Heterologous Expression of the Glutamine Synthetase Enzyme (GlnA) from *Escherichia coli* in Microalgae

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1. SUMMARY

The use of microalgae for biofuel production has opened a new window in the biotechnology research [1]. To date, several genomes of different species of microalgae have been sequenced and, through them, the identification of genes involved in metabolic processes with biotechnological potential has been identified [2-4]. One of the most important and interesting processes in microalgae is the generation of biomass, where the nitrogen metabolism plays an essential role [5]. In previous studies, it has been reported that the enhancement of the glutamine synthetase activity in higher plants has generated substantial increases in their protein content [6]. Hence, the aim of this work was to implement a pCAMBIA1302 vector-based genetic engineering strategy for the heterologous expression of Glutamine Synthetase gene (*glnA*) from *E. coli* in Microalgae. Here, it has been proved that *Scenedesmus spp* is an ideal target system for the genetic manipulation of Microalgae, because: (1) its sensitivity to the antibiotics, (2) its compatibility with the pCAMBIA-CaMV35S promoter for the expression of the proteins, and (3) its responses for biomass production. In addition, a BioBrick molecular tool or intermediate pCAMBIA-derived plasmid, the pCAMBIA::*Spec*(+), was successfully achieved by genetic engineering of the commercial original plasmid pCAMBIA1302. The expression cloning of the *glnA* gene into pCAMBIA1302 plasmid was successfully achieved, by using a double-substitution cloning strategy, which can be straightforward performed and screened by chemical selection (Spec) and visual characterization (GFP). Moreover, the heterologous GlnA protein expression in *Scenedesmus spp* recombinant clones was achieved and detected by Western-Blot analysis. Finally, the major finding in this study was the construction of the intermediary vector pCAMBIA1302::*Spec*(+) and the production of the recombinant pCAMBIA::*glnA Scenedesmus spp* clones.

2.   INTRODUCTION

2.1   MICROALGAE: STATE OF THE ART

Microalgae are a group of unicellular and multicellular oxygenic and photoautotroph microorganisms, classified within the Eukarya domain, which normally inhabit aquatic ecosystems, both seawater and continental water systems, and can be found in inland ecosystems as well. They possess chloroplasts with a double membrane like the higher plants, with the photosystem I and II, along with the pigments and accessories needed to carry out the photosynthesis process, such as chlorophyll, β-carotenes, astaxanthin, among others [7-10]. They are known for their role in the food chain and in the production of the atmospheric oxygen. Even more important is its biotechnological relevance for their ability to produce pigments, oils, vitamins, biofuels, and other products. Nowadays, some specific metabolic routes applied to biotechnological development are known and this knowledge is being used to develop genetic engineering strategies to enhance the efficiency of these processes.

2.1.1   Origin and Classification of the Microalgae

The origin of these microorganisms goes approximately from 1500 million years ago [8] and is presumed that their photosynthetic capacity was formed from a previous endosymbiotic event that took place millions of years ago in which a heterotrophic eukaryotic cell phagocyted a primitive cyanobacteria, generating a stable bond between the two and finally turning the latter into a plastid. Subsequently, cell lines were generated within the group of algae according to the type of microorganism that was phagocyted and the content of pigments and proteins (chlorophyll b, phycobiliproteins, β -carotene, fucoxanthin) [7-10].

These organisms are classified into two major clades: Chlorophyte and Streptophyte, in the first group the widest variety of green algae or with chlorophyll content is found, while the second group consist of the non- green algae, but is dominated by what is known as higher plants, an schematic representation can be

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seen in Figure 1 [7, 9]. The origin of these organisms is still in discussion; however the most accepted theory is the endosymbiotic, proposing that the green algae came from a phagocytosis of a primitive green alga by a heterotrophic protist and are classified as Chlorophyta. While the non-green algae were generated from the phagocytosis of a primitive red or brown algae and are classified as Rodophyta and Phaeophyta respectively [7-9, 11], Figure 2 shows a representation of the phylogenetic three of the microalgae and some related species [9, 11]. There are also other types of microorganisms that are considered as microalgae by some authors, such as cyanobacteria, the dinoflagellates and Euglenophyceae [9, 11], however this study will emphasize on the green, red and brown algae with a biotechnological interest, without issuing any concept about the global classification that has been determined for these microorganisms.
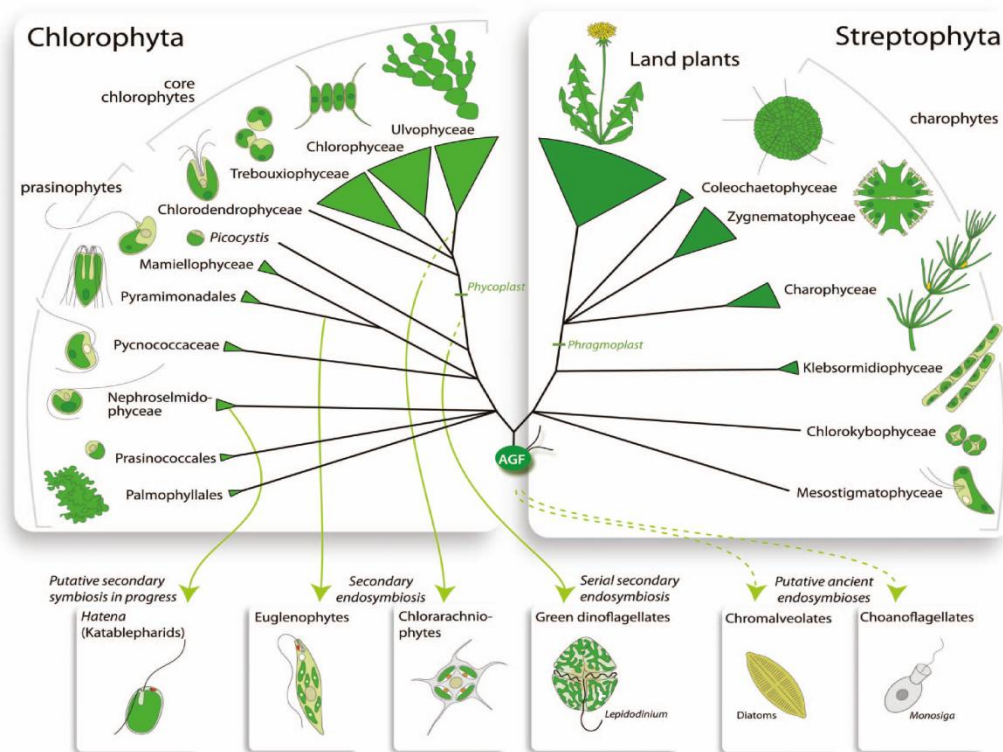
Figure 1. Phylogeny Classification and Gene Transfer in Green Algae
Taken from Leliaert F., et al., 2012.

2.1.2   Biological Characteristics of the Microalgae

Microalgae are microscopic eukaryotic organisms (protist), and as such, they possess the distinctive morphological characteristic of their realm: nucleus defined by a membrane and the presence of cytoplasmatic organelles like vacuoles, mitochondria and chloroplasts (Figure 3). However, this group of microorganisms possess a very particular set of features, being the most important one their ability to use the oxygenic photosynthesis. It's been suggested that the first primitive microalgae generated the oxygen necessary for the establishing of the atmosphere that we need to breathe and allows the sustaining of the oxygenic life as we know it today, and it is also one of the most complex and ingenious processes in nature. Oxygenic photosynthesis is the use of luminous energy for the fixation of inorganic carbon, mainly as CO_2, and the production of energy in form of ATP and NADPH plus oxygen [7-10, 12].
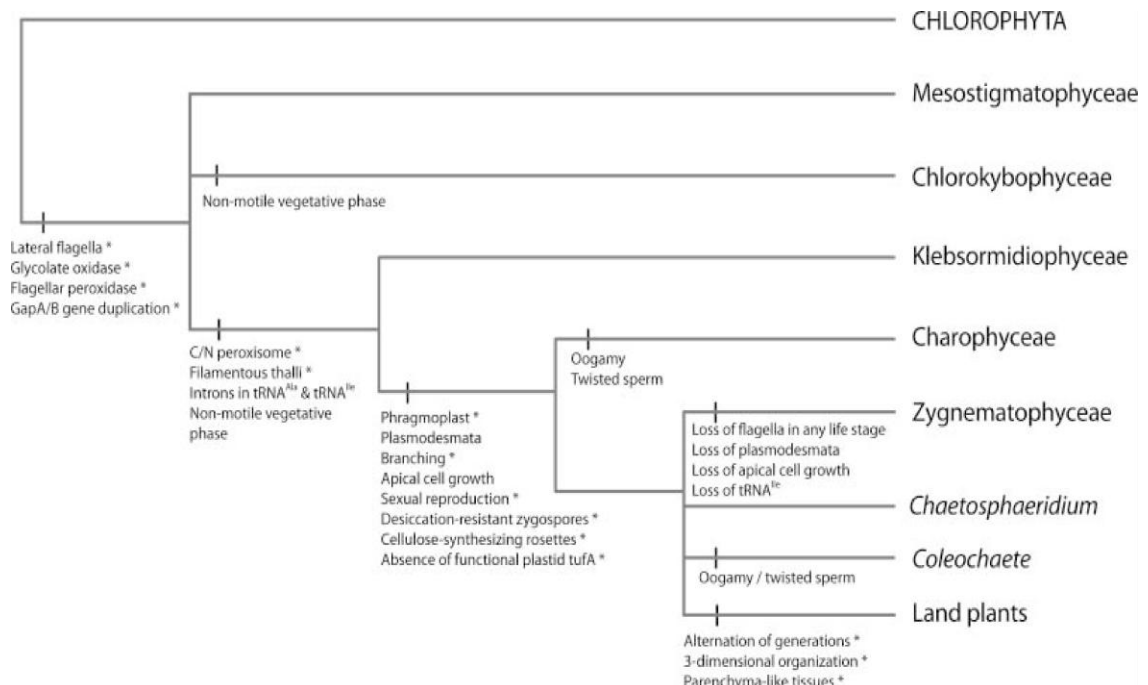
Figure 2. Phylogenetic Evolutionary Tree
Taken from Leliaert F., et al., 2012. * Indicate synapomorphic characters.

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Likewise, the different types of pigments that these organisms possess are of great importance because not they only give them a distinctive color, they also allow them to capture and use light at different wavelengths for photosynthesis, enabling them to colonize a wide range of environments. These pigments go from chlorophyll, xanthophyll, fucoxantin, β-carotene, phycobilins, astaxanthin, among others [1, 7, 9, 13, 14].

Additionally, microalgae have a rigid cell wall, composed mainly of polysaccharides and glyco-proteins, being cellulose the most common component. This structure has several functions, the most important are associated with resistance to osmotic stress and disruption by physical forces, other functions includes the regulation of the components that come in contact with the membrane, cell signaling, and adhesion in species that form aggregates or filaments. This structure gives these microorganisms an important versatility, allowing them to live in environments with extreme osmolarity, pH and temperature conditions, achieving a diversity both physiological and at the molecular level [8, 10, 15, 16].

Another characteristic of these organisms is their morphology, some microalgae can be found as an unicellular form as independent and motionless cells like *Chlorella spp.*, or aggregated into colonies like *Volvox spp.*, they can also form a structure type filaments called coenobia that consists on the adhesion between the cell wall of various cells of the same species like *Scenedesmus spp.*, these associations are often regulated by environmental conditions and other factors such as cellular signaling systems and others that are still unknown [7-10, 15].

2.1.3   Microalgae and Biotechnology

Microalgae are known for their wide range of tolerance to different metabolic environments due to the metabolic tools they possess, for example, the microalgae *Dunaliella salina* is able to tolerate environments with high concentrations of NaCl or *Chlamydomonas nivalis*, which is adapted to very low

temperatures and freezing and thawing cycles. Is this great adaptability that has positioned microalgae as an attractive object of study for many companies and biotechnology laboratories and which gave them the name of biofactories [1, 2, 17, 18].
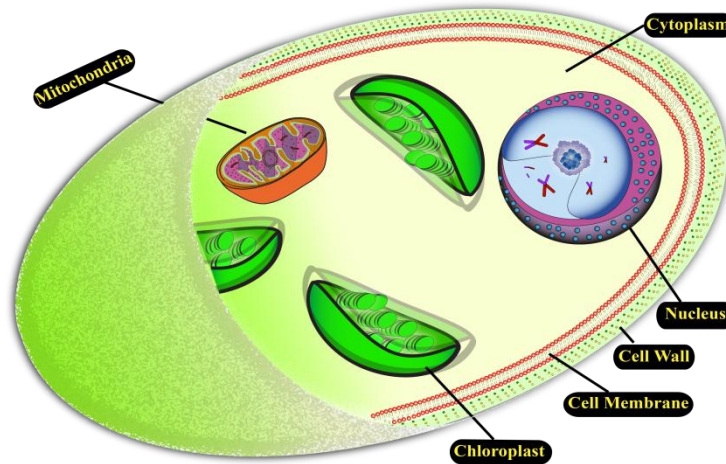
Figure 3. Main Structures in a Microalgal Cell

2.1.4   Use of Microalgae in Biotechnology

This interest in microalgae is not new, in fact there are historical records of the use of these organisms as a source of food and as a medicinal treatment, one of the earliest records goes back over 2000 years ago in China, where the cyanobacteria *Nostoc* was used to overcome a long period of food shortage. The use of microalgae has also been reported as a precursor to the development of weapons such as dynamite from *Diatomaceous earth* [1, 2, 17, 19].

In the 50's, microalgae were used as a protein supplement given the potential risk of food shortages by the uncontrolled growth of the world population, at the same time the early studies about its use for energy production and as catalysts for bioremediation reactions started. Some older traditions are still used as in the case of *Spirulina spp.* as a dietary supplement, or using *Porphyra umbilicalis* for the sushi wrapping in Asia [1, 2, 20, 21].

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The idea of using microalgae for biofuel production began to be mentioned around 1960 when the United States and Japan started research programs in this area in order to obtain information about a product with the characteristics of the oil, however due to the high production costs its commercial materialization was never achieved at that time [22].

2.1.4.1   Biomass Production

One of the most important and known biotechnological applications is the production of biomass from microalgae such as *Chlorella vulgaris* and *Scenedesmus obliquus* to be used to feed the cattle and as a human dietary supplement. The biomass of microalgae such as *Chlorella* and *spp. Gracilaria spp.* is used to generate energy by transforming it in combustible gases such as methane. This is possible by using the metabolic pathways that drive the anabolism of these microorganisms into the desired carbon accumulation pathways such as carbohydrates, lipids and proteins. One possibility to do this is by manipulating the concentration of the nutrients in the growth medium, like using growth mediums with a low content in nitrogen and phosphorous, or employing microalgae that are able to grow heterothrophically. Other mechanisms used to enhance the biomass yield include: modifying the light intensity for the growth, incubation temperature, cellular density of the inoculum, over-expression of the enzymes involved in this metabolic pathways, among others [2, 17, 19-25].

2.1.4.2   Production of Dietary Supplements

Microalgae have the potential to produce different types of compounds from their metabolism, an example of these products are vitamins and fatty acids that humans and animals cannot produce by themselves (essentials), but, can be produced from cultures of microalgae strains like *Chlorella spp.*, *Porphyridium spp.*, *Nannochloropsis spp.* and *Arthrospira spp.*, products like the riboflavin and the

omega 3 fatty acids, or even biopolymers such as agar that is widely used for preparation of bacteriological culture media from brown seaweed species like *Eucheuma spp.* or *Ascophyllum spp.* [1, 2, 12, 17, 20, 26].

2.1.4.3   Pigments Production

Dunaliella salina. and *Haematococcus pluvialis* are most commonly used for the production of a wide variety of pigments such as carotenoids and astaxanthin that are used in products for skin cancer prevention as a result of the solar radiation and weakness of the skin, or the phycobiliproteins, such as phycocyanin produced from *Spirulina platensis* which are used as colorants in the food, cosmetics or pharmaceuticals industry, or as fluorochromes for microscopy [1, 2, 13, 14, 21, 27].

2.1.4.4   Bioremediation

There are many other examples of the use of microalgae in the field of biotechnology, one of these is the use of *Chlorella spp.* and *Scenedesmus spp.* for the treatment of water and ecosystems with high concentrations of organic compounds like nitrogen, with a high removal efficiency within treatment plants, with the additional use of the resulting biomass as a food source or for the production of energy as previously mentioned. Additionally, it has been reported the use of the macroalgae *Sargassum spp.* for the removal of heavy metals such as chromium, cadmium, zinc, among others [1, 2, 17, 20].

2.1.4.5   Biofuels

Most of the commercially used microalgae are also grown for the production of lipids and fatty acids from species known to accumulate small chains of lipids which are then extracted from the cell in order to produce oil and biodiesel, the latter being of great importance in the energy industry as a supplement for petroleum

diesel, which is much more polluting because of its high sulfur content. Initially, this biodiesel was obtained from plants and crops. However this represented a nutritional risk due to the competition for the cultivable soil, being microalgae a possible solution to this problem by requiring less space and at the same time being more efficient in their growth from nonconventional sources like wastewater and by using CO_2 from industrial processes as the carbon source. Biodiesel production has been mainly reported for *Chlorella spp.*, *Chlamydomonas reinhardtii*, *Tetraselmis spp.*, *Scenedesmus spp.* and *Nannochloropsis spp.* In addition to the biodiesel production as an energy supplement, hydrogen production has also been achieved by metabolic engineering of cultures of microalgae [1, 2, 12, 17, 19, 20, 25, 28-35].

By summing up all these applications it would be possible to say that the greatest benefit or added value of using microalgae in the biotechnological processes mentioned above lies in its ability to successfully grow through the fixation of atmospheric CO_2 which if coupled with industrial processes producing high emissions of this greenhouse gas, such as thermal power plants, smelters, among others, can mean an approximate reduction in the emissions to the atmosphere of 50 and 70% [1, 2, 12, 17, 22, 33].

2.2   METHANE PRODUCTION FROM MICROALGAE: SUNCHEM

One of the most innovative technologies today is known as SunCHem. This process was designed by the PSI and the EPFL in Switzerland, and consists in the production of algal biomass by taking advantage of the nutrients contained in non-conventional sources like wastewater and CO_2 from the industries to generate a closed self-sustaining process for the methane production that can be used as a source of energy at home. It uses water as a solvent by reaching supercritical conditions of temperature and pressure of 421°C and 300 Bar respectively [36]. At this conditions water takes away all the organic compounds into solution and into a combustion process where all the carbon contained in the biomass is transformed into methane by using a catalyst like titanium or ruthenium [37-39]. However the reaction time has to

be carefully standardized to successfully convert all the carbon into methane and avoid the formation of intermediary products like furfurals and organic acids that lowers the yield of the process [37]. This process is quite simple and has been used both with open wells and bioreactors which gives a lot of versatility plus it's a process with a high added value due to the possibility to be coupled with wastewater treatment systems, emissions of greenhouse gases and contaminating material, to generate clean energy the process scheme can be seen in Figure 4 [36-39].
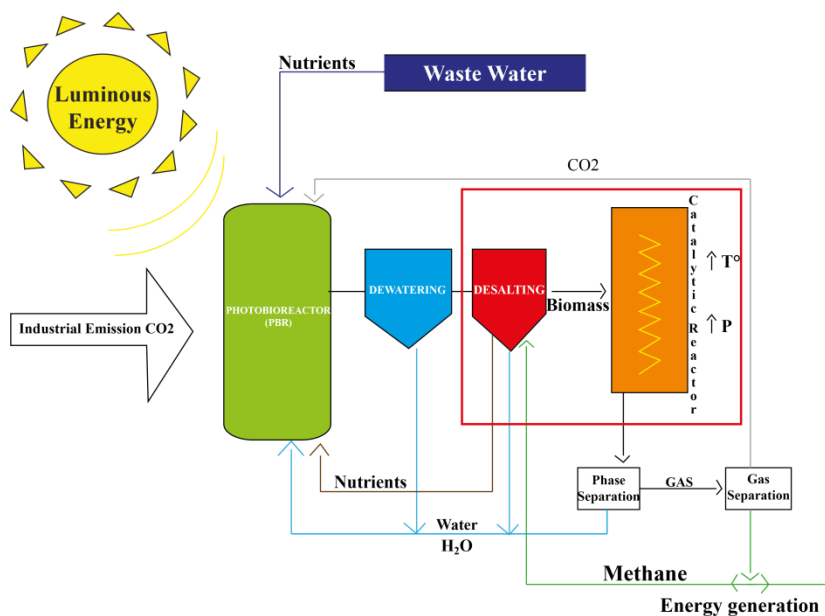
Figure 4. SunCHem Process Developed at the EPFL and the PSI

2.3   GENETIC ASPECTS OF MICROALGAE

Microalgae have similar genetic characteristics to eukaryotic organisms regarding their genetic conformation, structure and replication methods. They possess a defined nucleus where a molecule of double stranded DNA is condensed as chromosomes and whose size can vary between 30 MB and 120 MB as the specific case of *C. reinhardtii* or 138 Mb for *Volvox carteri*. Their genome can be haploid or diploid according to the species, and their average size is unknown because only some species have been sequenced while the rest are still in process [9, 40, 41].

The chromosomes are very similar to those of higher plants, they possess localized centromeres, and their number can vary between species, for instance the microalgae *Chlorella vulgaris* has 14 chromosomes, while *Chlamydomonas reinhardtii* has 17 [42].

Replication on these microorganisms is close to that of higher plants, the genetic material contained in the core is transferred according to Mendelian laws, while the content in the chloroplast and mitochondria follows an uniparental inheritance. They can be divide sexually or asexually depending on their genome, which can be haploid or diploid [43, 44].

Due to the size and complexity of the genome of microalgae, little information is known about their genetic characteristics; however some progress has been made in this field by studying the organelles such as the mitochondria and chloroplasts. The genome of the chloroplasts of algae are very similar to those of higher plants, is circular and is divided into four parts (quadripartite), two regions of single copy DNA, and two big regions of inverted repeats. Has autonomous semi-conservative replication, the GC content can vary between species between 20 and 75%, in the case of *C. reinhardtii* is 39%, and in this genome are most of the genes involved in photosynthesis [9, 41, 44, 45].

2.3.1   Microalgae as Genetically Modifiable Organisms

One of the major drawbacks in using microalgae for the generation of biotechnological products is the cost for the maintenance of photobioreactors (PBR) or in their performance on the production of oils or other compounds, this is because microalgae are also biological systems that respond to environmental conditions, and sometimes when there is insufficient light energy or a scarcity/excess of nutrients in the culture medium they change their metabolism and production yields making them uncompetitive when compared to the petrochemicals market and others [2, 46-50].

One of the most promising solutions has been the progress in the molecular techniques in microalgae for their genomes sequencing, the identification of the metabolic pathways and the enzymes involved in these processes along with the genes coding for these enzymes, plus the transformation methods that are being developed, the construction of vectors for the expression of recombinant proteins and the selectable markers, even the design of kits in order to transform them easily [2, 19, 45, 51-54].

The first approaches to microalgae transgenic technology occurred with the work of Dunahay et al for the production of transgenic strains of diatoms to improve the production of biodiesel through the use of non-coding sequences of the target microorganism, *Cyclotella cryptica* [55].

Clamydomonas reinhardtii (120 Mb genome) is being used as the role model for the genetic studies and is the first microalgae to be successfully transformed. Nowadays, different methods for the transformation of these microorganisms without affecting their viability, even transgenic algae have been constructed using genes from other species of algae to enhance the performance and production of certain components, in particular the production and storage of lipids and glucose excreted in the form of starch, production of pigments like astaxanthin, the synthesis of fatty acids and aminoacids for human nutrition among others [2, 13, 14, 41, 45, 46, 51].

2.3.2   Introduction of Genetic Material (DNA) in Microalgae

Given their distinctive genetic features, different strategies have been designed for their genetic manipulation, which depend on the objective to be achieved, since the foreign DNA can be either introduced into the cytoplasm, nucleus or chloroplasts [44, 53, 56]. The cell wall is one of the biggest barriers to transform microalgae; it prevents the passage of DNA into the nucleus making the process complicated. For this issue, several methodologies have been designed and tested successfully as explained in the following sections [56, 57].

One of these techniques consists in the cell wall degradation by enzymatic digestion using enzymes produced by themselves, like the autolysin. This technique is inefficient since the viability of the cells is greatly compromised, and some species aren't capable to survive without the cell wall [30, 31, 40, 53, 56].

The second and most widely used technique is the electroporation, this involves subjecting the cells to an electric shock opening small pores in the cell wall and membranes allowing the entry of DNA into the cell, is a technique that has proved to be efficient, plus it requires a relatively simple equipment. The necessary parameters should be standardized for the successful transfection of cells by using this technique, since the composition of the cell wall and the cell membrane vary among the different species. The following species have been transfected with this technique: *Dunaliella salina*, *Nannochloropsis oculata*, *Chlorella spp.* and for the expression of genes such as the Acetyl-CoA carboxylase (*ACC*), hygromycin resistance (*hpt)* among others [41, 53, 58-60].

There are additional methodologies, like mixing with DNA embedded in glass beads, the biobalistic technique using gold or platinum particles, or co-infection with *Agrobacterium tumefaciens*. The latter involves the insertion of the fragment of interest between the flanking viral sequences (vir) in the tumor inducing plasmid pTi. This technique has been widely used in higher plants and has been tested on *C. reinhardtii* for insertion of the gene *uidA* using GFP and hygromycin B resistance gene (*hpt*) as markers, making it a very promising technique, although it requires further studies to verify its efficiency in different microalgal species [41, 53].

2.3.3 Molecular Tools for the Genetic Modification of Microalgae

New strategies have been designed to overcome most of the known disadvantages regarding the genetic manipulation of microalgae and more than 30 species of microalgae have been transfected taking into consideration the phenotypic and genotypic characteristics of each one for the process [61, 62].

Additionally, new vectors were designed to enhance the efficiency in the expression of recombinant proteins, these include constitutive promoters such as SV40, CaMV 35S, the pSAD or inducible promoters such as the copper response promoter or the nitrate reductase promoter, among others which are specific for each species. For example, the CaMV 35S promoter (promoter of the cauliflower mosaic virus) was used for transformation and expression of amyloglucosidase phosphotransferase gene from *Streptomyces hygroscopicus* in *Chlorella vulgaris*, and the SV40 promoter was used for the expression of the *lacZ* gene of *E. coli* which proved to be a good marker for selection in *Gracilaria changii*. Another example is the use of the modified pUC18 vector with the hygromycin B resistance gene and in which a fragment of the promoter region of the β-tubulin gene of *Chlamydomonas* was inserted to express the gene for the hexose symporter protein of the microalgae *Chlorella kessleri* cloned into the same vector [41, 53, 58-60, 62].

Additional strategies for designing new selection markers are also being developed. Hygromycin B, zeomycin, sulfometuron methyl, kanamycin, spectinomycin, and even reporter genes like fluorescent markers, such as GFP (green protein fluorescent) and luciferase, have been integrated into vectors. All of them have been used in vectors for the transformation of different species of algae, such as *Porphyridium spp.*, *Laminaria spp.*, and *Phaeodactylum spp.* by the adapting of its sequences to the genetic systems in microalgae [2, 46, 58, 63].

More recently, a new issue related to the low protein expression, due to additional inserts in the gene, was revealed and consists on the change in the codons availability and their respective tRNAs present in the target cell. To address this issue, new guidelines have been developed. One, looking for heterologous genes, or gene redesigning in order to use the codons used in the target cell. Additionally, another proposal is the use of the flanking regions or the introns homologous regions from the cell itself to be transformed, as this can help to increase the success rate of the transfection and a better level of protein expression. One clear example is the transfection of *Chlamydomonas reinhardtii* for GFP-gene expression [46, 49, 60].

2.4   PURPOSES OF THE GENETIC MODIFICATION IN MICROALGAE

The strategy to be used must be determined depending on the final goal, as it must be decided whether to transform into the nucleus or the chloroplast, since the expression of recombinant proteins and stability of the mutant may vary according to the target that is chosen. Transfection into the nucleus results in a more stable clone but with a low protein expression, possibly due to causes such as RNA silencing or other epigenetic mechanisms of defense of the cells against foreign agents like virus and foreign DNA, while the chloroplast has been reported to yield higher levels of protein expression, but with less stable transfections [30, 31, 40, 44, 53].

Some difficulties in the correct expression of the protein are related to more common problems of eukaryotic organisms, such as appropriate promoter region for transcription in the target cell, the proper maturation of the protein and the transportation mechanisms inside the cell. All these drawbacks have been studied and discussed in prior publications and they all mentioned different strategies to overcome them. One of the main drawbacks is the correct folding in the process of final maturation of the protein, another one is the use of regions flanking the promoters in the target microorganism and the use of heterologous sequences considering the availability of different codons and their respective tRNA. a successful transfection can be achieved by analyzing these factors carefully [30, 51].

2.5   *Scenedesmus spp.* CLASSIFICATION AND FEATURES

Within the wide number of species of microalgae that have been discovered and studied till today, there are some that present unique features and applications, excelling over the others and making them attractive for the biotechnological industry. These microalgae are mostly distributed among the phylum Chlorophyta like *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella vulgaris*, *Scenedesmus obliquus*, *Tetraselmis suecica*. Some are also distributed among the Heterokontophyta like *Phaeodactylum tricornutum*, and the Rodophyta like *Gigartina stellata* and *Chondrus crispus* [9-11, 16, 20, 46].

One of the most studied microalgae from this group is *Scenedesmus spp.* It is classified in the Domain: Eukarya, Kingdom: Plantae, Phylum: Chlorophyta, Class: Chlorophyceae, Order: Sphaeropleales. There are several strains of this microalgae, and one of their most remarkable characteristics is their different morphology, ranging from the most common unicellular immotile form, to the creation of coenobia, aggregation like structures, comprising mostly of cells that attach one to each other and possess flagella at the ends granting it some motility. This morphology changes according to the environmental conditions as an adaptation response. The most common strains possessing this ability are *Scenedesmus obliquus* and *Scenedesmus dimorphus* [16, 64-66].

Scenedesmus spp. possess a cell wall composed mainly of celullose, glycoproteins, and algaenans organized in a trilaminar structure that provides great resistance to mechanical and chemical stress, even a protection against the activity of lytic enzymes. A high content of carbohydrates of 10-50%, lipids 21-42% and protein of 40-50%, depending on the strain and culture conditions. It's capable of fixating 15 to 25 % of atmospheric CO_2 into its biomass, possessing a high tolerance to strong light sources, high temperatures and salinity conditions, and has used for wastewater treatment for nitrogen and heavy metals removal [10, 16, 22, 33, 67, 68].

Scenedesmus spp. is mainly used for the production of feedstock due to its high protein content of up to 40% under certain conditions. Its high lipid content is used for biodiesel production under nitrogen starvation and has also been used for the production of bioethanol by fermenting its high carbohydrates content [66, 68-71].

2.6   NITROGEN METABOLISM

Nitrogen is an essential element for microalgae, and every organism known, and its assimilation into the cells vary according to the species, its requirements, the environmental conditions and the enzymatic machinery.

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Some metabolic pathways for the assimilation of nitrogen have been elucidated, from the fixing of atmospheric nitrogen (N_2) to the production of aminoacids, nucleotides, and other compounds necessary for growth and the sustaining of life.

One important step is the conversion of nitrogen as nitrate (NO_3^-) into ammonia (NH_3) by the nitrate reductase, as ammonia can be assimilated directly by the cells. Following the production of ammonia, it is used in different metabolic pathways, one of them involves an essential enzyme for the production of proteins and to maintain the nitrogen balance in the cells, the Glutamine synthetase [5, 72].

2.6.1   Glutamine Synthetase Enzyme

Glutamine synthetase is one of the key enzymes in the nitrogen metabolism, it catalyzes the formation of glutamine from glutamate by the amidation of the later and the use of a molecule of ATP, this enzyme is classified as a ligase used to form a carbon-nitrogen bond between glutamate and ammonia, or also called glutamate-- ammonia ligase (EC 6.3.1.2) (Figure. 5) [73].

The glutamine synthetase or GS is widely distributed among almost every organism known, and three different types of GS have been elucidated so far: the GSI that is commonly found in Prokaryotic organisms, GSII in Eukaryotes and GSIII in some specific types of prokaryotes. GSI is the most studied and known of the three, being the *E. coli* the most studied, with its structure and function determined. In *E. coli,* this enzyme is composed of twelve subunits, arranged as two opposing rings with six subunits each, thus creating twelve active sites that works like a bifunnel (Figure. 6). The mechanism for the formation of glutamine is described following the interactions illustrated by Eisenberg, et al. From the upper side of the bifunnel the ATP binds into the active site, while on the lower side the glutamate enters. By using one of two metal ions as cofactors (magnesium or manganese) the molecules are stabilized and the process starts, first the ATP binds to one of the metal ions, then the

glutamate binds to the new formed complex, next one phosphate is transferred to the glutamate generating an intermediate negatively charged pocket to which the ammonia binds, attacks the δ-carbon of the γ-glutamil phosphate intermediate, releasing the ADP molecule and forming glutamine [73-75].
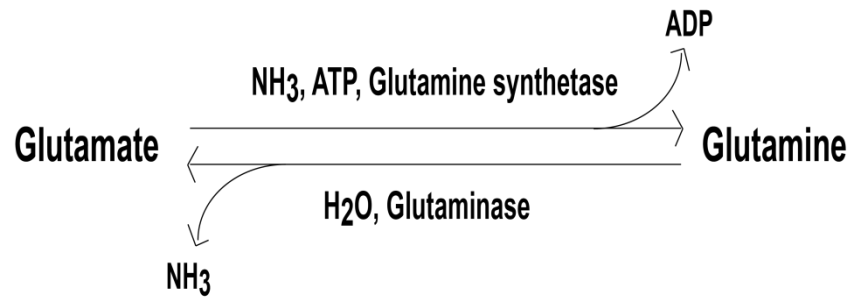
Figure 5. Glutamine Synthetase Enzymatic Mechanism
Glutamine synthetase is a key enzyme in the nitrogen metabolism. Glutamine is formed by the amidation of a molecule of glutamate with ammonium by using ATP. Adapted from Biochemistry by Mathews (3rd Edition).
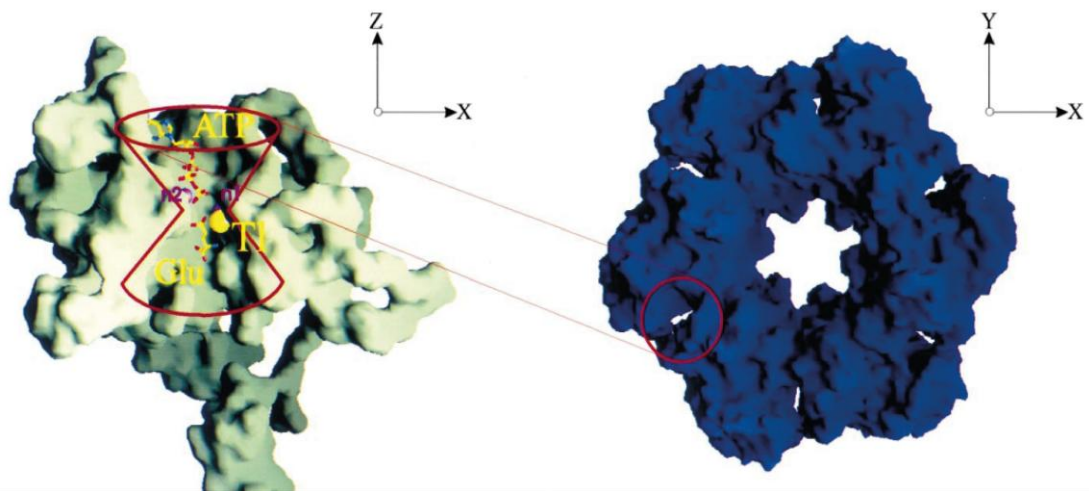
Figure 6. Structure Function Relationship of Glutamine Synthetase
The glutamine synthetase from *E. coli* consists of 12 subunits that are assembled as two opposing rings of 6 subunits that works like a Bifunnel, allowing the entrance of ATP from the top, gluta mate from the bottom and of the ammonium from one side. Taken from Eisenberg, et al, 2000

3.   AIMS OF THIS WORK

3.1   GENERAL AIM

To implement a pCAMBIA1302 vector-based genetic engineering strategy for the heterologous expression of Glutamine Synthetase gene (*glnA*) from *E. coli* in Microalgae.

3.2   SPECIFIC AIMS

3.2.1   To evaluate the experimental growth conditions in wastewaters and culture media of two microalgal species, *Chlorella spp and Scenedesmus spp*, and to evaluate their sensitivity to the antibiotics Kanamycin and Hygromycin B.

3.2.2   To design and develop pCAMBIA1302-derived constructs for the heterologous expression of the *E. coli* GlnA in Microalgae.

3.2.3   To transfect the GlnA enzyme of *E. coli* in microalgae using the final pCAMBIA1302::*glnA* construct and evaluate its expression by SDS-PAGE and Western Blot.

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Laboratory Strains, Primers, Plasmids and Antibodies

Table 1. Microalgae Wild Type Strains.

Strain	Characteristic(s)	Source or Reference
Scenedesmus spp.	Wild Type	donated by the Explora Park
Chlorella spp.	Wild Type	donated by the Greenbio Research group UdeM
Phaeodactylum tricornutum.	Wild Type	donated by the EPFL, Switzerland
Chlorella sorokiniana.	Wild Type	donated by the EPFL, Switzerland
Scenedesmus vacuolatus.	Wild Type	donated by the EPFL, Switzerland

Table 2. *Escherichia coli* Laboratory Strains.

Strain	Characteristic(s)	Source or Reference
DH5α	*Δ(lac)U169, endA1, gyrA96, hsdR17, Φ80Δ(lacZ)M15, recA1, relA1, supE44, thi-1*	Invitrogen
Top10	*F- mcrA, Δ(mrr- hsdRMS- mcrBC), Φ80Δ(lacZ)M15, ΔlacX74, recA1, araD139, Δ(araleu), 7697 galU, galK, rpsL (StrR), endA1, nupG*	Invitrogen

Table 3. Primers Used for the Cloning of *glnA* in *Scenedesmus spp.*

Usage	Name	Sequence (5'- 3')
***aad9* gene amplification and cloning in pCAMBIA1302**		
aad9	SpeCambia1302-F (*BstEII/SpeI*)	5'-GCGCGCGGTGACC/ACTAGTTTTTCGTTCGTGAATACATG-3'
	SpeCambia1302-R (*BstEII/ApaI*)	5'-GCGCGCGGTGACC/GGGCCCAATTAGAATGAATATTTCCC-3'
glnA* gene amplification and substitution cloning into pCAMBIA1302::*Spec		
glnA	glnA-F (*SpeI*)	5'-GCGCGCACTAGTGCTGAACACGTACTGACG-3'
	glnA-R (*ApaI*)	5'-GCGCGCGGGCCCAATTCACACGTGGTGGTGGTGGTGGTGGACGCTGTAGTACAGCTC-3'
pCAMBIA1302 Screening		
pCAMBIA1302	SeqCAMBIA1302-F	5'-ATGTGATATCTCCACTGACG-3'
	SeqCAMBIA1302-R	5'-CATCGCAAGACCGGCAAC-3'

Table 4. Cloning and Expression Vector Used in this Study.

Plasmid	Characteristic(s)[a]	Source or Reference
pCAMBIA1302	Cloning and Expression vector (10549 bp), Km^r, Hyg^r, with 6X Histidine tag	Cambia (Marker Gene)

[a]Km, Kanamycin; Hyg, Hygromycin

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Table 5. Cloning and Expression Vector Used in this Study.

Plasmid	Characteristic(s)[a]	Source or reference
For the *glnA* cloning and expression in microalgae		
pCAMBIA1302::*Spec*	*Spec-aad9* (1114 bp) cloned into pCAMBIA1302,Spec[r] / DH5α	This study
pCAMBIA1302::*glnA*	*E. coli* **glnA** (1410) cloned into pCAMBIA1302::Spec / DH5α	This study

[a]Spec, spectinomycin

Table 6. Antibodies Used in this Study.

Antibody	Dilution	Source or reference
Anti GlnA Chicken	1:5000	Agrisera
Goat anti-chicken IgY peroxidase conjugate	1:2000	Agrisera

4.1.2   Laboratory Equipments

Laboratory Device	*Manufacturer or Distributor*
Agarose gel (horizontal) electrophoresis chamber	Bio-Rad
Analytical balance	Adventurer OHAUS
Autoclave	Tuttnauer 3870 ELV
Benchtop centrifuge	Sigma 2-16PK
Bunsen burner	
Cell disruption ultrasound machine	Sonic Dismembrator
Electroporador	GenePulser Xcell Bio-Rad
Film cassette	Fujifilm
Fluorescence microscope	Nikon Eclipse 80i
Freezer (-80°C, ultra-low temperature freezer)	Panasonic VIP series
Freezer -20°C	NUAIRE
Gel documentation system	Bio-Rad
Heating block	LabNet Accublock
High Performance Liquid Chromatography (HPLC)	Agilent Technologies
Laminar flow hoods	NUAIRE
Luxometer	Light Meter
Magnetic mixer (with heat block)	Hiedolph

Microcentrifuge	Sigma 1-14
Micropipettes (p100, p200, p1000)	Nichiryo
Micropipettes (p2, p10)	RAININ
Microscope	ZEISS
Microwave	Whirlpool
Moisture analyser	Precisa XM-60
Nanodrop	Nanodrop Thermo-Scientific
Orbital Shaker	MaxQ450 Thermo-Scientific
PCR thermocycler	MJ Research PTC-100
pH meter	Hanna
Power source	Consort EV243
Refrigerator	LG
SDS-PAGE (vertical) electrophoresis chambers	Bio-Rad
Sonicator F550 sonic dismembrator	Fisher
Spectophotometer	Spectronic Genesys 2PC
Ultrasonic bath	Elma LC20H
Vortex	Genie 2

4.1.3 Laboratory Consumables

Product	*Manufacturer or Distributor*
Conical test tubes	Falcon
Nitrile gloves	Biofit
Nitrocellulose membrane	Whatmann
Parafilm	Pechiney
PCR tubes (0.2 mL)	Sarstedt
Disposable petri dishes	Filtracion y Analisis
Pipette tips (10 µL, 200 µL, 1000 µL)	Sarstedt and Gentech
Plastic cuvettes	Sarstedt
Slides and cover slides	Micro
Sterile filters (0.45 µM)	Millipore

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Vials (0.5 mL, 1.5 mL, 2.0 mL)	Gentech
Millipore filter paper	Millipore
X-ray films	Fujifilm

4.1.4 Chemicals

Products	_Manufacturer or Company_
Acetic Acid	Amresco
Ammonium Persulfate (APS)	Amresco
Calcium Chloride	Merck
Enhanced Chemiluminescence (ECL)	Invitrogen
Ethanol	Merck
Glycerin	Sigma
Isopropanol	Merck
Magnesium Chloride Hexahydrate	Amresco
Methanol	Sigma
Potassium Chloride	Merck
SDS	Amresco
Skimmed Milk Powder	Amresco
Sodium Acetate	Fluka
Sodium Chloride	Merck
Tris (tris-(hydroxymethil)-aminomethane)	Amresco
Tween-20	Sigma

4.1.5 Molecular Biology Reagents

Product	_Manufacturer or Company_
Acrylamide-(bis-)Acrylamide 40 %	Sigma
Agarose	Amresco
BSA (Bovine Serum Albumin)	Sigma
Coomassie Brilliant Blue R-250	Merck

DTT (Dithiothreitol)	Amresco
EDTA (Ethylenediaminetetraacetic Acid)	Amresco
EZ-Vision in Gel Solution, 10000X	Amresco
Hygromycin B	Amresco
Kanamycin Sulfate	Sigma
PMSF (Phenylmethylsulfonylfluoride)	Sigma
Ponceau Red S Staining Solution	Sigma
TEMED	Sigma
β-Mercaptoethanol	Sigma

4.1.6   Molecular Biology Kits

Kit	*Manufacturer or Company*
WizardSV Gel and PCR Clean-Up System	Promega
GeneJET Plant Genomic DNA Purification Mini Kit	Thermo Scientific

4.1.7   Molecular Biology Enzymes

Enzyme	*Manufacturer or Company*
Pfx Platinum DNA Polymerase	Invitrogen
Taq DNA Polymerase	Thermo Scientific
T4 DNA Ligase	Thermo Scientific

4.1.8   Restriction Enzymes

Enzyme		*Manufacturer or Company*
*Apa*I	G$^{\downarrow}$GGCCC	Thermo Scientific
*Bst*EII	G$^{\downarrow}$GTNACC	Thermo Scientific
*Nco*I	C$^{\downarrow}$CATGG	Thermo Scientific
*Spe*I	A$^{\downarrow}$CTAGT	Thermo Scientific

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4.1.9   Buffers and Solutions

LB (Luria-Bertani Broth) Medium

    10 g Peptone

    10 g NaCl

    5 g Yeast Extract;

    1 L Distilled Water

Bristol 3N Medium

    0.075 g $NaNO_3$

    0.0025 g $CaCl_2$-$2H_2O$

    0.0075 g $MgSO_4$-$7H_2O$

    0.0075 g K_2HPO_4

    0.0175 g KH_2PO_4

    0.0025 NaCl;

    1 L Distilled Water

CHU13 Modified Medium

    0.4 g $NaHCO_3$

    0.2 g/L KNO3

    0.04 g/L KH_2PO_4

    0.1 g/L $MgSO_4$-$7H_2O$

    0.08 g/L $CaCl_2$-$6H_2O$

    0.01 g/L Ferrum Citrate

    0.1 g/L Citric Acid

    0.50 mg/L Boron

    0.50 mg/L Manganese

    0.05 mg/L Zinc

    0.02 mg/L Copper

    0.02 mg/L Cobalt

    0.02 mg/L Molibdene

Solution for Competent E. coli *Cells Production*

Buffer IC: 15% Glycerin

75 mM/L $CaCL_2$

1 L Distilled Water

Solution for Plasmid Isolation from E. coli *(Miniprep)*

Buffer A: 25 mM Tris, pH 7.5

10 mM Na_2EDTA

50 mM Glucose

500 mL Distilled Water

Buffer B: 1% SDS

0.2 M NaOH

10 mL Distilled Water

Buffer C: 3 M Potassium Acetate, pH 5.0

500 mL Distilled Water

TAE Buffer (20X

800 mM Tris (96.8 g)

500 mM EDTA (146.13 g)

2.28% Glacial Acetic Acid (22.8 mL)

1 L Distilled Water

TAE Buffer (1X)

50 mL 20X TAE Buffer

950 mL Distilled Water

Agarose Gel Solution

0.8 % Agarose (4g)

500 mL TAE Buffer (1X)

5 µL EZ-Vision 10000X per 15 mL Agarose

TE Buffer

10 mM Tris

1 mM EDTA

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DNA Loading Buffer

   25% Sucrose (5g)

   0.1% Bromophenol Blue (20 mg)

   20 mL TE Buffer

SDS-PAGE Solutions

Resolving Gel Buffer: 1.5 M Tris (181.7 g), pH 8.8

                       1 L Distilled Water

Stacking Gel Buffer: 0.5 M Tris (60.6 g), pH 6.8

                       1 L Distilled Water

SDS Solution (10%):   10% SDS (10 g)

                       100 mL Distilled Water

APS Solution (10%):   10% APS (1 g)

                       10 mL Distilled Water

Running Buffer (10X TGS): 230 mM Tris

                           1.52 M Glycine

                           1% SDS

Running Buffer (1X TGS):   100 mL Running Buffer (10X TGS)

                           900 mL Distilled Water

Protein Sample Buffer (2X):

   6 mL Distilled Water

   4 mL Stacking Gel Buffer, pH 6.8

   4 mL 10% SDS Solution

   4 mL 100% Glycerol

   2 mL β-Mercaptoethanol

   A spatula tip of Bromophenol Blue

Coomassie Staining Solutions:

Staining Solution: 25% Ethanol (250 mL)

                       10% Acetic Acid (100mL)

                       0.05% Coomassie Brilliant Blue R-250 (50 g)

                       650 mL Distilled Water

Unstaining Solution: 25% Ethanol (250 mL)

        10% Acetic Acid (100mL)

        650 mL Distilled Water

TBS Buffer

 50 mM Tris, pH 7.6

 150 mM NaCl

TBS-Tween-20

 2.5 mL Tween-20

 500 mL TBS

Protein Transfer Buffer (Tank-Blotting)

 25 mM Tris

 190 mM Glycine

 20% Methanol (500 mL)

 2 L Distilled Water

Blocking Solution (5%)

 5% Skimmed Milk Powder (1 g)

 20 mL TBS-Tween-20

AP Buffer

 100 mM Tris (6.05 g), pH 9.5

 100 mM NaCl (2.92 g)

 5 mM MgCl 2 (0.51 g)

 500 mL Distilled Water

Chromogen Solution (Alkaline Phosphatase-Detection)

 50 µL NBT (0.5 g NBT in 10 mL of 70% Dimethylformiat)

 25 mL BCIP (0.5 g mL BCIP in 10 mL of 100% Dimethylformiat)

 10 mL AP Buffer

Chromogen Solution (Peroxidase-Detection)

 500 µL Chloronaphthol (1 Tablet dissolved in 10 mL of Methanol)

 10 µL H_2O_2

 10 mL PBS

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4.2 METHODS

4.2.1 Cloning and Expression of the Glutamine Synthetase Enzyme from *E. coli* in Microalgae

4.2.1.1 Bacterial and Microalgal Strains, Growth and Transformation

The bacterial and microalgal strains used in this study are listed (Tables 1 and 2), and plasmids are listed on Table 4. The *E. coli* strains employed in this study were grown in Luria-Bertani (LB) agar or broth at 37°C. All the microalgal strains employed in this study were grown in Wastewaters from two different sources (Enterprises: "*San Fernando*" and "*Locería Colombiana*") in Medellín Colombia, as well as in CHU13 agar or broth media, supplemented with 0.4 g/L $NaHCO_3$, under constant aeration with an air pump and illumination using cool fluorescent lamps with an intensity of 1000 lux, at 25°C. *E. coli* $CaCl_2$-competent cells were transformed with plasmids, following standard heat-shock protocols and then growing bacteria on LB agar, supplemented with 50 µg/mL Kanamycin.

4.2.1.2 Microalgae Transfection and GFP Fluorescence Determination

Electroporation was applied in order to transfect *Scenedesmus spp.* with the mock plasmid pCAMBIA1302, and the antibiotic sensitivity was checked employing a gradient of concentrations of Hygromycin B, and Kanamycin. The electroporation parameters were carried out following the described protocol by Jo-Shung Chang, et al., 2013, treating the cells with an osmotic solution (0.2 $molL^{-1}$ mannitol, 0.2 $molL^{-1}$ sorbitol and 10 % Glycerol) and using electroporation with one pulse of 2kV for 3ms and a plasmid DNA concentration of 50 ng/µL, then culturing in CHU13 agar supplemented with Kanamycin (100 µg/mL) and Hygromycin B (30 µg/mL). The positive clones were grown for 12 days in CHU13 agar and Broth, verified by colony PCR and the expression of the GFP gene was determined by fluorescence microscopy by checking the chlorophyll fluorescence with a Y-2 filter (Nikon) and the GFP fluorescence with a B-2 filter (Nikon).

4.2.2   Molecular Cloning of *glnA* into *Scenedesmus spp.*

4.2.2.1   Bioinformatics Analysis

In order to design and implement the genetic engineering strategy for the expression cloning of the glutamine synthetase (GlnA from *E. coli* K12), into microalgae by using pCAMBIA1302 vector, the *glnA* gene (Figure 13 and 14) sequence was retrieved from GenBank (http://www.ncbi.nlm.nih.gov/gene/948370) and uniprot (http://www.uniprot.org/uniprot/P0A9C5). Similarly, the mGFP5 and GFP sequences were retrieved from the CAMBIA plasmids information in GenBank (http://www.ncbi.nlm.nih.gov/nuccore/7638073). Both GFP genes were aligned for sequence analysis (http://multalin.toulouse.inra.fr/multalin/multalin.html) (Figure. 15).

4.2.2.2   Molecular Biology and Genetic Engineering Techniques

Primers designed for this study are listed on Table 3. The Bacterial DNA was extracted using the standard phenol-chloroform extraction method and was purified with the DNA purification kit (Promega). The microalgal DNA was extracted employing a combination of pretreatment with liquid nitrogen and glass beads following a GeneJet Plant DNA Purification Kit (Thermo-Scientific). The DNA amplification was carried out with the standard Polymerase Chain Reaction (PCR) working with the *Taq* Polymerase (Thermo-Scientific). For expression cloning, the Pfx platinum high fidelity polymerase was employed (Invitrogen). Both Polymerases were used as suggested by the commercial distributor. PCRs were carried out in 15 μL volume for screening and ordinary amplifications, and in 50 μL volume for expression cloning. PCR protocols for the *aad*9 and *glnA* amplification, and bacterial or microalgal colony screening were performed with an initial denaturation step (94°C, 5 min), followed by 30 cycles of amplification (94°C, 1 min; 60°C, 30 sec; and 72°C, 2 min), and a final elongation step (72°C, 10 min). For colony microalgae colony screening, cells were previously grown during 8 days, and harvested biomass was washed twice with distilled water and diluted 1/1000, before

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PCR amplification. All PCR products were purified with the wizard SV Gel and PCR clean-up system (Promega) and plasmids were extracted employing a conventional miniprep protocol. Purified plasmids or PCR products for sequencing were prepared and sent to MACROGEN USA, following their instructions.

Usually, recombinant cloning into pCAMBIA1302 vector is performed with the restriction enzymes *Nco*I and *Bst*EII. Nevertheless, the restriction analysis of the *glnA* gene sequence, using the Neb-Cutter Program: (http://tools.neb.com/NEBcutter2/index.php), detected one restriction site for each enzyme on the *glnA* gene (Annexe 9.7). Therefore, a substitution cloning strategy was designed by employing a Spectinomycin Resistance Cassette (*Spec* = *aad9* gene), which was amplified by PCR, and cloned into pCAMBIA1302 with the restriction enzyme *Bst*EII (Figure 9). After ligation with a T4 DNA ligase (Thermo-Scientific), the pCAMBIA1302::*Spec* construct was transformed into *E. coli* DH5α CaCl$_2$-competent cells for plasmid propagation. Positive clones were checked for their orientation by PCR. Subsequently, the *glnA* gene sequence from *E. coli* K12 was amplified by PCR and cloned by *Spec* substitution into the pCAMBIA1302::*Spec* by a double enzymatic restriction with *Spe*I and *Apa*I (Figure 10). Ligation was performed with a T4 DNA ligase (Thermo-Scientific) and transformation was done by using *E. coli* DH5α CaCl$_2$-competent cells for plasmid propagation. Positive clones were checked by colony PCR and a plasmid preps were done for *glnA* sequencing. Recombinant bacterial strains were preserved at -80°C with 20% glycerol. All plasmids and constructs generated in this study are depicted in Figure 11 and listed in Table 5.

4.2.2.3 pCAMBIA1302::*glnA* Electroporation into *Scenedesmus spp.*

Scenedesmus spp. transfection with the final construct pCAMBIA1302::*glnA* was carried out following the previously explained protocol for the mock plasmid (Section 4.2.1.2). The cells were plated on CHU13 agar and colonies were grown in CHU13 broth (30 μg/mL Hygromycin B and 100 μg/mL Kanamycin). Recombinant Microalgae were preserved at -80°C in CHU13 with 5% methanol and 10% DMSO.

4.2.2.4 <u>Protein Extraction, Immunoblotting and Quantification</u>

For the protein extraction, the microalgal cells were harvested by centrifugation at 7400g for 3 min, the supernatant removed and the wet biomass was determined using an analytical scale, the cells were resuspended with 200 μL of cold PEB buffer supplemented with 1 mM PMSF, lysed with a sonicator with 30% of its power until thawing and flash frozen again. The sample was subjected to four sonication/thawing cycles. In parallel, cells were also subjected to lysis by applying glass beads and flash freezing in liquid nitrogen. The sample was subjected to seven cycles of vortex and glass beads, flash freezing and thawing followed by a centrifugation step at 7400g for 3 minutes at 4°C. The pellet was checked to be grayish or white and the supernatant to have a dark green color for a complete rupture of the cells, the supernatant was pippeted into a new 1.5 mL vial. The proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and further transferred into a nitrocellulose membrane using a wet blotting system (Bio-RAD). The membrane was blocked with 10% skimmed milk for 2 hours and the proteins were detected by immunoblot analysis. The chicken anti-GlnA polyclonal antibody (Agrisera) was employed at a dilution of 1:5000, and the secondary antibody goat anti-Chicken IgG peroxidase conjugate (Agrisera) was used at a dilution of 1:2000. *E. coli* protein extract was set as a positive control while *Scenedesmus spp.* wild-type as a negative control. The binding activity was detected with an enhanced chemiluminiscence kit. The protein quantification was done with the modified Lowry assay [76], using Bovine Serum Albumin (BSA) for the standard curve.

4.2.2.5 <u>Statistical Analysis</u>

All data are reported as mean ± SD unless otherwise noted. *Scenedesmus spp.* antibiotic sensitivity to Hygromycin B and Kanamycin, protein concentration (mg/mL) and protein content (%) results were statistically analyzed using ANOVA with F-distribution. A p-value of <0.05 was considered statistically significant.

5. RESULTS

5.1.1 Growth Curves of Microalgae

Chlorella spp and *Scenedesmus spp.* were chosen for this study because of their availability in the laboratory. Both microalgal strains were grown successfully during 12 days, at low cell density, in wastewaters coming from two different sources in Medellín - Colombia: the first source was The Wastewater Treatment Plant "San Fernando", and the second one was The "Locería Colombiana", a Colombian Ceramic Manufacturing Industry (Data not shown). In addition, *Chlorella spp* and *Scenedesmus spp* were cultured in CHU13 broth during 8 days, and their growth curves were analyzed by measuring the O.D. at a λ = 680 nm each 24 hours (each day). Both Microalgae grew as expected, showing exponential growth curves, which are required for downstream experiments. In comparison with *Chlorella spp*, *Scenedesmus spp* exhibited a more optimal growth curve with higher O.D. values for each measure point (each day) (Figure 7).
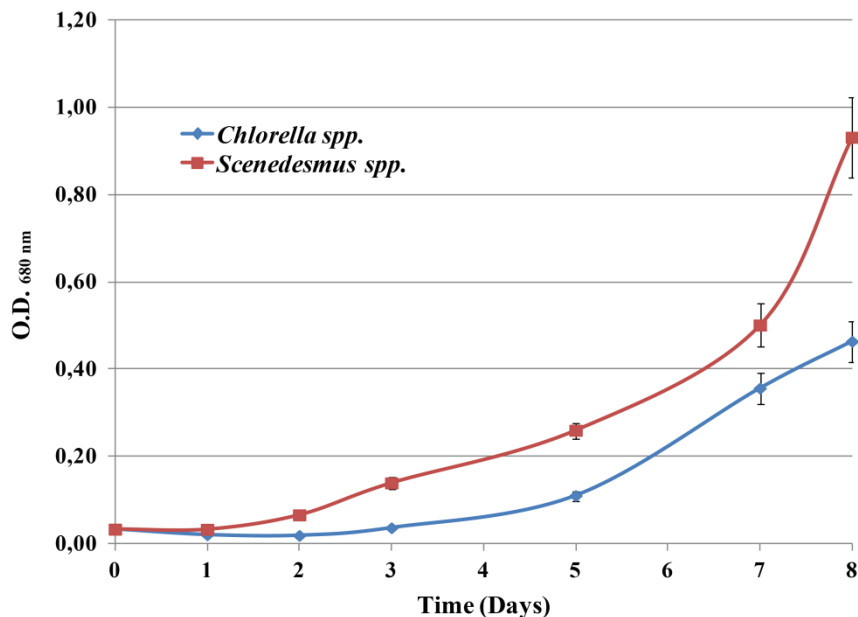
Figure 7. *Microalgae* **Growth in CHU13 Broth Medium**
Growth curves (O.D. at 680 nm) of *Chlorella spp.* and *Scenedesmus spp.* in CHU13 broth medium during 8 days. All the experiments were performed by triplicates under the same conditions.

Cells of both microalgal strains were observed under the microscope at the end of the experiment (8th day), showing the typical morphology of single units (*Chlorella spp*), and single cells and coenobia (*Scenedesmus spp*) (Figure 8).

Figure 8. *Microalgae* under the Microscope
Single cells of *Chlorella spp.* (left), and single cells and coenobia of *Scenedesmus spp.* (right) after growth on CHU13 medium during 8 days. Pictures were taken using a DS-1 Camera attached to a Nikon 80i Fluorescent Microscope, by using the 100x objective.

5.1.2 Sensitivity of *Scenedesmus spp* to Kanamycin and Hygromycin B.

In order to evaluate the inhibitory effect of the antibiotics Kanamycin and Hygromycin B on the cell growth of *Scenedesmus spp*, four Erlenmeyers, each with 100 mL of CHU13 broth medium, were inoculated with *Scenedesmus spp* until an O.D. value of 0.1, for each antibiotic. Three Erlenmeyers were treated with an end concentration of 100, 300, and 500 μg/mL (for Kanamycin), and 25, 50 and 100 μg/mL (for Hygromycin B). As a control, no antibiotics were added to the fourth Erlemmeyer. The growth curves of *Scenedesmus spp* with both antibiotics were analyzed by measuring their O.D. at a $\lambda = 680$ nm each 48 hours (each two days) (Figure 9). *Scenedesmus spp* shows a dose-dependent and differential sensitivity to both antibiotics. High concentrations of Kanamycin are able to inhibit completely *Scenedesmus spp* growth (Figure 9A), while Hygromycin B inhibition is not so efficient in liquid media, at the evaluated concentrations (Figure 9B). Antibiotic concentrations are summarized in the Table 7.

Results
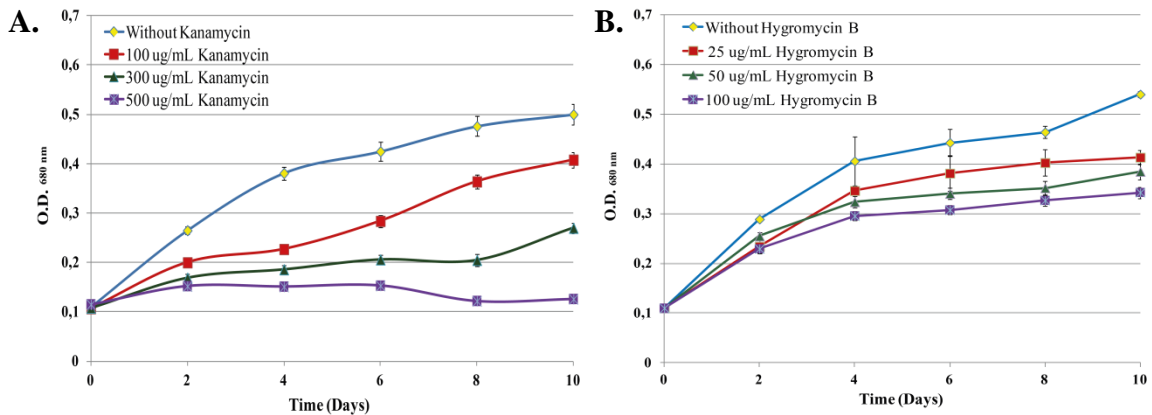
Figure 9. *Scenedesmus spp.* **Antibiotic Sensitivity.**
The growth behavior of *Scenedesmus spp*. under the effect of two different antibiotics is shown: (**A**) Kanamycin and (**B**) Hygromycin B. and the inhibitory doses were confirmed. These data were collected by performing an experiment with three replicates each under the same growth conditions.

Table 7. Statistic Test for the Antibiotic Sensivity of *Scenedesmus spp*. Kanamycin (up) and Hygromycin (down).

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	0,199	3	0,066	27,266	0,0000
Within groups	0,049	20	0,002		
Total (Corr.)	0,248	23			
Between groups	0,050	3	0,017	6,914	0,002
Within groups	0,058	24	0,002		
Total (Corr.)	0,109	27			

5.1.3   Transfection of *Scenedesmus spp*. with pCAMBIA1302 (MOCK)

In order to evaluate the capability of the Kanamycin and Hygromycin Resistance Genes, which are present on the pCAMBIA Expression Cloning Vectors, to induce recombinant antibiotic resistance in Microalgae, *Scenedesmus spp* was transfected by electroporation with the plasmid pCAMBIA1302, following the protocol previously reported by Suo-lian, 2013 [58]. Transfected cells of

Scenedesmus spp were grown on CHU13 agar medium, supplemented with different concentrations of both antibiotics: Kanamycin (Data not shown) and 25, 30, 40 and 50 µg/mL of Hygromycin B (Figure 10). 581 positive colonies were obtained from $1x10^8$ recipient cells, which accounts for a transfection efficiency of 0.06%. Later, twelve recombinant clones were selected for growth screening from the 30 µg/mL Hygromycin B plate. Seven of them were positive for growing and subsequently checked by PCR, using pCAMBIA1302 *mgfp5*-specific primers (Figure 11). In addition, their recombinant Green Fluorescence Protein (GFP) expression, which has been genetically engineered in pCAMBIA1302 for its efficient expression in Microalgae (Figure 12) was also confirmed by fluorescence microscopy (Figure 13).
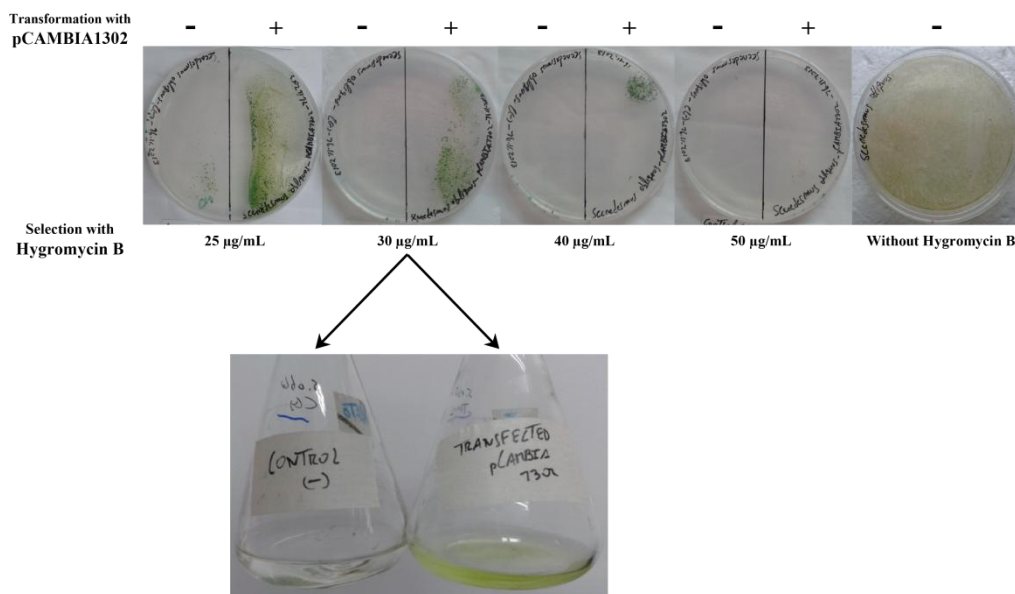
Figure 10. Transfection of *Scenedesmus spp.*

(**A**) *Scenedesmus spp* ($1x10^8$ recipient) cells were transfected with the pCAMBIA1302 plasmid and plated on CHU13 agar medium, supplemented with different concentrations of Hygromycin B: 25 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL. (**B**) One representative of seven recombinant colonies from the 30 µg/mL plate were subsequently checked for growth in CHU13 broth, supplemented with 30 µg/mL of Hygromycin B. One negative control colony was also taken from the plate without Hygromycin B and inoculated in CHU13 broth, supplemented with 30 µg/mL of Hygromycin B.
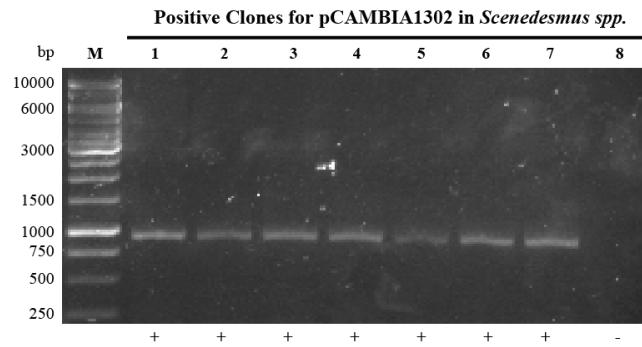
Figure 11. Screening by PCR of pCAMBIA1302-transfected *Scenedesmus spp* Colonies.
Agarose gel electrophoresis of the *gfp* gene (956 bp) amplified from pCAMBIA1302-transfected *Scenedesmus spp* cells. Lane 8: Negative Control (Non-transfected Microalgae).
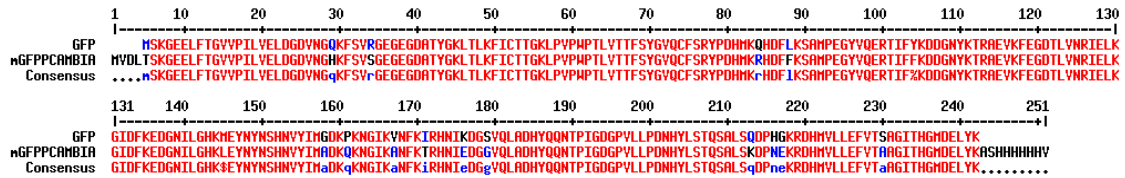
Figure 12. Alignment of the GFP and the pCAMBIA1302-GFP Protein Sequences.
The sequences of the GFP and the mGFP5, encoded in pCAMBIA1302 for expression in Microalgae, were aligned by using the online molecular tool Multalin (See 4.2.2.1 in Methods Section). Variations in a.a. are highlighted in blue, conserved a.a. are shown in red, and additional a.a. in the mGFP5 sequence are in black.
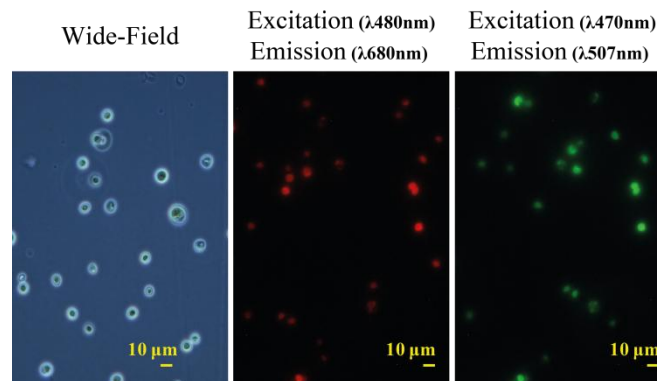
Figure 13. GFP Expression in *Scenedesmus spp.*

Visualization of transfected *Scenedesmus* cells: (**A**) under the wide-field of the microscope, (**B**) by the natural chlorophyl fluorescence, and (**C**) by the recombinant expression of the modified *gfp* gene on pCAMBIA1302. (B) and (C) are the same field under the microscope.

5.1.4 Genomic Context of the *glnA* Gene from *Escherichia coli* K12 Strain and Bioinformatics Analysis of the GlnA Protein for Expression Cloning in Microalgae

The location of the *glnA* gene on the *E. coli* chromosome, its genomic context and the organization of the *gln*-operon is depicted in Figure 14. The complete *glnA* gene and protein sequences of the *E. coli* K12 strain were retrieved from the NCBI and the KEGG databases (See 4.2.2.1 in Methods Section).
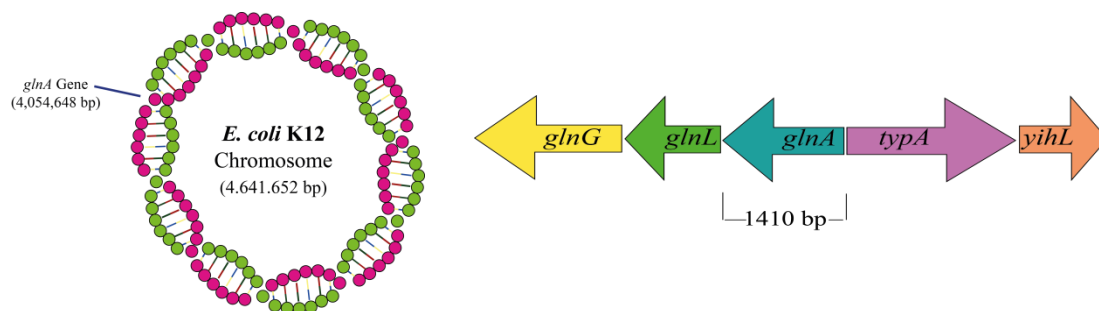
Figure 14. Location and Genomic Context of the Glutamine Synthetase Encoding Gene (*glnA*) on the Escherichia coli Genome.
The *glnA* gene is located on the indirect strand of the *E. coli* chromosome: Position: 4.05 Mb. The length of the glutamine synthetase gene (*glnA*) is 1410 bp. It belongs to the *gln*-operon and it is flanked by the *glnL* and *typA* genes on the *E. coli* K12 genome.

Given the fact that the Green Fluorescence Protein (GFP) has been genetically engineered in pCAMBIA1302 for its efficient expression in Microalgae (Figure 12); molecular strategy, which has been successfully confirmed here by fluorescence microscopy of *Scenedesmus spp* cells transfected with pCAMBIA1302 (Figure 13); similar changes were thought in this study (Figure 15), in order to ensure the efficient recombinant expression of the prokaryotic GlnA protein in Microalgae. Therefore, the forward and reverse primers for the complete amplification of the *glnA* gene from *E. coli* were designed and engineered to contain the following changes: Four a.a. at the N-ter, right after the starting Met (M), and 10 a.a. more at the end (C-ter), containing the 6xHis-Tag; thinking in an eventual purification step.

Results

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        1        10        20        30        40        50        60        70        80        90       100       110       120       130
        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
GlnA        METSAEHVLTMETLNEHEVKFVDLRFTDTKGKEQHVTIPAHQVNAEFFEEGKMETFDGSSIGGWKGINESDMETVLMETPDASTAVIDPFFADSTLIIRCDILEPGTLQGYDRDPRSIAKRAEDYL
mod_GlnA METVDLTSAEHVLTMETLNEHEVKFVDLRFTDTKGKEQHVTIPAHQVNAEFFEEGKMETFDGSSIGGWKGINESDMETVLMETPDASTAVIDPFFADSTLIIRCDILEPGTLQGYDRDPRSIAKRAEDYL
Consensus ....deTSAEHVLTMETLNEHEVKFVDLRFTDTKGKEQHVTIPAHQVNAEFFEEGKMETFDGSSIGGWKGINESDMETVLMETPDASTAVIDPFFADSTLIIRCDILEPGTLQGYDRDPRSIAKRAEDYL

        131       140       150       160       170       180       190       200       210       220       230       240       250       260
        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
GlnA        RSTGIADTVLFGPEPEFFLFDDIRFGSSISGSHVAIDDIEGAWNSSTQYEGGNKGHRPAVKGGYFPVPPVDSAQDIRSEMETCLVMETEQMETGLVVEAHHHEVATAGQNEVATRFNTMETTKKADEIQI
mod_GlnA RSTGIADTVLFGPEPEFFLFDDIRFGSSISGSHVAIDDIEGAWNSSTQYEGGNKGHRPAVKGGYFPVPPVDSAQDIRSEMETCLVMETEQMETGLVVEAHHHEVATAGQNEVATRFNTMETTKKADEIQI
Consensus RSTGIADTVLFGPEPEFFLFDDIRFGSSISGSHVAIDDIEGAWNSSTQYEGGNKGHRPAVKGGYFPVPPVDSAQDIRSEMETCLVMETEQMETGLVVEAHHHEVATAGQNEVATRFNTMETTKKADEIQI

        261       270       280       290       300       310       320       330       340       350       360       370       380       390
        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
GlnA        YKYVVHNVAHRFGKTATFMETPKPMETFGDNGSGMETHCHMETSLSKNGVNLFAGDKYAGLSEQALYYIGGVIKHAKAINALANPTTNSYKRLVPGYEAPVMETLAYSARNRSASIRIPVVSSPKARRIE
mod_GlnA YKYVVHNVAHRFGKTATFMETPKPMETFGDNGSGMETHCHMETSLSKNGVNLFAGDKYAGLSEQALYYIGGVIKHAKAINALANPTTNSYKRLVPGYEAPVMETLAYSARNRSASIRIPVVSSPKARRIE
Consensus YKYVVHNVAHRFGKTATFMETPKPMETFGDNGSGMETHCHMETSLSKNGVNLFAGDKYAGLSEQALYYIGGVIKHAKAINALANPTTNSYKRLVPGYEAPVMETLAYSARNRSASIRIPVVSSPKARRIE

        391       400       410       420       430       440       450       460       470       480       490       500       510   517
        |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+------|
GlnA        VRFPDPAANPYLCFAALLMETAGLDGIKNKIHPGEAMETDKNLYDLPPEEAKEIPQVAGSLEEALNELDLDREFLKAGGVFTDEAIDAYIALRREEDDRVRMETTPHPVEFELYYSV
mod_GlnA VRFPDPAANPYLCFAALLMETAGLDGIKNKIHPGEAMETDKNLYDLPPEEAKEIPQVAGSLEEALNELDLDREFLKAGGVFTDEAIDAYIALRREEDDRVRMETTPHPVEFELYYSVHHHHHHVSTP
Consensus VRFPDPAANPYLCFAALLMETAGLDGIKNKIHPGEAMETDKNLYDLPPEEAKEIPQVAGSLEEALNELDLDREFLKAGGVFTDEAIDAYIALRREEDDRVRMETTPHPVEFELYYSV..........
```

Figure 15. Alignment of the GlnA and the Mod_GlnA for Expression Cloning in Microalgae.
The GlnA and its modified copy (mod_GlnA) protein sequences were aligned by using the online molecular tool Multalin (See 4.2.2.1 in Methods Section). Variations in a.a. are highlighted in blue, conserved a.a. are shown in red, and the additional a.a. to be included for the heterologous GlnA expression in Microalgae are in black.

5.1.5 Strategy for the Insertion of the *E. coli glnA* Gene into pCAMBIA1302: Construction of a pCAMBIA1302::*Spec*(+) Intermediary Plasmid.

By using all the information obtained from the bioinformatics analysis, the pCAMBIA1302 vector map, and the *glnA* gene and GlnA protein sequences, a *mgfp5*-substitution cloning strategy was designed for the insertion of the modified *glnA* gene copy into pCAMBIA1302. The idea was to replace the *mgfp5* gene of pCAMBIA1302, by introducing the *mod_glnA* gene in frame for expression. Nevertheless, after several attempts, no positive results were obtained with this strategy (Data not shown). These persistent negative result can be explained by the fact that the big sizes of both pCAMBIA1302 Vector (10.540 bp) and the *mod_glnA* insert (1.440 bp) make difficult the experimental cloning duties and therefore the cloning efficiency. Moreover, given that GFP is a visual marker for the Mock-recombinant Microalgae cells, the selection of the pCAMBIA1302::*glnA*-recombinant cells becomes a difficult task, because non-transfected *E. coli* (DH5α) or *Scenedesmus spp* cells have the same cloning fenotype, making difficult the screening by visualization.

Consequently, in this study, it was necessary to design and construct an intermediary version of the pCAMBIA1302 vector for chemical selection of the recombinant DH5α cells (Figure 16). Therefore, the Spectinomycin resistance cassette (*aad9* or *Spec*) was amplified with a primer pair, containing the following adapters for cloning: 5'-*Bst*EII+*Spe*I-3' (Primer-F) and 5'-*Bst*EII+*Apa*I-3' (Primer-R) (Figure 17A). The amplified *Spec* fragment was then digested with the restriction enzyme *Bst*EII for non-directional cloning into pCAMBIA1302, which was also previously digested with the same restriction enzyme (*Bst*EII) in an unique *Bst*EII-restriction site, located immediately downstream of the *mgfp5* gene (Figure 17B).
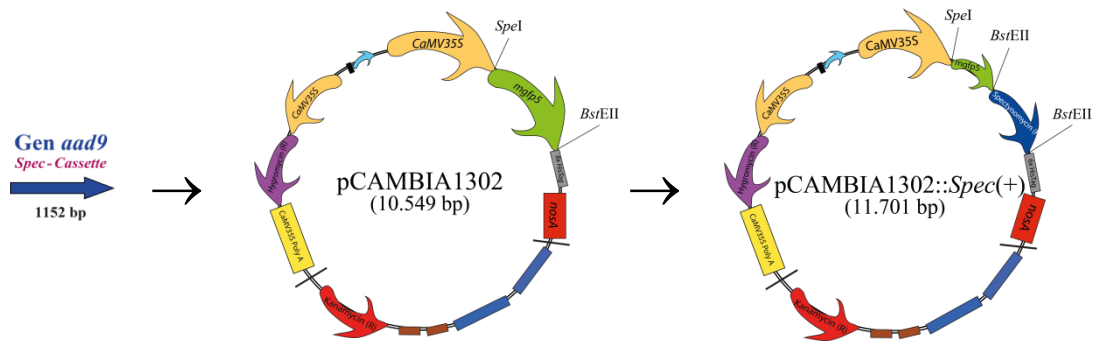
Figure 16. Construction of the pCAMBIA1302::*Spec* Intermediary Plasmid.
The Spectinomycin resistance cassette (*aad9* or *Spec*) was amplified and inserted into pCAMBIA1302, immediately downstream of *mgfp5*, by using the restriction enzyme *Bst*EII.
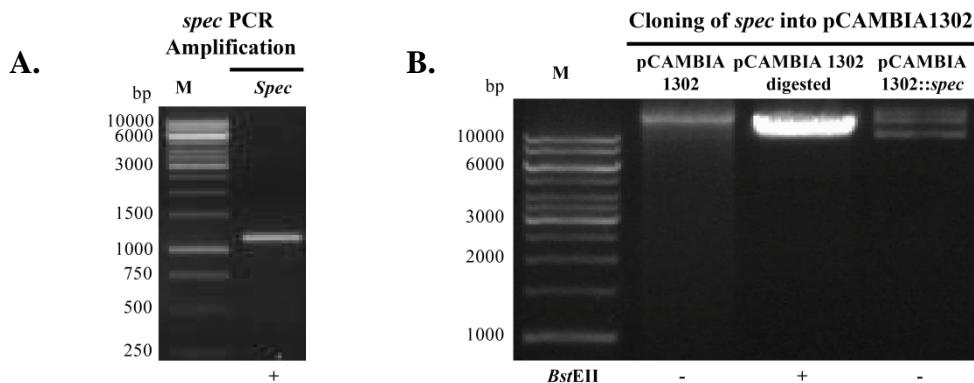
Figure 17. Spectinomycin (*Spec*) Gene Amplification and Cloning into pCAMBIA1302.
 (**A**)Separation by agarose gel electrophoresis of the PCR product from the Spectinomycin *aad*9 gene amplification, and (**B**) its cloning into the pCAMBIA1302 vector by digestion with *Bst*EII restriction enzyme followed by ligation with the T4 DNA ligase.

Results

The *Spec*-based chemical selection of the DH5α recombinant clones containing the intermediate pCAMBIA1302::*Spec* vector was highly efficient, since several *E. coli* positive colonies were obtained (Figure 18A). Furthermore, as mentioned above, the insertion of *Spec* into pCAMBIA1302 was done by non-directional cloning with *Bst*EII. For that reason, the orientation of the *aad9/Spec* gene was also confirmed by PCR (1.205 bp), employing a specific primer for the insert (SpeCAMBIA1302-F) and the specific primer for the vector (SeqCAMBIA1302-R) (Figure 18B). The correct identification and characterization of positive recombinant *E. coli* DH5α clones, containing the pCAMBIA1302::*Spec*(+), instead of pCAMBIA1302::*Spec*(-), is desired for the appropriate downstream localization of a restriction adapter *Apa*I, introduced in the SpeCAMBIA1302-R primer. This new *Apa*I site is required for the subsequent *glnA* gene insertion into pCAMBIA1302, and it was introduced because the *glnA* gene sequence from *E. coli* contains a non-desired *Bst*EII restriction site internally. Finally, the intermediary pCAMBIA1302::*Spec*(+) was achieved (Clone 22) for further experiments.
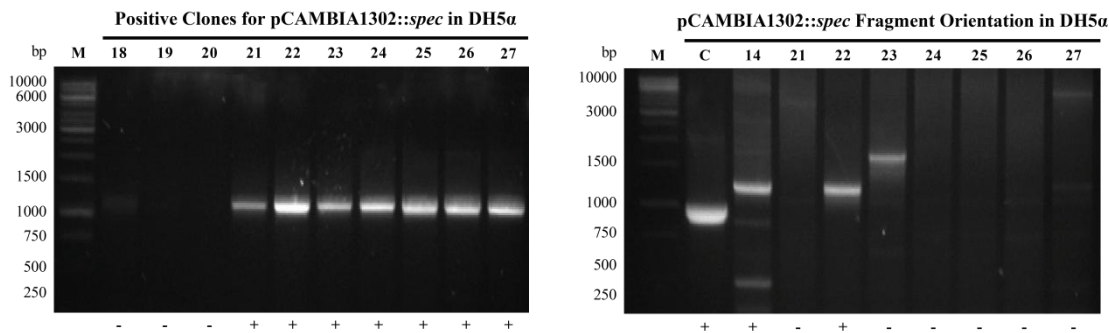
Figure 18. Screening for pCAMBIA1302::*Spec* Cloning.
(**A**) Colony PCR amplification for screening of the pCAMBIA1302::*Spec* plasmid, after transformation of *E. coli* DH5α competent cells. Representation of chosen positive colonies. (**B**) *Spec*-PCR amplification with the primer combination SpeCAMBIA1302-F and SeqCAMBIA1302-R, in order to check the right orientation of the cloned *Spec*-fragments. According to the result, clones 14 and 22 are pCAMBIA1302::*Spec*(+) and clones 21, 23,24,25,26 and 27 are pCAMBIA1302::*Spec*(-). For further experiments, the recombinant clone number 22 was chosen, expand in LB broth for minipreps and preserved in 20% Glycerol Stock at -80°C.

5.1.6   Strategy for the Construction of pCAMBIA1302::*glnA* for Recombinant GlnA Protein Expression in *Scenedesmus spp*.

In order to carry out the double (*mgfp5-Spec*) substitution cloning strategy, the intermediate pCAMBIA1302::*Spec*(+) plasmid was double-digested with the restriction enzymes *Spe*I (whose restriction site is located immediately upstream of *mgfp5* in pCAMBIA1302) and *Apa*I (whose restriction adaptor was introduced immediately downstream of *Spec*) (Figure 19). This double-digestion of the construct was able to release the *mgfp5-Spec* fragment, which was then separated from the vector backbone by running an Agarose-Gel Electrophoresis. Later, the electrophoretic band corresponding to the pCAMBIA1302 vector backbone was cutted out from the gel with a scalpel, under a minimal exposure to the UV light, and purified for further cloning experiments. On the other hand, the *glnA* gene was amplified by PCR from *E. coli* K12 genomic DNA (Figure 20A) and digested with the same restriction enzymes (*Spe*I and *Apa*I). Both, vector and insert were ligated and transformed into DH5α competent cells. Five positive clones were chosen and confirmed (Figure 20B). Clone # 1 was then chosen for preservation, minipreps and DNA sequencing (Macrogene, USA). Sequencing results, using the pCAMBIA1302 sequencing primers (Fwd and Rev), confirmed a 100% of identity between both *glnA* DNA sequences: the NCBI-annotated and the cloned *glnA* copy (Figure 22).
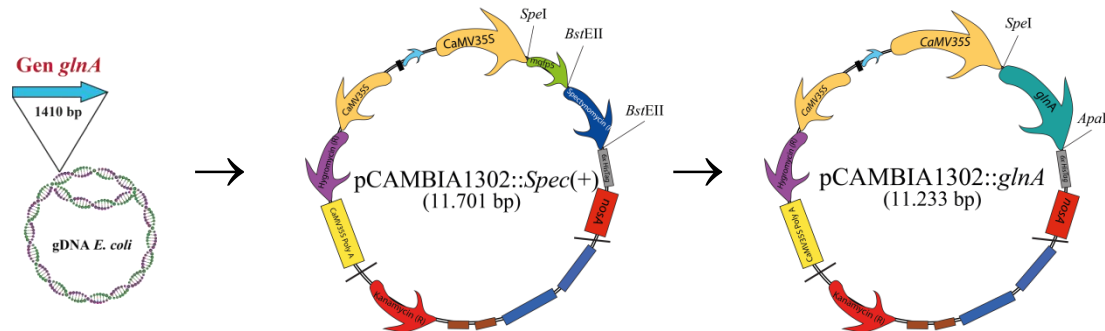
Figure 19. pCAMBIA1302::*glnA* Plasmid Construction.

The intermediary plasmid pCAMBIA1302::spec and the *glnA* amplified gene were digested with the enzymes *Spe*I and *Apa*I and ligated into the new plasmid.

A.

glnA PCR
Amplification

B. Positive Clones for pCAMBIA1302::*glnA* in DH5α
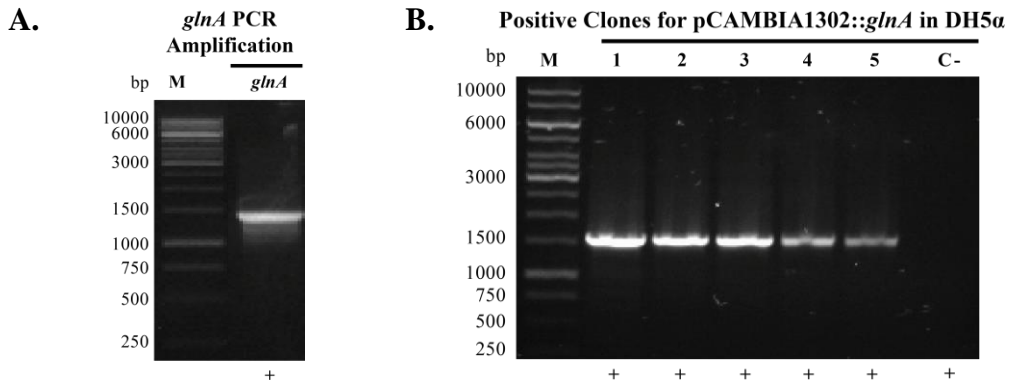
Figure 20. pCAMBIA1302::*glnA* Cloning

(**A**) Amplification of the *glnA* gene from *E. coli* K12 gDNA (1440 bp). (**B**) Separation by agarose gel electrophoresis of the Colony PCR amplification of the pCAMBIA1302::*glnA* in *E. coli* DH5α positive clones (1525 bp).

5.1.7   Transfection of *Scenedesmus spp*. with the pCAMBIA1302::*glnA* Vector

The pCAMBIA1302::*glnA* vector was transfected into *Scenedesmus spp.* by electroporation using the parameters and protocols already standardized. After 12 days of growth, cells were collected and some of the clones were confirmed by colony PCR (Figure 21) and fluorescence microscopy (Data not shown). Clones 6 (Figures 21) and clones 13 (Picture not shown) were selected for further experiments: protein extraction, SDS-PAGE, Wester-Blot and Lowry's Assays.

Positive Clones for pCAMBIA1302::*glnA* in *Scenedesmus spp.*
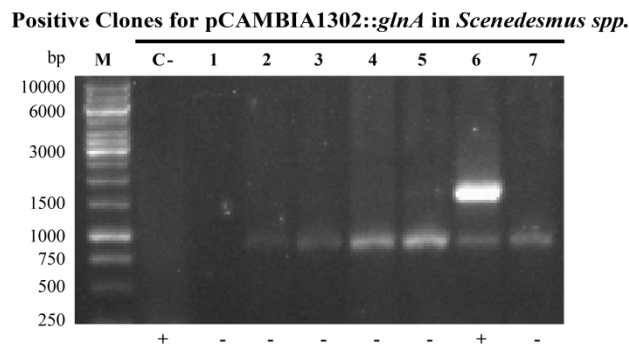
Figure 21. *Scenedesmus spp*. Transfected with pCAMBIA1302::*glnA*.

 (**A**) Separation by agarose gel electrophoresis of the PCR product from the *glnA* transfected *Scenedesmus spp*. (1653bp) colonies 1 - 7.

```
         1        10        20        30        40        50        60        70        80        90       100
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA              Start ATGTCCGCTGAACACGTACTGACGATGCTGAACGAGCACGAAGTGAAGTTTGTTGATTTGCGCTTCACCGATACTAAAGGTAAAGAAC
Rev_pCAMBIA_glnA  Start
Fwd_pCAMBIA_glnA  ATGGTAGATCTGACTAGTGCTGAACACGTACTGACGATGCTGAACGAGCACGAAGTGAAGTTTGTTGATTTGCGCTTCACCGATACTAAAGGTAAAGAAC
Consensus                    A.....GCTGAACACGTACTGACGATGCTGAACGAGCACGAAGTGAAGTTTGTTGATTTGCGCTTCACCGATACTAAAGGTAAAGAAC
                                          SpeI

         101      110       120       130       140       150       160       170       180       190      200
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   AGCACGTCACTATCCCTGCTCATCAGGTGAATGCTGAATTCTTCGAAGAAGGCAAAATGTTTGACGGCTCCTCGATTGGCGGCTGGAAAGGCATTAACGA
Rev_pCAMBIA_glnA
Fwd_pCAMBIA_glnA  AGCACGTCACTATCCCTGCTCATCAGGTGAATGCTGAATTCTTCGAAGAAGGCAAAATGTTTGACGGCTCCTCGATTGGCGGCTGGAAAGGCATTAACGA
Consensus         AGCACGTCACTATCCCTGCTCATCAGGTGAATGCTGAATTCTTCGAAGAAGGCAAAATGTTTGACGGCTCCTCGATTGGCGGCTGGAAAGGCATTAACGA

         201      210       220       230       240       250       260       270       280       290      300
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   GTCCGACATGGTGCTGATGCCAGACGCATCCACCGCAGTGATTGACCCGTTCTTCGCCGACTCCACCCTGATTATCCGTTGCGACATCCTTGAACCTGGC
Rev_pCAMBIA_glnA
Fwd_pCAMBIA_glnA  GTCCGACATGGTGCTGATGCCAGACGCATCCACCGCAGTGATTGACCCGTTCTTCGCCGACTCCACCCTGATTATCCGTTGCGACATCCTTGAACCTGGC
Consensus         GTCCGACATGGTGCTGATGCCAGACGCATCCACCGCAGTGATTGACCCGTTCTTCGCCGACTCCACCCTGATTATCCGTTGCGACATCCTTGAACCTGGC

         301      310       320       330       340       350       360       370       380       390      400
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   ACCCTGCAAGGCTATGACCGTGACCCGCGCTCCATTGCGAAGCGCGCCGAAGATTACCTGCGTTCCACTGGCATTGCCGACACCGTACTGTTCGGGGCCAG
Rev_pCAMBIA_glnA
Fwd_pCAMBIA_glnA  ACCCTGCAAGGCTATGACCGTGACCCGCGCTCCATTGCGAAGCGCGCCGAAGATTACCTGCGTTCCACTGGCATTGCCGACACCGTACTGTTCGGGGCCAG
Consensus         ACCCTGCAAGGCTATGACCGTGACCCGCGCTCCATTGCGAAGCGCGCCGAAGATTACCTGCGTTCCACTGGCATTGCCGACACCGTACTGTTCGGGGCCAG

         401      410       420       430       440       450       460       470       480       490      500
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   AACCTGAATTCTTCCTGTTCGATGACATCCGTTTCGGATCATCTATCTCCGGTTCCCACGTTGCTATCGACGATATCGAAGGCGCATGGAACTCCTCCAC
Rev_pCAMBIA_glnA  AACCTGAATTCTTCCTGTTCGATGACATCCGTTTCGGATCATCTATCTCCGGTTCCCACGTTGCTATCGACGATATCGAAGGCGCATGGAACTCCTCCAC
Fwd_pCAMBIA_glnA  AACCTGAATTCTTCCTGTTCGATGACATCCGTTTCGGATCATCTATCTCCGGTTCCCACGTTGCTATCGACGATATCGAAGGCGCATGGAACTCCTCCAC
Consensus         AACCTGAATTCTTCCTGTTCGATGACATCCGTTTCGGATCATCTATCTCCGGTTCCCACGTTGCTATCGACGATATCGAAGGCGCATGGAACTCCTCCAC

         501      510       520       530       540       550       560       570       580       590      600
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   CCAATACGAAGGTGGTAACAAAGGTCACCGTCCGGCAGTGAAAGGCGGTTACTTCCCGGTTCCACCGGTAGACTCGGCTCAGGATATTCGTTCTGAAATG
Rev_pCAMBIA_glnA  CCAATACGAAGGTGGTAACAAAGGTCACCGTCCGGCAGTGAAAGGCGGTTACTTCCCGGTTCCACCGGTAGACTCGGCTCAGGATATTCGTTCTGAAATG
Fwd_pCAMBIA_glnA
Consensus         CCAATACGAAGGTGGTAACAAAGGTCACCGTCCGGCAGTGAAAGGCGGTTACTTCCCGGTTCCACCGGTAGACTCGGCTCAGGATATTCGTTCTGAAATG

         601      610       620       630       640       650       660       670       680       690      700
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   TGTCTGGTGATGGAACAGATGGGTCTGGTGGTTGAAGCCCATCACCACGAAGTAGCGACTGCTGGTCAGAACGAAGTGGCTACCCGCTTCAATACCATGA
Rev_pCAMBIA_glnA  TGTCTGGTGATGGAACAGATGGGTCTGGTGGTTGAAGCCCATCACCACGAAGTAGCGACTGCTGGTCAGAACGAAGTGGCTACCCGCTTCAATACCATGA
Fwd_pCAMBIA_glnA
Consensus         TGTCTGGTGATGGAACAGATGGGTCTGGTGGTTGAAGCCCATCACCACGAAGTAGCGACTGCTGGTCAGAACGAAGTGGCTACCCGCTTCAATACCATGA

         701      710       720       730       740       750       760       770       780       790      800
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   CCAAAAAAGCTGACGAAATTCAGATCTACAAATATGTTGTGCACAACGTAGCGCACCGCTTCGGTAAAACCGCGACCTTTATGCCAAAACCGATGTTCGG
Rev_pCAMBIA_glnA  CCAAAAAAGCTGACGAAATTCAGATCTACAAATATGTTGTGCACAACGTAGCGCACCGCTTCGGTAAAACCGCGACCTTTATGCCAAAACCGATGTTCGG
Fwd_pCAMBIA_glnA
Consensus         CCAAAAAAGCTGACGAAATTCAGATCTACAAATATGTTGTGCACAACGTAGCGCACCGCTTCGGTAAAACCGCGACCTTTATGCCAAAACCGATGTTCGG

         801      810       820       830       840       850       860       870       880       890      900
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   TGATAACGGCTCCGGTATGCACTGCCACATGTCTCTGTCTAAAAACGGCGTTAACCTGTTCGCAGGCGACAAATACGCAGGTCTGTCTGAGCAGGCGCTG
Rev_pCAMBIA_glnA  TGATAACGGCTCCGGTATGCACTGCCACATGTCTCTGTCTAAAAACGGCGTTAACCTGTTCGCAGGCGACAAATACGCAGGTCTGTCTGAGCAGGCGCTG
Fwd_pCAMBIA_glnA
Consensus         TGATAACGGCTCCGGTATGCACTGCCACATGTCTCTGTCTAAAAACGGCGTTAACCTGTTCGCAGGCGACAAATACGCAGGTCTGTCTGAGCAGGCGCTG

         901      910       920       930       940       950       960       970       980       990     1000
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   TACTACATTGGCGGCGTAATCAAACACGCTAAAGCGATTAACGCCCTGGCAAACCCGACCACCAACTCTTATAAGCGTCTGGTCCCGGGCTATGAAGCAC
Rev_pCAMBIA_glnA  TACTACATTGGCGGCGTAATCAAACACGCTAAAGCGATTAACGCCCTGGCAAACCCGACCACCAACTCTTATAAGCGTCTGGTCCCGGGCTATGAAGCAC
Fwd_pCAMBIA_glnA
Consensus         TACTACATTGGCGGCGTAATCAAACACGCTAAAGCGATTAACGCCCTGGCAAACCCGACCACCAACTCTTATAAGCGTCTGGTCCCGGGCTATGAAGCAC

        1001 1010      1020      1030      1040      1050      1060      1070      1080      1090     1100
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   CGGTAATGCTGGCTTACTCTGCGCGTAACCGTTCTGCGTCTATCCGTATTCCGGTGGTTTCTTCTCCGAAAGCACGTCGTATCGAAGTACGTTTCCCGGA
Rev_pCAMBIA_glnA  CGGTAATGCTGGCTTACTCTGCGCGTAACCGTTCTGCGTCTATCCGTATTCCGGTGGTTTCTTCTCCGAAAGCACGTCGTATCGAAGTACGTTTCCCGGA
Fwd_pCAMBIA_glnA
Consensus         CGGTAATGCTGGCTTACTCTGCGCGTAACCGTTCTGCGTCTATCCGTATTCCGGTGGTTTCTTCTCCGAAAGCACGTCGTATCGAAGTACGTTTCCCGGA

        1101 1110      1120      1130      1140      1150      1160      1170      1180      1190     1200
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   TCCGGCAGCTAACCCGTACCTGTGCTTTGCTGCCCTGCTGATGGCCGGTCTTGATGGTATCAAGAACAAGATCCATCCGGGCGAAGCCATGGACAAAAAC
Rev_pCAMBIA_glnA  TCCGGCAGCTAACCCGTACCTGTGCTTTGCTGCCCTGCTGATGGCCGGTCTTGATGGTATCAAGAACAAGATCCATCCGGGCGAAGCCATGGACAAAAAC
Fwd_pCAMBIA_glnA
Consensus         TCCGGCAGCTAACCCGTACCTGTGCTTTGCTGCCCTGCTGATGGCCGGTCTTGATGGTATCAAGAACAAGATCCATCCGGGCGAAGCCATGGACAAAAAC

        1201 1210      1220      1230      1240      1250      1260      1270      1280      1290     1300
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   CTGTATGACCTGCCGCCAGAAGAAGCGAAAGAGATCCCACAGGTTGCAGGCTCTCTGGAAGAAGCACTGAACGAACTGGATCTGGACCGCGAGTTCCTGA
Rev_pCAMBIA_glnA  CTGTATGACCTGCCGCCAGAAGAAGCGAAAGAGATCCCACAGGTTGCAGGCTCTCTGGAAGAAGCACTGAACGAACTGGATCTGGACCGCGAGTTCCTGA
Fwd_pCAMBIA_glnA
Consensus         CTGTATGACCTGCCGCCAGAAGAAGCGAAAGAGATCCCACAGGTTGCAGGCTCTCTGGAAGAAGCACTGAACGAACTGGATCTGGACCGCGAGTTCCTGA

        1301 1310      1320      1330      1340      1350      1360      1370      1380      1390     1400
         |-----+----+---------+---------+---------+---------+---------+---------+---------+---------+---------|
E.coli-K12_glnA   AAGCCGGTGGCGTGTTCACTGACGAAGCAATTGATGCGTACATCGCTCTGCGTCGCGAAGAAGATGACCGCGTGCGTATGACTCCGCATCCGGTAGAGTT
Rev_pCAMBIA_glnA  AAGCCGGTGGCGTGTTCACTGACGAAGCAATTGATGCGTACATCGCTCTGCGTCGCGAAGAAGATGACCGCGTGCGTATGACTCCGCATCCGGTAGAGTT
Fwd_pCAMBIA_glnA
Consensus         AAGCCGGTGGCGTGTTCACTGACGAAGCAATTGATGCGTACATCGCTCTGCGTCGCGAAGAAGATGACCGCGTGCGTATGACTCCGCATCCGGTAGAGTT

        1401 1410      1420      1430      1440      1450      1460
         |-----+----+---------+---------+---------+---------+------|
E.coli-K12_glnA   TGAGCTGTACTACAGCGTCTAA Stop
Rev_pCAMBIA_glnA  TGAGCTGTACTACAGCGTCCACCCACCACCACCACCACGTGTGAATTGGGCCCGGTGACCA
Fwd_pCAMBIA_glnA
Consensus         TGAGCTGTACTACAGCGTC.A.
                                    6xHis-Tag       Stop    ApaI  BstEII
```

Figure 22. *glnA* Sequencing Result Aligned with the NCBI-Annotated *glnA* gene.

A summary of the tools designed and constructed here is depicted in Figure 23.
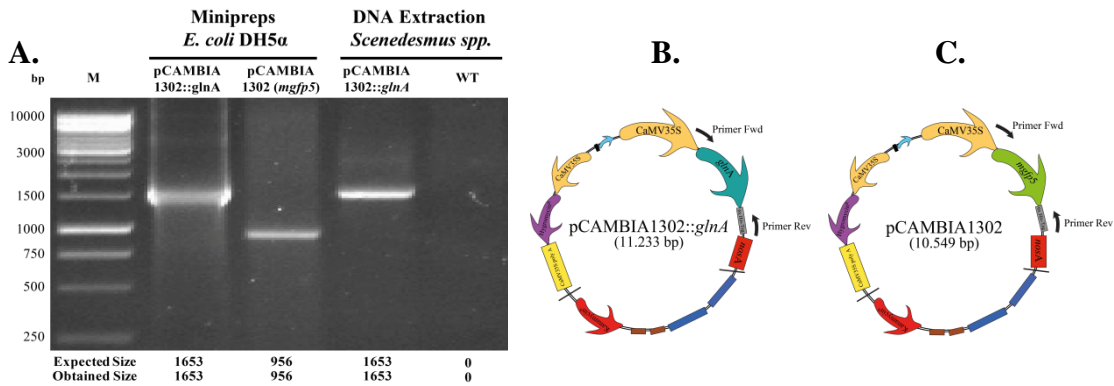
Figure 23. Summary of the pCAMBIA1302-Derived Constructs.
Sum up of the pCAMBIA1302-derived plasmids. After minipreps (*E. coli*) and DNA Extraction (*Scenedesmus spp*). (**A**) PCR were performed by using the pCAMBIA1302 primers. The *glnA* gene (1653 bp) was successfully amplified (Lanes 1 and 3), and the *mgfp*5 gene (956 bp) was also detected (Lane 2), according to the correspondent DNA templates (**B** and **C**). As expected, no bands were present on the negative control (*Scenedesmus spp* WT).

5.1.8   Protein Extraction and Confirmation of the GlnA Recombinant Protein Expression by SDS-PAGE and Immuno-Blot.

The WT and recombinant *Scenedesmus spp* cells (clones 6 and 13) were grown for protein extraction (Figure 24). After the extracting stage (Figure 24), the protein extract was subjected to SDS-PAGE, by using 8 mg of protein load per well for microalgae. A dilution of *E. coli* protein extract was used as positive control.
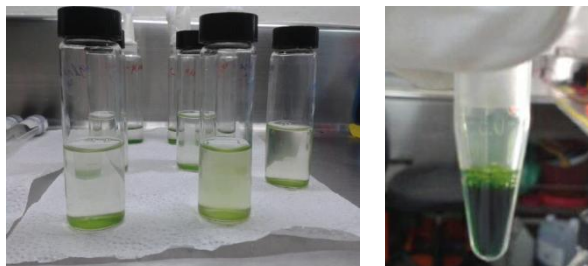
Figure 24. Protein Extraction from pCAMBIA1302::*glnA* Transfected *Scenedesmus spp*.
Transfected Clones 6 and 13, as well as the wild-type, were grown on 20 mL of CHU13 medium, supplemented with antibiotics for 10 days (Left) and subjected to a previously standardized protein extraction protocol (dark green color in the supernatant, on the Right).

Total microalgae proteins were separated by SDS-PAGE (Figure 25) and the heterologous expression of GlnA was subsequently confirmed by Western-Blot analysis (Figure 26), by using a specific polyclonal antibodies against prokariotic GlnA, and a secondary Goat anti-chicken peroxidase conjugated antibody for the developing.
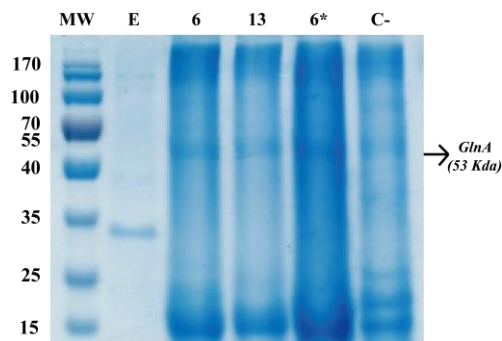
Figure 25. SDS-PAGE of Total Microalgae Protein.

The microalgae protein extract was separated on an SDS-PAGE using a 10% resolving gel and stained with Coomassie Brilliant Blue. The lanes are named as follows: **MW** (Molecular weight Pre-stained Page-Ruler protein ladder Fermentas), **E**: *E. coli* protein extract (Diluted 1/5), **6**: microalgal transfected clone 6 (first protein extract), **13**: microalgal transfected clone 13, **6***: microalgal transfected clone 6 (second protein extract); and **C-**: Wild type *Scenedesmus spp.* as negative control.
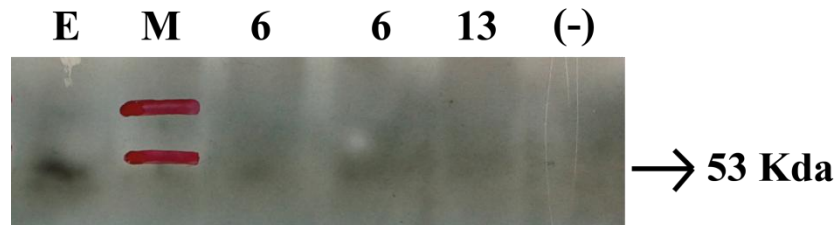
Figure 26. Detection of GlnA Protein Expression by Western-Blot.
The GlnA protein expression in *Scenedesmus spp* was confirmed by Western-Blot, by using 8 mg of total protein per well, a specific polyclonal antibody Hen anti-GlnA, and a secondary Goat anti-Hen peroxidase conjugated antibody. **M** (Molecular Weight Pre-Stained Page-

Results

Ruler Protein Ladder, Fermentas), **E**: *E. coli* total protein extract (Diluted 1/5), **6, 6*** and 1**3** are for the transfected colonies used in the different experiments, and **(-)**: Wild type *Scenedesmus spp.* as a negative control.

5.1.9   Protein Quantification from *Scenedesmus spp*

The protein content of *Scenedesmus spp* was quantified by Lowry's assay. The results showed that the total protein concentration for the transfected clones 6 and 13 was 0.72 ± 0.02 and 0.62 ± 0.005 mg/mL, respectively. For the wild-type, the same measurement showed a total protein concentration of 0.41 ± 0.02 mg/mL. Similar results were obtained when the protein content in percentage (%): clones 6 and 13 produced 68% $\pm$ 0.48 and 59% $\pm$ 2.09 of protein content respectively, while the wild-type produced a protein content of 40% $\pm$ 1.36 (Table 8). For the protein content results obtained here, the Anova showed statistically significant differences between both recombinant *Scenedesmus spp* clones (6 and 13), and the wild type, with a **p-value <0.05** (Table 9).

Table 8. Protein Content Determination for *Scenedesmus spp* after transfection.

Scenedesmus spp.	Protein Concentration (mg/mL)	Wet Biomass (mg/20mL)	Protein %
Wild type	$0,41 \pm 0.02$	21	$40\% \pm 1.36$
Clon 6	**$0,72 \pm 0.02$**	**21**	**$68\% \pm 0.48$**
Clon 13	**$0,62 \pm 0.005$**	**21**	**$59\% \pm 2.09$**

Table 9. Anova for Protein Concentration (mg/mL) and Protein Content (%)

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Concentration vs Treatment					
Between groups	0,144	2	0,072	303.668	0,000
Within groups	0,001	6	0,000		
Total (Corr.)	0,145	8			
Content vs Treatment					
Between groups	0,130	2	0,065	416.86	0,000
Within groups	0,001	6	0,000		
Total (Corr.)	0,131	8			

6. DISCUSSION

Recent studies on the microalgal genetics and molecular biology fields have allowed the design of new tools for the cloning and expression of different genes in several microalgal species. One remarkable outcome from these new tools is the pCAMBIA Vector Series for expression cloning in Microalgae and Plants. These vectors contain all the required elements for the correct cloning, selection, expression and purification of desired genes, transfected into microalgae. The use of strong promoters like CaMV35S, the availability of two antibiotic resistance genes, such as Kanamycin and Hygromycin B, and the presence of a highly efficient reporter gene like *mgfp5* (encoding for a modified version of GFP) opens a new window for the straightforward genetic engineering of this kind of organisms, contributing to increase the number of innovative and strategic biotechnological projects. Nowadays, Microalgae are used in the production of biofuels such as biodiesel, bioethanol and methane. All of them, directly related to the carbon content inside the cells. That is why, to enhance the carbon content in the cells by genetic and metabolic engineering emerges as the main focus for several studies. In contrast, for the protein content augmentation and biomass production, only few researches have obtained satisfactory results after the genetic manipulation of the Nitrogen Metabolism Pathways [6]. Hence, the focus of this research work was to induce the heterologous expression of the *glnA* gene from *E. coli* into Microalgae, by using the pCAMBIA1302 vector tools, and consider its impact on the protein content augmentation and biomass production.

Both microalgal strains used in this study, *Chlorella spp* and *Scenedesmus spp*, were selected because of their availability in the laboratory and their importance in biotechnology (i.e. production of dietary supplements for animals and biodiesel, among others products). For *Scenedesmus spp*, its lipid and protein content are well known and desired, making it suitable to be used for biotechnological process in the food and energy industries [20, 68, 77]. This microalgal strain grew properly in

Discussion

CHU13 (in 8 days of culture, they are able to reach O.D. 680 nm values of 0.9, which is equivalent to 6,53 x 10^6 Cells/mL). The growth curves obtained after the culture of both Microalgae in Wastewaters produced in two different Enterprises ("*San Fernando*" and "*Locería Colombiana*") in Medellín - Colombia, indicate their suitability to be used as cleaning machines in the city. However, due to the differences in the concentration and kind of chemical compounds present in both Wastewaters, the microalgal cell proliferation was more proficient in the "*San Fernando*" Wastewater than that from "*Locería Colombiana*". Despite these medium alternatives for the culture of Microalgae showed optimal results, they were not used here for downstream procedures, due to their heterogeneity in composition and high turbidity, which eventually makes difficult the analysis of the obtained results.

The scenario for the kanamycin and Higromycin B sensitivity was also evaluated for both Microalgae. The usefulness of Hygromycin B as a selectable marker for *Scenedesmus spp.* had not been reported in the literature, at the time. For this reason, a reference concentration range for this antibiotic, used previously to select *Chlorella spp* [78], was adopted and proved. A concentration of 30 μg/mL of Hygromycin B was then selected as optimal for the efficient selection of microalgal recombinant clones, after transfection with the original, or derived constructs from, pCAMBIA1302. Hygromycin B functions by inhibiting the polypeptide synthesis in the ribosomes, stopping the translocation mechanism.

The microalgal transfection with the pCAMBIA1302, by using the electroporation conditions suggested by Suo-Lian, 2013 was successfully achieved. The transfection efficiency was confirmed by screening of the recombinant microalgal colonies, after several washes with distilled water, using a standardized PCR protocol for the amplification of *mgfp5* gene. In addition, the recombinant expression of the mGFP from the mock (a modified version of GFP for expression in plants) was also successfully checked, as a visual marker, by fluorescence microscopy, confirming the high efficiency of the CaMV35S promoter for recombinant protein expression in Microalgae.

At the beginning of this work, a previous study had used the pCAMBIA1302 plasmid for the *E. coli glnA* gene over-expression in rice plants, reporting a 40% increased protein content [6]. Thus, the hypothesis of this research was that similar results could be obtain by the heterologous expression of the same gene in Microalgae, establishing the basis for further projects in our laboratory.

Since the *glnA* gene and the pCAMBIA1302 vector are both big in sizes, a substitution cloning strategy was selected as the best alternative for such a complex cloning procedure. Several attempts fails, when the *mgfp5* visual marker gene was target for substitution. Therefore, a *Spec* Cassette, as chemical selectable marker, was chosen for an intermediate double-substitution cloning strategy (*mgfp5-Spec*), profiting to introduce a couple of restriction adapters on its primers to facilitate the *glnA* gene cloning. The intermediate construct pCAMBIA1302::*Spec*(+) was successfully achieved, and technically it can be used in the future to clone and express many genes of interest in Microalgae. This achievement emerges as a new BioBrick element in the tool box for the genetic engineering of these organisms. Later, the introduction of the *E. coli glnA* gene into pCAMBIA1302 was straightforward, successfully achieved and confirmed, by the re-establishment of the *E. coli* DH5α sensitivity to Spectinomycin and by the absence of GFP-fluorescence ("*green signal*") inside the *Scenedesmus spp* cells under the microscope).

The heterologous protein expression of GlnA in *Scenedesmus spp* was checked by SDS-PAGE and Western-Blot, by using a chicken Anti-GlnA Polyclonal Antibody. The weak specific band on the Western-Blot picture indicates that, despite the *E. coli* GlnA recombinant protein is present in Microalgae, it is not enough over-expressed, as it would be desired. This low expression level can be explained by the fact that the CaMV35S promoter is mainly used in plants and perhaps is not so efficient in Microalgae. Nevertheless, this is a drawback to be confirmed and solved with further studies in the future.

Despite no measurements were taken in this study to evaluate the impact of the heterologous recombinant *glnA* gene expression in Microalgae, on its

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Carbohydrate or Lipid Contents, the Protein Content and Biomass Production were estimated in terms of concentration and percentage. As expected, the total protein content of 40% obtained here is in agreement with the previously reported protein content for *Scenedesmus spp* WT (Table 8) [24, 53, 79]. Hence, the protein content values obtained in this study for the pCAMBIA::*glnA* recombinant *Scenedesmus spp* clones (68% for Clone 6 and 59% for Clone 13) represent a 28% and 19% of additional protein content in the Microalgae, due to the heterologous expression of the GlnA from *E. coli*. These results indicate that the recombinant expression of GlnA do have a positive impact on microalgal Protein Content and Biomass production. In addition, it give insights into the alternative recombinant strategies, which can be properly used for the heterologous protein expression in these microorganisms.

Prospectively, the recombinant constructs and strains obtained here can be improved in the future, by considering new stronger specific promoters for gene "over"-expression in Microalgae. Moreover, the pCAMBIA::*glnA* recombinant microalgae can be used in SunCHem processes to produce Methane from wastewater more efficiently, through a higher Biomass Production.

Finally, the results obtained in this study showed the feasibility of using the pCAMBIA series vector to express successfully heterologous genes in Microalgae, for Protein Content Augmentation and Biomass Production.

7.   CONCLUSIONS

- *Scenedesmus spp* proved to be an ideal target system for the genetic manipulation of Microalgae, due to: (1) its sensitivity to the antibiotics, (2) its compatibility with the CaMV35S promoter for the expression of the proteins, and (3) its responses for Biomass Production.

- A BioBrick molecular tool or intermediate pCAMBIA-derived plasmid, the **pCAMBIA::*Spec*(+)**, was successfully achieved by genetic engineering of the commercial original plasmid pCAMBIA1302.

- The expression cloning of the *glnA* gene into pCAMBIA1302 plasmid was successfully achieved, by using a double-substitution cloning strategy, which can be straightforward performed and screened by chemical selection (Spec) and visual characterization (GFP).

- The heterologous GlnA protein expression in *Scenedesmus spp* recombinant clones was achieved and detected by Western-Blot analysis.

- The major finding in this study was the construction of the intermediary plasmid pCAMBIA1302::*Spec*(+) and the successful cloning of the *glnA* gene and its expression on *Scenedesmus spp*.

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9. ANNEXES

9.1 PCAMBIA1302 VECTOR MAP
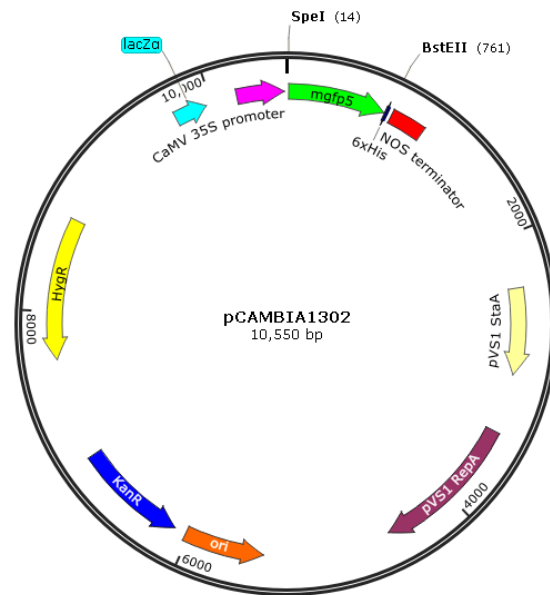
9.2 FULL-LENGHT MGFP5 PROTEIN SEQUENCE (251 a.a.)

M**VDLT**SKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
LVTTFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR
IELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKANFKTRHNIEDGGVQLADHYQQN
TPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYKASHHHHHHV
(**Protein Modification for Expression in Plants**; 6xHis-Tag).

9.3 FULL-LENGHT *mgfp5* GENE SEQUENCE (756 bp)

ATGGTAGATCTGACTAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAA
TTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACA
TACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCGTGGCCAACA
CTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAGCGG
CACGACTTCTTCAAGAGCGCCATGCCTGAGGGATACGTGCAGGAGAGGACCATCTTCTTCAAG
GACGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAACAGG
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(Start Codon; 6xHis-Tag; Stop Codon).

Annexes

9.4 FULL-LENGHT GlnA PROTEIN SEQUENCE (469 a.a.)

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9.5 FULL-LENGHT *glnA* GENE SEQUENCE (1410 bp)

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(Start Codon; Stop Codon).

9.6   PRIMER DESIGN FOR THE *glnA* EXPRESSION CLONING

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*Spe*I   *Bst*EII   *Nco*I   *Apa*I   (Start Codon; 6xHis-Tag; Stop Codon).

Minimum free energy of the structure= -0.61 kcal/mol

Minimum free energy of the structure= -6.22 kcal/mol

9.7   pCAMBIA1302::*glnA* PRIMER DESIGN

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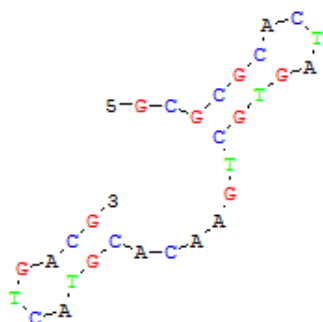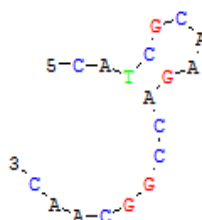`Spe`I   `Bst`EII   `Apa`I   `Bst`EII   (Start Codon; 6xHis-Tag; Stop Codon).

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Minimum free energy of the structure= -3.15 kcal/mol
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Minimum free energy of the structure= -1.00 kcal/mol
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9.8   FULL LENGHT Spec (*aad9*) GENE SEQUENCE (1114 bp) AND PRIMER DESIGN FOR CLONING INTO PCAMBIA1302

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Minimum free energy of the structure= -1.69 kcal/mol

Minimum free energy of the structure= -4.82 kcal/mol

Annexes
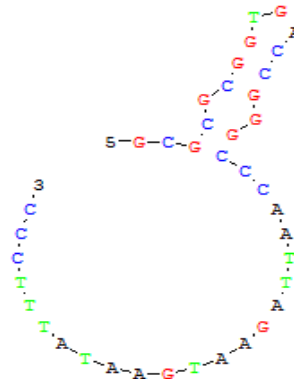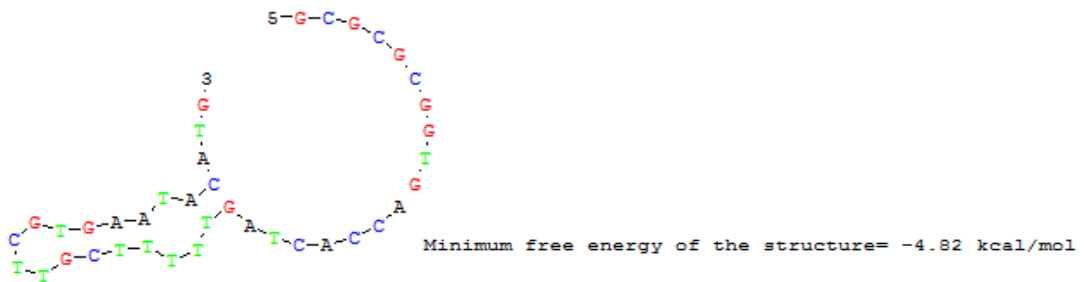
9.9 TRAINING INTERNSHIP IN THE EPFL - LAUSANNE, SWITZERLAND: THE SUNCHEM PROCESS

Wastewater treatment is a process that involves three stages for the complete removal of all organic and inorganic contaminants from the water bodies and making it suitable for human consumption. Most wastewater treatment plants finish their process at the secondary treatment stage leaving all the inorganic nitrogen and phosphorous in the water which can later induce eutrophication and environmental problems. Microalgae have been widely used in several biotechnological applications due to their features in using inorganic Carbon sources like CO_2 and the consumption of inorganic sources of Nitrogen and Phosphorous like NO_3, NO_2, and PO_3, making them an ideal microorganism for the third stage treatment in the wastewater treatment plants. At the same time, the microalgal biomass obtained from this processes can later be used in the production of biofuels such as Methane, as proposed in the SunCHem process developed in the Paul Scherrer Institute (PSI) and the École Polytechnique Fédérale de Lausanne (EPFL) in Switzerland. Following the increase in the protein content in Scenedesmus spp. the SunCHem process can be coupled as a strategy to enhance its yield, in order to do this, the microalgal strains and a non-conventional medium was evaluated for a future project involving the genetic engineering of the chosen strain.

9.9.1 Methodology

9.9.1.1 Microalgae Strains, Growth and Quantification

The microalgal strains analyzed in this study are listed on Table 1. Two different culture mediums were used, Bristol 3N and F/2 or M&M for the Chlorophyta and Diatoms respectively. They were all grown at 25 °C using LEDs for the light source with 16:8 light/dark cycles. The cell growth was quantified using a Neubauer Hemocytometer and all the counts were done by triplicates.

9.9.1.2 Hydrothermal Liquefaction Process (HTL) Aqueous Phase Analysis

The HTL aqueous phase was analyzed using IPC-OES for the presence of metals and their concentration, along with HPLC (Agilent technologies) using a Bio-Rad column HPX87-H for the quantification of organic acids and furfural and hydroxymethyl furfurals (HMF) and colorimetric kits for the determination of Nitrogen, Phosphorous, DOC and phenol (HACH).

9.9.1.3 *Phaeodactylum tricornutum* Growth on the HTL Aqueous Phase

The microalgae *Phaeodactylum tricornutum* was grown using a the HTL aqueous phase adjusted to the nitrogen conditions of the commercial mediums F/2 and M&M, the growth was followed for 14 days and the cell growth was determined.

9.9.2 Results

9.9.2.1 Microalgae Strains Growth in Commercial Medium Bristol 3N

Four microalgal strains, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Scedenesmus vacuolatus* which belong to the Chlorophyta and the diatom *Phaeodactylum tricornutum* were studied as suitable microalgae strains to be grown in a non-conventional medium.

Their growth was analyzed using Bristol 3N for the Chlorophyta and the F/2 and M&M culture mediums for the diatoms. The best growth was obtained with *Chlorella vulgaris* with a final cell density of 8,49 x 10^7 Cells/mL, but as an overall all the strains analyzed presented a good growth in this medium and a fast adaptability to it (Figure 27). However, *Phaeodactylum tricornutum* was the microalgae chosen for the Hydrothermal liquefaction because it has been previously used in the SunCHem, and its growth rate, composition and methane production have been previously standardized in this laboratory.

A.

B.

C.
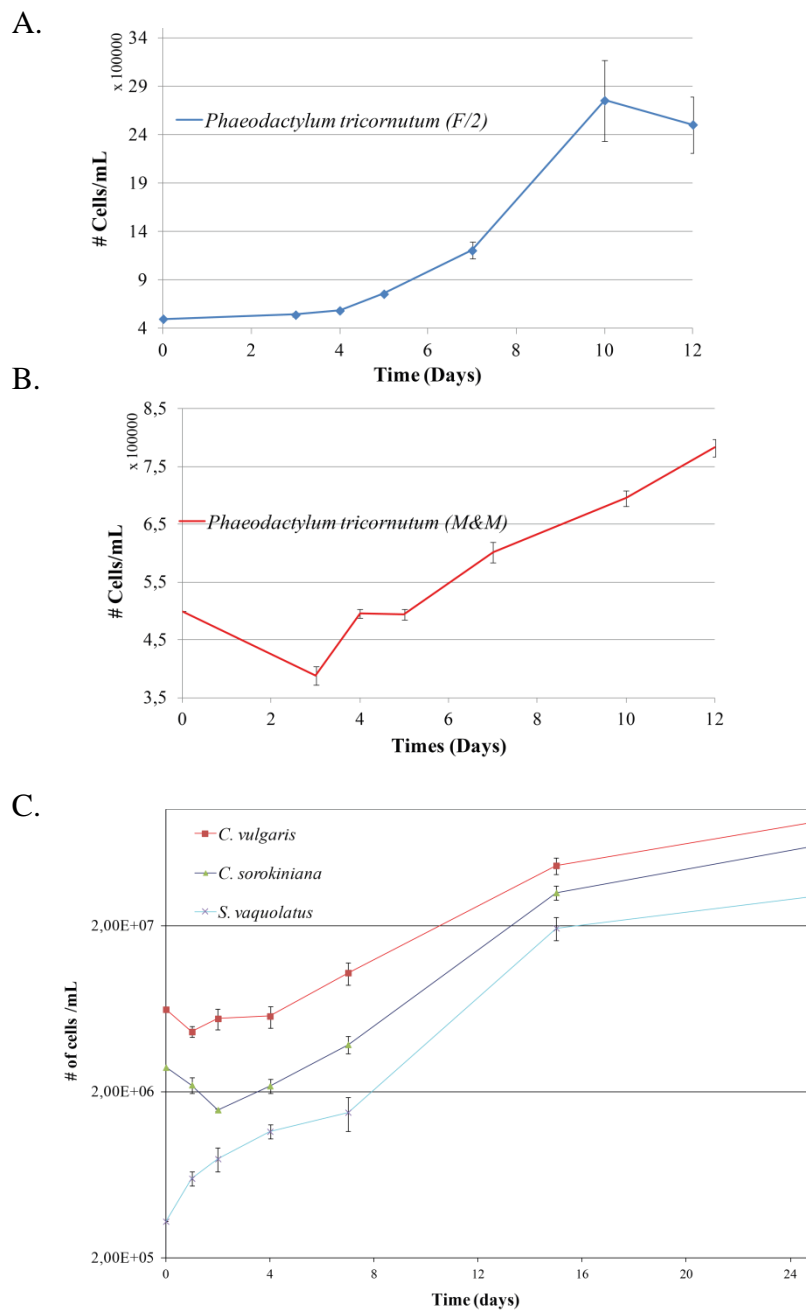
Figure 27. Growth of Microalgae in the Commercial Medium.

Phaeodactylum tricornutum was grown in F/2 and M&M medium (**A** and **B** respectively) while *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus vacuolatus* were grown in Bristol 3N (**C**) The experiments were done in triplicates**.**

9.9.2.2 HTL Aqueous Phase Characterization

The Aqueous phase of the Hydrothermal liquefaction process (HTL) was analyzed for its content in metals and organic acids by ICP-OES and HPLC respectively. The ICP-OES revealed the presence for all the trace metals that the microalgae need for its growth, but the concentrations were much lower when compared with the commercial F/2 and M&M mediums, additionally the presence of inhibitor metals like nickel was detected (Table 10). The HPLC analysis determined the presence of a wide variety of weak organic acids like Oxalic acid, Citric acid, Acetic acid, a high concentration of Ethanol but no presence of the HMF and furfurals inhibitors (Table 11).

Table 10. ICP-OES Test for Trace Metals Concentrations Analysis in the Aqueous Phase

Probe	Conz.	06022013 pH 5,3	11022013 pH 3	12022013 pH 3,4	14022013 pH 7,5	06022013 pH 5,3+Tr. HNO3	11022013 pH 3+Tr. HNO3	12022013 pH 3,4+Tr. HNO3	14022013 pH 7,5+Tr. HNO3	Kontr.H2O +Tr. HNO3	06022013 pH 5,3	11022013 pH 3	12022013 pH 3,4	14022013 pH 7,5
Cl	ppm	11.005,250	33.891,050	33.700,550	37.099,850	7.158,510	27.846,550	24.366,150	26.542,650	0,000	3.846,740	6.044,500	9.334,400	10.557,200
C	ppm	2.998,516	2.147,256	740,124	453,079	281,524	1.133,626	207,530	204,656	0,000	2.716,992	1.013,630	532,594	248,423
Na	ppm	145,680	189,503	176,729	181,807	143,131	183,272	185,481	160,188	0,000	2,549	6,231	-8,752	21,619
K	ppm	41,576	43,881	42,744	44,270	39,062	42,419	44,214	41,583	0,000	2,514	1,462	-1,470	2,687
P	ppm	20,701	32,042	35,554	21,148	17,934	29,208	36,232	18,915	0,000	2,767	2,834	-0,678	2,233
B	ppm	3,617	4,450	5,155	3,671	2,244	3,539	4,716	2,495	0,000	1,373	0,911	0,439	1,176
S	ppm	1,606	2,666	5,845	1,378	1,658	2,759	6,022	1,277	0,000	-0,052	-0,093	-0,177	0,101
Si	ppm	0,449	0,371	0,286	0,537	0,409	0,323	0,232	0,412	0,000	0,040	0,048	0,054	0,125
Ca	ppm	0,119	0,704	9,739	0,077	0,106	0,659	9,981	0,086	0,000	0,013	0,045	-0,242	-0,009
Mg	ppm	0,035	0,135	1,179	0,023	0,033	0,127	1,162	0,019	0,000	0,002	0,008	0,017	0,004
Ni	ppm	0,018	0,016	0,013	0,020	0,016	0,013	0,011	0,016	0,000	0,002	0,003	0,002	0,004
Fe	ppm	0,009	0,092	0,186	0,005	0,020	0,090	0,186	0,007	0,000	-0,011	0,002	0,000	-0,002
Ba	ppm	0,004	0,003	0,004	0,001	0,005	0,003	0,004	0,002	0,000	-0,001	0,000	0,000	-0,001
Co	ppm	0,001	0,001	0,001	0,001	0,000	0,001	0,001	-0,001	0,000	0,001	0,000	0,000	0,002
Cu	ppm	0,001	0,001	0,003	0,001	0,001	0,001	0,002	0,000	0,000	0,000	0,000	0,001	0,001
Mn	ppm	0,001	0,003	0,014	0,001	0,001	0,003	0,014	0,001	0,000	0,000	0,000	0,000	0,000
Zn	ppm	0,006	0,027	0,042	0,001	0,005	0,026	0,043	0,002	0,000	0,001	0,001	-0,001	-0,001
Mo	ppm	0,000	-0,001	0,000	0,000	-0,001	0,000	-0,001	0,000	0,000	0,001	-0,001	0,001	0,000

Table 11. HPLC Analysis of the Aqueous Phase Samples for Carbohydrates Degradation Products

/	Concentration (g/L)						
Compound	Oxalic acid	Citric Acid	Tartaric acid	Malic acid	Succinic Acid	Glycolic acid	Glycerol
Ret. Time (min)	6,905	8,075	8,624	9,557	11,532	12,262	13,57
Formula	$y = 4E\text{-}06x - 2,6361$	$y = 4E\text{-}06x - 1,5398$	$y = 4E\text{-}06x + 0,0024$	$y = 5E\text{-}06x + 0,0159$	$y = 5E\text{-}06x + 0,035$	$y = 9E\text{-}06x + 0,0301$	$y = 0,000004x + 0,0427$
2013,06,02	19,56	0,00	4,65	0,00	0,80	0,00	0,00
2013,11,02	25,59	0,00	4,00	0,00	0,70	0,00	0,50
2013,12,02	24,21	0,00	4,98	0,00	0,61	0,00	0,69
2013,14,02	12,11	0,00	3,11	0,00	0,79	0,00	0,00
2013,21,02	6,23	0,00	5,42	0,00	0,64	0,00	1,82

Table 11 *continued*. HPLC Analysis of the Aqueous Phase for Carbohydrates Degradation Products.

/	Concentration (g/L)						
Compound	Formic acid	Acetic Acid	Levulinic acid	Propionic acid	Ethanol	HMF	Furfural
Ret. Time (min)	13,877	15,081	15,574	17,58	22,481	29,19	43,713
Formula	$y = 9E\text{-}06x - 0,1381$	$y = 8E\text{-}06x + 0,113$	$y = 9E\text{-}06x + 0,0792$	$y = 7E\text{-}06x - 0,1375$	$y = 1E\text{-}05x + 0,3368$	$y = 5E\text{-}06x - 0,1113$	$y = 3E\text{-}06x - 0,2063$
2013,06,02	0,00	3,29	0,00	0,15	18,30	0,00	0,00
2013,11,02	0,00	2,65	0,00	0,00	29,53	0,00	0,00
2013,12,02	0,00	2,74	0,00	0,02	11,72	0,00	0,00
2013,14,02	0,00	3,63	0,00	0,86	13,21	0,00	0,00
2013,21,02	0,00	2,72	0,00	4,94	7,22	0,00	0,00

By using the HACH kits to determine the Nitrogen, Sulphur, Phosphorous and DOC concentration, a high levels of Nitrogen and Sulphur were detected, exceeding over 100 times the concentration found in the commercial mediums, which is a good indicative for the use of this non-conventional source for the growth of the microalgae. Phenol content was also determined using HACH kits but the concentration was much lower than the inhibitory doses (Table 12).

Table 12. Aqueous Phase Samples Physico-Chemical Parameters Determination (mg/mL)

Sample	Total Nitrogen	Ammonium NH4-N	Nitrate NO3-N	COD	Phosphate PO4-P	Phenol	pH
2013,06,02	5900	3760	700	43000	840	9,65	5,26
2013,11,02	4900	3860	500	52000	1990	10,05	2,96
2013,12,02	4900	3640	210	35000	1640	10,55	3,35
2013,14,02	6500	3860	180	34000	1090	11	7,49
2013, 21,02	7250	2640	1400	56000	1980	11	6,87

9.9.2.3 *Phaeodactylum tricornutum* Growth in the HTL Aqueous Phase

Using the information obtained above, a growth strategy was designed for the culture of *Phaeodactylum tricornutum* in a modified version of the HTL aqueous phase, adjusting its nitrogen concentration to fit the M&M and the F/2 mediums nitrogen content (Table 13). The growth showed by the diatom suggest a long adapting phase, possibly due to the presence of the organic acids and some inhibitors in lower concentration, but after the sixth day the microalgae showed a good and fast growth until the tenth day for the F/2 conditions and the thirteen day for the M&M conditions, being the best growth results for the latest (Figure 28).

Table 13. Comparison Between Nitrogen and Phosphorous Concentrations in the Standard Medium and the HTL Process Water.

	Standard Medium M&M	Standard Medium F/2	HT aqueous phase (2013,06,02)	HT aqueous phase (2013,11,02)
N (mg/L)	300	54,4	5900	4900
P (mg/L)	65	1,12	840	1990
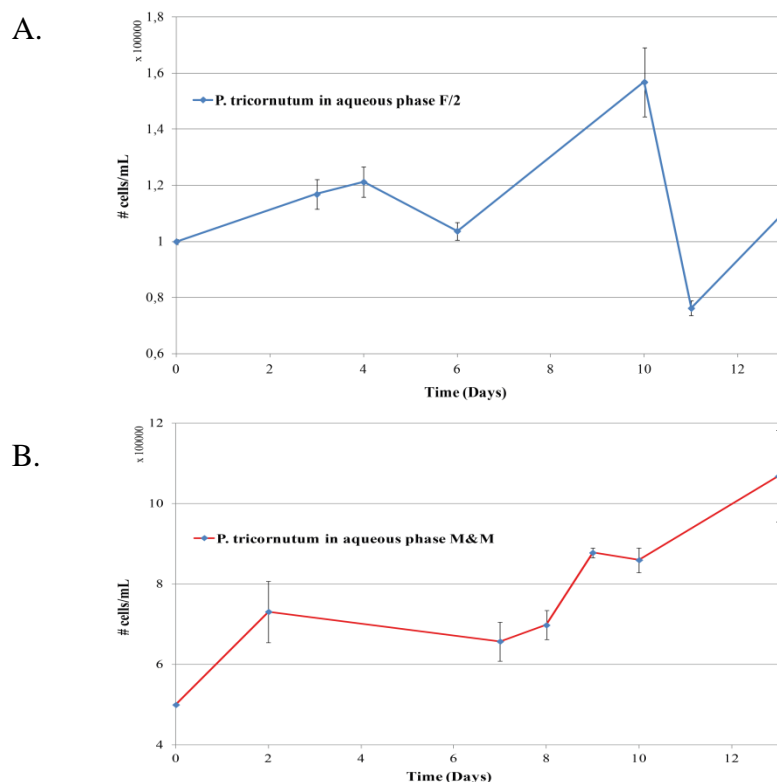
Figure 28. Growth of *Phaeodactylum tricornutum* in the HTL Aqueous Phase.
Phaeodactylum tricornutum was grown using the HTL aqueous phase, adjusted to meet the nitrogen concentrations of the commercial mediums: (**A**) F/2 medium and (**B**) M&M medium. The experiments were performed by triplicates.

9.9.3 Discussion

As a secondary result of this work, and by taking the results obtained with the genetic strategy implemented, a selection and analysis of the growth of different strains in a non-conventional medium was performed for its final conversion to methane using the SunCHem process developed at the PSI and the EPFL. This process is the result of the work and research that is being conducted in the biofuels field worldwide, and as an important notice, the use of microalgae for the production of biofuels by itself is not profitable, which is why this process works in synergy with different sub-processes that aims to obtain as much components from the microalgae as possible by reusing the non-conventional culture medium and its own energy production as a closed system. That's why the final objective in this study, was to

analyze the use of one of these wastes, in this case the aqueous phase of this process as a suitable growth medium to be re-used in the process for the biomass production. The reactors used for this processes are usually made of a metal alloy which can produce leaching of this metals to the medium and inhibit the growth of microorganisms, in this case the reactor is a stainless steel batch reactor (316SS) which is mainly composed of nickel, then after analyzing the metal content in the aqueous phase there was evidence of the presence of nickel in it (Table 10), this was also expected as the reaction condition are harsh and produce the leeching of this metals among others like titanium, and the unused catalyst itself [36, 37]. After analyzing the results obtained by HPCL during this study, one of the main issues found was the high content of weak organic acids and Ethanol (Table 11) as a sub-product from the incomplete combustion of the biomass during the process, there're several researches that explains the reason behind this situation, such as a short reaction time, low temperature or low pressure used in the reaction, this incomplete combustion leads to intermediate carbon products like the ones mentioned above along with more dangerous one like HMF and furfurals that are known to have an inhibitory effect on the microalgae [37, 80]. Finally the aqueous phase was analyzed for its nitrogen, phosphate and sulfate content, probably unused by the microalgae during their growth. The results were impressive, as a great amount of this compounds were being concentrated in this waste and had the potential to be re-used (Table 12). The interpretation of all the different elements found so far lead to the developing of an adjusted aqueous phase based on the nitrogen concentration on two of the used commercial mediums for the growth of *Phaeodactylum tricornutum,* the medium had to be adjusted by dilution over 100 times due to the high concentrations of the main components like nitrogen and phosphate. By seeing the results obtained from this growth (Figure 28), a remarkable fact is the long adaptation phase that this diatom presents in this medium possibly due to the presence of some organic acids that alters its growth, as well as the presence of the inhibitory nickel and the low concentration of the trace metals. Nevertheless, the growth obtained in the M&M medium adjusted conditions shows a good growth for this diatom up until some point

where the most reasonable explanation is the exhausting of the nutrients [81]. In conclusion, these study showed the possibility of using the genetic engineering strategy implemented previously for the enhancing of the carbon content of *Phaeodactylum tricornutum* and the functionality of using the aqueous phase of the HTL process as a suitable culture medium taking into account the physic-chemicals parameters and metals concentrations in it.

9.9.4   Concluding Remarks

The growth rate of the four different microalgae strains was fast and highly adaptable to the commercial mediums. The analysis of the different HT aqueous phase samples showed a high amount of salts and essential elements for the growth of the microalgae, such as nitrogen, sulphate, phosphate, organic carbon, some metal traces, among others. This finding shows the importance of considering the HTL aqueous phase as a growth media for the microalgae in the SunCHem process. The HPLC analysis showed a high concentration of small organic acids such as oxalic, succinic, acetic and tartaric acid as well as high concentrations of ethanol but no signs of HMF and Furfural in the HT aqueous phase which indicates a good potential to use this as organic carbon sources for the microalgal biomass production. The growth experiments showed a reduced growth of *P. tricornutum* in the aqueous phase compared to both F/2 and $M_{\&}M$ commercial media. The growth behavior in the recycled HT medium can be explained from the presence of low concentration of nutrients and the effect of possible inhibitors in the medium. This is why the lag phase of the microalgae was extended. The aqueous phase is a suitable and economically viable strategy for the microalgae biomass generation in the SunChem process if the correct dilutions are performed to guarantee a low concentration of inhibitors and sufficient amount of nutrients for the microalgal growth.

10. PARTICIPATION IN SCIENTIFIC EVENTS

10.1 INTERNATIONAL SCIENTIFIC EVENTS

i. **Alejandro Gómez,** Alejandro Acosta, Mariluz Bagnoud, Christian Ludwig and Gustavo Gámez**.** *Implementation of a genetic engineering strategy for the enhancing of the glutamine synthetase enzyme from E. coli in Scenedesmus spp.* 10th Recruitment Simposyum. Marburg - Germany, 2014. **(Oral Presentation).**

ii. **Alejandro Gómez,** Alejandro Acosta, Mariluz Bagnoud, Christian Ludwig and Gustavo Gámez**.** *Implementation of a genetic engineering strategy for the enhancing of the glutamine synthetase enzyme from E. coli in Scenedesmus spp.* IX Congreso Nacional de Micro y Macro Algas, Viña del Mar - Chile, 2014. **(Oral Presentation).**

iii. **Alejandro Gómez,** Alejandro Acosta, Mariluz Bagnoud, Gustavo Gámez, Céline Terrettaz And Christian Ludwig. *Implementation of a genetic transformation strategy to improve the biological CO_2 capture in Chlorella spp. and Scenedesmus spp*. 5th Swiss Microbial Ecology (SME) Meeting. Murten - Switzerland, 2013. **(Poster).**

10.2 COLOMBIAN NATIONAL SCIENTIFIC EVENTS

i. **Alejandro Gómez,** Alejandro Acosta, Mariluz Bagnoud, Christian Ludwig and Gustavo Gámez**.** *Implementation of a genetic engineering strategy for the enhancing of the glutamine synthetase enzyme from E. coli in Scenedesmus spp.* VII Jornadas de Investigación y Extensión Escuela de Microbiología. Medellín - Colombia, 2014. **(Oral Presentation).**

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11. CURRICULUM VITAE

PERSONAL INFORMATION

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LANGUAGES

Spanish: native language, English: good.

ACADEMIC FORMATION

➢ **Master Student in Microbiology and Bioanalysis (Last Semester).**

University of Antioquia. Medellín, Colombia. 2012 - 2014.

"Heterologous Expression of the Glutamine Synthetase Enzyme (GlnA) from *Escherichia coli* in Microalgae: Impact on Protein Content and Biomass Production"
Supervisor: Prof. Dr. Gustavo Gámez. BSc, MSc.

➢ **Microbiologist.**

University of Antioquia. Medellín, Colombia. 2006 - 2011.

"Efects of the Quorum Sensing molecules: Tyrosol and Farnesol, on the Production of Ethanol and the Celular Viability of *Saccharomyces cerevisiae".
Supervisor: Carlos Mejia. BSc, MSc.

➢ **High School in Natural Sciences**.

"Jose Maria Berrio School". Sabaneta, Colombia. 1999 - 2005.

Curriculum Vitae

PROFESSIONAL EXPERIENCE

➢ **Researcher**. January - April of 2013. Institute of Environmental Engineering (IIE - ENAC). École Polytechnique Fédérale de Lausanne (EPFL). Lausanne - Switzerland.

➢ **Researcher** (Internship). 2010-2011. Institute of Genetic and Functional Genomics. University of Greifswald. Greifswald - Germany.

➢ **Research Assistant**. 2009. Biotransformation Research Group. School of Microbiology, University of Antioquia. Medellín - Colombia.

AREAS OF STRENGTH KNOWLEDGE AND LABORATORY TECHNIQUES

➢ Microbiology, Biochemistry, Genetic Engineering, Bioprocess, Quality Assurance, Food Microbiology, Soil Management and Contaminated Waters, Biocontrol Agents for Agriculture.

➢ Research experience in genetic engineering, DNA extraction, PCR, genotyping by PCR, cloning, production of mutants, protein production and purification, restriction enzyme digestions, staining with antibodies (DIFs), microscopy (optical, confocal), western and northern blot, microalgae growth and genetic engineering of microalgae.

➢ Management of bioreactors and photobioreactors, biofuel production, determination of reducing sugars, liquefaction and saccharification of glucose rich compounds.

PARTICIPATION IN RESEARCH PROJECTS

➢ **Enhancing of the glutamine synthetase enzyme activity of *E. coli* in *Scenedesmus spp.*** School of Microbiology. University of Antioquia. Medellín - Colombia. 2014.

➢ **A sustainable production of algal biomass integrating wastewater treatment Potentials, Possibilities and Limitations in Colombia**. Institute of

Environmental Engineering (IIE - ENAC). École Polytechnique Fédérale de Lausanne (EPFL). Lausanne - Switzerland. 2013.

➢ **Genotyping of pilus islet-1, pilus islet-2, RD10-PsrP and PavB in *S. pneumoniae* clinical isolates from Germany**. Institute for Genetics and Functional Genomics. Greifswald University. Greifswald - Germany. 2010 - 2011.

➢ **Ethanol Production from Cassava flour using a Simultaneous Enzymatic Hydrolysis and Fermentation System (SEHF)**. School of Microbiology, University of Antioquia. Medellín - Colombia. 2008 - 2010.

FELLOWSHIPS AND AWARDS

➢ **Honor Tuitions** (Three Full-Tuition Scholarship Awards for 2008-1, 2008-2, 2009-1 Semesters). University of Antioquia. Medellín - Colombia.

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12. ACKNOWLEDGMENTS