**Chlamydomonas reinhardtii as Host in Production of Recombinant Proteins for Medical Uses**

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**Abstract.** Microalgae as a new preferred bioreactor system have many advantages compared with other bioreactor system as it is edible and does not contain endotoxins for human consumption. The green microalga species *Chlamydomonas reinhardtii* possesses many beneficial properties that have made it as model organism in photosynthetic related studies, biofuel production and also as eukaryotic host in heterologous proteins production. Recombinant proteins expressed in *C. reinhardtii* have been produced for the medical applications as hormones, vaccines, and antibodies. Production of these therapeutic proteins in microalgae has enhanced the yield of the proteins expressed compared to the traditional cell tissue culture methods which normally are more expensive in the cost of production. In this review, the recombinant microalgae developed by genetic transformation of both nuclear and chloroplast genomes are discussed, recombinant proteins that had been produced for medical purposes in *C. reinhardtii* are presented and the transformation methods as well as strategies for better protein expression in *C. reinhardtii* are also discussed.

**Keywords:** algae; therapeutics; transformation; recombinant protein; genetic engineering

**INTRODUCTION**

Popularity on the research of microalgae-based proteins have been on rise for both health and environmental reasons. Microalgae are single celled organisms which some of them have the potential to generate biofuel, as they have the capability to produce lipids (Jiang *et al.*, 2014), the ability to produce a wide range of valuable metabolites (Priyadarshani & Rath, 2012) and many others. It has also been used widely as a model organism in photosynthetic related studies (Gerloff-Elias *et al.*, 2005; Im *et al.*, 2003). Lately, microalgae has been used as a model organism to produce complex recombinant proteins as it has established methods and stable transformation system for the expression of foreign genes. For instance, hepatitis B surface antigen gene (HBsAg) was produced efficiently under the control of ubiquitin promoter in the nuclear genome of green alga *Dunaliella salina* (Geng, Wang, & Wang, 2003). Other than that, *Dunaliella tertiolecta*, as a model organisms from the marine green alga, together with *Chlamydomonas reinhardtii* was transformed by using particle bombardment in the chloroplast genome to produce several recombinant enzymes from five different classes such as xynalase, α-galactosidase, phytase, phosphate anhydrolase, and β-mannanase (Georgianna *et al.*, 2013).

One of the most studied microalgae in producing complex recombinant protein is *C. reinhardtii*. *C. reinhardtii* as many other alga is considered as safe (GRAS) for human consumption (Harris, 2009; Mayfield *et al.*, 2007; Rosales-Mendoza *et al.*, 2012). It is as an edible model organism that has a haploid genome where any modification in its genotype can be directly observed phenotypically. It has a wide potential to produce biopharmaceutical products which requires minimal

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C. reinhardtii for production of protein

purification processes and potentially to be consumed orally. In addition, it is also highly scalable, where the scale-up production culture takes within a few weeks only. Microalgae can be cultured from one cell to 1000 s of liters within only a week, and can be transferred to a wide open pond within a few months (Rasala & Mayfield, 2015). This can reduce cost in comparison with animal cell culture which cost higher in terms of maintaining the culture medium.

C. reinhardtii has been successfully utilized to produce human antibody protein (Mayfield et al., 2007) and act as potential malarial edible vaccines (Dauvillé et al., 2010). Several complex recombinant protein products have been produced in this microalgae either by transferring the plasmid harboring-respected genes into the nuclear or chloroplast genomes for pharmaceuticals and medical related purposes. C. reinhardtii nuclear transformation involves the integration of foreign genes into the nuclear genome by non-homologous recombination whereas the chloroplast transformation involves integration of the inserted genes by homologous recombination. The nuclear transformation was initially conducted to study the functions of the Chlamydomonas nuclear genes by mutant populations. Most of the recombinant protein products on the other hand, are produced by transferring the respected gene into chloroplast genome. In 2009, a study by Tran, et al., shows that the chloroplast genomes of C. reinhardtii are able to synthesize a full length of IgG1 human monoclonal antibody (mAb), 83K7C. The antibody has been shown to works efficiently against the anthrax toxin in animal models, similarly functioning with the one produced in the cell lines of Chinese hamster ovary (CHO) cells.

The preferred techniques for the transformation of plasmids harboring-the respected gene into the C. reinhardtii are biolistic bombardment and also electroporation other than glass beads technique. In some cases, silicon whiskers have also been used to introduce the foreign genes into the host. The biolistic bombardment is carried out by coating the recombinant DNA with tungsten or gold particles, and bombarded into the cells or tissues by applying a high-velocity microprojectiles of helium gas in a vacuum chamber (Kikkert et al., 2003). This technique that initially tested in C. reinhardtii is improved and later, being applied in many other types of algae. The transformation efficiency of recombinant plasmid into the selected host by electroporation technique depends on the concentration of DNA used, voltage, electric pulse and amplitude, salt concentration, temperature and capacitance (Fromm et al., 1985). Other factors such as codon optimization, promoters, selection system and other regulatory elements also plays very important roles in affecting the transformation rates and protein expression efficiency.

THE PRODUCTION OF RECOMBINANT PROTEINS BY CHLOROPLAST GENOME TRANSFORMATION

The plants cells in general have three genomes and there are two genomes that are commonly transformable; the nuclear genome and the chloroplast (plastids). The production of new valuable plant-derived proteins in the chloroplast of eukaryotic organisms are still relatively few in the biotechnology related field. Some valuable proteins had been successfully produced in the chloroplast of tobacco plants (Fromm et al., 1985) and maize (Quist & Chapela, 2001). Researches showed that the usage of transgenic C. reinhardtii chloroplast as a bioreactor to produce recombinant proteins is feasible. Initially, chloroplast-transformed algae was first succeed in the chlorophytes (green algae), and later it has been applied in rhodophyte (red algae), phaeophytes (brown algae), euglenoids, diatoms and dinoflagellates (Barrera & Mayfield, 2013). The genetic transformation in the chloroplast genome is adopted for many beneficial properties its carry. The chloroplast genome provides the possibility of producing foreign polycistrons and the ability to reconstructing the entire metabolic pathways in a single transformation event (Su et al., 2005). Other than that, the transformation in the plastids offered higher expression levels (Oey et al., 2009), protected within the operons and reduced the possibility of the transgene from being transmitted by pollen (Bock, 2014).

Most of the products produced by the chloroplast were soluble and active biologically (Rasala & Mayfield, 2015). In 2005, Su et al., 2005 showed that using the chloroplast of C. reinhardtii, the expression of prokaryotic allophycocyanin gene containing alpha subunit apcA and beta subunit apcB fragments was correctly translated, providing a basis for the expression of new pathways or therapeutic proteins involving multiple genes produced by the C. reinhardtii chloroplast. The level of the gene expression reached up to 2-3% (w/w) of total soluble protein (TSP). The gene inserted in the nuclear genome for the expression of multiple genes are time-consuming and more complex to achieved (Cosa et al., 2001) due to monocistronic translation of nuclear mRNAs (Su et al., 2005). The expression of the allophycocyanin gene was carried out under the control of atpA promoter and rbcL terminator.

In a different study, a proteinaceous antibiotic, plyGBS derived from a phage infecting group B streptococcus (GBS) was produced by Oey et al., 2009 against the pathogenic group A and group B streptococci from the plastid genome.GBS infections can lead to neonatal sepsis, and meningitis. The protein is highly expressed and stably produced in the chloroplast genome. In this study, it is proven that the bacterial operon can be expressed in a single integration route as well as the protein produced by the chloroplast genome functions efficiently against the targeted bacteria.
While Dauvillée et al., 2010, developed vaccine antigens for malaria disease from the Plasmodium vaccine antigens fused to the granule bound starch synthase (GBSS). The gene which encodes for P. falciparum MSP1 was constructed for codon bias of Chlamydomonas. The production of transgenic starch by the chloroplast of C. reinhardtii was under the control of RBCS2 and HSP70A promoters. The study found that the immunoglobulin G of mice immunized with the expressed protein inhibits the development of the plasmodial species. A recent study by Bertalan et al., 2015 used C. reinhardtii chloroplast as a machinery to express MPT64, a secreted protein from members of Mycobacterium tuberculosis. This protein is detected in patients with tuberculosis. The gene of MPT64 is inserted into the multiple cloning site (MCS) of the pMM2 plasmid by Golden Gate cloning method. The marker-free selection procedure using psbA deletion mutant Fud7 was used in introducing the new transformation vectors with variable promoter (labelled as 16S MPT, psbA MPT, psbD MPT, atpA MPT), UTR combinations, tags and etc. This study provides an efficient method for the generation of stable recombinant proteins production by green microalga C. reinhardtii chloroplast.

Based on the studies above and table below (Table 1), the production of recombinant proteins in chloroplast genome method is sustainable and very cost-effective. The production of recombinant proteins for the enzymes and biopharmaceuticals industry however, is still very limited to a very small number of plants species. Thus, developing the methodologies for optimizing the tissue culture, its selection process and regenerating especially, are very crucial in order to enhance both chloroplast genome genetic engineering studies and the commercialization of its products.

THE PRODUCTION OF RECOMBINANT PROTEINS BY NUCLEAR GENOME TRANSFORMATION

The production of recombinant proteins in the nucleus of algae genome is less common compared with the chloroplast genome. This may be due to the lower expression levels of proteins in algal nuclear systems as reported in many studies, despite its ability to introduce foreign genes into the genome by many established methods. The lower expression levels may be caused by the transgene silencing in the protein production by nuclear genome (Shaver et al., 2010; Wu-Scharf et al., 2000). The reasons behind these effects on the protein expression by nuclear genome are not yet clear. However, in the production of biopharmaceutical products which the glycosylated proteins are needed, the nuclear genome protein expression are more preferred, as chloroplast are lack of enzymatic structure for N-glycosylation process (Mathieu-Rivet et al., 2014). Moreover, nuclear genome system are correlated with eukaryotic expression systems, allowing effective folding of complex proteins, and posttranslational modifications like glycosylation and disulfide bond formation (Rasala & Mayfield, 2015). The manipulations of the transformation systems such as the enhancement of the strains (Neupert et al., 2009), promoter technologies, selection system, and the inclusions of intron helps to increase the efficiency of protein expression (Walker et al., 2005).

In 2013, Hou et al. conducted a research on the production of organic selenium (Se) in green algae by constructing the expression of human solenoprotein known as Sep15 in the nuclear genome of C. reinhardtii. Se is an important nutrient naturally produced in rocks and soils. Solenoprotein is the main component of Se in vivo and its deficiency can lead to other diseases to human being (Hou et al., 2013). Some cases related to selenium deficiency in human has been reported. Se deficiencies with the combination of coxsackie virus infections can cause Keshan disease which was early found in the Republic of China, where the patient’s body (mostly under the aged of 15), became more susceptible to illness caused by other infectious diseases and potentially fatal (Chen et al., 1980). Other than that, it also causes Kaschin-Beck disease which effects joint cartilage and epiphyseal plate cartilage of limbs which causes the structural shortening of the fingers, toes, shorten the limbs with consequence of growth retardation (Tan et al., 2002). In this research the fragments of wild-type human Sep15 with open reading frame (ORF) and solenocyteine insertion sequence (SECIS) constructs from either human Sep15 or C. reinhardtii algal solenoprotein W1 was used to produce the very first product of human solenoprotein in algal nuclear system. The expression vector pH124 with ampicillin and zeocin resistant was used under the control of the hybrid promoter Hsp70-RbcS2. It is shown that the protein was successfully expressed in the cells from the nuclear genome by glass beads transformation method.

In a study carried out by Rasala et al., in 2012, the nuclear genome of C. reinhardtii was manipulated to express a recombinant xynalase from Trichoderma reesei in order to develop the methods for industrial enzyme secretion which currently is in high demand. The foot-and-mouth-disease-virus 2A (FMDV 2A) self-cleavage peptide fused to the antibiotic resistance was used for this Ble2A nuclear expression system. It is proven that linked construct of Ble2A and xynalase 1 (xyn 1) on the same ORF enhance the xynalase activity in cells lysate by about 100 fold. The electroporation transformation method was applied where the plasmid was first linearized with a single restriction enzyme and followed by double digestion with pHyg and Xyn1. The usage of double digestion shows an improvement of the transformation efficiency.

The research done by Eichler-Stahlberg et al., in 2009 produced recombinant protein of human erythropoietin (crEpo) which adapted to nuclear codon usage of
### Table 1. Therapeutic proteins produced in nuclear vs chloroplast expression system

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression strategy</th>
<th>References</th>
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<tr>
<td>CRY 1-1 with ARG7 selectable marker gene</td>
<td>nuclear</td>
<td>(Neupert, Karcher, &amp; Bock, 2009)</td>
</tr>
<tr>
<td>Human Erythropoietin (crEpo)</td>
<td>nuclear</td>
<td>(Eichler-Stahlberg et al., 2009)</td>
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<tr>
<td>Major Surface Protein (MSP1) and Granule-bound starch synthase (GBSS)</td>
<td>nuclear</td>
<td>(Dauvillée et al., 2010)</td>
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<tr>
<td>and GBSS-AMA1</td>
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<tr>
<td>MDV 2A fused to ble-2A-GFP</td>
<td>nuclear</td>
<td>(Rasala et al., 2012)</td>
</tr>
<tr>
<td>Fluorescent proteins; blue mTagBFP, cyan mCerulean, green CrGFP,</td>
<td>nuclear</td>
<td>(Rasala et al., 2013)</td>
</tr>
<tr>
<td>yellow Venus, orange tdtdTomato and red mCherry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human selenoprotein (Sep15)</td>
<td>nuclear</td>
<td>(Hou et al., 2013)</td>
</tr>
<tr>
<td>cas9/sgRNA</td>
<td>chloroplast</td>
<td>(Jiang et al., 2014)</td>
</tr>
<tr>
<td>apcA and apcB</td>
<td>chloroplast</td>
<td>(Su et al., 2005)</td>
</tr>
<tr>
<td>lysins</td>
<td>chloroplast</td>
<td>(Oey et al., 2009)</td>
</tr>
<tr>
<td>Herper simplex virus D (HSV8-scFv)</td>
<td>chloroplast</td>
<td>(Mayfield &amp; Franklin, 2005)</td>
</tr>
<tr>
<td>Classical Swine Fever Virus (CSFV) structural protein E2 gene</td>
<td>chloroplast</td>
<td>(He et al., 2007)</td>
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<tr>
<td>Malaria transmission blocking vaccine candidates</td>
<td>chloroplast</td>
<td>(Gregory et al., 2012)</td>
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<tr>
<td>Plasmodium falciparum surface protein pfs25 and pfs28</td>
<td>chloroplast</td>
<td>(Gregory et al., 2013)</td>
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<tr>
<td>Whole cell oral vaccine of pfs25 fused to cholera toxin (CtxB)</td>
<td>chloroplast</td>
<td>(Gregory et al., 2013)</td>
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<tr>
<td>EctA (ectoine)</td>
<td>chloroplast</td>
<td>(Lunde, 2012)</td>
</tr>
<tr>
<td>Transgene encoding E7 oncoprotein of HPV-16</td>
<td>chloroplast</td>
<td>(Demurtas et al., 2013)</td>
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C. reinhardtii. The gene was artificially divided into two exons and RBCS2 introns sequences was integrated within the sequences to enhance the expression of transgene in nuclear genome. This research is very significant to investigate the expression at post-translational level of transgene expressed in the nuclear algal system. This will encourage further study on the better expression level of recombinant proteins in nuclear algal genome to meet the commercial needs.

THE TRANSFORMATION METHODS

There are many methods that have been used to transform the transgene into the host organisms. Amongst them are the biolistic bombardment, electroporation, glass beads, bio-active beads and silicon carbide (SiC) whiskers method. Since 1980s the electroporation methods are widely used in higher plants transformation, where the genes of interest are inserted into monocot and dicot plant cells. Electroporation methods are preferred as it is convenience, low cell toxicity, high efficiency and applicable to wide range of plants (Fromm et al., 1985). While Biolistic are used as an alternative to do the transformation works. The biolistic can be used to transfer the transgene into many kind of intact cells and tissues from a wide range of organisms. The biolistic process are also known as microprojectiles, bombardment, particle bombardment, particle acceleration, or ballistics. The technology is patented by several patents such as E. I. du Pont de Nemours and Co. and also Powderject Vaccines, Inc. (Kikkert et al., 2003). Biolistic bombardment have been applied in many kind of organisms such as bacteria, yeast, algae, plant cells/tissues, and animal cell culture (Heiser & Ph, 2000). Higher plants such as transgenic maize and wheat have been also successfully produced by using this technique (Wright et al., 2001). However this technology processes is more tedious and time-consuming technique (Bock, 2014) in comparison with some other techniques. There are several factors that influencing the efficiency of the process such as microprojectile’s constitution, size, concentration and amount and the methodology used for the DNA coating (Armaele et al., 1990). Particle bombardment and electroporation are the most commonly used in the microalage transformation. Whereas, glass beads that are use in unicellular eukaryotic algae transformation posses more convenient methods and inexpensive equipments needed for the transformation works (Wang et al., 2010). However it is more suitable for those microalgae that have thin walls for the penetration of the plasmids DNA. Other than that, bio-active beads techniques have the potential for transferring larger molecules of DNA as shown previously in yeast cells transformation studies (Liu et al., 2004). Among the plant species that was succefully transformed by using this techniques are the green unicellular algae Platymonas subcordiformis (Cui et al., 2012), tobacco (Liu et al., 2004), and rice (Liu et al., 2004; Wada et al., 2009).

In 2010 a research carried out by Wang et al., 2010 a rhodophyte, Porphyra haitanensis was transformed by using glass beads agitation under the control of SV40 promoter for its conchospores. The conchospores and the plasmid containing lac Z was agitated for 10, 15, 20 and 25 seconds, together with the glass beads before spreaded on the glass slides for the detection of the transformants (Chart 1). The blue cells of conchospores that expressing lac Z gene and the green fluorescence from egfp gene was screened in P. haitanensis that was successfully transformed. Based on this research, it was found that the transformation rate was best at 15 to 25 s agitation. This methods are suitable to be used in this red seaweed as it is lack of the spore walls. The longer time of agitation may be needed to penetrate other kind of conchospores that contains walls. The chart below shows the general methods for the glass beads agitation.
Electroporation methods are commonly used for the transformation of bacteria, yeast, plants protoplasts, and tissue cell cultures for gene and cell-based therapy. This method works for fungal species *Podophyllum zanthii* (Vela-Corcía *et al.*, 2015) and also in higher monocot and dicot plants such as carrot, maize and tobacco protoplasts (Fromm *et al.*, 1985). This methods can be applied in any plant protoplasts. It is simpler, fast and cheap to compare with the biolistic methods (Rivera *et al.*, 2012). Other than that transformation by electroporation was done in green microalgae such as *Scenedesmus obtusus* (Guo *et al.*, 2013) and *C. reinhardtii* (Walker *et al.*, 2005). Guo and his colleagues in 2013 developed an efficient electroporation method for transforming *S. obtusus* by using chloramphenicol resistance gene (CAT) and green fluorescent protein gene (gfp) as the selectable marker inserted within the plasmid. High efficiency transformation with stable propagation of transformants were obtained while optimizing the important parameters of the electroporation methods such as pulse voltage, pulse duration, amount of plasmid and the concentration of solution used.

In the research conducted by Gan *et al.*, 2004, another rhodophyte, a Malaysian red algae *Glaucaria changii* taken from Morib, Banting was used to improve the transformation methods for particle bombardment in microalgae. This rhodophyte is the most plenteous agarophytic seaweeds in Malaysia. Agar is mostly extracted from the red algae which is widely used for the biotechnological research. *G. changii* has a potential to meet the commercial demands as it has the ability to produce good agar grade and it also has the ability to cope with the harsh mangrove environments for growth. One µg of plasmid construct *psv40-lac Z* was coated with gold particles before bombarded by using Biolistic PDS-1000/He system to the thalli of the algae. For the optimization, 4482, 6206, 7584, and 8963 KPa rupture disc pressures was used. The particle bombardment techniques are suitable to be use for many kind of the walled species algae.

Last three years, Liu *et al.*, developed a simpler new tools for transformation in chlorella species. It is function to weakens the cell wall of chlorella and make it competent for the uptake of foreign DNA. A simpler methods for the transformation in this green algae was developed in order to meet the biotechnological and pharmaceutical industries demands. *Chlorella ellipsoidea* SD0801 strain was used in this study, carrying the plasmid pSP-Ubi-GUS. This plasmid harboring zeocin-resistance gene and beta-glucuronidase (GUS) reporter gene. During the transformation salmon sperm DNA was used as a carrier for the DNA plasmid. Upon the treatment of the chlorella cells with enzyme cellulase, the thickness of the cell walls was checked for the ability of the cells to do the transformation activities. This techniques are suitable for chlorella as it has a thinner structure of cell walls. It is also applicable for other kind of microalgae that shares similar properties for further research.

**STRATEGIES TO ENHANCE THE EXPRESSION OF ALGAL RECOMBINANT PROTEINS**

There are many factors that affecting the protein expression and accumulation of a recombinant protein. These includes the selection of host organism, the plasmids construct, the transformation methods, the screening process, and the growth conditions of the algae harboring the protein of interest. Besides of the transformation systems, the codon bias, promoters and other regulatory elements used in the cloning process also plays a very important role in affecting the protein expression and accumulation (Barrera & Mayfield, 2013). Thus putting initial well-planned strategies is needed to ensure an appreciable expression of the recombinant proteins. Other than that, the expression level will also be depending on the cells age, the size of the gene of interest, and the sequence content. Many kind of approaches and different elements in the plasmids are developed by companies to simplify the process of protein expression in algae for various purposes such as in enzymes and biofuels production. The codon bias are used in many researches involving *C. reinhardtii* where the gene of interest resembles the nuclear (Ruecker *et al.*, 2008) or chloroplast (Gregory *et al.*, 2012, 2013; Tran *et al.*, 2009) of *C. reinhardtii* to optimize the expression. The codon bias are to be considered not only in genomic level but also in organelles level as it can be vary from one organelles to another. For instance, the GC content of nuclear genome from *C. reinhardtii* composed of 62 % of overall coding sequences, 48% in mitochondrial and 38% in chloroplast genome respectively (Barrera & Mayfield, 2013).

Other than that, promoter also plays a very crucial role in affecting protein expression level. In *C. reinhardtii* nuclear genomes, the most common promoters that are used are HSPV 70 A and rbc S2 or the usage of both heterologous algal promoter as in the study from Walker *et al.*, in 2005. While in the chloroplast *C. reinhardtii* genome, Atp A promoter is commonly used. The protein tags, and selection marker gene will makes the screening process of the gene of interest easier. The selectable marker genes which are often an antibiotic resistance helps to ensure the production of a stable successfully produced transformants. For instance the ble selectable marker is used for the selection of *C. reinhardtii* that has been transformed to select the transformants that are resistance to the specific antibiotic zeomycin/bleomycin. The usage of protein tags helps to remove all the unnecessary components for further purification steps of the protein.
CONCLUSIONS

Nowadays it is possible to transfer the genes from an organism to the selected host organisms that are quite distant evolutionarily via biological and physical methods. In general the expression of the microalgal recombinant proteins are varies. Many studies have been carried out to explore the potential of *C. reinhardtii* as a model organism in producing complex recombinant proteins. Microalgal systems both in chloroplast and nuclear genome are a promising tools to produce cost-effective high value recombinant proteins for pharmaceutical and health industry in the near future as rapid progress in overcoming its challenges have been made to enhance the production level of the bio-products. The transformation system, regulatory elements, the constructs and the selection systems could be optimized in order to develop more efficient strains for the applications of the diverse algae species. The development of a simpler and efficient transformation strategies are very much in need to enhance the production of the transformant harboring the protein of interest. The products resulted from these researches have to be complement with the increasing of the society needs. These usage of microalgae as a platform in protein production is very ideal to guarantee a scalable, lower costs, safer and non-toxic alternatives for the recombinant proteins production to be used in medical and pharmaceutical industries for various purposes such as for the diseases treatment, supplements and also health care.

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