazine, glufosinate, glyphosate, oxyfluorfen and diuron, also induce the production of antioxidant enzymes, like superoxide dismutase, peroxidase and catalase in *Chlorella* (Romero *et al.* 2011); and ascorbate peroxidase, glutathione reductase, and glutathione S transferase in *Scenedesmus* (Geoffroy *et al.* 2002). These antioxidant enzymes limit lipid peroxidation, DNA damage, and protein degradation. Heavy metals also induce the production of antioxidant enzymes in microalgae (Arunakumara & Xuecheng 2008).

Thus, many algal enzymes, which could have different biotechnological uses, respond to specific environmental or anthropogenic stimulus. The knowledge of these different responses in enzyme production is very important, when the aim is to co-produce an algal enzyme in a biorefinery.

ALGAL ENZYMES PRODUCTION METHODS

Production in biorefineries

Even if the production potential of many algae derived products is very high, culture and processing technologies are required to be optimized, to make large-scale production profitable (Gimpel *et al.* 2015). It is estimated that only the 20 – 40 % of the algal biomass can be utilized to produce biofuels; and the remaining 60 – 80 % contains high value coproducts, like organic pigments, nutraceuticals, omega-3 fatty acids, enzymes, algal meal, among others (Subhadra & Edwards 2011). This offers the possibility of produce, from algal biomass, high quantities of biofuels to low cost and low quantities of coproducts of high value.

The product extraction steps, in a biorefinery that uses algal biomass, depends of the number of coproducts that are intended to obtain. Each step involves an additional cost, that is why the design of the biorefinery and the market analysis is of great importance when the aim is to go further than a pilot scale. In order to assess the environmental impact of the algal biorefinery, the water, land and carbon footprints should be measured too. Life cycle models, and fuzzy linear programing have been successfully used for this propose (Ubando *et al.* 2012).

Generally, the first extraction step is for the main product of the biorefinery. This one could be fatty acids or starch, when the aim is to produce biodiesel or bioethanol. Several methods have been described for the extraction of lipids and starch; however, not all are suitable for a subsequent enzyme recuperation. For example, the hydrothermal liquefaction process allows the production of high amounts of algae-oil, but the process involves high temperatures (250 °C to 350 °C) and pressure (1500 to 3000 psi), which denature all the enzymes; also, it consumes a fraction of the nutrients (e.g. nitrogen) of the biomass, avoiding its recycling (Frank *et al.* 2013). Other methods involve the use of organic solvents (e.g. ethanol, chloroform, hexane; Ramluckan *et al.* 2014), but have many drawbacks related with energy consumption. Recently, it has been proposed the use of switchable solvents (*N, N*-dimethylcyclohexyl-amine) to extract lipids directly from liquid cultures of algae (Samori *et al.* 2012), showing promising results; although, it must be account the toxicity of *N, N*-dimethyl-cyclohexyl-amine. Thus, alternative solvents like α-pinene, d-limonene and p-cymene are under consideration, (Dejoye *et al.* 2013).

For starch recuperation, similar methods using organic solvents, acid pretreatments or even genetic modified yeast are used for bioethanol production (Aikawa *et al.* 2013; Scholtz *et al.* 2013); but no matter which method is used, it is important planning the utilization of the residual biomass. When the aim is to isolate enzymes, can be used the following methods of extraction.

Generally, crude enzymes are extracted using buffers, which protect them from denaturation and preserve its activity; for algal enzymes, the buffer of Davison (1987) and Davison & Davison (1987) has been successfully used for the isolation of photosynthetic enzymes and kinase of brown algae. For green microalgae, the buffer of Gerard & Driscoll (1996), is suitable for isolation of ribulose 1,5 bisphosphate carboxylase oxygenase (Bischof *et al.* 2002). For lipoxygenase extraction from cyanobacteria cells, the use of phosphate buffer (pH 7) enriched with nonionic detergents (e.g. Brij 99 0.1 %) is appropriate (Beneytout *et al.* 1989).

Extrusion with French pressure disrupt the unicellular algae cells without denaturing enzymes; this method has been used for the isolation of acetyl-CoA synthetase, acetate kinase, phosphotransacetylase, citrate synthase, isocitrate dehydrogenase, isocytrate lyase, urease, urea amidolyase, malate synthase, NADP linked glyceraldehyde 3 P dehydrogenase, ribulose 5 P kinase, NADP linked malic dehydrogenase, sedoheptulose l,7 diphosphatase, fructose l,6 diphosphate, glucose 6 P dehydrogenase and P fructokinase (Duggan & Anderson 1975; Leftley & Syrett 1973; Pearce & Carr 1967).

Ultrasonic disruption can break down the algal cells, and did not affect enzyme activities; this method was used for the isolation of isocitric acid dehydrogenase, malic acid dehydrogenase, α-ketoglutaric acid dehydrogenase and succinic acid dehydrogenase from blue-green algae (Smith *et al.* 1967). Other methods, like immunoprecipitation can be used for enzyme isolation. Superoxide dismutase from blue-green algae was extracted with antibodies, and precipitate with ammonium sulfate at 33 % (Asada *et al.* 1975). The methods of cell disruption for enzyme isolation, are also useful for lipid or starch extraction, thus can be successfully incorporated in a biorefinery design.

Genetic overexpression and transgenic technology

Genetic engineering seeks to improve the production of enzymes or metabolites. A variety of transformation methods have been used to transfer DNA into microalgal cells: including agitation in the presence of glass beds or silicon carbide whiskers, electroporation, biolistic microparticle bombardment (the prefer one for chloroplast transformation; Purton *et al.* 2013), and *Agrobacterium tumefaciens*-mediated genetic transfer (Radakovits *et al.* 2010). Algae can be transformed in nuclear, as well as in chloroplast genomes.

Some algal genes, which codifies for enzymes, have been object of genetic manipulation seeking for the overexpression of the enzyme. The acetyl Co-A carboxylase, involved in fatty acids production, was successfully overexpressed in *Cyclotella cryptica* Reimann, Lewin & Guillard (Dunahay *et al.* 1995). Also, some enzymes related to β-oxidation of fatty acids can be inhibited, with the aim of accumulate lipids (Michinaka *et al.* 2003), however little is known about this in algae.

Transgenic microalgae can be used to produce specific fatty acids. Ideal fatty acids for biodiesel production should be 12:0 and 14:0; the chain lengths of fatty acids are determined by acyl-ACP thioesterases; transgenic or overexpression of these enzymes into algae, could modify its fatty acids production (Radakovits *et al.* 2010). Other important enzyme, which should be object of manipulation, is ADP-glucose phosphorylase (AGPase); this enzyme is key in the process of starch production, and have been successfully overexpressed in plants (Zabawinski *et al.* 2001).

The transgenic methodologies for algae manipulation, are well developed for several models, like *Chlamydomonas reinhardtii*, *Volvox carteri* Stein, *Phaeodactylum tricornutum* Bohlin, *Dunaliella salina* (Dunal) Teodoresco and *Haematococcus pluvialis* Flotow (Walker *et al.* 2005). Also, the DNA genomes of algal viruses (e.g. phaeovirus Es V-1; Delaroque *et al.* 2001) represent a source of strong promoters that potentially could be exploited to produce heterologous proteins (or enzymes) in algal cells (Walker *et al.* 2005). The metabolic pathways engineer in algae, seeking the accumulation of lipids or starch is very complex, and involves the overexpression or inhibition of many enzymes. Beer *et al.* (2009) indicate that, when the aim is to increase lipid production, at least five steps should be achieved which involves the overexpression of enzymes related to fatty acids production, inhibition of the enzymes of the β-oxidation and lipid hydrolysis pathways, regulation of the activity of the desaturases, and control of the fatty acids length by thioesterases.

The transcription factor engineering of target enzymes, which lead to overexpression, is a very promising strategy since it may avoid the limitations of other commonly used approaches, like biochemical stress or even genetic engineering; however, it is still a novel concept to be investigated systemically (Courchesne *et al.* 2009). Classical promoters used for heterologous expression of proteins in algae are those of photosystem I complex protein, HSP70A or RBCS2 (Fisher & Rochaix 2001; Schroda *et al.* 2000). In addition, bacterial or viral promoters are other tool for heterologous protein expression in algae. Some heterologous enzymes expressed

are: neomycin phosphotransferase and nitrate reductase using the nopaline synthase promoter of *Agrobacterium tumefaciens* (Hall *et al.* 1993); chloramphenicol acetyl transferase using the cauliflower mosaic virus 35S promoter (CaMV 35S; Tang *et al.* 1995), β-glucuronidase and hygromycin phosphotransferase with the same promoter (Kumar *et al.* 2004) and aminoglycoside 3'-phosphotransferase using CaMV 35S (Díaz-Santos *et al.* 2013). In summary, the overexpression of certain enzyme genes, could be very helpful for metabolic engineering in algae, for bioactive compounds production, or to serve as biofuels prime matter.

TARGET ENZYMES FOR INDUSTRIAL PRODUCTION

Carbonic anhydrase

Carbonic anhydrases (CAs) are ubiquitous metalloenzymes (with Zn^{2+} active site) found in prokaryotes and eukaryotes in different isoforms: the α-CAs (present in vertebrates, eubacteria, algae and cytoplasm of green plants); β-CAs (predominantly in eubacteria, algae and chloroplasts of both mono as well as dicotyledons), γ-CAs (mainly in archaea and some eubacteria) and δ- and ζ-CAs (both discovered in marine diatoms). These enzymes have an important role in several different metabolic processes such as acid–base regulation, respiration and transportation of carbon dioxide and bicarbonate (Chegwidden *et al.* 2000).

In recent years, there has been an increasing interest in the utilization of these enzymes in CO_2 caption and storage processes (Di Fiore *et al.* 2015); since it is possible to convert the captured CO_2 into various benefic by-products through enzymatic catalysis, among them acrylates, polycarbonates, stable carbonate storage polymers, methane and building materials (Beckman 1999). To improve the process of sequestration, some biological methodologies, also called "bio-mimetic" CO_2 capture systems have been implemented as economic and sustainable technologies (Di Fiore *et al.* 2015).

As previously described, all CAs in vertebrates belong to α-CAs. These enzymes have been found in a periplasmic location of *Chlamydomonas reindhardtii* (Fukuzawa *et al.* 1990). This alga has proved to present homologous sequences with human isozymes, particularly CA I, CA II and CA III (Fukuzawa *et al.* 1990). Among α-CAs enzymes, CA-II is the most studied form of CA isozyme because of its functions and uses in human health. CA II was successfully used as liver protector, and to improve the preservation of transferred organs, because it regulates pH changes caused by cold ischemia (Bejaoui *et al.* 2015). Given the homology between algal and human α-CAs, and its uses in medicine, algal biomass could be used as an alternative source of these enzymes.

Thus, CAs are promising enzymes used for CO_2 sequestration and transferred organs protection. Even though they are mostly isolated from vertebrates, human erythrocytes and other animals, algae also share some isotypes like CA I, CA II and CA III (Fukuzawa *et al.* 1990). More studies are required to explore the isotypes present in other algae species; since several need to fix carbon from environment, many isotypes might still be ignored. The study of this enzyme might offer a new byproduct to biorefinery initiatives and environmental benefits in the frame of climate change.

Hydrogenases

Hydrogenases (H_2ases) are enzymes that catalyze the reversible oxygenation of molecular hydrogen. They play a central role in microbial metabolism and are important enzymes in the photobiological H_2 production (Vignais *et al.* 2001). There are three classes of hydrogenases: the [Fe]-H_2ases, [NiFe]-H_2ases and the metal-free H2ases, according to the catalytic nucleus of the enzyme (Gutekunst *et al.* 2006).

These enzymes are proposed to be used for hydrogen production. Biological H_2 production offers distinctive advantages for environmental protection over existing physico-chemical methods. The hydrogen production by bio-photolysis, involves the conversion of water and sun energy into hydrogen and oxygen. This process utilizes microorganisms, generally microalgae and/or cyanobacteria, from photosynthetic reactions (Dutta *et al.* 2005; Gutekunst *et al.* 2006). The production of hydrogen by microalgae is based on the consequent transfer of electrons due to activation of photo-system 1 (PS1); the electrons flow to ferredoxin and after to the hydrogenase, all the process occurs in thylakoid-like structures (Martín-Gil & Martín-Gil 2004). The ferredoxin serves as an electron donor to [Fe]-H_2ase in the electron Fe/chain of the green algae chloroplast (Contreras *et al.* 2008).

It is known that some algae are hydrogen producers. The main algal model in bio hydrogen production research are *Chlamydomonas*, showing very satisfactory results (Martín-Gil & Martín-Gil 2004). Some species that are been object of research in the bio hydrogen photoproduction field are *Spirulina* sp. and *Anabaena* sp. (Dutta *et al.* 2005). In addition, Contreras *et al.* (2008) reported that *Chlorella vulgaris*, *Chlamydomonas angusta* (Dujardin) Diesing (as *C. angustae*) and *C. reinhardtii* have the capacity to produce hydrogen by photoproduction. Unfortunately, the production of biohydrogen from algae resulted in very

low yields; thus, more research is needed to improve the process, if the goal is to reach commercial levels.

Lipoxygenases

Lipoxygenases (LOXs) are defined as enzymes that specifically catalyze dioxygenation of unsaturated fatty acids containing a 1, 4-*cis*, *cis*-pentadiene system and produce *cis*, *trans*-conjugated monohydroperoxides as primary products. These enzymes produce the oxylipins 13-hydroperoxy-9, 1 1-octadecadienoic acid (13-HPODE) and 9-hydroperoxy- 10, 12-octadecadienoic acid (9-HPODE) when linoleic acid is used as substrate (Beneytout *et al.* 1989). Many oxylipin pathway products are involved in defensive mechanisms, e.g. plant produce different jasmonates and volatile compounds (mainly from octadecanoids and hexadecanoids; Weber 2002) which activate defense mechanisms against insects and pathogens; also, the hydroxy derivatives of linoleic acid exhibit antifungal or antimicrobial properties (Blée 2002).

LOXs enzymes are the initial and key enzymes in oxylipin pathway; however, even if these enzymes are present in different organisms, some studies suggest a polyphyletic origin (Chen *et al.* 2015), and some LOXs from algae exhibit interesting properties. The red alga *Pyropia haitanensis* (Chang & Zheng) Kikuchi & Miyata has a LOX with multiple catalytic sites, and based on the analysis of their different reaction products, Chen *et al.* (2015) found that this enzyme is a multifunctional enzyme with lipoxygenase, hydroperoxidase and dihydroxiperoxidase activities. Multifunctional LOXs are very scarce in nature, but marine algae probably contain 5R, 8R, 9S, 12S, and 15S lipoxygenases that act on eicosanoic (C20) polyunsaturated fatty acids, as well as ω3, ω6, ω9 and ω10 lipoxygenases that act on octadecanoids (C18) (Gerwick *et al.* 1999). Thus, the oxylipin metabolism in red algae and diatoms featured both, animal- (eicosanoid) and plant-like (octadecanoids) oxylipins, which are involved in immunity mechanisms (Bouarab *et al.* 2004). This dual metabolism in red algae, employs a high diversity of LOXs, making them a rich source of oxylipins; also, algae are considered the richest group of life forms in oxylipins diversity (Gerwick *et al.* 1999).

Many algal LOXs are non-specific, and catalyze different polyunsaturated fatty acids, ranging from C18 to C22 in *Pyropia haitanensis*, e.g. γ-linoleic acid, arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid, producing different oxylipins (Chen *et al.* 2015). In the same report, found 16 different products from 4 polyunsaturated acids using only one algal LOX (PhLOX2). This high diversity of products can be used in biotechnological applications. In the knowledge that oxylipins are involved in the defensive mechanisms of algae, like the protection against herbivorous amphipods in *Dictyopteris* sp. (Schnitzler *et al.* 2001), and the toxicity of diatoms-aldehydic oxylipins against bacteria, fungi and even invertebrates (Adolph *et al.* 2004), the use of algal LOXs for the production/induction of oxylipins opens a huge opportunity in the field of biocontrol.

Nitrilase

Nitrilases are versatile nitrile-metabolizing enzymes used as an alternative of nitrile hydrolysis instead of strong acids or base catalyst (Djambaski *et al.* 2009). Nitriles are cyanide-substituted carboxylic acids with general structure R-CN. These compounds are synthesized on a large scale and are used as solvents, in plastic industry, synthetic rubber and as a starting material for pharmaceuticals, herbicides and other industrially important chemicals (Gupta *et al.* 2010). In addition, most nitriles are highly toxic and their biochemical degradation has been considered as an efficient way for detoxification of industrially polluted waters and soils (Djambaski *et al.* 2009).

In plants, nitrilases are involved in the process of cyanide detoxification, in the catabolism of cyanogenic glycosides and presumably in the catabolism of glucosinolates (Piotrowski 2008). Cyanide is a metabolic by-product in the biosynthesis of plant hormone ethylene, and nitrilases transform the intermediate detoxification product β-cyanoalanine into asparagine, aspartic acid and ammonia (Piotrowski 2008). Meanwhile, nitrilases were found in many algal species, and has been associated with the biosynthesis of indole-3 acetic acid from indole-3-acetonitrile (Kiseleva *et al.* 2012). Algal nitrilases have an active site with a cysteine residue, which acts as a nucleophile for substrate attack when its sulfhydryl group undergoes unprotonated (Zhang *et al.* 2014a). Few studies of cyanide detoxification using algae are available, but the results obtained are promising. It was found that *Chlorella pyrenoidosa* Chick has the capacity to transform cyanide into β-cyanoalanine and γ-glutamyl-β-cyanoalanine, and use it as a carbon and nitrogen source (Gurbuz *et al.* 2004). Also, algae require less nutrients for growing than some bacteria and fungi, and some species like *Scenedesmus obliquus* (currently *Tetradesmus obliquus* (Turpin) Wynne) can detoxify until 400 mgL^{-1} of cyanide in 68 h (Gurbuz *et al.* 2004). Cyanide is commonly used in mining and it is present in high quantities in its wastewater, and the use of *S. obliquus* has been recommended as a viable process

for gold mine effluents bio treatment (Gurbuz *et al.* 2009). Algae containing nitrilases, or free-nitrilases could be a good option for bioremediation processes, nevertheless, more research is needed in this field for enhance nitrilases production in algae.
Free nitrilases from *Pseudomonas fluorescens* (Sohoni *et al.* 2015), and *Acidovorax facilis* (Zhang *et al.* 2014b), among other bacterial nitrilases have been successfully produced, but few studies have reported the production of algae-based nitrilases. Heterologous expression of nitrilases in bacteria could be a good strategy for the production in large-scale; however here is a lack in the knowledge of algae-nitrilases production.

Nitrogenase

This enzyme is essential for atmospheric nitrogen fixation, being the cyanobacteria the main organisms that accomplish this important function in ecosystems (Fay 1992). Nitrogenase is an enzymatic complex, conformed by two components, the dinitrogenase (Mo-Fe protein), which is a tetramer α2β2 that have their subunits codified by *nif*D and *nif*K genes, and the dinitrogenase reductase (Fe protein) which is a dimer of two identical subunits, codified by nifH gene (Bergman *et al.* 1997; Fay 1992). The last one collects electrons from external donors (ferredoxin or flavodoxin) and transfers them, in an ATP dependent process, to dinitrogenase, that utilizes the reducing power to convert N_2 into ammonium.
The nitrogenase from cyanobacteria have similar characteristics to other N_2 fixing bacteria nitrogenases. For example, *Gloethece* sp., *Plectonema* o *Pseudanabaena* which have the genes nifH, nifD and nifK, organized in a single operon, similar to some diazotrofic bacteria, such as Azotobacter or Rhizobium sp. (Soto-Urzúa & Baca 2001). Some cyanobacteria can differentiate specialized cell forms, in response to environmental signals (Soto-Urzúa & Baca 2001). For example, when nitrogen sources (NH_4^+, NO_3^-) are limited, some cells from thallus change into heterocytes (Whitton & Potts 2012). On the other hand, when nitrogen sources are available, the nitrogenase synthesis and heterocyte formation are repressed (Graham *et al.* 2009).
The heterocytes are specialized structures for nitrogen fixation, inside them is generated an anaerobic environment suitable for N_2 reduction by nitrogenase (Böhme 1998). However, some species of cyanobacteria have no heterocyst and utilize different mechanisms of protection against oxygen, to ensure the nitrogenase catalysis (Berman-Frank *et al.* 2003). These mechanisms can involve spatial and temporal separation of the photosynthetic, respiratory and N_2 fixation processes (Berman-Frank *et al.* 2003). Some diazotrophic species, like those from the genus *Anabaena* and *Nodularia* present a clear spatial separation between N_2 fixation (that occurs in heterocyst) and photosynthesis that occurs in the rest of the vegetative cells (Fay 1992). Temporal differentiation can occur controlling the expression of the photosynthetic and nitrogen fixation genes; thus, the first ones (*psaA*, *psbA* genes) are expressed with several hours of difference with respect to the genes *nifHDK* in a circadian rhythm (Bergman *et al.* 1997).
Cyanobacterial nitrogen fixation has acquired great ecologic relevance in many natural and artificial ecosystems. For example, in rice fields it allows the input of 5 g of N per m^2/year, and in shallow lakes from polar areas, where atmospheric nitrogen fixation is extremely important, it can ensure the input of 1.5 g per m^2/year (Megías *et al.* 2011). In oceanic ecosystems, some cyanobacteria, like *Trichodesmium* sp. and *Richelia intracellularis* Schmidt (in association with diatoms) are some of the main nitrogen fixators, and the amount of nitrogen fixed is only comparable to the nitrate input (Bergman *et al.* 2012). At global scale, it is estimated that the oceanic nitrogen fixation is similar to the terrestrial fixation (100-200 Tg per year), and *Trichodesmium* can fix 60-80 Tg N annually. The great nitrogen fixation power of cyanobacteria could be very valuable for ecosystems and human activities, like rice production, where can incorporate 70-110 $Kg \times N \times ha^{-1}$ per culture cycle (Megías *et al.* 2011).

Phosphatases

Phosphatases are a group of enzymes that release inorganic phosphorous from organic sources, making it available for plants, algae and fungi, among others. In marine algae, alkaline phosphatases are mainly produced, due to the pH of water (8.3); these phosphatases are not free-enzymes, they are localized in the external part of the alga (cell wall, external membrane or periplasmic space; Hernández *et al.* 1994). Alkaline phosphatase (AP) activity from the algal genus *Gelidium*, *Cladophora*, *Polysiphonia* and *Stypocaulon* can be useful for environmental monitoring because they are very sensitive to phosphorous availability (Hernández *et al.* 2002). AP of single algal cells, as *Scenedesmus quadricauda* (currently *Desmodesmus communis* (Hegewald) Hegewald) and *Asterionella formosa* Hassall, could be an indicator of the internal P status (Litchman & Nguyen 2008). Generally, when there is high P availability, phosphatase activity is low, and vice versa (Pettersson 1980); however, some authors pointed that the increase or decrease in phosphatase activity, depending to P

concentration, may not be immediately, and could be related to other variables (Litchman & Nguyen 2008).

Phosphatases can be helpful for water monitoring. AP activity of *Chlorella vulgaris* has been proposed as a biosensor for heavy metals (Cd^{2+} and Zn^{2+}) contaminated water, which is important for constant monitoring of water bodies (Durrieu & Tran-Minh 2002). In the same way, *Scenedesmus quadricauda* and *Anacystis nidulans* (currently *Aphanothece nidulans* Richter) showed a decrease in their alkaline phosphatases when they were exposed to Ni^{2+}, Zn^{2+} and Cd^{2+} (Awasthi 2012), and *Arthrospira platensis* Gomont when it was exposed to Cd^{2+} and Hg^{2+} (Tekaya *et al.* 2013). The sensitivity of AP to heavy metals contamination, and the possibility of using immobilized algal cells, opens the opportunity of easy and rapid detection of contamination by heavy metals in water bodies.

Some algal phosphatases have important biological roles, which can be used for biotechnological applications. Mannitol is one of the principal reserve of carbohydrates in brown algae, and can represent up to 20-30 % of their dry weight (Reed *et al.* 1985); this polyol is synthetized in two enzymatic steps. First, fructose 6-phosphate is reduced into mannitol 1-phosphate by the mannitol 1-phosphate dehydrogenase, and subsequently the phosphate group is released from mannitol by the mannitol 1-phosphate phosphatase (Iwamoto & Shiraiwa 2005). Mannitol is a poly alcohol that serves as an osmoprotectant in algae (Dittami *et al.* 2011), and has many medical and industrial applications. It can be used as a neuroprotective compound for Parkinson treatment (Shaltiel-Karyo *et al.* 2013), and to control the raised intracranial pressure and renal protection in diverse surgeries (Shawkat *et al.* 2012).

Groisillier *et al.* (2014) characterized the genes of mannitol phosphatases of *Ectocarpus silicolosus* (Dillwyn) Lyngbye (EsM1Pase 1 and EsM1Pase 2), and comparing it to previous data of mannitol phosphatases of the red algae *Caloglossa continua* (Okamura) King & Puttock and the green microalga *Micromonas* sp. They suggest that brown algal-sequences, and their orthologues, represent an additional family of phosphatases, with new substrate specificity within the haloacid dehydrogenase superfamily. Cloning these genes into bacteria for its heterologous expression is possible (Libertador *et al.* 1998); thus, specific algal phosphatases could be used for specific aims as, an alternative production way for mannitol from fructose.

Therefore, algal phosphatases do not only have a great ecological importance, due to their relevance in aquatic phosphorous cycle, they also have been successfully used as phosphorous availability and heavy metal biosensors, which are important in the field of biomonitoring. Further applications of this algal enzyme could be their use for advanced aims, like the production of mannitol from fructose.

Thiolase

Thiolases are wide-distributed enzymes that can be found in prokaryotic as well as eukaryotic organisms including algae (Kursula *et al.* 2002). This enzyme family can be further subdivided into catabolic and metabolic thiolases. Both can catalyze a Claisen type condensation of two acetyl-CoA molecules to generate acetoacetyl-CoA (Reiße *et al.* 2014). Hence, these enzymes are key in forming extended carbon skeletons from the universal metabolic precursor acetyl-CoA into others of biotechnological interest. Biosynthetic thiolases are utilizing only acetyl-CoA and acetoacetyl-CoA as substrates (Kursula *et al.* 2002). In contrast, catabolic thiolases display a varied substrate spectrum which includes larger substrates such as 3-ketodecanoyl-CoA (Staack *et al.* 1978), involved in the lipid metabolism.

Thiolases are found in different algal organelles. In *Mougeotia*, thiolase and the acyl-CoA-oxidizing enzyme are located exclusively in the peroxisomes, meanwhile, in *Bumilleriopsis* all enzymes of the fatty-acid fl-oxidation pathway (included thiolases) are constituents only of the mitochondria (Winkler *et al.* 1988). In *Euglena gracilis* Klebs and *Eremosphaera*, thiolase and acyl-CoA-oxidizing enzymes were found both in the peroxisomes as well as in the mitochondria (Graves & Becker 1974; Winkler *et al.* 1988).

There has been a growing interest in thiolases for the uses of their products, because they are key enzymes in the biosynthesis of several sterols (e.g. ergosterol), by the mevalonic acid pathway (Lopes *et al.*, 2013). Ergosterol is the predominant sterol of fungi and algae (Miller *et al.* 2012), it is produced from acetyl CoA and has interest since it has shown to have a suppressive effect on cancer growth and tumors (Kobori *et al.* 2007). Some efforts have sought to improve thiolase activity to obtain higher ergosterol yields; nevertheless, Dequin *et al.* (1988) showed that high thiolase activity are not sufficient to obtain a high level of sterol in yeast. Acetoacetyl-CoA thiolase leads to inhibition when high amounts of ergosterol are available, increasing its activity (among 3 hydroxy 3 methylglutaryl-CoA synthase/reductase) at low ergosterol quantities (in *Saccharomyces cerevisiae*; Servouse and Karst 1986). Acetoacetyl-CoA thiolase, called thiolase II, regulates the production of isoprenoids via meva-

lonate (Soto *et al.* 2011); and is a key enzyme for carotenoids biosynthesis in *Cyanophora*, *Galdieria*, *Cyanidium*, *Mesostigma* and *Euglena*, among others; however, green algae have lost this metabolic pathway (Lohr *et al.* 2012). The metabolic engineering for isoprenoids in algae is attracting the attention, mainly for their uses in industry (Tippmann *et al.* 2013). The production of acetoacetyl-CoA by thiolase II and 3-ketothiolase, can be used to produce polyhydroxybutyrate, an important precursor of biodegradable plastics (Ojumu & Solomon 2004); and for the production of biobutanol (Reiße *et al.* 2014). Thus, thiolases from algae, are important since they are key enzymes in sterols and isoprenoids biosynthesis, and can be used in the fabrication of bioplastic and biofuels, under the strategy of enzymatic cascades.

FURTHER PERSPECTIVES

Algae can be considered an excellent prime matter for biorefinery and are high-biomass producers that can serve as bioenergetics. In addition, algae produce different bio compounds like pigments, toxins and enzymes that can be considered as high value byproducts. In this review, we summarized the knowledge of different algal enzymatic complexes, which exhibit interesting properties for industry, bio remediation and bio monitoring representing an opportunity for biotechnological development. Bioprospection of algal enzymes will open an avenue into new biocatalyst discovering, that can be produced in photoreactors from algae, or produced by heterologous expression in bacteria. The use of algae for enzyme production have the advantage that they can be grown without carbon or nitrogen sources, and can consume small quantities of toxic compounds or heavy metals. Despite the potential of algae as source of novel enzymes, there is a lack of knowledge on this field. However, some efforts have been made to procure the understanding of metabolic insights in algae and the enzymes involved on it. In the future, algal enzymes could be helpful in different industrial activities, and could serve for environmental protection.

Table 1. Algal enzymes with potential industrial or biotechnological uses

Enzyme	Sources	Actual or potential uses	References
Carbonic anhydrase	*Chlamydomonas reindhardtii*	Transferred organs protection CO_2 sequestration	Fukuzawa *et al.* (1990); Bejaoui 2015
Hydrogenases	*Chlamydomonas reinhardtii* *Spirulina* sp. *Anabaena* sp. *Chlorella vulgaris* *Chlamydomonas angutae*	Bio hydrogen production	Martín-Gil & Martín-Gil 2004 Dutta *et al*. 2005 Contreras *et al*. 2008
Lipoxygenases	*Pyropia haitanensis* *Dictyopteris* sp.	Production of oxylipins (can be used like bio defense mechanisms for plants and biocontrol)	Chen *et al*. 2015 Gerwick *et al.* 1999 Schnitzler *et al.* 2001
Nitrilases	*Thalassiosira pseudonana* *Phaeodactylum tricornutum* *Ectocarpus siliculosus* *Micromonas* sp. *Chlorella variabilis* *Volvox carteri* *Micromonas pusilla* *Chorella pyrenoidosa* *Scenedesmus obliquus* *Pseudomonas fluorescens* *Acidovorax facilis* *Synechocystis* sp.	Degradation of nitriles, cyanide detoxification. Gold mine effluents bio treatment	Kiseleva *et al.* 2012 Gurbuz *et al.* 2004 Gurbuz *et al*. 2009 Sohoni *et al*. 2015 Zhang *et al*. 2014b
Nitrogenase	*Gloethece* sp *Plectonema* sp. *Pseudoanabaena* sp. *Anabaena* sp. *Calothrix* sp. *Nodularia* sp. *Nostoc* sp. *Mastigocladus* sp. *Stigonema* sp. *Trichodesmium* sp *Richelia intracellularis*	Atmospheric N_2 fixation, useful for agriculture practices	Soto-Urzúa & Baca 2001 Fay 1992 Bergman *et al*. 1997 Megías *et al*. 2011 Bergman *et al*. 2012
Phosphatases	*Gelidium* sp. *Cladophora* sp. *Polysiphonia* sp. *Stypocaulon* sp. *Scenedesmus quadricauda* *Asterionella formosa* *Chlorella vulgaris* *Anacystis nidulans* *Arthrospira platensis* *Eimeria silicolosus* *Caloglossa continua* *Micromonas* sp	Phosphorous availability bioindicator Heavy metals contamination bioindicator Mannitol production	Hernández *et al.* 2002 Litchman & Nguyen 2008 Durrieu & Tran-Minh 2002 Awasthi 2012 Tekaya *et al*. 2013 Iwamoto & Shiraiwa 2005
Thiolases	*Mougeotia* sp. *Bumilleriopsis* sp. *Eremosphaera* sp. Euglena gracilis	Sterols production Biobutanol production Isoprenoids production	Winkler *et al*. 1988 Graves & Becker 1974

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