Universidad de Huelva

Departamento de Ingeniería Química, Química Física y Ciencias de los Materiales

Optimización de herramientas moleculares para la manipulación genética de microalgas y su aplicación en procesos de bioflocculación

Memoria para optar al grado de doctora
presentada por:

Encarnación Díaz Santos

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Bajo la dirección de los doctores:

Rosa María León Bañares

Javier Vigara Fernández

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UNIVERSIDAD DE HUELVA

FACULTAD DE CIENCIAS EXPERIMENTALES
DEPARTAMENTO DE QUÍMICA Y CIENCIA DE LOS MATERIALES
"PROFESOR JOSÉ CARLOS VÍLCHEZ MARTÍN"

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TESIS DOCTORAL

"OPTIMIZATION OF MOLECULAR TOOLS FOR GENETIC MANIPULATION OF MICROALGAE AND THEIR APPLICATION IN BIOFLOCCULATION PROCESSES"

"OPTIMIZACIÓN DE HERRAMIENTAS MOLECULARES PARA LA MANIPULACIÓN GENÉTICA DE MICROALGAS Y SU APLICACIÓN EN PROCESOS DE BIOFLOCULACIÓN"

PROGRAMA DE DOCTORADO
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MEMORIA PRESENTADA PARA OPTAR AL GRADO DE DOCTORA POR:

Encarnación Díaz Santos

Trabajo presentado bajo la dirección de:

Dra. Rosa María León Bañares
Dr. Javier Vigara Fernández

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Directores:

Dra. Rosa María León Bañares **Dr. Javier Vigara Fernández**

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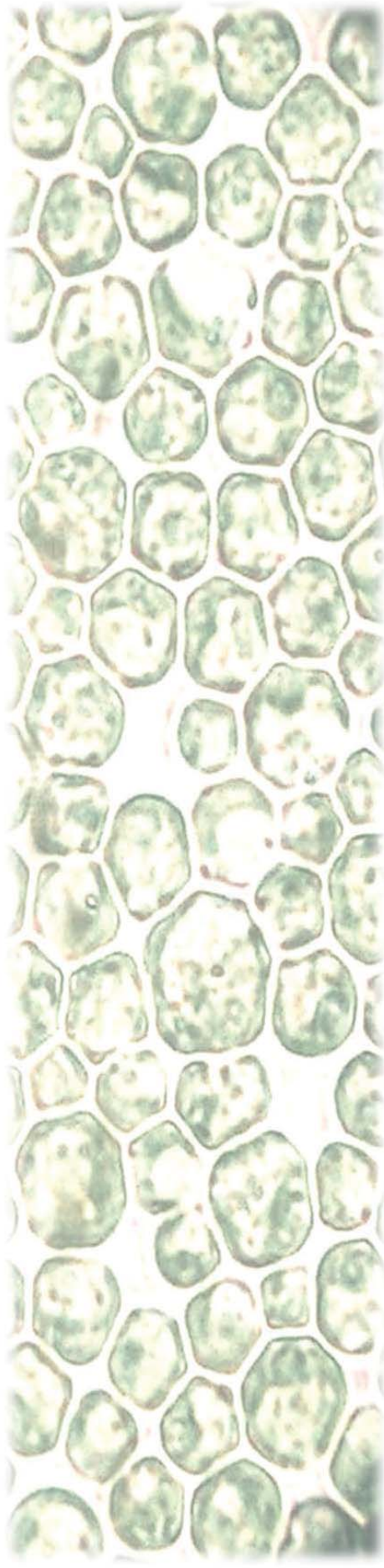
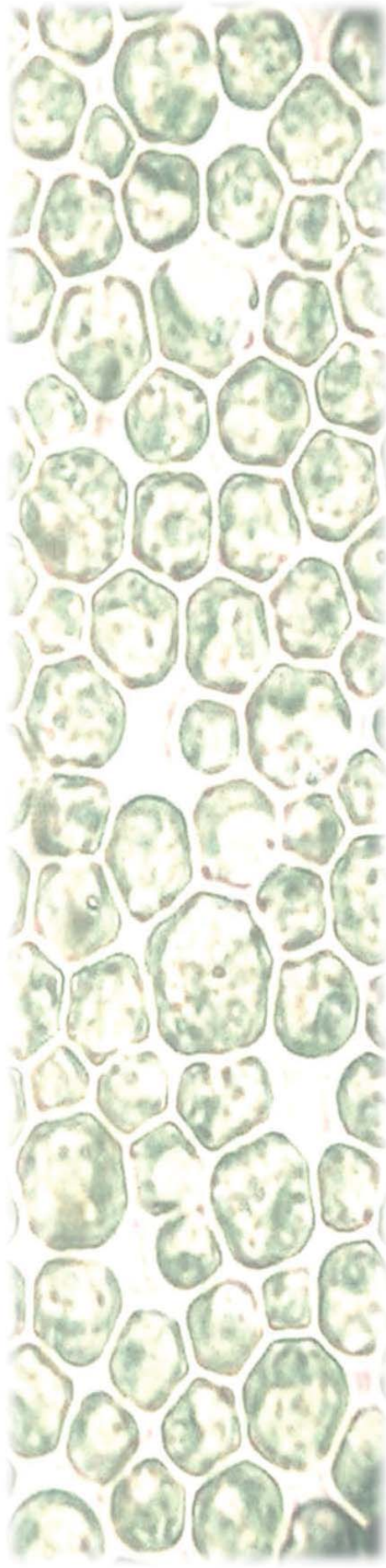


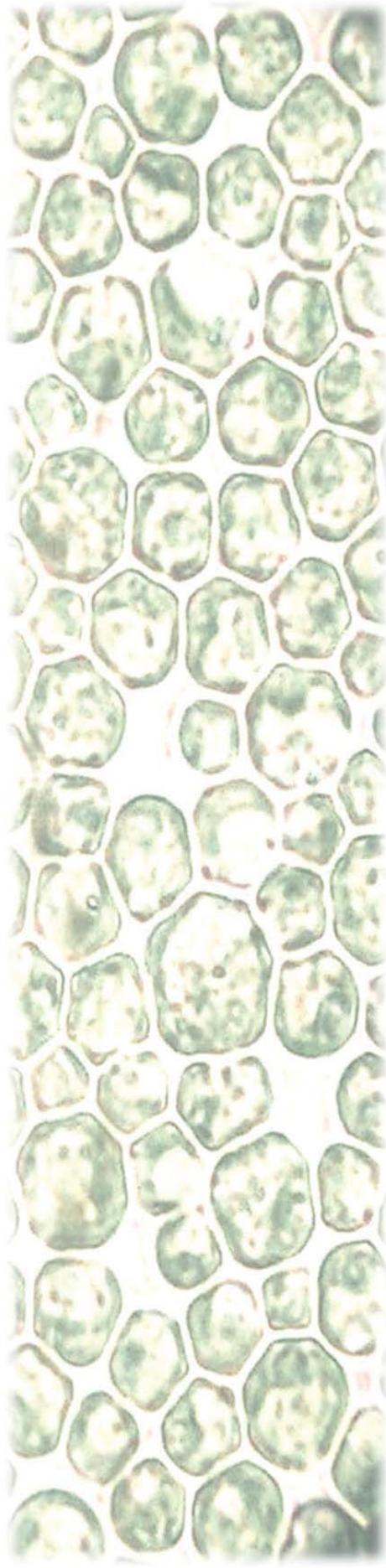
Abstract



Microalgae are a highly diversified group of eukaryotic and photosynthetic microorganisms with a relevant biotechnological potential because of they are producers of numerous bioproducts used in feed, cosmetic, pharmaceutical and bioenergy industry. Among the high-added value substances produced by microalgae, can be highlighted antioxidant carotenoid pigments (ь-carotene, lutein, astaxanthin), vitamins (A, B2, B6, B12, C, pholic acid), polysaccharides, fatty acids (ω3 and ω6) or triacylglycerols. And, in last decades microalgae have acquired an enormous industrial and commercial interest. Despite of all of these microalgae properties, the use of the genetic engineering is necessary to improve the yield of these microorganisms and achieve an economically feasible and environmentally friendly microalgal industry. In this aspect, the harvesting of the microalgal biomass becomes particularly important because is a critical step which accounts for about 20-30% of the total production cost and its improvement is necessary. In this thesis, the optimization and the development of molecular tools are performed for genetic manipulation of microalgae and their further application for the improvement of bioflocculation methods to harvest microalgal biomass. In **Chapter 2**, in *Chlamydomonas reinhardtii* transformants obtained by genetic transformation with glass beads method, the efficiency of two different heterologous promoters fused to paromomycin resistance gene from *Agrobacterium tumefaciens*, the *APHVIII* gene, is evaluated. These promoters are: the *CaMV 35S* promoter of cauliflower mosaic virus and the nopaline synthase promoter, *NOS*, from *Agrobactereium tumefaciens*. The hybrid promoter *HSP70S/RBCS2* from *Chlamydomonas reinhardtii* is used as a control. In addition, transcript and protein levels are also analysed. Among the three promoters used, the higher values of both efficiency and transcript and protein, are found for the *NOS* promoter, followed by the *CaMV 35S* promoter and the *HSP70S/ RBCS2* respectively. Although the use of heterologous promoters is an efficient tool for the expression of exogenous genes in algae, looking for strong endogenous promoters, may be a viable alternative to find an efficient and universal promoter for the majority of the microalgae species. In **Chapter 3**, the technique known as Promoter Trapping, for identifying endogenous promoters, is developed in *Chlamydomonas reinhardtii* which is genetically transformed using the *APHVIII* as the marker gene, without any promoter preceding it. Transformants which exhibit a stronger phenotype of paromomycin resistance, are selected and analysed by inverse PCR and subsequent sequencing. The genomic region preceding the gene marker inserted, is determined. In the transformants analysed, the marker gene was integrated in intragenic regions and therefore its expression was due to

an adequate insertion in frame with the endogenous genes. Some of the new identified promoters were used to drive the expression of the *APHVIII* gene, obtaining high efficiencies. In **Chapter 4,** the efficiency of the two heterologous promoters and the *APHVIII* promoterless checked in Chapter 2 and 3, are also used for genetic transformation of the industrially important microalga *Chlorella sorokiniana*. Firstly, a cheap, simple and feasible method of electroporation is performed for this microalga, defining 2.5 kV of electric field strength and 3 electric pulses as the suitable parameters. In a second part of the experiment, the efficiencies of *CaMV 35S* and *NOS* promoters are evaluated. *Chlorella sorokiniana* cells were electroporated with the chosen promoters and using the optimal parameters, and several transformants were analysed. The results showed the best efficiency values using the *CaMV 35S* promoter, in contrast with the results obtained for *C. reinhardtii*, confirming thus, that the heterologous promoters currently used are strongly specie-dependent. In the following chapters, physiological and molecular tools are developed and optimized to the improvement of bioflocculation methods for the microalgal biomass harvesting. In **Chapter 5**, the highly self-flocculating yeast *Saccharomyces bayanus var. uvarum* and its flocculating factors released into the culture medium during the fermentation, are used to induce flocculation in two microalgae: *C. reinhardtii* and *Picochlorum sp.* HM1. The addition of *Saccharomyces* cultures and flocculating factors induced cell aggregation in both microalgal species, resulting in maximum recovery efficiency values of 95% and 75% for *Chlamydomonas* and *Picochlorum* respectively. With these positive results and in order to induce self-flocculating phenotypes in *C. reinhardtii* cells, in **Chapter 6**, a wild type strain of this chlorophyte is genetically transformed with the *FLO5* flocculin gene, a dominant gene which induces self-flocculation phenotypes in the highly self-flocculating yeast *Saccharomyces bayanus var. uvarum*. Three *Chlamydomonas* transformants, CrFLO511, CrFLO513 and CrFLO520, were identified having integrated the flocculin gene into their genome and exhibiting self-flocculating phenotypes.

Resumen



Las microalgas son un grupo muy diverso de microorganismos fotosintéticos y eucariotas. Poseen un alto potencial biotecnológico debido a que son productores de numerosos compuestos bioactivos, de interés tanto en alimentación y cosmética como en farmacia o industria bioenergética. Entre ellos cabe destacar sustancias antioxidantes, como los pigmentos carotenoides (b-caroteno, luteína, astaxantina), vitaminas (A, B2, B6, B12, C, ácido fólico), polisacáridos, ácidos grasos (ω3 y ω6) o triacilgliceroles. Es por ello, que en las últimas décadas las microalgas han adquirido un importante interés industrial y comercial. A pesar de todas las buenas propiedades que poseen, se hace necesario el uso de herramientas moleculares, para la mejora del rendimiento de estos microorganismos en algunas de las fases del proceso y así poder lograr una industria microalgal económicamente viable en todas sus etapas, competitiva con otros sectores, sostenible y respetable con el medio ambiente. En este aspecto, la recogida de biomasa de microalgas se vuelve particularmente importante, debido a que es una etapa crítica en el proceso de producción representando en la actualidad alrededor del 20-30% del coste total. Por tanto, su mejora se hace necesaria para poder abaratar dichos costes. En la presente tesis doctoral, se lleva a cabo el desarrollo y la optimización de herramientas moleculares para la manipulación genética de microalgas y su posterior aplicación en la mejora de los métodos de recogida de biomasa microalgal, a través de procesos de bio y auto-floculación. En el **Capítulo 2**, en transformantes de *Chlamydomonas reinhardtii* obtenidos mediante transformación genética con perlas de vidrio, se evalúa la eficiencia de dos diferentes promotores heterólogos fusionados al gen de resistencia a paramomicina procedente de *Agrobacterium tumefaciens*, el gen *APHVIII*. Dichos promotores son el promotor *CaMV 35S* del virus del mosaico de la coliflor y el promotor *NOS* de la nopalina sintasa de *Agrobactereium tumefaciens.* El promotor híbrido *HSP70S/RBCS2* propio de *Chlamydomonas* es utilizado como control. Además, los niveles de transcrito y proteína APHVIII también son analizados. De entre los tres promotores utilizados, los valores más elevados tanto de eficiencia como de transcrito y proteína, son encontrados para el promotor *NOS*, seguido por el promotor *CaMV 35S* y el *HSP70S/RBCS2* respectivamente. Aunque el uso de promotores heterólogos es una herramienta eficiente para la expresión de genes exógenos en microalgas, la búsqueda de promotores endógenos fuertes, podría ser una alternativa viable para encontrar un promotor universal y eficiente para la expresión de exogenes en el mayor número de especies de microalgas. En el **Capítulo 3**, se desarrolla la técnica denominada como Promoter Trapping, para la identificación de promotores endógenos en *Chlamydomonas reinhardtii*. Para ello, se

transforman genéticamente células de dicha microalga, utilizando el *APHVIII* como gen marcador, sin ningún promotor que lo preceda. Los transformantes que exhiben un fenotipo más fuerte a la inserción del gen de resistencia a paramomicina, *APHVIII*, son seleccionados y mediante PCR inversa y posterior secuenciación, se determina la región genómica que precede a dicho gen y por tanto si se encuentra bajo la influencia de alguna región promotora. En la mayoría de los transformantes analizados, el gen marcador se encontró inserto en regiones intragénicas y por tanto su expresión era debida a una adecuada inserción en fase de lectura con los genes endógenos. Algunos de los promotores identificados fueron utilizados para conducir la expresión del gen *APHVIII* obteniéndose eficiencias elevadas. En el **Capítulo 4**, tanto los promotores heterólogos como el gen *APHVIII* sin ningún promotor precedente, utilizados en los capítulos 2 y 3, se emplean para la transformación genética de *Chlorella sorokiniana*, una de las microalgas actualmente con mayor potencial y relevancia a nivel industrial. En una primera fase de la experimentación, se lleva a cabo la puesta a punto del método de transformación génica. En este caso, la electroporación fue el método elegido por tratarse de un sistema barato, sencillo y fácilmente reproducible. Los parámetros óptimos de electroporación se establecieron tras varias rondas de transformación para verificar la reproducibilidad del método. Como consecuencia de ello, se establecieron los siguientes parámetros óptimos: 2,5 kV de intensidad de campo eléctrico y 3 pulsos eléctricos. En una segunda fase, utilizando éstos parámetros de electroporación, células de *C. sorokiniana* son transformadas con los promotores heterólogos previamente elegidos, fusionados al gen *APHVIII* y su eficiencia es evaluada. Los resultados mostraron los mejores valores de eficiencia utilizando el promotor *CaMV 35S*, en contraste con los resultados obtenidos para *C. reinhardtii*, donde el promotor *NOS* resultó ser el más eficiente, confirmando de este modo, que los promotores heterólogos que se utilizan en el presente estudio son fuertemente dependientes de cada especie. En los siguientes capítulos, tanto herramientas fisiológicas, como moleculares son desarrolladas y optimizadas para la mejora de los métodos bioflocculación que pueden ser adecuados para una recogida eficiente de biomasa de microalgas. En el **Capítulo 5**, la levadura altamente floculante *Saccharomyces bayanus var. uvarum* y sus factores floculantes que son liberados al medio de cultivo durante la fermentación, son empleados para inducir bioflocculación en dos microalgas: *C. reinhardtii* y *Picochlorum sp.* HM1. La adición tanto de cultivos de *Saccharomyces* como de dichos factores purificados, indujo agregación en las células de ambas especies, resultando en valores máximos de eficiencia de recuperación de biomasa del 95% y 75% para

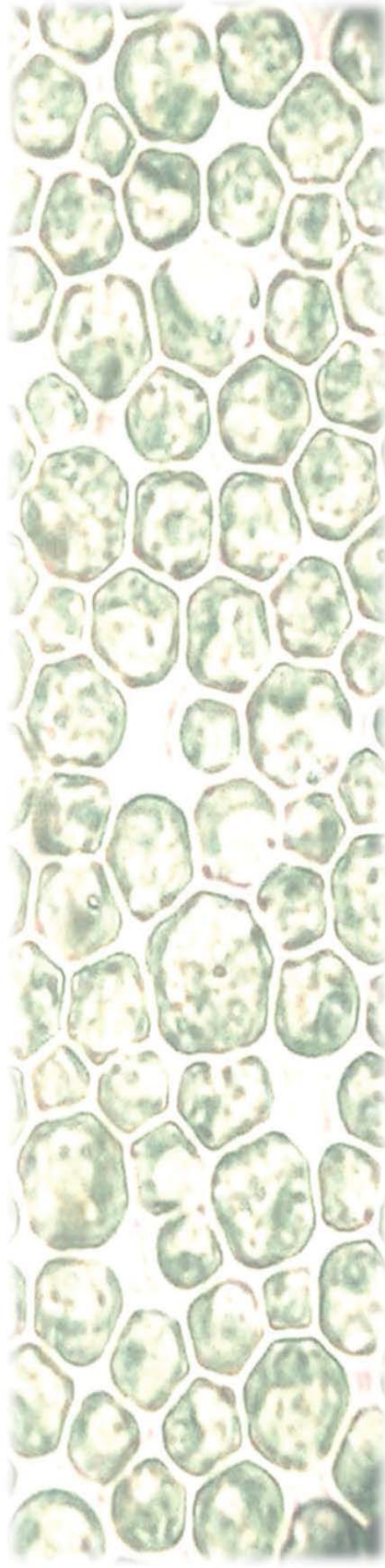
Chlamydomonas y *Picochlorum* respectivamente. Con estos resultados positivos obtenidos y con el fin de inducir fenotipos auto-floculantes en células de *C. reinhardtii*, en el **Capítulo 6**, una cepa silvestre de esta clorofita, es transformada genéticamente con uno de los genes dominantes responsables de la floculación en la levadura *Saccharomyces bayanus var. uvarum*, el gen *FLO5*. De entre los muchos transformantes de *C. reinhardtii* analizados, tres de ellos: CrFLO511, CrFLO513 y CrFLO520, son identificados habiendo integrado establemente en su genoma el gen de floculación y exhibiendo fenotipos de auto-floculación. Estos resultados determinan que aunque la maquinaria génica de regulación de los genes *FLO* es compleja, la inserción de un único gen en el genoma de una microalga no floculante, puede inducir floculación, abriendo así puertas al entendimiento de los genes que pueden estar implicados en la auto-floculación de microalgas con este fenotipo.

Chapter 1

Introduction

Thesis Outline

Objectives



1. INTRODUCTION

1.1. Microalgae and their almost endless potential

Microalgae are a big and heterogeneous group of unicellular, microscopic and photosynthetic microorganisms capable of capturing light and using its energy to convert CO_2 into sugars and oxygen. They are wildly distributed in all existing earth ecosystems, can be found in both marine and freshwater environments and they largely contribute to the global oxygen production, 50%-87%. It is estimated that more than 50,000 microalgal species exist, but only a limited number, of around 30,000, have been studied, highlighting the diatoms (*Bacillariophyceae*), the green algae (*Chlorophyceae*) and the golden algae (*Chrisophyceae*), the three most abundant classes (Mata et al., 2010; Priyadarshani et al., 2012; Enzing et al., 2014).

Microalgae can be consider as cell factories (León et al., 2004), producers of a wide range of bioactive compounds with an important commercial interest for human such as proteins, carbohydrates, lipids, or secondary metabolites with applications ranging from health, food for human consumption, aquaculture and animal feed, to colouring agents, cosmetics and biofuels (Fig. 1) (Priyadarshani et al., 2012; Skjånes et al., 2013).

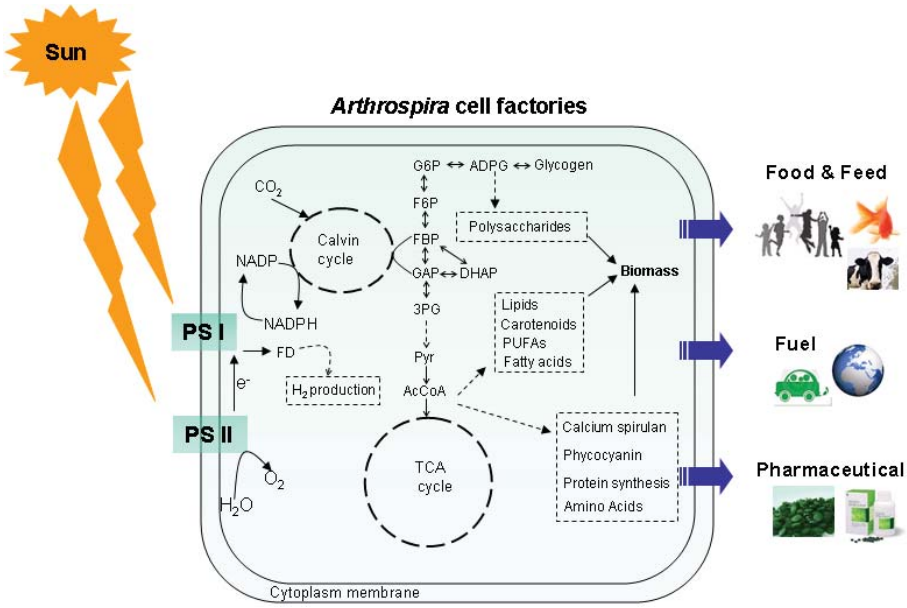


Fig.1. Example of a microalga cell factory. Schematic pathway diagram of the *Arthrospira* core metabolic process for the production of the chemical building blocks used as the precursors of bioactive compounds **(**Klanchui et al., 2012).

Human food

Although nowadays the use of microalgae for human food is subjected to several strict quality controls, algal biomass is worldwide sold as health food in different forms such as tablets, capsules and liquids. Microalgae are considered as a functional food due to their high content in fatty acids and antioxidants pigments and because they are a rich source of carbohydrates, protein, enzymes, fibres, many vitamins and minerals such as vitamin A, C, B1, B2, B6, niacin, iodine, potassium, iron, magnesium and calcium (Priyadarshani et al., 2012). Furthermore, microalgae are added to pasta, snacks, salsas, marmalades, mayonnaises, or drinks which can be found with an increasing frequency in supermarkets (Fig. 2). The main species used in human feeding are: *Spirulina maxima*, *Chlorella vulgaris*, *Dunaliella salina* and *Haematococcus pluvialis* (Pulz et al., 2004; Spolaore et al., 2010; Graziani et al., 2013).

Fig. 2. An example of a mayonnaise which contents microalgae extracts, found in a Portuguese supermarket.

Aquaculture and animal feed

Microalgae are widely used as essential live feeds and supplements in the aquaculture, in fact, 30% of the world biomass production is sold for animal feed applications (Becker et al., 2004). Microalgae are required for feed of molluscs, rotifers, shrimps or small fish larvae, being the first step in the aquatic food chain (Brown et al., 1997). Furthermore, they are also used as a source of natural pigments for the culture of prawns, salmonids and ornamental fish (Priyadarshani et al., 2012) and as a supplement for animal feed. The most frequently required species in this field are: *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira* (Spolaore et al., 2006).

Wastewaters treatment

Microalgae have a great potential in phycoremediation, mainly in treatment of wastewaters derived from municipal, agriculture and industrial activities (Pittman et al., 2011). Microalgae cultures offer a solution to convectional tertiary and quandary treatments due to the ability of these microorganisms to use inorganic nitrogen and phosphorus for their growth. And also, for their capacity to remove heavy metals, as well as some toxic organic compounds (Abdel-Raouf et al., 2012). Some of the microalgae with ability to remove nitrogen and phosphorus from different water streams belongs to the genus *Chlorella*, *Chlamydomonas*, *Scenedesmus*, *Arthrospyra* or *Phaeodactylum* (Cai t al., 2013).

Cosmetics and Pharmaceutical

The high content in antioxidant pigments and vitamins makes microalgae potential candidates to the development of cosmetics. Microalgae extracts can be mainly found in face and skin care products (anti-aging cream, refreshing or regenerant care products, emollient and as an anti-irritant in peelers). Microalgae are also represented in sun protection and hair care products (Spolaore et al., 2005). Besides, microalgae synthesize bioactive molecules such as ω-3 fatty acids or carotenoids as well as other secondary metabolites used as antiinflamatory, anticoagulant, antiangiogenic, antimutagenic, anticancer and antiviral drugs (Mimouni et al., 2012; Skjånes et al., 2013). Among the most important species used for cosmetic and pharmaceutical industry are: *Chlamydomonas reinhardtii*, *Chlorella sp*., *Phaeodactylum tricornutum*, *Porphyridium sp*. or *Haslea ostrearia.*

Biofuels

In the last decades, due to fossil fuels depletion, microalgae have been considered a potential feedstock for the production of 3rd generation biofuels, such as biohydrogen, bioethanol or biodiesel (Kleinová et al., 2012; Slade et al., 2013). Although there are still certain economic constraints, the biofuel production from renewable sources can reduce fossil fuel dependency and assist to maintain the healthy environment and economic sustainability and biofuel from microalgal biomass can provide an alternative source (Alam et al., 2012). In table 1 the most used species and their oil content are cited.

Table 1. Oil content of microalgae (Alam et al., 2012).

Microalgae specie	Oil content (% dry weight)
Botryococcus braunii	25 - 75
Chlorella sp.	28 - 32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16 - 37
Dunaliella primolecta	23
Isochrysis sp.	25 - 33
Monallanthus salina	20
Nannochloris sp.	20 - 35
Nannochloropsis sp.	31 - 68
Neochloris oleoabundans	35 - 54
Nitzschia sp.	45 - 47
Phaeodactylum tricornutum	20 - 30
Schizochytrium sp.	50 - 77
Tetraselmis suecica	15 - 23

1.2. Harvesting of microalgal biomass

Harvesting of microalgae biomass and its separation from the culture medium is a critical step in the microalgae production industry, which accounts for more than 30% of the total production cost (Salim et al., 2011; Zitelli et al., 2006). This has promoted research to improve microalgae harvesting methods. Classical physical methods of biomass recovery like filtration, centrifugation or gravity sedimentation (Molina Grima et al., 2002), and chemical flocculation by metallic cations, methanol or pH changes (Papazi et al., 2010; Scholz et al., 2011; Wu et al., 2012) are widely used. Many authors have also tested the capacity of diverse polymers as polyacrylamide grafted starch (St-g-PAM), chitosan or superabsorbent polymers (SAPs) to induce flocculation of microalgae (Farid et al., 2013; Martín del Campo et al., 2013). And, in the recent years, the scientific advances in bioflocculation processes, with flocculating factors and self-flocculating microorganisms such as bacteria, yeasts and microalgae, has largely increased (Salim et al., 2011; Guo et al., 2013; Alam et al., 2014; Díaz-Santos et al., 2014).

Self-flocculation mechanisms

Self-flocculation of microorganisms is a biological process in which cells are spontaneously aggregated together to form large flocs. This mechanism has been thoroughly studied in brewing or wine fermentative yeastdue to the high importance of these mechanisms for the wine and brewing industry. Furthermore, yeast flocculation is important for bioethanol production, biofilms formation or in pharmaceutical industry (Kondo et al. 2002; Verstrepen et al., 2006; Domímgues et al., 2000). Flocculation is a complex process in which there are multiple factors involved. Besides genetic characteristics of the strains, different genetic and physiological parameters, affect flocculation (Soares, 2010). Among the main physiological properties involved in yeast flocculation, pH, temperature, agitation, CO_2, O_2 and ethanol content, cell size, cell age and culture medium (mainly salt and glucose amount) are the highlighted. It has been confirmed that flocculation in yeast is induced by nutrient starvation, stress thermal and/or stress fermentation, thus, yeast cells gain flocculation properties in response to adverse conditions (Smit et al., 1992; Domímgues et al., 2000; Soares, 2010).

The genetic basis of yeast flocculation is quite complex and has been the object of several investigations. The main studies suggest that a family of subtelomeric genes called *FLO*, encode specific cell surface glycoproteins, like-lectins called flocculins which are responsible for flocculation (Govender et al., 2008). The flocculins in the cell walls of flocculating cells are capable of binding selectively specific carbohydrate residues of adjacent yeast cells. This mechanism of adhesion cell-cell is mainly mediated by Ca^{2+} (Miki et al., 1982; Verstrepen et al., 2003). The structural gene *FLO1* is the best known and its expression appears to be exclusively required for cell-cell adhesion. *FLO5, FLO8, FLO9* and *FLO10* dominant genes are highly homologous to *FLO1* (Fig. 3) and these five subtelomeric dominant genes causes flocculating phenotypes in non-flocculating strains at different levels of intensity strain-dependent (Govender et al., 2008; Bester et al., 2012).

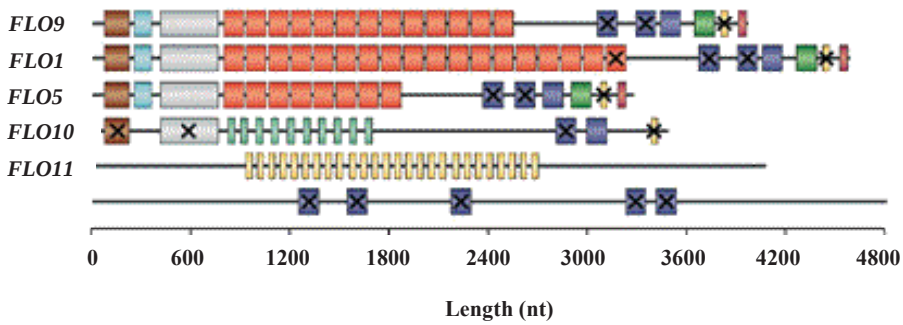


Fig. 3. Five *FLO* dominant genes and their related genomic structure (Verstrepen et al. 2004).

Biomass recovery by bioflocculation or self-flocculation is a promising sustainable and friendly environmentally strategy, but in microalgae the studies about these biological mechanisms are still scanty (Guo et al., 2013). To date several spontaneously flocculating microalgae have been identified, emphasizing the species *Ankistrodesmus falcatus*, *Tetraselmis suecica*, *Scenedesmus obliquus*, *Chlorella vulgaris* or *Ettlia texensis* (Guo et al., 2013; Alam et al., 2014; Salim et al., 2014). However, the compounds involved in the self-flocculation of microalgae and the genes responsible for the bioflocculation mechanisms In microalgae remain poorly understood. Alteration of the cellular surface charges, excreted or cellular surface-attached extracellular polymers are some of the mechanisms proposed to explain self-flocculation in microalgae (Kobayashi et al., 1996; Konno et al., 1993). And, in *Scenedesmus obliquus* AS-6-1, *Chlorella vulgaris* JSC-7 (Fig. 4) and *Ettlia etenxis* the self-flocculation factors have been physical, chemical and microscopically characterized and all of them could be closely related with the characteristics of the yeast flocculation factors (Guo et al., 2013; Alam et al., 2014; Salim et al., 2014).

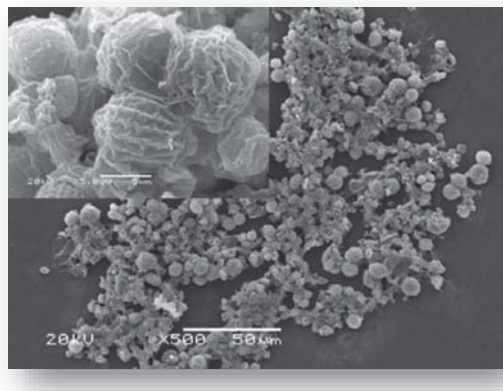


Fig. 4. An example of *C. vulgaris* JSC-7 self-flocculating cells (Alam et al**.,** 2014).

1.3. Genetic engineering of microalgae

Although microalgae have recently attracted worldwide attention because of their great biotechnological potential, including the production of biofuels, nutraceutical, therapeutic and chemical drugs and animal feeding, as was previously mentioned in section 1.1, it is important to engineer solutions for their improvement and the achievement of new and economically feasible production systems, as well as the identification of new strains with desirable biotechnological features. Genetic engineering through expression of exogenous genes or silencing of endogenous ones, could enhance the productivity of bioproducts obtained from microalgae as well as improve processes such as biomass harvesting or products

extraction (Radakovits et al., 2010; Rasala et al., 2014). However, the development of these molecular techniques has certain constraints such as the need of an efficient transformation method for each microalga species or a universal vector and promoter for the robust expression of exogenous genes, which have not been t found yet. Furthermore, while much has been written about the potential of transgenic microalgae, little of that potential has yet to be commercialized due to the numerous obstacles found nowadays for transgenic products (Rasala et al., 2014).

Genetic transformation of microalgae

Historically, the green microalgae *Chlamydomonas reinhardtii* has been considered the model for genetic manipulation and its successfully genetic transformation was firstly reported in 1989 by complementation of mutations with the corresponding homologous genes (Fernández et al., 1989; Debuchy et al., 1989). Over the time, other many species have been genetically transformed, belonging to *Chlorophyta*, *Rodophyta*, *Phaeophyta* algae, diaotms, euglenids, and dinoflagellates and to date, more than 30 different strains of microalgae have been successfully transformed (Radakovits et al., 2010). The main genetically modified species are cited in table 2.

Table 2. Different genetically transformed microalgae species (Enzing et al., 2014).

Microalgae specie	Stability of transformation
Chlamydomonas reinhardtii	Stable
Volvox carteri	Stable
Dunaliella salina	Stable
Dunaliella viridis	Stable
Haematococcus pluvialis	Stable
Chlorella sorokiniana	Stable
Chlorella kessleri	Stable
Chlorella ellipsoidea	Stable
Chlorella vulgaris	Transient
Ostreococcus tauri	Stable
Cyanidioschyzon merolae	Stable
Porphyra yezoensis	Transient
Porphyra miniata	Transient

Kappaphycus alvarezii	Transient
Gracilaria changii	Transient
Porphyridium sp.	Stable
Gracilaria	Stable
Phaeodactylum tricornutum	Stable
Navicula saprophila	Stable
Cylindrotheca fusiformis	Stable
Cyclotella cryptic	Stable
Thalassiosira weissflogii	Transient
Nannochloropsis sp.	Stable
Amphidinium sp.	Stable
Symbiodinium	Stable
Microadriaticum	Stable
Euglena gracilis	Stable

The transformation methods developed for genetic transformation of microalgae are very diverse and in the most of cases are strongly dependent of each specie, their cell surface or plasmatic membrane composition and the chosen genetic material, varying the transformation efficiencies according to these main parameters. Among the most used methods to transfer DNA into microalgal cells, glass beads agitation method, biolistic microparticle bombardment, electroporation and *Agrobacterium tumefaciens*-mediated gene transfer are found.

Glass beads method

This is a simple and repetitive method in which microalgal cells are exposed to the chosen DNA, in presence of glass beads and the membrane fusion agent polyethylene glycol (PEG) during agitation. The first report found in microalgae was for the freshwater microalgae *Chlamydomonas reinhardtii* in 1990 (Kindle et al., 1990).

Biolistic

In microparticle bombardment method, the DNA chosen to be transferred into the microalgal cells is recovering gold or tungsten particles, which are projected into the cells using a gun device (El-Sheek, 1999). This methods was firstly reported for diverse diatoms in the 90s (Apt et al., 1996).

Electroporation

Electroporation is a transformation method in which an electric field is applied to the microalgal cells to induce formation of transient pores in the cellular surface and allow the entrance of exogenous DNA.

The first efficient electroporation was achieved for *Chlamydomonas reinhardtii* in 1991 (Brown et al., 1991).

Agrobacterium tumefaciens-mediated gene transfer method

Is a natural tool to transform microalgal cells in which a segment of DNA from *Agrobacterium* Ti plasmid, known as T-DNA is transferred into microalgal cells so that the T-DNA integrates randomly into the nuclear chromosomes. The first report of a stable genetic transformation by *A. tumefaciens* in algae was conducted in the marine red seaweed *Porphyra yezoensis* (Cheney et al., 2001). In table 3, the main characteristics of the current genetic transformation methods for algae are described.

Table 3. Genetic transformation methods in algae (Qin et al., 2012).

Transformation method	Characteristics
Glass beads	The efficiency is highly dependent on many elements and this method is technically challenging
Biolistic	Exogenous DNA can be introduced into various cells and tissues. Diversified vectors can be applied to overcome the genetic background insufficiency of the substances. The manipulation is controllable and mature. But it requires specialised and high cost equipment.
Electroporation	It has simple procedure, and is used universally to different genera but constrained in brown algae.
Agrobacterium tumefaciens-mediated gene transfer	The efficiency is highly dependent on many elements and this method is technically challenging.

Silicon carbon whiskers method	It overcomes the cell wall's obstruction of exogenous DNA compared to glass beads method and is inexpensive. But it requires strict safeguards to avoid the inhalation hazard.
Microinjection	Whereas it is a highly efficient and low cost method but it has complicated and delicate procedure
Artificial transposon method	Exogenous gene could be directionally integrated into receptor's genome.
Recombinant eukaryotic algal Viruses	It has potential application in brown algae but still needs extensive and comprehensive fundamental studies.
Trans-conjugation	It is mainly used in cyanobacteria and rarely used at present.
Natural and induced transformation	It is mainly applied to cyanobacteria and rarely used at present.

Besides the choice of a suitable transformation method for genetic manipulation in microalgae, one of the crucial parts in determining the stability and frequency of exogenous DNA expression in microalgae is the availability and selection of strong promoters. Moreover, it is necessary the selection of marker genes for identifying transformants (Qin et al., 2012).

The promoters most frequently used to drive gene expression in microalgae are: endogenous promoters such as the Rubisco small subunit (*RbcS2*), the phytoene desaturase or the nitrate reductase promoters. Heterologous promoters such as the maize ubiquitin (*Ubi1*) promoter, *CaMV 35S* and *SV40* (Enzing et al., 2014) have also been used in microalgae, with different degree of success.. The cauliflower mosaic virus 35S promoter (*CaMV 35S*), is a typical promoter for strong expression in higher plants and works well in

several microalgal strains such as *C. reinhardtii* and *C. sorokiniana* (Díaz-Santos et al., 2013), while the *SV40*, the simian virus 40 promoter, a polyomavirus promoter, has been shown to work in *H. pluvialis* and in *C. reinhardtii.* Most recently, a heterologous promoter from the *A. tumefaciens* nopaline synthase (*NOS*) have also been used to transform *C. reinhardtii* cells and some species of *Chlorella* genus (Talebi et al., 2013; Díaz-Santos et al., 2013). Many different selectable marker genes have been reported in microalgae for transformants selection (León et al., 2004; Walker et al., 2005). The most frequently used are those which confer resistance to antibiotics such as paromomycin, geneticin, phleomycin or kanamycin (Talebi et al., 2013; Vila et al., 2013). Successful selection of transformants on the basis of functional complementation of metabolic mutants has also been achieved (Qin et al., 2012). In table 4, adapted from León et al. 2004 the main promoters and marker genes used in genetic transformation of microalgae are shown.

Table 4. Main promoters and marker genes in genetic transformation of microalgae.

Promoter	Gen resource	Transformed microalgae	Marker
Nopaline synthase (*NOS*)	*Agrobacterium tumefaciens*	*Chlamydomonas sp.*,*Amphidinium sp.* and *Symbiodinium sp.*	*NptII* + *Nia1*
Cauliflower mosaic virus 35S	Cauliflower mosaic virus	*Chlamydomonas*	*NptII* *Cat*
Fucoxanthin chlorophyll-a or -c binding protein	*Phaeodactylum tricornutum*	*Amphidinium* and *Symbiodinium* *P. tricornutum*	*Gus* *Ble*
RBCS2 (Rubisco)	*Chlamydomonas*	*Chlamydomonas*	*Glut1/Hup1* *Ble/NptII/Gfp/Gus* *ϵ-frustulin* *Ble+Luc*

			Gfp+Ble *AphVIII* *Ble* *Cry1-1* *Ars*
Heat shock protein Hsp70B + Hsp70A	*Chlamydomonas*	*Chlamydomonas*	
Photosystem I complex protein (PsaD)	*Chlamydomonas*	*Chlamydomonas*	*Ble/Arg7/PsaD*
Chlamyopsin (Cop)	*Chlamydomonas*	*Chlamydomonas*	*Gfp+Cop*
β2-Tubulin	*Chlamydomonas*	*Chlamydomonas*	*AphVIII*
Acetyl CoA carboxylase (Acc1)	*Cyclotella cryptica*	*C. cryptica* and *Navicula saprophila*	*NptII*
p1′2′	*A. tumefaciens*	*Amphidinium* and *Symbiodinium*	*Hpt*
Chlorophyll-ab binding (CabII-1)	*Chlamydomonas*	*Chlamydomonas*	*Nia1*

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2. THESIS OUTLINE

The present doctoral thesis is framed within the research work entitled "OPTIMIZATION OF MOLECULAR TOOLS FOR GENETIC MANIPULATION OF MICROALGAE AND THEIR APPLICATION IN BIOFLOCCULATION PROCESSES." This thesis is presented as a compendium of scientific publications arranged in chapters including a general introduction (**Chapter 1)**, and five main chapters, three of which (**Chapter 2**, **Chapter 3** and **Chapter 5)** have been published in peer-reviewed international scientific journals and other two (**Chapter 4** and **Chapter 6)** are in process to be published. Also, the main general conclusions obtained from this work are summarised at the end of the manuscript.

In **Chapter 1**, an overview of microalgae and their extraordinary biotechnological potential for food, pharmaceutical, cosmetic or biofuels industry, is described. Special attention is paid to the harvesting of microalgae biomass due to the relevance of this step in the microalgal industry. In addition, the importance of the genetic engineering to improve biotechnological properties of microalgae is presented and the main molecular tools available are described. In **Chapter 2** and **Chapter 3** different approaches to obtain efficient exogenous and endogenous promoters potentially applicable to different microalgal strains are presented and validated in the model chlorophyte *Chlamydomonas reinhardtii,* A method for the genetic transformation of *Chlorella sorokiniana*, one of the current species most industrially relevant, is then stablished and the efficiency of the heterologous promoters obtained is also evaluated in this species as described in **Chapter 4**. As we have already stated, harvesting of microalgal biomass is a crucial step of the microalgal productive process and the molecular tools developed in previous chapters can provide a new approach to deal with problems of microalgal harvesting. In **Chapter 5**, induction of bioflocculation in microalgae by the highly self-flocculating yeast, *Saccharomyces bayanus var. uvarum* and the flocculating factors that this yeast excretes into the culture medium during the fermentative phase of growth has been studied. And, finally combining the new genetic manipulation tools stablished in the first three chapters with the results of bioflocculation obtained in the fourth chapter, a molecular strategy to induce self-flocculation is approached and described in **Chapter 6**. Self-flocculating transformants of *Chlamydomonas reinhardtii* have been obtained by the genomic nuclear insertion of a gene responsible for flocculation in yeast.

3. OBJECTIVES

The main objectives aimed for this doctoral thesis are:

- To evaluate the efficiency of two heterologous promoters , the cauliflower mosaic virus 35S RNA promoter (*CaMV 35S*); and the promoter of the nopaline synthase from the Ti plasmid of *Agrobacterium tumefaciens* (*NOS*), to direct the expression of transgenes in the model chlorophyte *Chlamydomonas reinhardtii* using the aminoglycoside 3'-phosphotransferase VIII from *Streptomyces rimosus* (*APHVIII*) as selectable marker gene. **(Chapter 2)**

- To optimize a method for the identification of new strong endogenous promoter sequences in microalgae, based on the random insertion of the *APHVIII* promoterless gene in the algal genome and the subsequent identification of the flanking regions by inverse PCR. **(Chapter 3)**

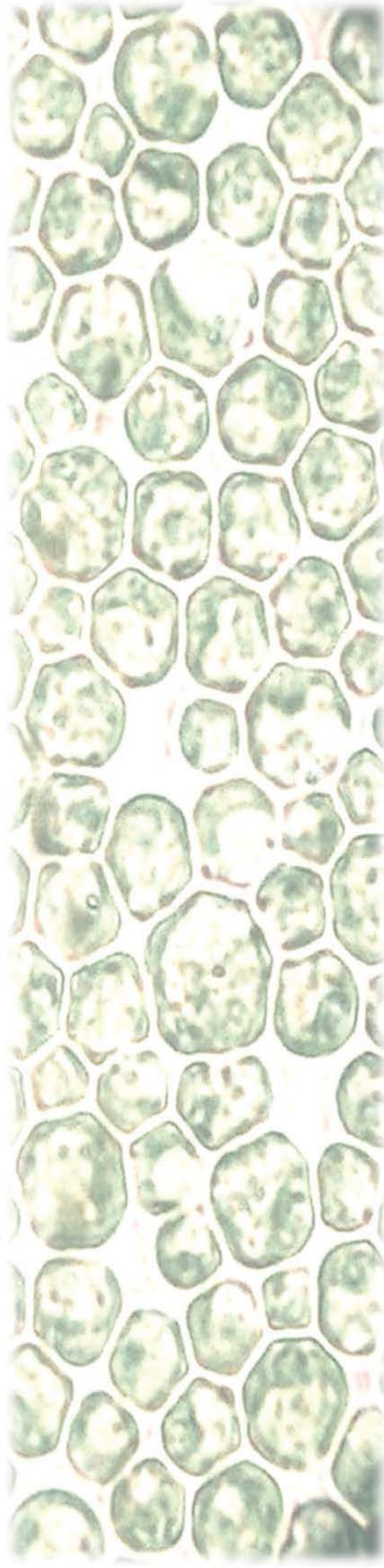
- To stablish a feasible and stable genetic transformation method for the industrial chlorophyte *Chlorella sorokiniana* and evaluate the efficiency of the cauliflower mosaic virus 35S RNA promoter (*CaMV 35S*), the promoter of the nopaline synthase from the Ti plasmid of *Agrobacterium tumefaciens* (*NOS*) and the hybrid promoter *HSP70A/RBCS2 from Chlamydomonas reinhardtii*, to conduce the expression of the paromomycin resistance gene (*APHVIII*). **(Chapter 4)**

- To gain more insights in the origin of microorganism-induced microalgal flocculation by identifying protein factors able to induce aggregation of microalgae and evaluate the effect of the flocculating yeast *Saccharomyces bayanus var. uvarum* CECT 1969 and the proteins released into the culture medium by this flocculating yeast, on two species: the model freshwater microalga *Chlamydomonas reinhardtii* and the novel marine microalga *Picochlorum sp.* HM1. **(Chapter 5)**

- To obtain self-flocculating transformants of *Chlamydomonas reinhardtii* by the nuclear expression of a flocculin gene from the highly self-flocculating yeast *Saccharomyces bayanus var. uvarum*. **(Chapter 6)**

Chapter 2

"Efficiency of Different Heterologous Promoters in the Unicellular Microalga Chlamydomonas reinhardtii."



This chapter has been published as: ***Díaz-Santos E**, de la Vega M, Vila M, Vigara J, León R. Efficiency of different heterologous promoters in the unicellular microalga Chlamydomonas reinhardtii. Bioresourc Technol.* ***2013****;29(2):319-328.*

PUBLICACIONES

El artículo "*Efficiency of Different Heterologous Promoters in the Unicellular Microalga Chlamydomonas reinhardtii*" que forma parte del Chapter 2 ha sido retirado de la tesis debido a restricciones relativas a los derechos de autor. Dicho artículo ha sido sustituido por la referencia bibliográfica, así como por el enlace al texto completo (solo miembros de la UHU), enlace a la revista y resumen.

Díaz Santos, E., Vega Naranjo, M. de la , Vila Spínola, M., Vigara Fernández, J., León Bañares, R.M.: "Efficiency of Different Heterologous Promoters in the Unicellular Microalga Chlamydomonas reinhardtii". Biotechnology Progress. Vol. 29, n. 2, págs. 319–328, (2013). DOI: 10.1002/btpr.1690

Enlace al texto completo del artículo (solo para miembros de la UHU):
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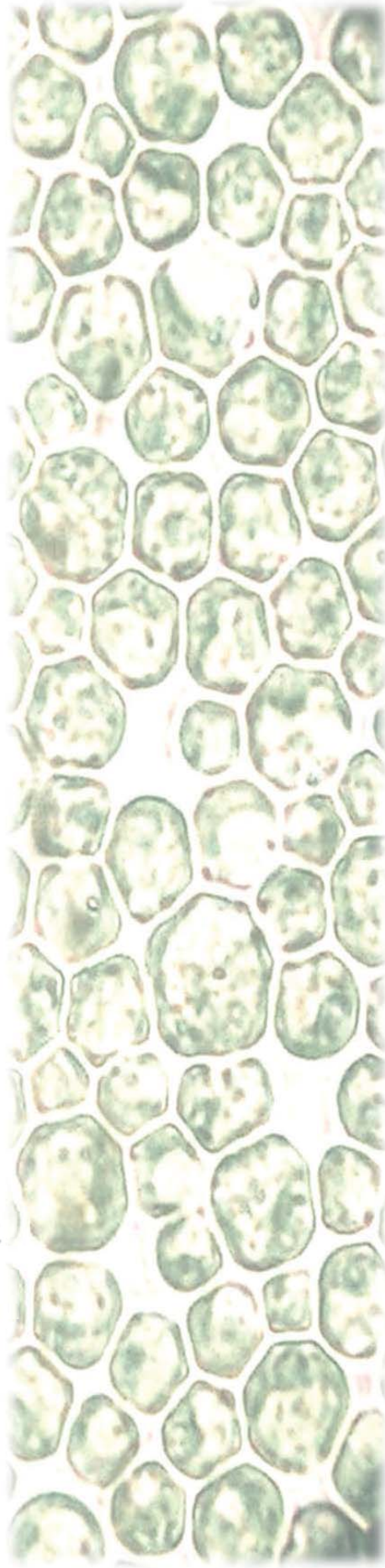
RESUMEN:

Despite the biotechnological interest of microalgae, no robust and stable methods for genetic transformation of most microalgal strains exist. The scanty and disperse data about the efficiency of heterologous promoters in microalgae and the use of different transformation methods, DNA quantities and reporter genes in the existing studies makes very difficult a real comparison of their efficiency. Using Chlamydomonas reinhardtii as a host, we have evaluated the efficiency of the heterologous promoters of cauliflower mosaic virus 35S (CaMV 35S) and Agrobacterium nopaline synthase (NOS) genes. These promoters were fused to the paromomycin conferring-resistance aminoglycoside 3'-phosphotransferase encoding gene (APHVIII), and C. reinhardtii was transformed by the glass beads agitation method. The transformation efficiency and the APHVIII transcript and protein levels were evaluated in a series of transformants for each promoter. The chimeric promoter HSP70A/RBCS2 and the promoter-less APHVIII marker gene were used for comparison. We found significantly higher transformation efficiencies and higher level of APHVIII expression in those transformants harboring the NOS promoter than in those transformed with CaMV 35S promoter. The NOS promoter, widely used for genetic manipulation of higher plants, has been very rarely used for the transformation of microalgae. The results shown here suggest the possibilities of this heterologous promoter as an efficient system for the genetic manipulation of microalgae.

Chapter 3

"Promoter Trapping in Microalgae Using the Antibiotic Paromomycin as Selective Agent."



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Article

Promoter Trapping in Microalgae Using the Antibiotic Paromomycin as Selective Agent

Marta Vila [1], Encarnación Díaz-Santos [1], Marta de la Vega [1], Herminia Rodríguez [2], Ángeles Vargas [2] and Rosa León [1,*]

[1] Biochemistry Laboratory, Experimental Sciences Faculty, University of Huelva, Huelva 27071, Spain; E-Mails: marta.vila@dqcm.uhu.es (M.V.); encarnacion.diaz@dqcm.uhu.es (E.D.-S.); marta.delavega@dqcm.uhu.es (M.V.)

[2] Institute of Plant Biochemistry and Photosynthesis, CIC Cartuja, University of Seville and CSIC, Seville 41092, Spain; E-Mails: hrm@us.es (H.R.); avargas@us.es (A.V.)

* Author to whom correspondence should be addressed; E-Mail: rleon@uhu.es; Tel.: +34-959-219951; Fax: +34-959-219942.

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Abstract: The lack of highly active endogenous promoters to drive the expression of transgenes is one of the main drawbacks to achieving efficient transformation of many microalgal species. Using the model chlorophyte *Chlamydomonas reinhardtii* and the paromomycin resistance *APHVIII* gene from *Streptomyces rimosus* as a marker, we have demonstrated that random insertion of the promoterless marker gene and subsequent isolation of the most robust transformants allows for the identification of novel strong promoter sequences in microalgae. Digestion of the genomic DNA with an enzyme that has a unique restriction site inside the marker gene and a high number of target sites in the genome of the microalga, followed by inverse PCR, allows for easy determination of the genomic region, which precedes the *APHVIII* marker gene. In most of the transformants analyzed, the marker gene is inserted in intragenic regions and its expression relies on its adequate insertion in frame with native genes. As an example, one of the new promoters identified was used to direct the expression of the *APHVIII* marker gene in *C. reinhardtii*, showing high transformation efficiencies.

Keywords: *APHVIII*; *Chlamydomonas reinhardtii*; microalgae transformation; paromomycin; promoter trapping

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1. Introduction

In recent years, the number of genetically modified microalgal species has been slowly increasing, but it is still scanty. The lack of specific promoters and good methods for DNA delivery inside the nucleus are the main drawbacks to achieving efficient transformation of many of these species. The first and best studied transformed microalga is *Chlamydomonas reinhardtii*, which is still the favorite model system. This freshwater chlorophyte was transformed in 1989 by the complementation of a mutant form of the *NIT1* gene with the corresponding homologous nitrate reductase gene [1]. The genetic transformation of the diatoms *Cyclotella criptica* and *Navicula saprophila* was first reported in 1995 [2] and soon thereafter reports describing the transformation protocols for *Phaeodactylum tricornutum* appeared [3]. Since then, a significant number of selectable markers, promoters and new procedures for efficient introduction of DNA into the *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum* nucleus have been developed [4]. Nevertheless, the number of transformed species has timidly increased, and the majority of the work on transgenic microalgae is still being performed with the model species, especially with the chlorophyte *Chlamydomonas reinhardtii*. In this system, easy procedures for genetic manipulation of nuclear and plastidic genomes and a complete kit of molecular and bioinformatic tools have been developed [5–9].

Heterologous promoters have allowed transient or low efficient expression of marker genes in some species of green microalgae [10,11]. However, at present, the best transformation efficiencies and the most stable transformants are obtained with endogenous promoters [12]. As concerns the classical microalgal transformation systems referred, *Chlamydomonas* and *Phaeodactylum*, there is a wide collection of strong constitutive homologous promoters, such as the rubisco small subunit *RBCS2* promoter fused to the heat shock protein (*HSP70A*) promoter of *C. reinhardtii* or the fucoxanthin chlorophyll binding protein (*FCPA*) promoter of *P. tricornutum*, which are extensively used to drive the expression of heterologous proteins in these microalgae [13]. However, the isolation of endogenous promoters for the genetic transformation of other chlorophytes has only been described in *Chlorella sorokiniana* [14], *Dunaliella* [15,16], *Haematococcus pluvialis* [17], *Ostreococcus tauri* [18] and *Nannochloropsis* [19,20]. In the three first cases, the transformation was based on complementation of deficient mutants with a homologous gene of nitrate reductase, for *Chorella sorokiniana* and *Dunaliella*, and of phytoene desaturase, for *Haematococcus pluvialis*. In some species, endogenous strong promoters, controlling genes that encode abundant proteins, did not yield good transformation efficiencies. Walker *et al.* [12] isolated the flanking regions of *Dunaliella tertiolecta RBCS2* genes. These promoters were shown to drive the expression of bleomycin resistance gene (*BLE*) in the related microalga *Chlamydomonas reinhardtii* [12] and in the own *Dunaliella tertiolecta*, but with very low efficiency [16]. This shows that much more work is necessary to develop transformation systems with endogenous constitutive promoters that allow an efficient and stable expression of transgenes in the wide range of microalgal strains.

The choice of highly active endogenous promoters to drive the expression of transgenes is thus a critical first step in the development of efficient transformation systems in microalgae. New promoters are generally isolated by chromosomes walking based on gene encoding sequences or retrieved from the genome sequence data, if available. Promoter trapping method has also allowed the isolation and characterization of a good number of novel promoters in higher plants [21,22]. The method basically

consists on the generation of a collection of transformants with random insertions of a promoter-less reporter gene, and the subsequent sequencing by different strategies of the genomic region preceding the marker insertion in the transformants selected. However, in microalgae only a few examples of promoter trapping have been reported [23,24].

A good number of marker/reported genes have been isolated for *Chlamydomonas reinhardtii*, some of which have been successfully used for screening of differentially regulated genes [25] and for identification of new genes by forward [26–28] or reverse [29] genetic approaches. In 1997, Haring and Beck [23] described the identification of new promoters in *Chlamydomonas* using the promoter-less radial spoke protein (RSP3) as selectable marker. However, this system involves the necessary use of a mutant strain as a host. In the present work, we propose the use of the promoter-less gene *APHVIII* from *Streptomyces rimosus*, which encodes for an aminoglycoside 3′-phosphotransferase and provides resistance to the antibiotic paromomycin, for promoter trapping in microalgae. The *APHVIII* gene has been shown to work under the control of endogenous promoters in *Chlamydomonas* [30] and in the closely related species *Gonium pectorale* [31]. In addition, we have shown that the antibiotic paromomycin can be used as selective agent in a good number of chlorophytes. In this work, we have fused the *APHVIII* gene with the 3′ UTR region of the nopaline synthase and we have used this construction for transformation of *Chlamydomonas reinhardtii*. We demonstrate that random insertion of the *APHVIII* promoterless gene and subsequent sequencing of the marker flanking region allows the easy and quick identification of new promoter sequences. The method is validated in the model chlorophyte *Chlamydomonas reinhardtii* and the possibility of using it on other microalgae is discussed.

2. Results

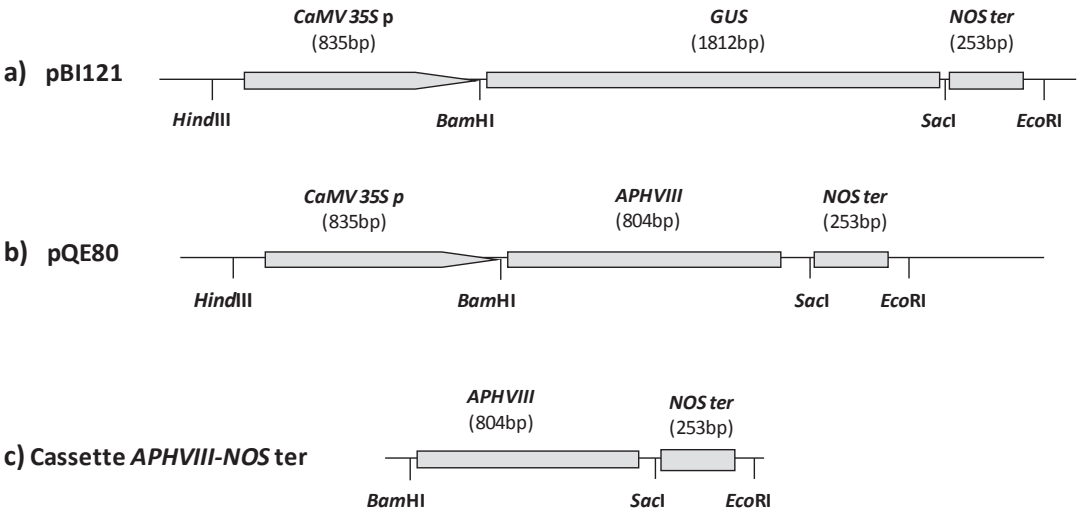
2.1. Construction of a Promoter-Less Cassette Suitable for Promoter Trapping in Microalgae

To synthesize the *APHVIII* promoter-less construction, the *CaMV 35S* p-*GUS*-*NOS* ter cassette was excised from the binary vector pBI121 [32], and subcloned between the *Hind*III and *Eco*RI sites of pQE80 plasmid (Qiagen). The cassette, which contains the *35S* promoter of the Cauliflower mosaic virus, the β-glucuronidase (*GUS*) gene and the *NOS* terminator region from *Agrobacterium tumefaciens*, was modified by exchanging the *GUS* gene by the A*PHVIII* gene from *Streptomyces rimosus*, previously obtained by PCR amplification from the pSI103 plasmid [33]. The resulting construction contains the *APHVIII* gene flanked by the *CaMV 35S* promoter and the *NOS* terminator region. The *APHVIII* promoter-less cassette was obtained from this construction by digestion with *Bam*HI and *Eco*RI (Figure 1).

The reporter gene chosen, *APHVIII*, has proved to be a good marker gene for *Chlamydomonas* [30], and other chlorophytes. It has very stable activity and provides an efficient screening system. Here we carry out the molecular characterization of the transformants obtained with the *APHVIII* promoter-less construction to check if the expression of the marker gene is the result of *in vivo* fusion with endogenous promoters. The *APHVIII* gene is simultaneously used as selectable and reporter gene. The 3′ UTR of *RBCS2*, which has been reported to have a certain antisense promoter activity [30], was avoided as terminator region; instead, we used the nopaline synthase terminator region. In this way, we

ensure that the paromomycin tolerance phenotype in the obtained mutants is due to endogenous promoters and not to the reverse promoter activity of 3′ *RBCS2* UTR.

Figure 1. Schematic structure the *APHVIII-NOS* ter promoter-less cassette and the constructions generated during its construction. The *CaMV 35S* p-*GUS-NOS* ter cassette was excised from the binary vector pBI121 (**a**); The *GUS* gene was substituted by *APHVIII* (**b**) and finally the *APHVIII-NOS* ter promoter-less cassette excised by digestion (**c**). The more relevant restriction sites are indicated.



2.2. Transformation of C. reinhardtii *with the* APHVIII *Promoter Trapping Construction and Analysis of Transformants*

C. reinhardtii was transformed with 150 ng of the *APHVIII-NOS* ter cassette excised by digestion with *Bam*HI and *Eco*RI restriction enzymes from the pQE80-*CaMV 35S* p-*APHVIII-NOS* ter plasmid (Figure 1). A control with an equivalent quantity of the linearized pSI103 plasmid was also included. This plasmid designed by Sizova and coworkers [30] contains the *APHVIII* gene flanked by the heat shock protein 70A (*HSP70A*), the rubisco small subunit 2 (*RBCS2*) promoters and the 3′ untranslated region of the *RBCS2*. The transformation rate observed in *Chlamydomonas reinhardtii* transformed with the linearized pSI103 control is 1.8×10^{-6} transformants per cell and µg of DNA while the transformation with the promoter-less cassette was 2.6×10^{-7}, which means about 14% of the transformation rate observed for the control plasmid (Table 1).

Twenty transformation reactions with 150 ng of the trapping cassette allowed the isolation of 70 paromomycin-resistant transformants that were cultured in TAP medium with increasing quantities of paromomycin over three days. 100 µL of a well-grown culture of each mutant were inoculated in 3 mL tubes of TAP medium, containing 30, 40 and 50 µg mL^{-1} of paromomycin. Five per cent of the transformants lost their resistance to the antibiotic and were discarded; about 24 transformants (35%) were able to survive in the presence of 50 µg mL^{-1} of paromomycin. Ten transformants were selected

among those that grew more vigorously in the presence of paromomycin (50 μg mL^{-1}) for further molecular analysis.

Table 1. Comparison between the efficiency of transformations with the promoter-less *APHVIII-NOS* cassette and the control pSI103 plasmid. The number of transformants obtained per reaction (mean value and error) and the transformation rate are shown for the linearized pSI103 plasmid and for the *APHVIII-NOS ter* cassette. About 150 ng of DNA and 10^8 cells were used for each transformation.

Transformation cassette	*N* of transformants per reaction	Transformation rate (transformants $cell^{-1}$ μg^{-1} DNA)
Linearized pSI103	28 ± 5 (*n* = 3)	1.8×10^{-6}
APHVIII-NOS ter cassette	4 ± 2 (*n* = 20)	2.6×10^{-7}

2.3. Molecular Characterization of the Selected Transformants

DNA from the obtained transformants was isolated by rapid DNA isolation method as described in the experimental section to check integration of the marker gene in the genome. A simple Real-Time qPCR based method, previously validated by Gonzalez-Ballester *et al.* [29] and based on *NIA1* (nitrate reductase) as a unique-copy reference gene, was used to determine the *APHVIII* copy number in each transformant. Briefly, the method consists on comparing the Ct value for *APHVIII* relative to the Ct for amplification of *NIA1* in each transformant and in a selected control transformant with a unique copy of the *APHVIII* gene.

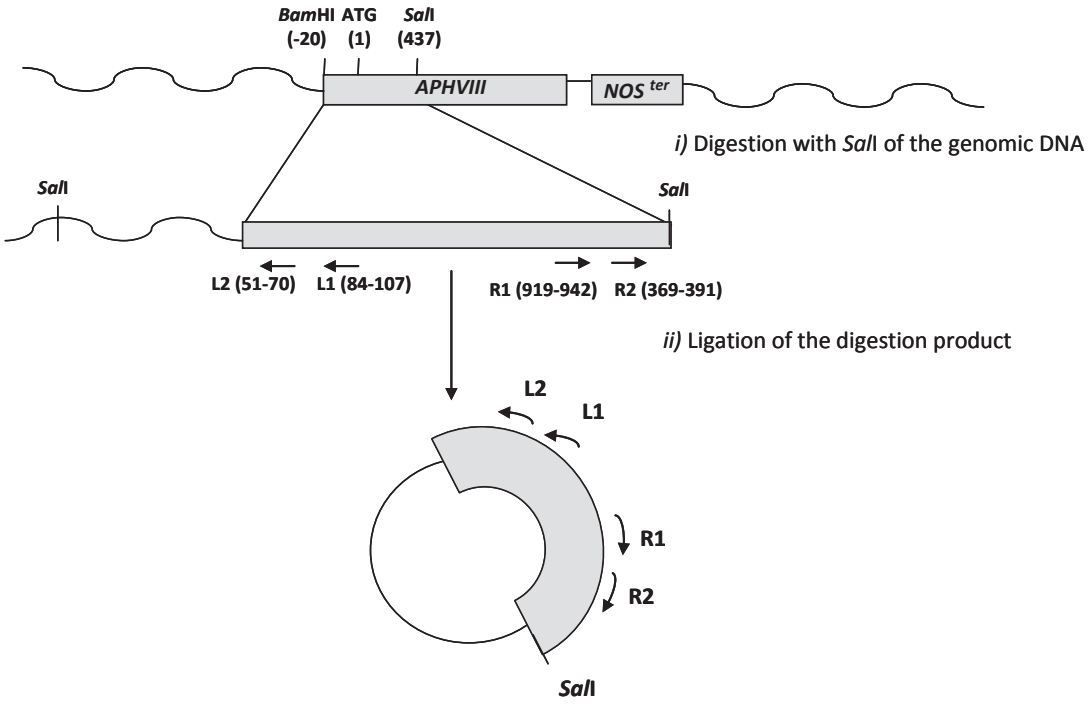
$$\frac{X_{APHVIII,mut}}{X_{NO_3^-,mut}} = \frac{X_{APHVIII,cont}}{X_{NO_3^-,cont}} \cdot 2^{(CtAPHVIII,con-Ct\ APHVIII,mut)-(CtNO_3^-,cont-CtNO_3^-,mut)} \quad (1)$$

We found that practically all of the mutants analyzed (80%) had a single copy of the tag. The use of small quantities of DNA in the transformation experiments has been described to avoid or minimize multiple insertions [29].

The putative endogenous promoter region responsible for the expression of the *APHVIII* was identified amplifying by inverse PCR and sequencing the region upstream the marker gene, in seven of the paromomycin resistant transformants selected. All the selected transformants showed resistance to high concentrations of the antibiotic paromomycin (50 μg mL^{-1}) and had a single marker gene insertion. The genomic DNA located upstream the marker gene was isolated by an inverse nested PCR and sequenced, as described in materials and methods using primers ParaL2 and ParaR2 (Figure 2).

In all cases, the sequence obtained by amplification through inverse PCR included a short fragment homologous to the *APHVIII* gene from *Streptomyces rimosus*, which confirmed specificity, and a fragment that showed high identity with the genome of *C. reinhardtii*. These fragments were compared with the last version of the *C. reinhardtii* genome database at the joint genome institute (DOE-JGI) [34] by the BLAST tool. The insertion site and the protein encoded by the interrupted gene, when available, are shown in Table 2.

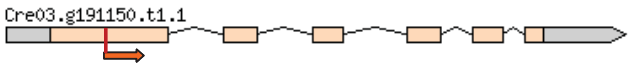
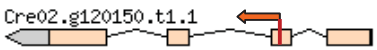
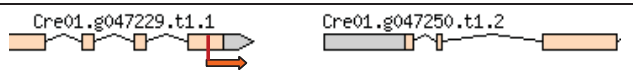
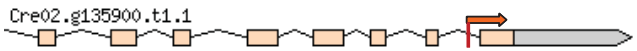
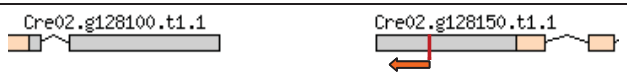
Figure 2. Scheme of the inverse PCR strategy used for amplification of the regions preceding the *APHVIII* marker insertion. The approximate sites for primers hybridation and restriction enzymes digestion are indicated.



We found that in all the transformants analyzed the insertion took place in intragenic regions, preferably inside the protein encoding region. Only in two of the analyzed transformants (1–14 and 4–15) the marker gene was inserted within the promoter region. In these two cases, the sense of the *APHVIII* insertion is reverse to the sense of the coding region directed by the interrupted promoter (Table 2), suggesting that the promoter regions in transformants 1–14 and 4–15 could have an antisense promoter activity.

We have found a variety of functions for the proteins encoded by the genes localized in the insertion sites of the analyzed transformants. For example, transformant 4–5 showed an insertion in the last intron of the gene encoding for the 1b light intermediate chain of flagellar dynein protein. In transformant 4–15 the insertion took place within the promoter region of the gene encoding one short chain dehydrogenase. Curiously, in transformant 3–4 the marker gene was located in the second exon of the ribulose biphosphate carboxylase small chain whose promoter is, alone or fused to the *HSP70A* promoter, the most widely constitutive promoter used to express foreign genes in *Chlamydomonas reinhardtii* [33]. In transformants 1–14, 2–6, and 3–8 the insertion took place within genes encoding proteins with unknown functions. In transformant 2–9 the *APHVIII* marker gene was inserted in the first exon of a protein predicted as an ubiquitine regulatory protein (UBIRP).

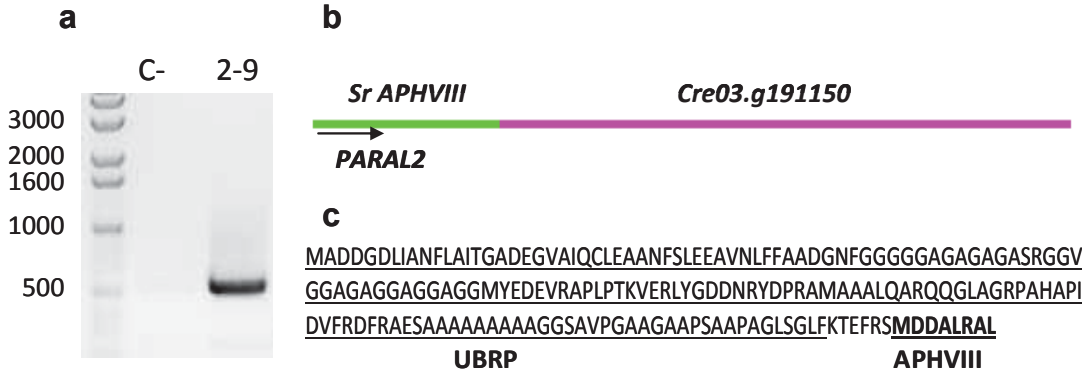
Table 2. Molecular analysis of some obtained mutants. Localization of the marker insertion site and identification of the protein encoded by the interrupted gene, when available, are shown. The arrows indicate the site and the sense of the insertion. In the scheme of the insertion site: grey color represents the untranslated region, orange color are the exons and lines the introns, as represented in the *Chlamydomonas* genome database [34].

Mutant	Insertion site	Nearest protein	Scheme of the detailed insertion site
1–14	1:5437700	No functional annotations for this locus (Cre01. g039718)	
2–6	3:7536446	No functional annotations for this locus (Cre03. g210150)	
2–9	3:5019967	Predicted Ubiquitine regulatory protein (Cre03. g191150)	
3–4	2:6042257	Ribulose bisphosphate carboxylase small chain (Cre02. g120150)	
3–8	11:6509508	No functional annotations for this locus (Cre01. g047250)	
4–5	2:8218509	Dynein 1b light intermediate chain; D1bLIC (Cre02. g135900)	
4–15	2:7024090	Predicted dehydrogenase short chain (Cre02. g128150)	

2.4. Testing the Promoter of the 2–9 Transformant

The genomic region preceding the marker gene in transformants that exhibit high antibiotic tolerance should drive the expression of foreign genes with high efficiency. As an example, the 5′ untranslated region isolated from the transformant 2–9, which corresponds to the promoter region of protein Cre03.g191150, predicted as an ubiquitine regulatory protein (UBIRP), was used to drive the expression of the marker *APHVIII* gene in *C. reinhardtii*. Details about the marker insertion site in this transformant are shown in Figure 3. The second round of the inverse PCR allowed the isolation of a DNA fragment of about 500 bp. The first 30 nucleotides of this sequence corresponded to the *APHVIII* marker, confirming the specificity of the amplification. The following nucleotides of the fragment had 100% homology with the protein Cre03.g191150 of chromosome 3 as shown in Figure 3. Detailed analysis of the sequence confirmed that the *APHVIII* marker gene is in the same reading frame that the predicted ubiquitine regulatory protein.

Figure 3. Detailed molecular characterization of transformant 2–9. The genomic DNA fragment amplified during the second round of the iPCR preformed in 2–9 transformant was separated by agarose gel electrophoresis (**a**). The product was sequenced and analyzed using the BLAST tools of the NCBI and the *Chlamydomonas* genome data (**b**). The amino acid sequence translated from the amplified sequence shows that the predicted UBIRP and APHVIII proteins are in the same reading frame (**c**). The efficiency of the nuclear transformations and the relative fold difference between the *APHVIII* transcript level in transformants of *Chlamydomonas reinhardtii* obtained with *UBIRP-APHVIII* and with pSI103 plasmid (**d**) are also shown.



d

Transformation plasmid	N of transformants per reaction	Transformation efficiency (transf cell^{-1} μg^{-1}DNA)	*APHVIII* Transcript level
pSI103	140± 18 (n=3)	1.4 10^{-6}	1
*UBIRP*p-*APHVIII-RBCS2* ter	60± 6 (n=2)	6.0 10^{-7}	0.7

The promoter of the ubiquitine regulatory protein was amplified by PCR with the primers UBIRP-F and UBIRP-R (Table 3) and fused to the *APHVIII* gene by inserting it among the *Not*I/*Bst*BI restriction sites of pSI104-PLK plasmid [35]. The obtained plasmid was used for transformation of

Chlamydomonas reinhardtii by the glass beads method as described in materials and methods. The pSI103 [30] plasmid in which the *APHVIII* gene is under the control of *HSP70A/RBCS2* promoter was used for comparison. The efficiency of the new identified promoter was high, reaching 60 transformants per reaction and per µg of DNA (which means a transformation efficiency of about 6.0×10^{-7} transformants per µg DNA cell^{-1}), though it was lower than that observed for the plasmid pSI103 which was about 1.4×10^{-6}, with 140 transformants per µg DNA (Figure 3). In this case, about 1 µg of plasmid DNA was used for each transformation.

Table 3. Nucleotide sequences of primer pairs used for PCR amplifications.

Primer	Sequence (5′→3′)	Uses
APH8-F*BAM*	CGCCCTCCCCGGATCCGAAGAA	Amplification of the *APHVIII* gene from pSI103 plasmid
APH8-R*SAC*	ACCCACGAGCTCCAACCCTACCC	
NRfor	GCGCTGCCCTCCGTCACCTTCC	Estimation of the number of *NR* genes by qPCR
NRre	CAGCCGCACGCCCGTCCAGTAG	
Parafor	GAGGATCTGGACGAGGAGCGGAA	Estimation of the number of *APHVIII* genes and the *APHVIII* transcript level by qPCR
Pararev	CCCTCAGAAGAACTCGTCCAACAGC	
qUbqL-for	GTACAGCGGCGGCTAGAGGCAC	Housekeeping gene for estimation of the *APHVIII* transcript level
qUbqL-rev	AGCGTCAGCGGCGGTTGCAGGTATCT	
ParaR 1	GTGGAGGGTGGTGGGGACGAGAGG	Identification of the region upstream the marker gene by iPCR
ParaR 2	GGTGTCCGTTCGATCGCAGTCTC	
ParaL1	GCCCACCACCCCGAAGCCGATAAA	
ParaL2	GGCCCCATCCTCCACAACAA	
UBIRP-F	GCTGCCCGCGACTGTGATGTA	Amplification and subcloning of the promoter identified in mutant 2–9
UBIRP-R	GGGCCGCTGCTGCACCAAACGC	
UBIRP-F*Not*I	GGCGGCCGCGACTGTGATGTA	
UBIRP-R*Bst*BI	GGTTCGAAGCTGCACCAAACGC	

Several random transformants obtained with *URRP-APHVIII* and *HSP70A/RBCS2-APHVIII* constructions were chosen for determination of the number of *APHVIII* gene copies. In three one-copy transformants of each type total mRNA was isolated as described in the experimental section and the *APHVIII* transcript level was calculated on the basis of the relative quantification analytical method (ΔΔCt), using *UBQL* as internal standard. The *APHVIII* transcript level in transformants obtained with the *UBIRP-APHVIII* construction was 0.7 fold the *APHVIII* transcript level found in control transformants obtained with the pSI103 plasmid. The presence of additional regulatory sequences, such as the *HSP70A* promoter and the first intron of the *RBCS2* gene, fused up and downstream the *RBCS2* promoter in the pSI103 plasmid may contribute to the relative higher efficiency of expression as compared to the *UBIRP* alone. Previous studies have demonstrated a synergistic effect of the combined use of the *HSP70A* and *RBCS2* promoters with the *RBCS2* first intron to drive transgene expression in *Chlamydomonas* [30,33].

3. Discussion

3.1. Choice of the Selectable Marker Gene for Promoter Trapping

In promoter trapping experiments, reporter genes lacking a promoter are randomly inserted into a position of the genome where its transcription relies on its adequate insertion in a region adjacent to an endogenous genomic promoter or in frame with a native gene. This strategy has been widely used in higher plants allowing the screening of differentially regulated genes or monitoring gene expression profiles [21,22,36]. In *Chlamydomonas reinhardtii* the argininosuccinate lyase (*ARG7*) [24] or the radial spoke protein (*RSP3*) [23] genes were successfully used for promoter trapping, on the basis of restoring the ability to grow in the absence of arginine or the motility in *arg7* and *pf-14* mutants, respectively. Other marker genes, such as versions of *GFP* [37] or luciferase [38] adapted to *Chlamydomonas* nuclear codon usage, or the arylsulphatase [25] are available for *Chlamydomonas* and have been successfully used for easy assay of promoters activity. However, the most powerful selectable markers are those conferring resistance against antibiotics such as bleomycin (*BLE*) and paromomycin (*APHVIII*). The *APHVIII* gene has been widely used for transformation of *Chlamydomonas*, making possible optimization of promoters [30] and preparation of knockout insertional mutants [26] but, to date, no promoter trapping system based on this marker has been described.

Here, we show that the selection of vigorous transformants under increasing antibiotic concentrations allows the identification of strong constitutive promoters. We chose *APHVIII* as marker gene because it provides a stable phenotype, the antibiotic paromomycin lacks the mutagenic activity described for other antibiotics, such as bleomycin [29]. It is an exogenous gene easy to identify by PCR, and selection of transformants does not rely on restoring phenotypes of mutant hosts, such as the systems based on *RSP*3 or *ARG7* genes. Furthermore, certain frequency of transformation has already been reported with promoter-less *APHVIII* genes in *Chlamydomonas* [30]. The use of an exogenous promoter avoids the possibility of homologous recombination, which has been reported to happen at low frequency (0.01%–0.7%) in *Chlamydomonas* [23]. We observed that in 100% of the transformants the insertion took place in intragenic regions, while in promoter trapping experiments preformed in higher plants, a high percentage of insertions were reported to occur in intergenic regions [36,39]. The authors explain reporter expression in these transformants from cryptic promoter activity in such regions or from the promoter activity of unannotated genes.

To identify the promoter DNA in each of the transformants chosen, we used an inverse nested PCR over digested and relegated genomic DNA (Figure 2). Other strategies widely used for this purpose in higher plants or microalgae include: Thermal asymmetric interlaced PCR [40], adaptor ligation-mediated PCR [36], rapid amplification of 5′ complementary DNA ends (5′ RACE) [23], or RESDA PCR [41]. Tonooka and Fujishma [42] have recently offered a critical review of these methods to walk along genomes. Similar inverse PCR strategy has been successfully used for identification of the regions flanking the *APHVIII* gene in insertional mutants of *Chlamydomonas reinhardtii* [43].

3.2. Application to Other Microalgal Species

Strong constitutive promoters, which drive the expression of abundant proteins, such as the RBCS2 [33] or the PSAD [44] in *Chlamydomonas* or the fucoxanthin chlorophyll binding protein (FCP) in

Phaeodactylum [3] have been widely used to drive the expression of heterologous proteins in these microalgae. However, the promoters of the corresponding genes of other species are not necessarily as efficient. Walker and coworkers *et al.* [12] isolated two *RBCS* genes in *Dunaliella tertiolecta* and used their promoters to drive the expression of the gene encoding bleomycin resistance (*BLE*) in the own *D. tertiolecta*. They only recovered one stably transformed *Dunaliella* line [16]. Therefore, a wise selection of strong promoters based on marker genes should be necessary to identify the strongest promoters in each species. In preliminary experiments, not shown here, we have determined the lethal doses of paromomycin for other chlorophytes, showing that this antibiotic can be a useful selective agent for many freshwater green microalgae and thus the *APHVIII* gene could be used as marker gene in many other microalgae. Following the strategy described here, *APHVIII*-based systems could be designed for promoter trapping in other species.

Identification of promoters once the region preceding the marker has been sequenced is not always an easy task. Eukaryotic promoters are typically located upstream of the gene open reading frame and most of them contain the sequence TATAAA (the TATA box) which binds a TATA binding protein, and assists RNA polymerase to initiate transcription. However, this characteristic element is not always present in all promoters and often other regulatory elements far away from the transcriptional start site can be important to enhance promoter activity. Furthermore, the marker gene can be expressed not only when inserted following a promoter region, but also when inserted in frame with a native gene. The availability of the whole genome sequence and a large number of annotated proteins in *Chlamydomonas* is a great advantage, which has made possible a detailed study of the insertion site in several of the paromomycin-resistant transformant obtained. In other microalgal strains, for which the genome sequence is not available, it should be necessary to isolate several transformants and check the ability of the region preceding the marker gene to act as a promoter in all of them, to have higher probabilities to isolate a real promoter. However, the growing number of microalgal genomes sequenced will surely facilitate the identification of promoters in these species. Beside *Chlamydomonas* [45] and *Phaeodactylum* [46], the nuclear genome sequencing project of other microalgae such as the small *Ostreococcus tauri* [47], *Micromonas* [48] and more recently *Nannochloropsis gaditana* [20], have been completed, and many other are in progress [49].

4. Experimental Section

4.1. Microorganisms and Culture Conditions

Chlamydomonas reinhardtii cell-wall deficient strain 704 (*cw15*, *arg7*, mt^+) was kindly provided by Dr. Roland Loppes [50] and cultured photomixotrophically in liquid or agar solidified TAP medium [51] at 25 °C under continuous white light irradiation of 100 μE m^{-2} s^{-1}. The *Escherichia coli* strain used for *in vivo* amplification of DNA was DH5α, cultured in LB medium as previously described [52].

4.2. Nuclear Transformation of Chlamydomonas reinhardtii *with the* APHVIII *Promoter-Less Cassette*

Transformants for promoter trapping were generated by transformation of the *Chlamydomonas reinhardtii* strain 704 (*cw15*, *arg7*, mt^+) with a DNA cassette containing the aminoglycoside

3′-phosphotransferase gene (*APHVIII*) from *Streptomyces rimosus*, followed by the nopaline synthase (*NOS*) terminator region from *Agrobacterium tumefaciens*.

Transformation was carried out using the glass-bead method of Kindle [53], with minor modifications. *C. reihardtii* cells were grown until the middle of the exponential phase of growth (about 1.6×10^6 cells mL^{-1}), harvested by centrifugation and resuspended in fresh TAP medium to obtain a 100 fold concentrated cell suspension. The concentrated cell suspension (0.6 mL) was added to a conical tube containing 0.3 g of sterile glass beads (0.4–0.6 mm diameter), 0.2 mL of 20% polyethylene glycol (MW8000) and the indicated quantities of the chosen cassette or plasmid. Cells were vortexed for 8 s and resuspended in 50 mL of fresh sterile TAP medium where they were incubated overnight. After this incubation in the absence of antibiotic, the cells were pelleted and spread onto TAP solid medium plates with paromomycin (30 μg mL^{-1}). Transformed colonies were visible after 4 or 5 days.

4.3. Isolation of Genomic DNA

The pellet obtained after centrifugation of 2 mL of *Chlamydomonas* culture was resuspended in 300 μL of lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2% SDS, 0.3 M NaCl), vigorously vortexed for 10 min and incubated in ice for 2 min. DNA was extracted with phenol/chloroform and precipitated with absolute ethanol, overnight at −20 °C. The pellet was washed with ethanol 70%, dried and resuspended in 40 μL of 5 mM Tris-HCl, pH 8 [36].

4.4. RNA Extraction and Reverse Transcription

Isolation of total RNA was performed with the RNAeasy plant MiniKit of Qiagen according to instructions of the manufacturer. Single strand cDNA was synthesized from total RNA according to the SuperScript II RNaseH-reverse transcriptase manual (Invitrogen) and used as substrate for Real Time-PCR reactions.

4.5. Standard Polymerase Chain Reaction

The PCR amplification was performed from 1 μL of genomic DNA in a total volume of 25 μL containing 10 pmol of each primer, 0.2 mM dNTPs, 0.5 U *Taq* DNA polymerase from Biotools (B & M Labs, Madrid, Spain), 2.5 μL of specific 10× buffer (containing 2.5 mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO). The PCR program was: 0.5 min at 96 °C, 0.5 min at annealing temperature, and 1.5 min at 72 °C for 30 cycles.

4.6. Inverse PCR

Inverse PCR was preformed by digesting 500 ng of RNAase treated genomic DNA with the restriction endonuclease *Sal*I, which has a unique restriction site inside the *APHVIII-NOS* cassette and has a high number of target sites in the genome of *Chlamydomonas*. The resulting digestions were precipitated by adding ethanol 95% and sodium acetate at a final concentration of 100 mM and resuspended in 8 μL of water. The obtained digestion products were ligated with T4 ligase overnight at 16 °C and used as a template for nested PCR (Figure 2) using different pairs of inverted primers

(Table 3). Each PCR reaction was carried out at the standard conditions described before, excepting that cycling conditions were 1× (96 °C, 5 min), 35× (95 °C, 1 min; 60 °C, 1 min; 72 °C, 3 min), 1× (72 °C, 10 min).

4.7. Analysis of the Insertion Sites

The products amplified by inverse PCR were separated by agarose gel electrophoresis. The fragments obtained were isolated with a gel extraction kit (Qiagen) and sequenced. The resulting sequences were analyzed by comparison with *Chlamydomonas* genome database [34].

4.8. Quantitative Real-Time PCR. Transcript Level Analysis and Determination of the Number of Copies of the APHVIII *Gene Integrated in the Genome of* Chlamydomonas

Real time PCR was performed on a Mx3000P Multiplex Quantitative PCR System from Stratagene using the Brilliant SYBR Green QPCR Master Mix (Agilent). Each determination was carried out in triplicate using genomic DNA as template, and 10 pmoles of the indicated primers in a final volume of 25 µL. Cycling conditions were: 10 min at 95 °C for activation of the hot start Taq polymerase and 40 cycles for the melting (30 s at 95 °C), annealing (30 s at 60 °C) and extension (30 s at 72 °C). The fluorescence measurement was made at the end of the annealing step. A dissociation curve (30 s at 95 °C, 30 s at 55 °C and 30 s at 95 °C) was applied at the end of the amplification reaction to check possible formation of dimmers.

For transcript level analysis, the ubiquitine ligase gene (*UBQL*, GenBank BU648530), which encodes the ubiquitine ligase protein, was used as housekeeping gene. Expression of this gene was previously checked to be constitutive under the different conditions used [41]. Primers efficiencies were 1.04 and 1.00 for *APHVIII* and *UBQL*, and yield amplicons 359 bp of 161 bp, respectively.

$$\frac{X_{test}}{X_{control}} = 2^{(CtAPHVIII - CtUBQL)_{cont} - (CtAPHVIII - CtUBQL)_{test}} \quad (2)$$

For determination of the number of *APHVIII* copies, nitrate reductase (*NIA1*) was used as one copy gene reference. Using specific primers with very similar efficiencies for *APHVIII* and *NIA1* (Table 3) the number of integrations was calculated using the ΔΔCt method [54]. Efficiencies of the primers were previously determined [26].

5. Conclusions

Promoter trapping, which has been widely used for the selection of regulable promoters induced under certain nutritional or environmental conditions, can also allow the selection of strong endogenous promoters. We have demonstrated that transformation with the promoterless *APHVIII* gene from *Streptomyces rimosus*, which encodes for an aminoglycoside 3′-phosphotransferase and provides resistance to the antibiotic paromomycin, and subsequent isolation of the most robust transformants enables easy identification of novel promoter sequences in *Chlamydomonas*. This strategy could be applied to many chlorophytes sensitive to paromomycin.

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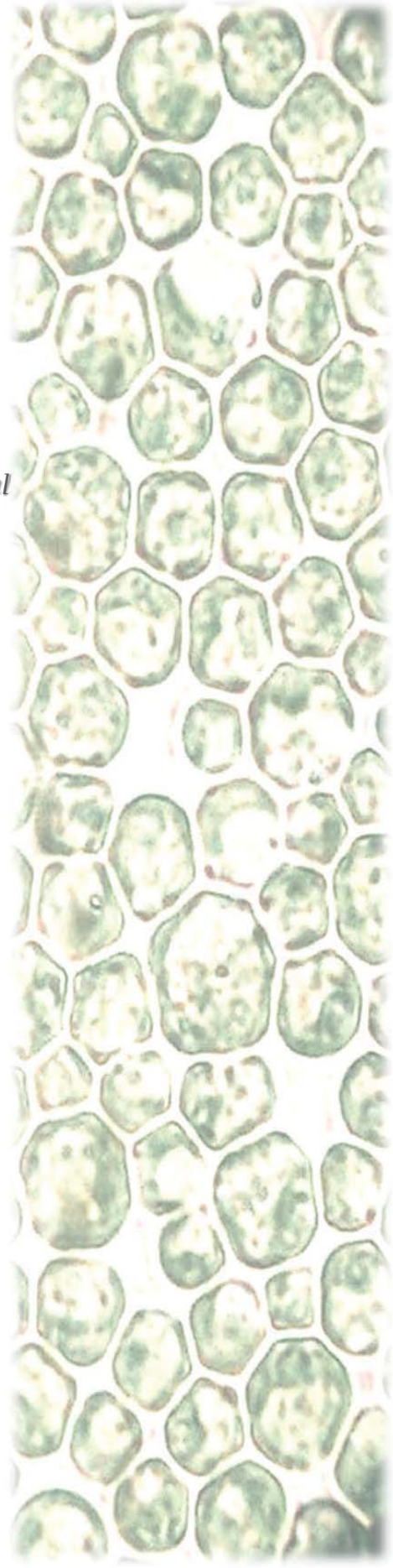
Samples Availability: Available from the authors.

Chapter 4

"Stable Nuclear Electrotransformation of the Industrial Microalga Chlorella sorokiniana Using Different Heterologous Promoters."

The manuscript of this chapter is in process to be published.



Article

Stable Nuclear Electrotransformation of the Industrial Microalga *Chlorella sorokiniana* Using Different Heterologous Promoters

Encarnación Díaz-Santos, Marta Vila, Javier Vigara and Rosa León

Biochemistry Laboratory, Experimental Sciences Faculty, University of Huelva, Marine International Campus of Excellence (CEIMAR), Huelva 27071, Spain.

Abstract: Currently, the chlorophyte *Chlorella sorokiniana* is one of the most robust microalgae with a relevant biotechnological importance because of its constitutively high content of lutein, its high lipid content, sustainable for biodiesel production and its capacity to grow fast in high irradiance conditions. Despite all these good features of *C. sorokiniana*, studies about its genetic manipulation to improve their properties and makes it more profitable for the microalgal industry are scanty. In the present work, a suitable genetic electrotransformation method for *Chlorella sorokiniana* is developed. Using the optimal electroporation conditions: 2.5 kV of electric field strength and 3 electric pulses and the paromomycin resistance gene as a marker, the efficiency of three heterologous promoters, the cauliflower mosaic virus 35S RNA promoter (*CaMV 35S*), the promoter of the nopaline synthase from the Ti plasmid of *Agrobacterium tumefaciens* (*NOS*) and the hybrid promoter *HSP70A/RBCS2* from *Chlamydomonas reinhardtii* was tested. It was found stable transformants with the three tested promoters, obtaining the highest efficiency values with *CaMV 35S* promoter followed by *NOS* and *HSP70A/RBCS2* promoter respectively.

Keywords: Electroporation; *Chlorella sorokiniana*; microalgae transformation; nopaline synthase; *CaMV 35S*

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1. Introduction

The genus *Chlorella* comprises unicellular, flagellar-less eukaryotic chlorophyte algae widespread in freshwater and marine environments. *Chlorella* species are considered model organisms to study the genetics and molecular biology of photosynthesis and chloroplast development (Chen et al., 2001; Talebi et al., 2013), and many of them have an important biotechnological potential due to their high content in essential amino acids, carbohydrates, minerals, vitamins, fatty acids and bioactive compounds, being widely used in aquiculture as feed for rotifers and in human health as vitamin supplement (Chan et al., 2012). *Chlorella sorokiniana*, is a robust industrial species and one of the fastest growing eukaryotic microalgae, with a high maximal specific growth rate of 0.27 h^{-1}, an optimal growth temperature of 37 ºC and tolerant to high irradiance levels. All these characteristics make of *C. sorokiniana* an adequate species for large-scale cultivation in meridional regions of high irradiance (Cuaresma et al., 2009). Furhtermore, *C. sorokiniana* has been considered as one of the best candidates for sustainable biodiesel production due to its high lipid content, biomass productivity and its high growth rate at different culture conditions (Li et al., 2013, 2014; Shriwastav et al., 2014). Recently, the ability of this microalgae to grow using human urine as a source of nutrients has been proven, being of great interest in urban wastewater treatment coupled to biodiesel production (Zhang et al., 2014). Its potential in phytoremediation and its ability to incorporate metals as selenium with antioxidant properties have been also confirmed (Cuaresma 2011). Other significant characteristic of *Chlorella sorokiniana*, that makes it a strain with a relevant industrial importance, is its constitutively high content of lutein, a carotenoid pigment very important in aquaculture, poultry farming, pharmaceutical or cosmetic industry as well as in human health as a nutraceutical against macular degeneration, cataracts or early atherosclerosis (Cordero et. al, 2011). All these important properties make *Chlorella sorokiniana* very attractive candidate to be genetically manipulated.

Historically, the genetic manipulation of microalgae have been focussed on a few model microalgal species. The first transformed and best studied microalgae was *Chlamydomonas reinhardtii,* that remains, after more than twenty years, the favorite model system for which a complete kit of molecular and bioinformatic tools have been developed (León et al., 2007; Specht et. al, 2010). In the last decade, the increasing interest on microalgae has stimulated research on the genetic transformation of species with applied interest and successful stable transformation of species such as *Dunaliella tertioleta* (Walker et. al, 2005), *Nannochloropsis* sp. (Kilian et. al, 2011), *Nannochloropsis gaditana* (Radakovits et. al, 2012; Jinkerson et. al, 2013) or Scenedesmus (Guo et. al, 2013; Dautor et al., 2014) has been reported. Several transformation procedures have been described for the genetic manipulation of *Chlorella* species (Table 1). El-Sheekh et al. in 1999 and Talebi and co-workers in 2013 developed a biolistic method to transform *Chlorella vulgaris*, currently known as *Coccomyxa sp.* This strain was also transformed by electroporation (Chow et al., 1999; Koo et al., 2013) and via *Agrobacterium tumefaciens* (Chan et al., 2012). Other species

such as *Chlorella zofingiensis* and *Chlorella ellipsoidea* have been genetically manipulated to express different transgenes by electroporation and biolistic (Chen et al., 2001; Liu et al., 2014). However, the first and the only study about the genetic transformation of *Chlorella sorokiniana* was published in 1997 by Dawson et al. It describes a biolistic method to introduce the nitrate reductase gene from *Chlorella vulgaris* into *Chlorella sorokiniana* and recover transformants by functional complementation of NR-deficient mutants.

In the present work, a simple, cheap and stable electrotransformation method is developed for genetic transformation of *Chlorella sorokiniana* intact cells. Furthermore different heterologous promoters, such as the cauliflower mosaic virus 35S RNA promoter (*CaMV 35S*), the promoter of the nopaline synthase from the Ti plasmid of *Agrobacterium tumefaciens* (*NOS*) and the hybrid promoter *HSP70A/RBCS2* from *Chlamydomonas reinhardtii* are tested to conduct the expression of the paromomycin resistance aminoglycoside 3`-phosphotransferase *(APHVIII)* gene from *Streptomyces rimosus*

2. Materials and Methods

2.1. Chlorella strain and culture conditions

C*hlorella sorokiniana* used in this study was obtained from the culture collection of the Institute of Vegetal Biochemistry and Photosynthesis, IBVF, (Seville, Spain) and cultured in liquid or agar solidified Seuoka medium (Harris et al., 2009) at room temperature, under continuous white light irradiation of 100 $\mu E\ m^{-2}\ s^{-1}$ and air bubbling supplied with 2% of CO_2.

2.2. DNA constructions with different heterologous promoters

The DNA constructions used in the present work to transforming *Chlorella sorokiniana* cells were achieved by Díaz-Santos et al. (2013), using the cauliflower mosaic virus 35S RNA promoter (*CaMV 35S*), the promoter of the nopaline synthase from the Ti plasmid of *Agrobacterium tumefaciens* (*NOS*) and the hybrid promoter *HSP70A/RBCS2* fused to the paromomycin resistance gene, aminoglycoside 3`-phosphotransferase *(APHVIII)* from *Streptomyces rimosus* as a selective marker gene (Sizova et al. 2001).

2.3. Electrotransformation of Chlorella sorokiniana

Genetic transformation of *C. sorokiniana* cells was performed by electroporation using a Gene Pulser™ electroporator (BioRad). A *Chlorella* culture was grown to a density of 1-2 x 10^6 cells/mL and centrifuged at 4000 rpm for 5 min. The pellet obtained was washed with a glycerol solution 50 mM and resuspended in a 1:100 total volume. Cell suspension was kept on ice for 20-30 min and 800 µL were added to a 0.4 cm gap electroporation cuvette previously chilled on ice for 10-15 min. For each electroporation, 20-40 µg of plasmid DNA were added to the cuvette and mixed with the cell suspension. Electrotransformation was

conducted at a voltage of 2.5 kV cm^{-1} and 3 electrical pulses. Capacitance, resistance and pulse duration were assumed by the electroporator. Electroporated cells were kept on ice for 5 min, transferred to 50 mL of fresh Seuoka medium and cultured for 24 h post-electroporation. After 24 h, cells were pelleted and spread on selection agar plates containing 100 μg mL^{-1} of paromomycin. The agar plates were kept in the incubation chamber under continuous white light irradiation of 100 μE m^{-2} s^{-1} at 25 ºC. Transformed colonies appeared after about 15 days and these were picked up on new selection plates and used for molecular analysis. Each electroporation experiment was run in triplicate (n=3) excepting when heterologous promoters were used in which experiments were run five times (n=5).

2.4. Small-scale genomic DNA extraction

Chlorella sorokiniana genomic DNA extraction was performed as previously described for *Chlamydomonas reinhardtii* by Vila et al. (2013).

2.5. RNA extraction and reverse transcription

RNA total extraction was performed using the RNAeasy plant MiniKit of Qiagen according to instructions of the manufacturer. To synthesize cDNA from the total RNA was used the SuperScript II RNaseH- reverse transcriptase manual (Invitrogen).

2.6. Molecular analysis

The genomic DNA isolated from the transformants cells was subjected to a PCR analysis to determine the integration of the different plasmid constructions into the *Chlorella* transformants genome. The PCR amplification was run from a sample of 1 μL of DNA genomic in a total volume of 25 μL containing 10 ρmol of each primer, 0.2 mM dNTPs, 0.5 U *Taq* DNA polymerase from Biotools (B&M Labs, Madrid, Spain), 2.5 μL of specific 10x buffer (containing 2.5 mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO). The PCR program was: 1 pre-denaturation cycle at 96 °C for 0.5 min; followed by 30 cycles of denaturation at 96 ºC for 1 min, annealing at 61 ºC and 0.5 min at 72 °C for extension; and a final cycle at 72 ºC for 5 min. To verify the gene expression a sample of 1 μL of cDNA was subjected to PCR analysis as describe above. PCR products were analysed by 2% agarose gel electrophoresis.

2.7. Chlorella sorokiniana crude extract preparation

Chlorella sorokiniana cell suspension harvested by centrifugation at the end of the exponential phase of growth (approximately 1 g mL^{-1} fresh weight cells) was resuspended in a total volume of 1 mL g^{-1} fresh weight cells of TBS buffer, containing: Tris-HCl 20 mM, 140 mM NaCl, pH 7.4. The cells were disrupted by sonication 10 times for 15 s with a micro-sonicator and the lysate was centrifuged twice at 14,000 rpm for 15 min. The supernatant obtained was used as extract crude source.

2.8. Protein determination

The total protein concentration in the microalga crude extracts was determined with the Bio-Rad Bradford assay according to the manufacture protocol, using bovine serum albumin (BSA) as a standard.

2.9. SDS-PAGE

Polyacrylamide gel electrophoresis in denaturing conditions was performed on a 10% acrylamide gel, in SDS-electrophoresis buffer pH 8.3 (SDS 1 g L^{-1}, Trizma base 3 g L^{-1} and Glycine 14.4 g L^{-1}). The gel was stained with colloidal CBB G-250 (BioRad).

2.10. Western Blot analysis

Proteins separated by SDS-PAGE were electroblotted onto a polyvinylidene difluoride membrane (PVDF) using the Multiphor II Novablot system (Pharmacia LKB) according to the manufacturer's instructions, washed with TBS buffer and blocked overnight using nonfat dry milk with 2% of Tween 20. Inmunodetection was performed using polyclonal antibodies anti-APHVIII raised as described Díaz-Santos et al. (2013) as primary antibody to a dilution 1:5,000 and alkaline phosphatase (AP)-conjugated goat-anti-rabbit IgG (SIGMA), at 1:10,000 dilution, as secondary antibody.

3. Results and Discussion

3.1. Development of a suitable electrotransformation method for Chlorella sorokiniana

Several microalgal species from the genus *Chlorella* have successfully transformed employing particle bombardment or biolistic and electroporation methods (Table 1). However for *Chlorella sorokiniana* a unique report describing its genetic transformation
has been published (Dawson et al., 1997).

Table 1. Transformation methods of different *Chlorella* species.

***Chlorella* specie**	**Transformation method**	**Antibiotic**	**Promoter/plasmid**	**Reference**
Chlorella sorokiniana	Biolistic	-	Nitrate reductase	Dawson et al., 1997
Chlorella kessleri	Biolistic	Kanamycin	*CaMV 35S*	El-Sheekh et al.,1999
Chlorella vulgaris	Electroporation	Hygromycin B	*pIG121-Hm*	Chow et al., 1999
Chlorella ellipsoidea	Electroporation	Geneticin	*CaMV 35S, Ubi 1*	Chen et al., 2001
Chlorella vulgaris	Agrobacterium	Hygromycin	*CaMV 35S*	Chang et al., 2012
Chlorella ellipsoidea	Cellulolytic enzymes	Zeocin	*pSP-Ubi-GUS*	Liu et al., 2012
Chlorella vulgaris	Biolistic	Kanamycin	*CaMV 35S*	Talebi et al., 2013
Chlorella vulgaris	Electroporation	Hygromycin	*CaMV 35S*	Koo et al., 2013
Chlorella zofingiensis	Electroporation, Biolistic	Norflurazon	*PDS, NIT, RBCS*	Liu et al., 2014
Chlorella sorokiniana	Electroporation	Paromomycin	*CaMV 35S, NOS, RBCS*	Current study

In this report, the transformation of *Chlorella sorokiniana* by particle bombardment with the nitrate reductase gene from *Chlorella vulgaris* as selectable marker is described. In the current work, the genetic transformation of *Chlorella sorokiniana* is approached by electroporation or electrotransformation, which is a cheaper and simpler technique than particle bombardment.

In order to stablish a suitable electrotransformation procedure for *C. sorokiniana*, a preliminary experiment was performed in which different parameters, involved in the electroporation efficiency, were tested. The electric field strength and the electric pulses as well as the osmotic pressure of the resuspension solution were assayed. These preliminary assays were run using the promoterless-*APHVIII* gen from *Streptomyces rimosus*, which encodes an aminoglycoside 3`-phosphotransferase that confers resistance to the antibiotic paromomycin, and has been successfully used for the previous transformation of *Chlamydomonas reinhardtii* (Díaz-Santos et al., 2013). The expression of the promoterless-*APHVIII* gene relies on its insertion in a region adjacent to an endogenous genomic promoter or in frame with a native gene, avoiding the influence exogenous regulatory regions, which should be further optimized to improve

transformation efficiency. The sensitivity of *C. Sorokiniana* to the antibiotic paromomycin was previously studied and a lethal doses of 100 µg mL was established (Díaz-Santos et al., 2013). The main results are summarized in table 2, where the number of *Chlorella* transformants obtained for several combinations between the electric field strength and the number of pulses can be observed.

Table 2. Number of transformants in each electroporation condition tested (n=3).

Electric field strength (kV cm^{-1})	Pulses	Number of transformants per transformation reaction
2.5	1	0
2.5	2	80 ± 13
2.5	3	800 ± 46
3	1	2 ± 0.4
3	2	5 ± 1
3	3	350 ± 22

The highest transformation efficiency was obtained for an electric field strength of 2.5 kV cm^{-1} and 3 pulses. Comparing our results with the studies previously reported by other authors, it can be concluded that the optimal field strength is strain-dependent, being for example 1.8 kV cm^{-1} for *Chlorella vulgaris* or 4 kV cm^{-1} for *Chlorella zofingiensis* (Chow et al., 1999; Liu et al., 2014). Regarding to the cellular resuspension solution, firstly, a solution of Tris HCl with glycerol 10 mM was used to resuspend the cell culture but the electroporation events were not successfully executed due to errors in the electroporator system. The salts present into the resuspension medium may had a negative influence in the electric field supplying and the electroporator system was blocked. The Tris HCl with glycerol 10 mM solution was then replaced by a Glycerol 50 mM solution, eliminating the salts into the medium and increasing the glycerol concentration to preserve the cell osmolarity, getting under these conditions electroporation events successfully.

Optimal variables for the electroporation of *Chlorella sorokiniana* are summarized in Table 3. These parameters were used in subsequent genetic transformation experiments.

Table 3. Optimal conditions for electroporation of *Chlorella sorokiniana*.

DNA amount	20-40 µg
Cell resuspension solution	Glycerol 50 mM
Electroporation cuvette gap	0.4
Electric field strength	2.5 kV
Pulses	3

Although particle bombardment has been successfully employed for the transformation of *Chlorella sorokiniana* (Dawson et al., 1997), and it is the most effective method for the transformation of chloroplasts (Koo et al., 2013), this method requires specific instruments and expensive consumables such as gold or tungsten particles, making it highly expensive (Liu et al., 2014). Furthermore, the selectable marker chosen by Dowson and co-workers is only applicable for molecular complementation of NR-deficient *C. Sorokiniana* mutants and no to wild type microalgae. According to the results obtained in these preliminaries experiments performed for *Chlorella sorokiniana*, it is possible to stablish an alternative method to the particle bombardment for the genetic transformation of this microalgae, a cheaper and simpler method, the electrotransformation.

3.2. Electrotransformation of Chlorella sorokiniana with different heterologous promoters

The choice of highly active promoters is one of the most important factors for the development of efficient transformation systems to ensure the high expression of heterologous proteins in microalgae. In the *Chlorella* genus, several studies have been focused in the use of the cauliflower mosaic virus *35S* RNA promoter (*CaMV 35S)* as a feasible promoter to express transgenes (El-Sheekh et al., 1999; Talebi et al., 2013). Other authors have described transformation methods using the promoter from maize plant, *Ubi1* or some endogenous promoters such as the promoter of the phytoene desaturase, the nitrate reductase and the ribulose-1,5-bisphosphatecarboxylase/oxygenase (Chen et al., 2011; Liu et al., 2014). And, many different studies have reported the expression of marker genes which confer resistance to antibiotics such as geneticin, phleomycin or kanamycin (Talebi et al., 2013). In the present study, three heterologous promoters have been tested to conduce the expression of the paromomycin resistance gene (*APHVIII*) in *Chlorella sorokiniana* cells: the cauliflower mosaic virus *35S* RNA promoter (*CaMV 35S*), the nopaline synthase promoter (*NOS*) from *Agrobacterium tumefaciens*, and the hybrid promoter *HSP70A/RBCS2* from the microalgae *Chlamydomonas reinhardtii*.

Three different genetic constructions, based in the promoters described above, have been used for the electroporation experiments: *HSP70A/RBCS2p-APHVIII-RBCS2ter* which contains the *APHVIII* gene under the control of the promoter *HSP70A/RBCS2*, *CAMV35Sp-APHVIII-NOSter* containing the marker gene under the control of the cauliflower mosaic virus 35S RNA promoter and *NOSp-APHVIII-NOSter* which contains the *APHVIII* gene under the control of the nopaline synthase promoter. The optimized conditions, selected in the previous experiments described in section 3.2, were utilized for electroporation. Approximately 15 days after electroporation, paromomycin resistant transformants were observed for all constructions tested (Fig. 1).

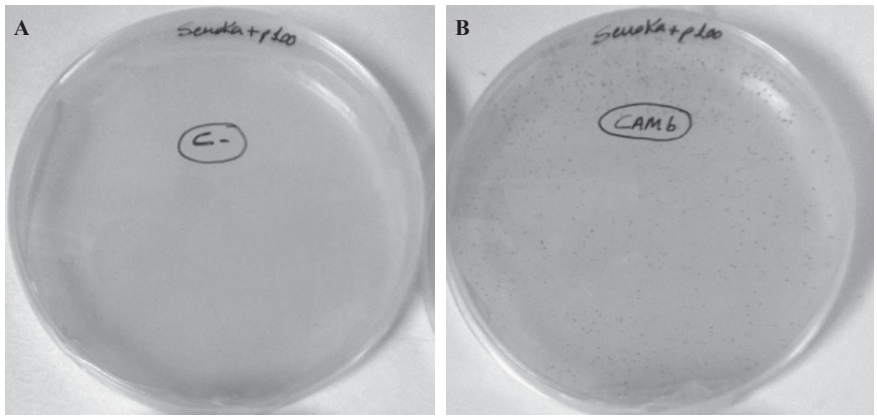

Fig.1. Picture of paromomycin resistant colonies of *Chlorella sorokiniana* on selective agar plates after electroporation with *CAMV35Sp-APHVIII-NOSter*. (A) Negative control, cells of *C. sorokiniana* without *APHVIII* gene. (B) *C. sorokiniana cells* with *CAMV35Sp-APHVIII-NOSter* integrated into the genome.

The transformants obtained were picked up and subcultured several times in agar plates containing 100 $\mu g\ mL^{-1}$ of paromomycin in order to select the stable transformants and discard the transient transformants. The results described in table 4 showed that the number of transformants obtained using the paromomycin resistance gene under the control of the promoters *CaMV 35S* and *NOS* was higher than the transformants obtained with the hybrid promoter *HSP70/RBCS2*. Furthermore, the best transformation efficiency among the heterologous promoters was obtained in the case of the cauliflower virus promoter *CaMV 35S*, being around 1.5-fold higher than the obtained efficiency using the nopaline synthase promoter from

Agrobacterium and more than 3-fold higher than the efficiency with the hybrid promoter from *Chlamydomonas*.

Table 3. Transformation Efficiency. The number of transformants is the media of five independent transformation experiments (n=5). In all of cases 20 µg of DNA were used.

Genetic construction	Number of transformants per transformation reaction	Transformation efficiency (transformants $cell^{-1}$ μg^{-1} DNA)
CAMV 35Sp-APHVIII-NOSter	917 ± 45	4.6×10^{-7}
NOSp-APHVIII-NOSter	694 ± 26	3.5×10^{-7}
HSP70A/RBCS2p-APHVIII-RBCS2ter	252 ± 31	1.3×10^{-7}

In contrast to the results obtained for *Chlamydomonas reinhardtii* transformed with *CaMV 35S* and *NOS* promoters in which *NOS* was the most efficient (Díaz-Santos et al., 2013), for *Chlorella sorokiniana* the best efficiencies are obtained using the cauliflower mosaic virus promoter *CaMV 35S*, even higher than the efficiencies found using the *HSP70/RBCS2* from *Chlamydomonas reinhardtii*, a phylogenetically close microalgae to *Chlorella*. As previously described in literature, the *CaMV 35S* promoter is the most commonly used for genetic manipulation of high plants and according to several reports can also be used in microalgae transformation (Chen et al., 2001) including *Chlorella* species such as *Chlorella zofingiensis*, *Chlorella vulgaris*, o *Chlorella ellipsoidea* (Table 1) and also *Chlorella sorokiniana* with the data obtained in the current work. Furthermore, although weaker than the *CaMV 35S* promoter, in *Chlorella sorokiniana* the nopaline synthase promoter from *Agrobacterium* could be also used to express transgenes, as was also described for *Chlorella vulgaris* in 2013 by Talebi et al.

These results indicate the heterogeneity and specificity of different promoters to conduce the expression of transgenes in different chlorophytes species, being difficult to find a universal promoter for the genetic transformation of all microalgae.

3.3 Molecular characterization of Chlorella sorokiniana putative transformants

3.3.1. DNA PCR analysis

A total of 20 paromomycin resistant transformant colonies, were randomly selected, picked and inoculated into liquid media (TAP paromomycin 50 ug mL-1) until exponential growth. At this stage, genomic DNA from each transformant was extracted and the presence of the *APHVIII* gene analysed by PCR as described in Material and Methods. Figure 2A shows PCR products with the expected size (360 bp) in the transformants examined, confirming the presence of the introduced gene into the *Chlorella sorokiniana* transformed cells.

3.3.2. RT-PCR analysis

To obtain further information about the effect of the introduced construction containing the *APHVIII* gene in the genome of the *Chlorella sorokiniana* transformants, the transcription to mRNA was examined by RT-PCR as described in Material and Methods section. Figure 2B shows an example of the presence of the transcript *APHVIII* gene, with an expected molecular size, around 360 bp in the selected transformants. For all transformants checked the same result was found. These data confirm the successful nuclear integration of the *APHVIII* gene into the genome and its later expression in the *Chlorella sorokiniana* transformed cells.

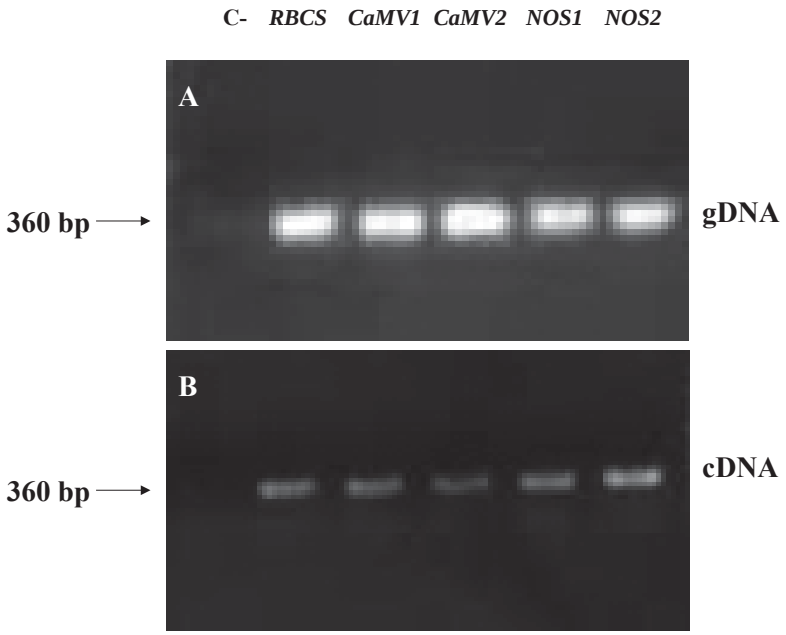

Fig. 1. PCR analysis of genomic DNA and complementary DNA in the analysed transformants using *APHVIII* primer pair. Line C- is a negative control. The subsequent lines show a 360 bp fragment corresponding to the *APHVIII* gene into the genome of the some analysed transformants using the heat-shock-protein/rubisco promoter (*RBCS2*), the *CaMV 35S* promoter (*CaMV1* and *CaMV2*) and the nopaline synthase promoter (*NOS1* and *NOS2*) respectively (A) and after retrotranscription in cDNA (B).

3.3.3. Western Blot analysis

In addition, a Western Blot analysis of the protein extracts from the transformed cells was performed to test the expression of the protein product from the introduced *APHVIII* gene. As was shown in figure 3, the antibodies anti-APHVIII, as previously described (Díaz-Santos et al., 2013), revealed the presence of an electrophoretic band of approximately of 29 kDa corresponding to the APHVIII protein, in all transformants tested.

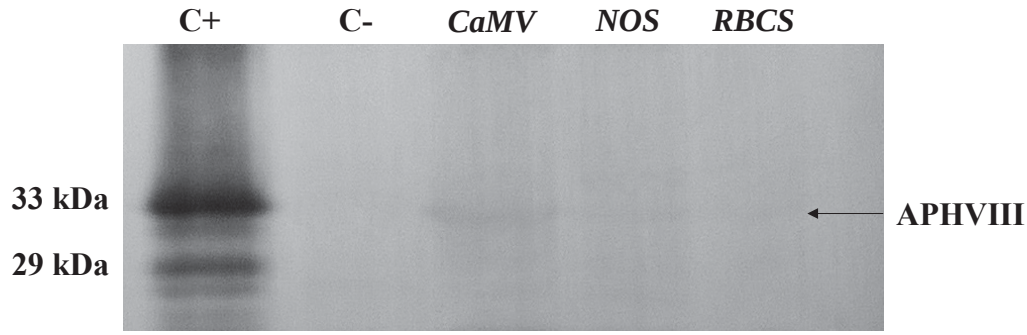

Fig. 3. Immunoblot analysis showing the 32 KDa APHVIIII protein product from *C. sorokiniana* cells transformed with the *APHVIII* gene under the control of *CaMV 35S* (*CaMV*), *NOS* and *HSP70A/RBCS2 (RBCS)* promoters. Total protein extracts were electrophoresed and immunoblotted as described in Material and Methods. The APHVIII protein was detected by anti-APHVIII polyclonal antibodies and alkaline phosphatase-conjugated goat anti-rabbit IgG. A positive control corresponding to 10 mg of the purified APHVIII protein (C+) and a negative control consisting on protein extracts from untransformed *C. sorokiniana* cells (C-) have also been included.

These results obtained at three molecular levels: DNA, mRNA and protein, confirm that the *APHVIII* gene under the control of different promoters is not only successful integrated into the *Chlorella sorokiniana* genome but is also transcribed and translated into protein.

4. Conclusions

In conclusion, the results reported in the current work clearly revealed a feasible technology of electroporation as a method for a stable genetic transformation of *Chlorella sorokiniana* intact cells, for which only particle bombardment was early described. Indeed, the cauliflower mosaic virus 35S RNA promoter, *CaMV 35S*, seems to be the most effective promoter to conduct the expression of foreign genes in *Chlorella sorokiniana*, as occurs in other *Chlorella* strains studied.

Therefore, the establishment of a cheap, simple and suitable method for genetic manipulation of *Chlorella sorokiniana* gives it a more relevant importance in biotechnology, being possible the development of new molecular technologies to increase the biotechnology potential of this microalgae, for example, as biological factory of added-value compounds or alternative system for the overexpression of proteins with a high commercial and biomedical importance.

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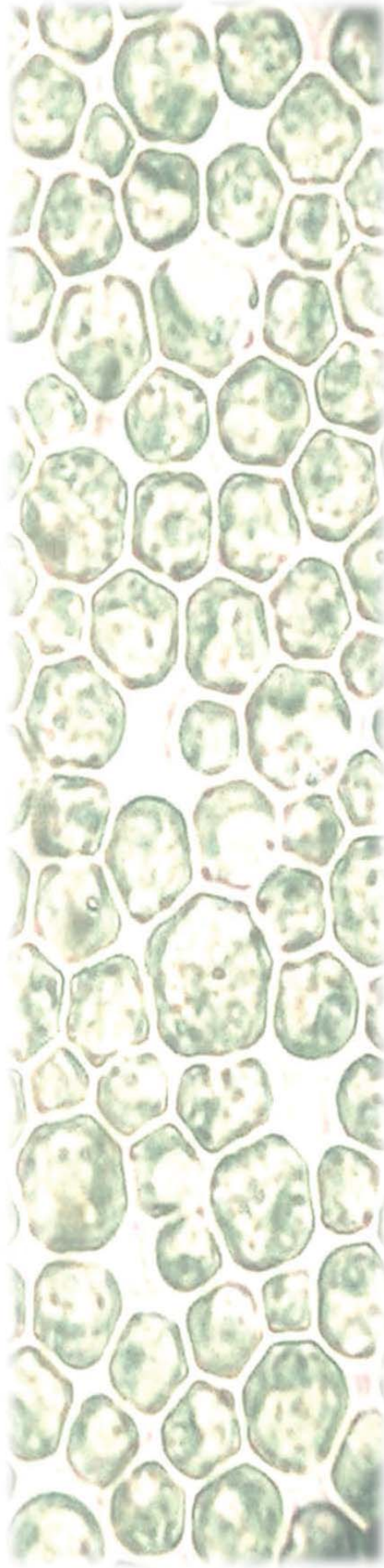
Chapter 5

"Study of Bioflocculation Induced for Saccharomyces bayanus var. uvarum and Flocculating Protein Factors in Microalgae."



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Study of Bioflocculation Induced for *Saccharomyces bayanus var. uvarum* and Flocculating Protein Factors in Microalgae

Encarnación Díaz-Santos, Marta Vila, Marta de la Vega, Rosa León and Javier Vigara

Biochemistry Laboratory, Experimental Sciences Faculty, University of Huelva, Marine International Campus of Excellence (CEIMAR), Huelva 27071, Spain.

Abstract: Autoaggregation of flocculent microalgae in response to stressing conditions is poorly understood, but it is a promising approach to induce the aggregation of microalgae into flocs and make microalgal harvesting a straightforward and cheap procedure. The effect of the self-flocculating yeast strain *Saccharomyces bayanus var. uvarum* on two chlorophytes: the model freshwater microalga *Chlamydomonas reinhardtii* and the novel marine microalga *Picochlorum sp.* HM1, has been investigated. The addition of *Saccharomyces* induces cell aggregation in both microalgal species studied, being the flocculating effect caused by anaerobically grown yeasts almost two-fold the effect of standard aerobically grown yeast. In order to gain more insights in the origin of yeast-induced microalgal flocculation, proteins released into the culture medium by the flocculent yeast *Saccharomyces bayanus var. uvarum* during the fermentative phase of growth were isolated and their ability to induce flocculation was tested. Addition of 0.1 mg mL^{-1} of concentrated flocculating excreted proteins resulted in recovery efficiency values of 95% and 75% for *Chlamydomonas* and *Picochlorum* respectively. The flocculating activity of some plant lectins on the chosen chlorophytes was also evaluated.

Keywords: flocculation; glucanase; lectins; microalgae; yeast

1. Introduction

Microalgae constitute a highly heterogeneous group of photosynthetic microorganisms with a capital ecological importance [1] and an enormous biotechnological potential, which has experimented a renewed interest in the last years [2-4]. More than 5,000 tons of dry algal biomass are produced and marketed per year with an average value of 1.25 billion of euros [5]. Microalgae and their products are used in aquaculture, as additives for animal feed, in human nutrition, and for production of high-added value compounds used as nutraceuticals or dietetics complements [2, 4, 6, 7]. Furthermore, the possibility of using microalgae as feedstock in the production of biofuels, like methane, biohydrogen or biodiesel is being intensively studied [8 -11].

Differently from the production of high-added value nutraceutical or dietetic compounds, algal-based biofuels have to compete with the price of fossil fuels. And despite the enthusiasm generated, the cost for microalgae production, harvesting and processing is very far for being competitive for the production of biodiesel. Harvesting of microalgae biomass and its separation from the culture medium is a critical step, which accounts for about 20-30% of the total production cost [12-14]. Classical physic methods of biomass recovery like filtration or centrifugation are energy intensive and only economically feasible for high-value products [15].

Different ways to induce aggregation of individual microalgae into flocs of larger size and to make microalgal harvesting a straightforward and cheap procedure have been proposed. Addition of trivalent cations, cationic polymers or pH adjustment, which reduce the electrostatic repulsion among cells, are the most common chemical procedures to induce flocculation [14, 16]. But the chemicals added are an additional cost, make difficult reusing the growth medium, can disrupt the cells causing release of internal metabolites and can interfere with further downstream processes.

Many microorganisms can spontaneously flocculate. This mechanism is called self or autoflocculation and is a complex process in which multiple factors are involved [17].

Flocculation of yeasts has been widely studied, due to its relevance for industrial applications such as brewing or wine fermentations or bioethanol production [18]. In yeasts, a family of subtelomeric genes called *FLO*, which encode specific cell surface lectins-like glycoproteins, known as flocculins, are responsible for flocculation [19]. The flocculins in the cell walls of flocculating cells are capable of binding selectively specific carbohydrate residues of adjacent yeast cells. This mechanism of cell-cell adhesion is mainly mediated by highly specific carbohydrates and Ca^{2+} [20, 21]**.** Recent studies suggest that certain cell wall-remodelling enzymes such as glucanases can also have an important role in yeast flocculation [22].

Naturally bioflocculating microalgal species, such *Scenedesmus obliquus*, or *Tetraselmis suecica* [13] have been described. Furthermore, bioflocculation of non-flocculating microalgal species induced by

addition of these flocculating species has been proposed as a promising pre-concentration step in harvesting of microalgae [23]. But unlike yeasts, for which the molecular basis of flocculation is starting to be unravelled, the mechanisms underlying aggregation of flocculating microalgae are poorly understood. Spontaneous aggregation in flocculating microalgae is usually mediated by extracellular polymer substances excreted into the culture medium. Water-soluble extracts of the marine microalga *Skeletoma marinoi* have been reported to induce flocculation of *Nannochloropsis oculata* [24]. And characterization of the flocculating agent isolated from the self-flocculating microalga *S. obliquus* AS6a revealed that self-flocculation of this microalga was mediated by cell wall associated polysaccharides [25]. Agglutination of microalgal cells during sexual reproduction has been studied in certain species. In *Chlamydomonas*, when gametes of opposite mating type are mixed, they immediately undergo cell-cell adhesion via specific hydroxyproline-rich glycoproteins called agglutinins. The sequence and structure of these agglutinin proteins and the sequence of the genes which encode them have been characterized [26].

Agglutinins and flocculins, as well as many other proteins with agglutinating ability, are lectins. These proteins reversibly and non-enzymatically bind specific carbohydrates and play an important role in a high diversity of mechanisms in which cell-cell or cell-molecule interactions are involved [27, 28]. They have been isolated from a diversity of organisms like plants, animals, algae, fungi or microorganisms. Plant lectins are one of the most extensively studied lectins from natural resources due to their high diversity in structure and function. They are involved in physiologic plant mechanisms such as plant defence against predators and pathogens or symbiotic interactions between host plants and symbiotic microbes and also have been used in biomedical studies as histochemical reagents to label cells or used to agglutinate erythrocytes in identifying blood serotypes. Some well-known examples are Concanavalin A, from the legume *Canavalia ensiformis,* UEAI lectin from *Ulex europeaus,* or WGA, wheat germ agglutinin, a cereal lectin [27, 28].

The aim of this work is to gain more insights in the origin of microorganism-induced microalgal flocculation by identifying protein factors able to induce aggregation of microalgae. We have investigated the effect of the flocculating yeast *Saccharomyces bayanus* var. uvarum CECT 1969 and the proteins released into the culture medium by this flocculating yeast, on two chlorophytes: the model and freshwater microalga *Chlamydomonas reinhardtii* and, the novel and marine microalga *Picochlorum sp.* HM1. The study has been completed evaluating the flocculating activity of some plant lectins on the chosen chlorophytes.

2. Materials and Methods

2.1. Materials

Concanavalin A (CON A), *Dolichos biflorus* Agglutinin (DBA), Peanut Agglutinin (PNA), Soybean Agglutinin (SBA), *Ulex europeaus* Agglutinin I (UEA I), Wheat germ Agglutinin (WGA) and *Ricinus communis* Agglutinin I (RCA I), were supplied by Vector Laboratories Inc; U.S.A. The lectins listed were provided as 1 mg of salt-free lyophilized powders and were reconstituted in 0.5 mL of 10 mM HEPES saline buffer, pH 8.4, 0.1 mM Ca^{2+}, except RCA I which was supplied as a 2 mg mL^{-1} solution.

2.2. Microorganisms and culture conditions

2.2.1. Microalgal strains

Freshwater *Chlamydomonas reinhardtii* cell-wall deficient strain 704 (Cw15, Arg7, mt+) was kindly provided by Dr. E. Fernández from the University of Córdoba and cultured photomixotrophically in liquid TAP (Tris-Acetate-Phosphate) medium [29] at 25 ºC under continuous white light irradiation of 100 μE m^{-2} s^{-1}. Saline *Picochlorum sp.* HM1, isolated from the marshlands of Huelva [30], was cultured in F/2 medium with filtered sea water at pH 8 as reported Guillard and Ryther (1962) [31].

2.2.2. Yeast strain

Saccharomyces bayanus var. uvarum CECT 1969 was kindly supplied by the Department of Genetic (University of Seville) and cultured at 28 ºC, initial pH 4.5, in YPD (Yeast Extract Peptone Dextrose) medium containing the following components: peptone (20 g L^{-1}), yeast extract (10 g L^{-1}) and glucose (20 g L^{-1}) dissolved in 1 L of demineralized water. For aerobic growth, cotton capped Erlenmeyer flasks were aerated by shaking at 150 rpm. For anaerobic cultures, yeasts were grown in well-sealed Erlenmeyer flasks at low shaking speed. The pH of the culture medium and the optical density at 620 nm were monitored along fermentation. At the stationary phase of growth, pH of the medium increased from 4.5 to around 6. Cultures at this stage were used for flocculation assays or for the isolation of the proteins released to the culture medium.

2.3. Obtaining proteins from Saccharomyces bayanus fermentation supernatant

Supernatant was separated from a *Saccharomyces* fermentative culture, grown as indicated in section 2.2.2, by centrifugation at 9500 rpm in a refrigerated centrifuge. Ethanol was added to a final concentration of 10% (v/v) to limit microbial contamination and pH of the obtained supernatant was adjusted to 7 with 1 M NaOH. The supernatant was stored at -20 ºC until further purification. Proteins were precipitated by addition of three volumes of methanol and incubated overnight at 4 ºC [32]. Precipitated proteins were collected by centrifugation at 9500 rpm and dissolved in one volume of demineralized water at 1/10 of the

initial volume. The same procedure was performed for a YPD culture medium without yeast inoculum, used as a negative control.

2.4. Protein determination

Protein content of the yeast culture supernatant was measured with the Bio-Rad Bradford assay according to the manufacture protocol, using bovine serum albumin (BSA) as standard.

2.5. SDS-PAGE

Denaturing polyacrylamide gel electrophoresis was carried out on a 10% acrylamide gel, in SDS-electrophoresis buffer pH 8.3, containing: SDS 1 g L^{-1}, Trizma base 3 g L^{-1} and Glycine 14.4 g L^{-1}. After migration, the gel was stained with colloidal CBB G-250.

2.6. Flocculation assays

Flocculation experiments were run in small cylindrical glass tubes (20 mL) with a total final volume of 10 mL, or in polystyrene cuvettes (4 mL) with a total volume of 3 mL, when lectins were used as flocculating agents. The initial optical density of the microalgal cultures was measured at 660 nm and it was adjusted to a value of 1 with the suitable volume of the culture medium. At the beginning and at the end of each flocculation assay, temperature and pH were measured to ensure that they were 25 ºC and 7.5, respectively.

After addition of the flocculating agent each tube was vortexed vigorously for 8 s and subsequently left without agitation during the settling period. To follow the kinetic of flocculation and to evaluate the recovery efficiency for each flocculating agent in both microalgal strains, small culture aliquots were withdrawn from the top part of the tube and the OD_{660} was measured in a spectrophotometer (Ultrospec 3100 pro) at 0, 15, 45, 90, 180 and 360 min after addition of the flocculating agents, according to Papazi et al. (2010) [33] with minor modifications. For lectins, the absorbance was directly followed in the polystyrene cuvettes. Each flocculation assay was run in triplicate (n=3) to test the reproducibility of the experiment.

2.7. Determination of the recovery efficiency

In order to compare the ability of the different flocculating agents tested to induce flocculation in *Chlamydomonas reinhardtii* and *Picochlorum sp.* HM1, the *recovery efficiency* was calculated according to the following equation, adapted from Salim et al. (2011, 2012) [13,23]:

$$Recovery\ efficiency\ (\%) = \left[1 - \frac{\frac{OD_{660}\,(t)}{OD_{660}(t_0)}}{\frac{OD_{c660}(t)}{OD_{c660}(t_0)}}\right] \times 100 \quad (1)$$

where OD_{660} (t_0) and OD_{660} (t) are the absorbances of the samples taken at time zero and at time t, respectively, and OD_{c660} (t_0) and OD_{c660} (t) are the absorbances of the control at time zero and at time t respectively.

3. Results and Discussion

3.1. Microalgal flocculation induced by Saccharomyces bayanus

The spontaneous flocculation of some yeast strains in response to stressing conditions is well known, but, to our knowledge, their ability to induce flocculation in microalgae cultures has not been previously explored. We have selected the industrial and highly flocculating strain, *Saccharomyces bayanus var. uvarum* CECT 1969, to check its ability to induce aggregation of microalgae from the genus *Chlamydomonas* and *Picochlorum*. Flocculation experiments were carried out using yeasts grown in standard aerobic conditions and yeasts after seven days of growth in fermentative anaerobic conditions as detailed in Material and Methods section. Three different concentration ratios of *Saccharomyces*:non-flocculating microalgae (1:9, 3:7 and 5:5) were studied.

The recovery efficiencies were calculated as described in section 2.7 of Materials and Methods and the time course evolution followed for both microalgal species (Fig. 1).

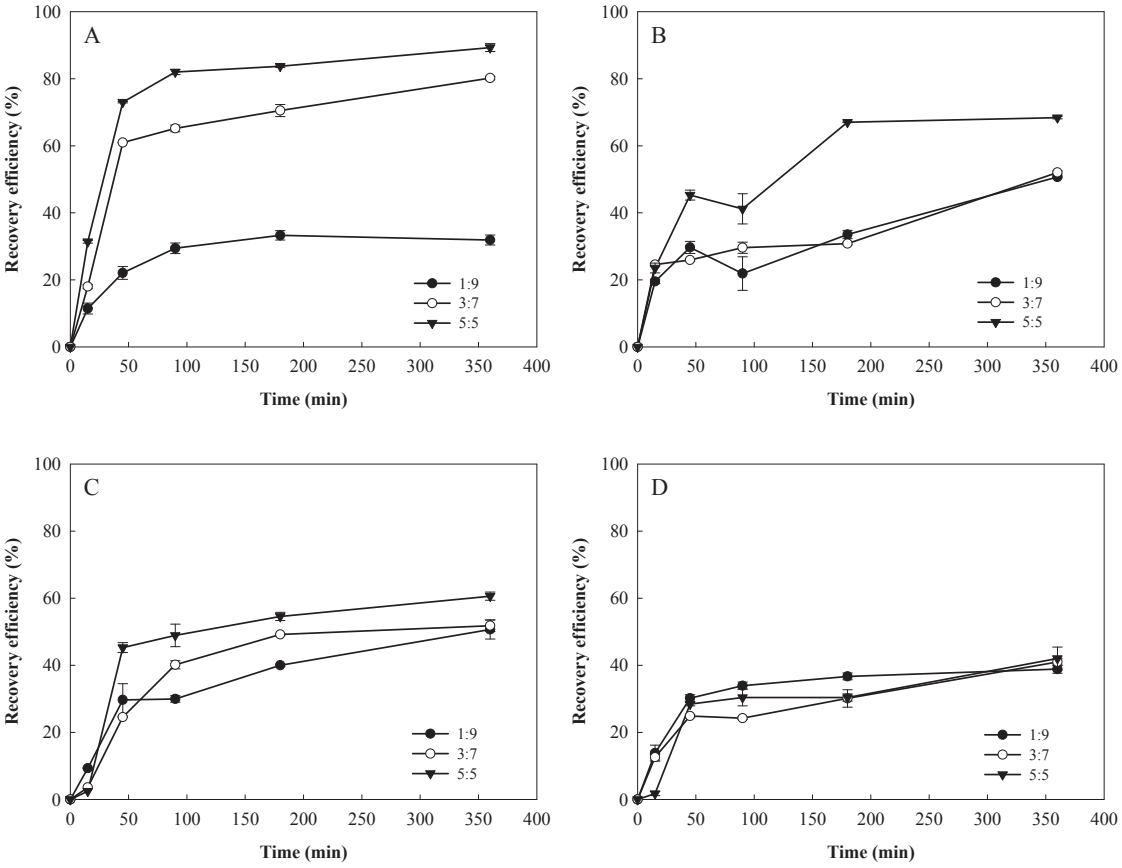

Fig. 1. *Saccharomyces bayanus* var. *uvarum* as flocculent agent for microalgae. *Saccharomyces bayanus* var. *uvarum* cultures grown in anaerobic (A, C) and aerobic (B, D) conditions were added to *Chlamydomonas reinhardtii* (A, B) and *Picochlorum sp.* HM1 (C, D) cultures, at different *Saccharomyces*:non-flocculating microalgae ratios (1:9, 3:7, 5:5). The recovery efficiency was determined along the assay time as detailed in Materials and Methods. Errors bars denote standard deviations between triplicate measurements (n=3).

Using *Saccharomyces* cultures grown in fermentative anaerobic conditions, the values of recovery efficiency for *Chlamydomonas* were approximately 65% and 80%, 90 min after applying concentration ratios of 3:7 and 5:5 of *Saccharomyces*:non-flocculating microalgae respectively, reaching values of 80%

and 90% after 6 h of assay (Fig. 1A). In the experiments with aerobically grown yeast, the efficiencies did not increase over 70% after 6 h (Fig. 1B). At 90 min the recovery efficiency of *Chlamydomonas* microalgal cells treated with anaerobically grown yeast is 2-fold the value observed when bioflocculation is induced with standard aerobically grown yeast.

Similar results were found for flocculation of *Picochlorum*, although the efficiency values were lower. While with anaerobic yeast cultures the recovery efficiencies were ranging between 50-60% with medium and high amount of added yeast culture respectively (Fig. 1C), for aerobic *Saccharomyces* cultures the recovery efficiency values did not reach more than 40% (Fig. 1D). These results show that the addition of *Saccharomyces bayanus var. uvarum* is able to induce flocculation of *Chlamydomonas* and *Picochlorum* cells, increasing the recovery efficiencies in both of cases, but higher recovery efficiencies were observed for *Chlamydomonas,* indicating the specificity of the bioflocculation induced by *Saccharomyces*.

For both microalgal species, the highest recovery efficiencies were observed with yeast cultures grown in fermentative conditions in agreement with the fact that in most flocculating yeast strains, flocculating phenotype only becomes evident at the end of exponential fermentative phase of growth [17]. Additionally, it has been reported that in this phase some *Saccharomyces* strains release into the extracellular medium mannoproteins and glycolytic enzymes involved in cell wall synthesis, playing also an important role in the flocculation [34, 35].

It is well stated that in yeast, flocculation is mediated by aggregating lectinic factors, like flocculins, which recognize glycosidic residues on the surface of the adjacent cells with high specificity [34]. The different recovery efficiencies obtained for *Chlamydomonas* and for *Picochlorum* indicate the cell-cell specificity described above.

3.2. Isolation and identification of proteins with flocculating activity from Saccharomyces bayanus fermentation supernatant

Once established the ability of *Saccharomyces* anaerobic cells to induce flocculation of the microalgal species studied, we aimed to evaluate if the proteins released to the culture medium by the yeast during the anaerobic growth phase also have this ability. We submitted the *Saccharomyces* fermentative supernatant to a series of purification steps, detailed in Materials and Methods, in order to precipitate, separate and identify the proteins which are present in the culture broth.

A sample from the protein preparation obtained was submitted to a SDS-PAGE. Proteins precipitated from YPD culture medium without yeast inoculum was run in parallel as a control. The SDS-PAGE gel showed two protein bands which are not present in the control culture medium, corresponding to proteins released to the culture medium by *Saccharomyces* during the fermentative phase of growth; the major protein with approximately 40 kDa, and other less abundant protein with about 20 kDa (Fig. 2). Both of

them were identified by PMF (Peptide Mass Fingerprinting) at the proteomics service of the University of Córdoba. Results are presented in figure 2. The highest band was identified as Scw10 protein from *Saccharomyces* strains, with a C.I.% of 100. The lower band showed homology with an ADP ribosylation factor.

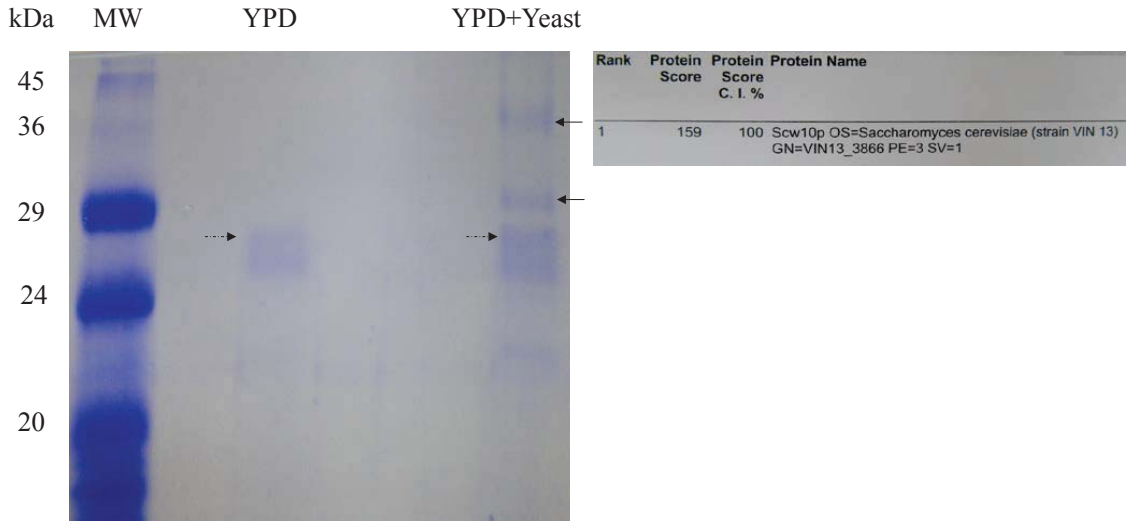

Fig. 2. Analysis of proteins released into the culture medium by *Saccharomyces bayanus var. uvarum* after the fermentation. 6 µg of proteins precipitated from the supernatant obtained from *Saccharomyces* cultures grown in fermentative conditions (lane YPD+Yeast) and from the same medium without yeast inoculum (lane YPD), were separated by SDS-polyacrylamide gel (SDS-PAGE). Filled arrows indicate the position of the proteins found exclusively in the medium in which yeasts were grown. The higher band was identified as Scw10p from *Saccharomyces* strains. Discontinuous arrows indicate the position of the proteins found in both mediums, with and without yeast inoculum.

Using *Saccharomyces* genome database (http://www.yeastgenome.org) and NCBI BLAST tools, it was confirmed that the major protein is a soluble cell wall protein with similarity to glucanases, involved in cell-cell interactions during yeast mating. It has been described that a Scw10 Scw4 double mutant of *Saccharomyces* exhibits defects in mating [36-38]. And, interestingly, in 2012 it was discovered that certain cell wall-remodelling enzymes like glucanases, can also have a relevant role in yeast flocculation [22].

3.3. Microalgal flocculation induced by proteins isolated from the supernatant of Saccharomyces bayanus in fermentative conditions

The ability of the proteins isolated from the supernatant of *Saccharomyces* grown in fermentative conditions to induce flocculation of the microalgae *Chlamydomonas* and *Picochlorum* was tested as described in Material and Methods. Six different concentrations of protein preparations were studied, adding 0.1, 0.25, 0.5, 1, 2 and 3 mg of protein to 10 mL of microalgal cultures. The recovery efficiency for both microalgal species was followed along the time for different quantities of protein preparation (Fig. 3).

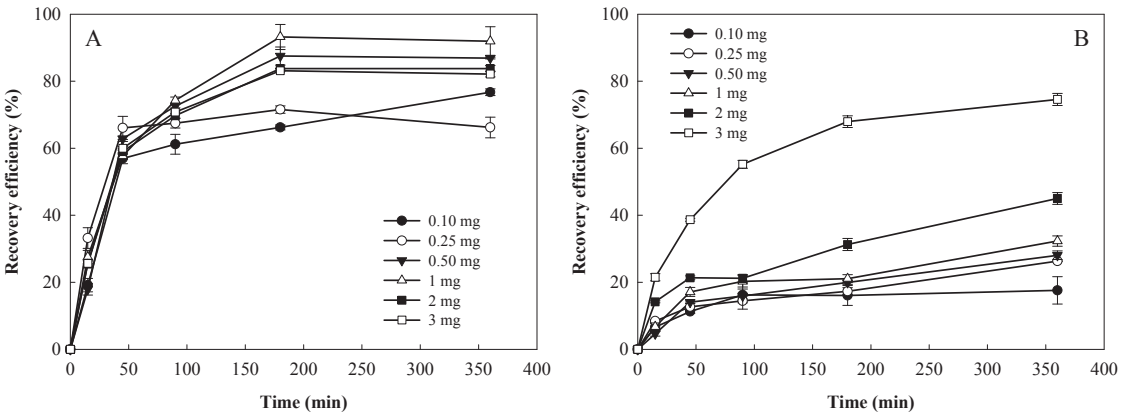

Fig. 3. Effect of proteins isolated from *Saccharomyces* cultures, grown in fermentative conditions, on *Chlamydomonas reinhardtii* and *Picochlorum sp.* HM1 aggregation. Increasing doses (0.1, 0.25, 0.5, 1, 2 and 3 mg) of protein preparation precipitated from the culture medium of *Saccharomyces bayanus* var. *uvarum* grown in fermentative conditions were added to 10 mL of *Chlamydomonas reinhardtii* (A) and *Picochlorum sp.* HM1 (B) cultures. The recovery efficiency was determined along the assay time. Errors bars denote standard deviations between triplicate measurements (n=3).

The results show that for *Chlamydomonas reinhardtii* the optimal dose of yeast protein preparation was 1 mg in 10 mL of microalgae culture, with a recovery efficiency of 75% in 90 min, increasing to almost 95% in 180 min, while a slight decrease of efficiency was observed for higher concentrations of protein preparations (Fig. 3A). In *Picochlorum sp.* HM1 the recovery efficiency increased significantly from 32% to 75% when the yeast proteins dose increased from 1 to 3 mg in 10 mL of culture (Fig. 3B). In both microalgae, flocculation was clearly induced upon addition of extracellular yeast protein preparations, but

the maximum recovery efficiencies reached were slightly lower for *Picochlorum*, and higher quantity of protein preparation was necessary in this case, indicating again the specificity of the flocculating proteins for their binding site [39, 27].

In figure 4 a comparison is shown between the flocculating ability of the flocculating industrial yeast *Saccharomyces bayanus var. uvarum* CECT 1969, grown in aerobic and anaerobic conditions, and the flocculating proteins excreted into the culture medium by the yeast in the fermentative growth phase supernatant. The effect of the YPD culture medium without yeast inoculum has also been included as a negative control. The highest flocculation activity in both microalgae was observed for the *Saccharomyces* proteins reaching recovery efficiency values of 95% and 75%, for *Chlamydomonas* and *Picochlorum,* respectively. No flocculation activity was found for the YPD culture medium. These results confirm the flocculating activity of the extracellular proteins identified in this work, which are released by *Saccharomyces bayanus* when it is grown at fermentative conditions.

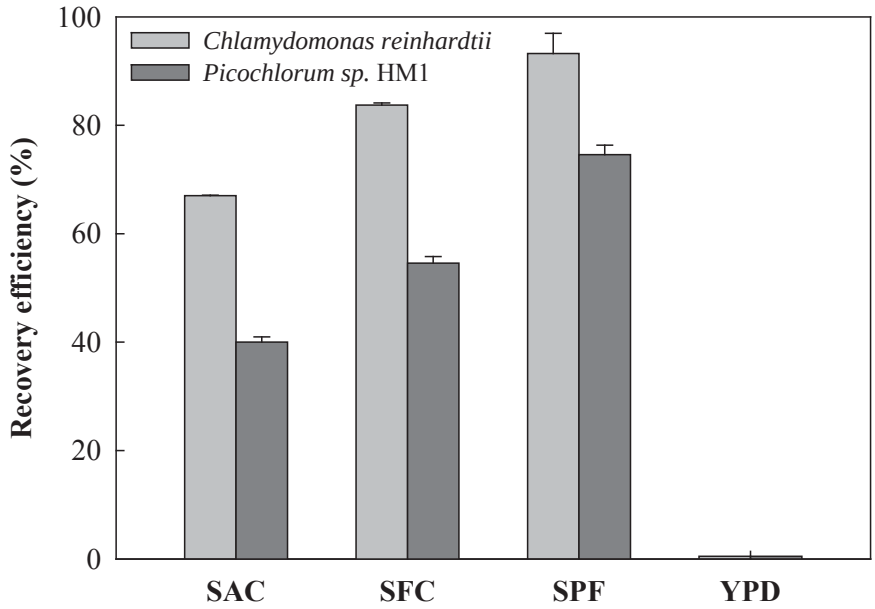

Fig. 4. Comparison between the flocculating ability of *Saccharomyces bayanus var. uvarum* cultures and the proteins obtained from their culture medium as described in Fig. 3. Optimal quantities of *Saccharomyces* cultures grown in aerobic (SAC) and fermentative conditions (SFC) and of *Saccharomyces* protein factors (SPF) were added to *Chlamydomonas reinhardtii* (light grey) and *Picochlorum sp.* HM1 (dark grey) cultures respectively. A YPD medium sample was added as a negative control. The recovery

efficiencies were determined after 180 min. Errors bars denote standard deviations between triplicate measurements (n=3).

Although studies about the use of proteins precipitated from the fermentative yeast supernatant to induce microorganism flocculation are scanty, Van Den Brent (1997) [32] reported the induction of flocculation in *Pediococcus* by proteins from different yeast strains grown in fermentative conditions.

Flocculation experiments were completed with microscopic studies. Micro-photographic images from control and aggregated microalgal samples were taken using a digital camera (CANON Power Shot, 10-megapixel) coupled to an optical microscope (OLYMPUS CX41). In figure 5, an example can be observed showing the aggregation of *Picochlorum* cells into big flocs after addition of the protein preparation, obtained from the supernatant of anaerobically grown yeasts. Similar response was observed for microscopic analysis of both microalgae, *Picochlorum* and *Chlamydomonas* cells, with the other flocculating agents (data not shown).

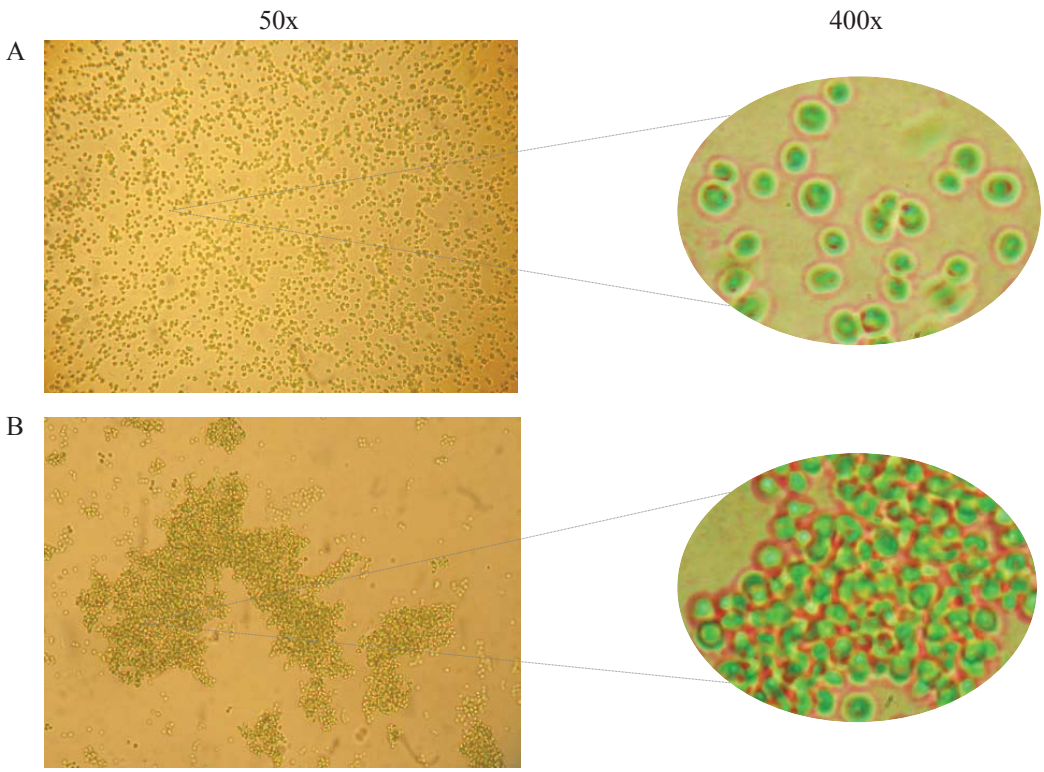

Fig. 5. Microscopic analysis. *Picochlorum sp.* HM1 cells before (A) and after (B) addition of 3 mg of proteins isolated from fermentative cultures of *Saccharomyces bayanus var. uvarum*.

3.4. Microalgal flocculation induced by plant lectins

Although lectins are a diverse group of carbohydrate-binding proteins found in organisms from all kingdoms of live [27], the majority of previous biological, biochemical and molecular studies have focused on plant lectins.

Seven different plant lectins (CON A, DBA, PNA, SBA, UEA I, WGA, RCA I) were selected and their ability to induce the flocculation of *Chlamydomonas* and *Picochlorum* cells was tested**.** Two different concentrations of each lectin (0.1 mg mL^{-1} and 0.01 mg mL^{-1}) were added to the microalgal cultures in the test cuvettes and the flocculation experiments were carried out as described in Materials and Methods.

The recovery efficiencies for the freshwater microalga *Chlamydomonas reinhardtii* treated with the selected lectins, are showed in figure 6. Among the seven tested plant lectins only Concanavalin A (CON A) succeeded to induce flocculation in *Chlamydomonas* cells reaching a recovery efficiency of 90% at a concentration of 0.1 mg mL^{-1}, after 60 min. At a concentration of 0.01 mg mL^{-1} no flocculation was observed. Regarding the other lectins tested in *Chlamydomonas*, the flocculation effectiveness did not reach values higher than 25%. Surprisingly, neither of the lectins tested had the ability to induce flocculation in the marine trebouxiophyceae *Picochlorum* (data not shown) at least not at the concentrations tested.

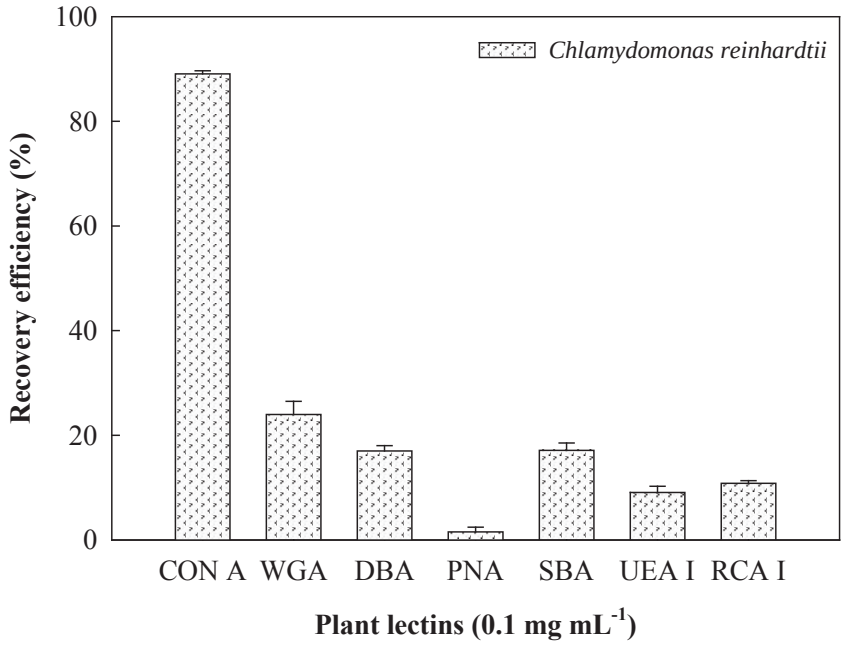

Fig. 6. Effect of plant lectins on *Chlamydomonas reinhardtii* flocculation. Recovery efficiency in *Chlamydomonas reinhardtii* was determined 60 min after addition of the different lectins (0.1 mg mL^{-1}) Concanavalin A (CON A), Wheat germ Agglutinin (WGA), *Dolichos biflorus* Agglutinin (DBA), Peanut Agglutinin (PNA), Soybean Aglutinin (SBA), *Ulex europeaus* Agglutinin I (UEA I) and *Ricinus communis* Agglutinin I (RCA I). Errors bars denote standard deviations between triplicate measurements (n=3).

The different effect of Concanavalin A on the two microalgal strains tested, confirms the high specificity of lectins for a specific recognition carbohydrate motif. The existence of CON A binding sites on the flagellar surface of gametes [40] and vegetative cells [41] of *Chlamydomonas* has been previously reported. These authors quantified the amount of CON A necessary to bind gamete and vegetative cell flagella and also determined to which flagellar glycoproteins CON A binds. They observed that the *Chlamydomonas* gametes were agglutinated by 0.01-0.1 mg CON A mL^{-1} while vegetative cells were agglutinated only by a high concentration, above 0.1 mg CON A mL^{-1} in agreement with our results, which showed that only CON A at a concentration of 0.1 mg mL^{-1} is able to induce flocculation of *Chlamydomonas* vegetative cells.

4. Conclusions

In this work it has been demonstrated that aggregation of microalgae can be induced by the flocculating yeast species *Saccharomyces bayanus* and by the proteins released into the culture medium during its fermentative growth. The ability of yeasts to induce flocculation is especially important during the anaerobic fermentative phase of growth. Similar effect can be achieved adding small quantities of some well-known plant lectin proteins, such as Concanavalin A. Different responses have been observed for the two microalgal species studied, confirming the specificity of the interactions between the flocculating agents and the recognition motifs of microalgal cell surface. Flocculation is much more easily induced, by of all the flocculating factors tested, in the cell wall deficient strain *Chlamydomonas reinhardtii*. The bioflocculating agents evaluated in this study were compared with bioflocculating methods cited in the bibliography (Table 1). The recovery efficiencies observed in the current work are in the same range or even higher than the recovery efficiencies found in the references listed in table 1.

Table 1. Comparison of the effect of different bioflocculating agents on microalgae

Bioflocculating agent	Microalgal species	Recovery efficiency (%)	References
Flocculating agent from *Paenibacillus sp.* AM49	*Chlorella vulgaris*	83	Hee-Mock Oh et al. (2001)
Ankistrodesmus falcatus	*Chlorella vulgaris*	>15	Salim et al. (2011)
Scenedesmus obliquus	*Chlorella vulgaris*	>30	Salim et al. (2011)
Tetraselmis suecica	*Neochloris oleoabundans*	>60	Salim et al. (2011)
Y-PGA (poly y-glutamic acid) from *Bacillus subtilis*	*Chlorella protothecoides*, *Chlorella vulgaris* LICME 001 and *Botryococcus braunii* LICME 003	>95	Zheng, H. et al. (2012)
Y-PGA (poly y-glutamic acid) from *Bacillus subtilis*	*Chlorella vulgaris*, *Nannochloropsis oculata* LICME 002 and *Phaeodactylum tricornutum*	>90	Zheng, H. et al. (2012)
Flocculating agent from *Chlorella vulgaris* JSC-7	*Chlorella vulgaris* CNW11 and *Scenedesmus obliquus*	>90	Alam, M. A. et al. (2014)
***Saccharomyces bayanus var. uvarum* 1969**	*Chlamydomonas reinhardtii*	90	Current study
***Saccharomyces bayanus var. uvarum* 1969**	*Picochlorum sp.* HM1	60	Current study
Flocculating agent from *Saccharomyces*	*Chlamydomonas reinhardtii*	>95	Current study
Flocculating agent from *Saccharomyces*	*Picochlorum sp.* HM1	>75	Current study
Concanavalin A from *Canavalia ensiformis*	*Chlamydomonas reinhardtii*	>90	Current study

Identification of the proteins excreted into the culture medium by fermentative yeasts and study of their ability to induce flocculation of microalgae is essential for a better understanding of the nature of yeast-microalgae interactions. The involvement of protein factors in specific interactions between microalgae and yeast cells opens doors to the easy genetic manipulation of flocculating process in microalgae as it has already happened in yeasts. Genetically modified non flocculating yeasts, expressing flocculating proteins in their cell wall, in which aggregation of cells is achieved, are encouraging examples.

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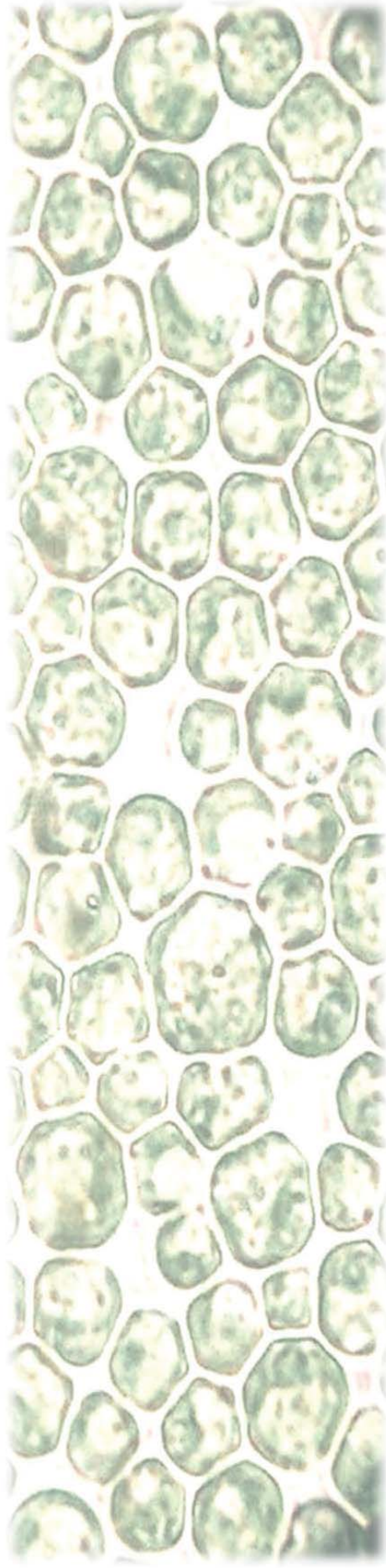
Chapter 6

"Induction of Self-flocculation in Chlamydomonas reinhardtii by Nuclear Genomic Integration of a Flocculin Gene from the Flocculating Yeast Saccharomyces bayanus var. uvarum."

The manuscript of this chapter is in process to be published.



Article

Induction of Self-flocculation in *Chlamydomonas reinhardtii* by Nuclear Genomic Integration of a Flocculin Gene from the Flocculating Yeast *Saccharomyces bayanus var. uvarum*

Encarnación Díaz-Santos, Marta Vila, Javier Vigara, Rosa León

Biochemistry Laboratory, Experimental Sciences Faculty, University of Huelva, Marine International Campus of Excellence (CEIMAR), Huelva 27071, Spain.

Abstract: Harvesting of biomass is one of the most limiting steps in the microalgae industry. The high economic costs make that the studies about suitable and economically efficient methods are increasing. Self-flocculation ability in microalgae cells is a promising strategy to make microalgal biomass recovery a straightforward and cheap procedure, although the studies about this approach are still limited in microalgae. In yeast, this mechanism of self-flocculation and their involved genes are well understood. In the current work, a gene responsible of self-flocculating phenotypes, *FLO5*, from the highly self-flocculating yeast *Saccharomyces bayanus var. uvarum*, is integrated into de genome of the microalgae *Chlamydomonas reinhardtii* by nuclear transformation. Three transformants: CrFLO511, CrFLO513 and CrFLO520 are chosen, having stably integrated the *FLO5* flocculin gene and showing self-flocculation phenotypes. The results showed a faster flocculation in the three obtained transformants than in the *C. reinhardtii* wild type cells.

Keywords: Self-flocculation; microalgae harvesting; *Chlamydomonas reinhardtii*; *Saccharomyces*; *FLO5*

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1. Introduction

Self-flocculation on microorganisms is a biological process in which cells are spontaneously aggregated together to form large flocs. Over the years, flocculation have been extensively studied in yeasts, in which this process has a relevant role during the fermentation in wine or beer industry to separate cells from the medium (Verstrepen et al., 2003; Garcia-Sanchez et al., 2012; Díaz-Santos et al., 2014). Also yeast flocculation has been applied for bioethanol production (Zhao and Bai 2009). In yeasts, the main factors involved in flocculation processes have been genetically studied and identified. The genetic basis of flocculation has been the object of several investigations. These studies suggest that a family of subtelomeric genes, *FLO1*, *FLO5*, *FLO9*, and *FLO10*, encode specific lectins that are responsible for flocculation (Govender et al., 2008). Also *FLO8* and *FLO11* genes have been identified (Soares 2011). Although the regulation of these genes is quite complex, some authors suggest that the only cloning of dominant *FLO* genes as *FLO1* or *FLO5* induce self-flocculation phenotypes in non-flocculating yeasts (Bidard et al., 1994; Kobayashi et al., 1996). Other organisms such as fungi or bacteria have flocculating ability and are used for harvesting of microalgae by bioflocculation (Zhou et al., 2013; Wang et al., 2012). In contrast, the knowledge on self-flocculating microalgae is still limited (Guo et al., 2013). Several spontaneously flocculating microalgae have been identified, emphasizing the species *Ankistrodesmus falcatus*, *Tetraselmis suecica*, *Scenedesmus obliquus*, *Chlorella vulgaris* or *Ettlia texensis* (Guo et al., 2013; Alam et al., 2014; Salim et al., 2014). However, the involved compounds in the self-flocculation of microalgae are not clear, and studies about the responsible genes are not found, but some parameters and mechanisms such as cellular surface charges, extracellular polymeric substances attached to the cellular surface or excretion of these polymers produced by microalgal cells, are proposed by some authors (Kobayashi et al., 1996; Konno et al., 1993). And, in *Scenedesmus obliquus* AS-6-1, *Chlorella vulgaris* JSC-7 and *Ettlia etenxis* the self-flocculation factors have been physical, chemical and microscopically characterized (Guo et al., 2013; Alam et al., 2014, Salim et al., 2014).

Chlamydomonas reinhardtii, a model microalgae in which the genome is totally sequenced, the genetic base is wildly studied and its genetic transformation is well stablished, could be a good candidate to study the molecular and genetic base of the self-flocculation mechanisms using the genetic engineering tools.

The main objective of the current work is to induce self-flocculating phenotypes in *Chlamydomonas reinhardtii* cells by nuclear insertion of the flocculin gene *FLO5*, from the strongly flocculating yeast *Saccharomyces bayanus var. uvarum*, in order to obtain a greater understanding of the factors and mechanisms involved in the spontaneous flocculation process in microalgae.

2. Materials and Methods

2.1. Microorganisms and culture conditions

2.1.1. Chlamydomonas strain

Freshwater *Chlamydomonas reinhardtii* cell-wall deficient strain 704 (Cw15, Arg7, mt+) was kindly provided by Dr. E. Fernández from the University of Córdoba and cultured photomixotrophically in liquid TAP (Tris Acetate Phosphate) medium (Harris et al., 1989) at 25 ºC under continuous white light irradiation of 100 μE m^{-2} s^{-1}.

2.2.2. Saccharomyces strain

Saccharomyces bayanus var. uvarum CECT 1969 was kindly supplied by the Department of Genetic (University of Seville) and cultured in YPD (Yeast Extract Peptone Dextrose) medium containing the following components: peptone (20 g L^{-1}), yeast extract (10 g L^{-1}) and glucose (20 g L^{-1}) dissolved in 1 L of demineralized water, at 28 ºC, pH 4.5 and aerated by shaking at 150 rpm. For solid medium agar at 1% was added.

2.2. Genetic material

In this work, two genes were used to carry out the co-transformation experiments: the aminoglycoside 3'-phosphotransferase encoding gene (*APHVIII*) isolated from *Streptomyces rimosus* which confers resistance to paromomycin and a flocculin encoding gene (*FLO5*) isolated from the flocculating yeast *Saccharomyces bayanus var. uvarum* CECT 1969 which

2.3. Genomic DNA extraction from Saccharomyces bayanus var. uvarum

The extraction of genomic DNA from *Saccharomyces* was performed following the protocol described by Hoffman et al. in 1987 with minor modifications. Three milliliters from a yeast culture grown to saturation were collected by centrifugation and resuspended in 1 mL of distilled water. Cells were transferred to a 1.5 mL microfuge tube, pelleted by centrifugation and resuspended in 0.2 mL of Hoffman solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA). Then, 100 μL of 0.5 mm glass beads were added. The mix was vortexed twice 1 minute each and 0.2 mL of phenol/chloroform were added and the mix was vortexed again twice. 0.2 mL of Hoffman solution and 0.2 mL of phenol/chloroform were added again and centrifuged. The aqueous layer was taken in a microfuge and incubated overnight at -20 ºC with ethanol. Finally, the mix was centrifuged and the pellet was resuspended in elution buffer and treated with RNasa. An aliquot was used as sample for subsequent PCR reactions.

2.4. Small-scale genomic DNA extraction from Chlamydomonas reinhardtii

Two milliliters of *C. reinhardtii* culture were pelleted by centrifugation and cells were resuspended in 300 μL of lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 % SDS, 0.3 M NaCl), vigorously vortexed

for 5 min and incubated in ice for other 5 min. DNA was extracted with phenol/chloroform and precipitated with absolute ethanol, overnight at -20 ºC. The pellet was washed with 70 % ethanol, dried and resuspended in 40 µL of 5 mM Tris-HCl, pH 8.0. Quantification of the genomic DNA obtained and assessment of its purity were done using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific).

2.5. PCR amplifications of APHVIII and FLO5 genes

The PCR reactions for the *APHVIII* gene amplification were performed as was described by Vila et al. in 2013 with minor modifications. In this case, an elongation time of 1 min at 72 ºC was achieved.

For the *FLO5* gene amplification the PCR reactions were run from a sample of 1 µL of DNA genomic in a total volume of 25 µL according to the Certamp Kit for complex amplifications manufacture protocol (Biotools, B&M Labs, Madrid, Spain).

2.6. RNA extraction and reverse transcription

The RNA total extraction was performed using the RNAeasy plant MiniKit of Qiagen according to instructions of the manufacturer. To synthesize cDNA from the total RNA was used the SuperScript II RNaseH- reverse transcriptase manual (Invitrogen).

2.7. Nuclear co-transformation of Chlamydomonas reinhardtii

Nuclear co-transformation was carried out using the glass-bead method of Kindle (1990) with minor modifications. *C. reinhardtii* cells were grown until the middle of the exponential phase of growth (about 1.6×10^6 cells mL^{-1}), harvested by centrifugation and resuspended in fresh TAP medium to obtain a 100 fold concentrated cell suspension. The concentrated cell suspension (0.6 mL) was added to a conical tube containing 0.3 g of sterile glass beads (0.4–0.6 mm diameter), 0.2 mL of 20% polyethylene glycol (MW8000) and the indicated quantities of *APHVIII* and *FLO5* genes. Cells were vortexed for 8 s and resuspended in 50 mL of fresh sterile TAP medium where they were incubated overnight. After this incubation in the absence of antibiotic, the cells were pelleted and spread onto TAP solid medium plates with paromomycin (30 µg mL^{-1}). Transformed colonies were visible after 4 or 5 days.

2.8. Self-flocculation assays

Flocculation experiments were run in small cylindrical glass tubes (20 mL) with a total final volume of 10 mL. The initial optical density of the microalgal cultures was measured at 660 nm and it was adjusted to a value of about 1 with the suitable volume of the culture medium. At the beginning and at the end of each flocculation assay, temperature and pH were measured to ensure that they were 25 ºC and 7.5, respectively. The *Chlamydomonas* cultures were left without agitation during all the settling period. To

follow the kinetic of sedimentation and calculate the self-flocculation percentage in each transformant and the wild type, small culture aliquots were withdrawn from the top part of the tube and the OD_{660} was measured in a spectrophotometer (Ultrospec 3100 pro) at 0, 15, 45, 90, 180 and 360 min after starting the experiment. Each flocculation assay was run in triplicate (n=3) to test its reproducibility.

2.9. Determination of the self-flocculation percentage

In order to test the self-flocculation ability of each transformant and the *Chlamydomonas* wild type, the self-flocculation percentage was calculated according to the following equation adapted from Alam et al. (2014):

$$Self\text{-}flocculation\ \% = (1 - (A/B)) \text{ x } 100 \quad (1)$$

where *A* is the OD_{660} taken at each sampling time and *B* is the OD_{660} taken at time zero.

2.10. Morphological analysis

Microscopy pictures were taken from the *Chlamydomonas reinhardtii* wild type cells and the transformants using a digital camera (CANON Power Shot, 10-megapixel) coupled to an optical microscope (OLYMPUS CX41).

3. Results and Discussion

3.1. Co-transformation of Chlamydomonas reinhardtii with the APHVIII and FLO5 genes

C. reinhardtii cells were co-transformed with 1 µg of *APHVIII* gene and 3 µg of *FLO5* gene by glass beads agitation method as described in Material and Methods section. The *APHVIII* gene was selected as marker gene because of confers resistance to the paromomycin antibiotic in *Chlamydomonas* reinhardtii at a concentration of 30 µg mL^{-1}, as was indicated in a paromomycin sensitivity test performed by Díaz-Santos et al. in 2013, and the transformants could be selected. The *FLO5* gene from the flocculating yeast *Saccharomyces bayanus var. uvarum* was selected to induce the self-flocculation ability in *C. reinhardtii* wild type cells. It was obtained by several PCR reactions from the genomic DNA of the yeast as described in Material and Methods section. Paromomycin resistant transformants obtained in each co-transformation reaction were subcultured several times in plates containing solid TAP medium, supplemented with 30 µg mL^{-1} of paromomycin. For this co-transformation an average of 100 resistant colonies per transformation was obtained which means an efficiency of 1 x 10^{-6} transformant per cell and µg of *APHVIII* gene and per 3 µg of *FLO5* gene. The 50% of these transformants were selected and well-grown in liquid TAP medium for the subsequent molecular analysis.

Studies of genetic co-transformation by glass beads agitation method in *Chlamydomonas* cells are found in bibliography from the 90s when Gumpel and Purton (1994) co-transformed the arginine-requiring *Chlamydomonas* strain 363 using the plasmid *pARG7.8* together with *pSP108*, *pSP115* or *pSP124*. Also, Lumbreras and co-workers in 1998 used the arginine-requiring gene *ARG7* and the bleomycin resistance gene *ble* to co-transform *C. reinhardtii* cells. More recently, León and co-workers in 2007 used the plasmid *pSI103* containing the paromomycin resistance gene together with the plasmid *pSI104tp bkt1* containing the ь-carotene ketolase gene. In our case, the paromomycin resistant gene *APHVIII* and the flocculin encoding gene *FLO5* are chosen for co-transformation experiments. Although the genetic basis of *FLO* genes family responsible for flocculation in yeasts is quite complex, with many genes and transcription factors involved, and has been the object of several investigations (Teunissen et al., 1995, Govender et al., 2008), there are some authors who confirm that the only presence of *FLO1* or *FLO5* dominant genes is enough to induce self-flocculating phenotypes in non-flocculating yeasts (Bidard et al., 1994; Sieiro et al., 1996). Nowadays, studies in which a gene from the *FLO* family is cloned by nuclear transformation into the genome of a microalgae to induce a self-flocculating phenotype, are not found.

3.2. Molecular analysis of the co-transformants

The obtained stable transformants were grown into liquid TAP medium containing paromomycin until reach the growth exponential phase. In this stage, the genomic DNA from each transformant was isolated by the rapid DNA isolation method and an aliquot was used for PCR reactions of *APHVIII* and *FLO5* genes as described in Material and Methods section. PCR analysis by electrophoresis gel showed that in all analysed co-transformants a fragment with the expected size, 360 bp, was found using the primer pair for amplification the *APHVIII* gene (Fig. 1).

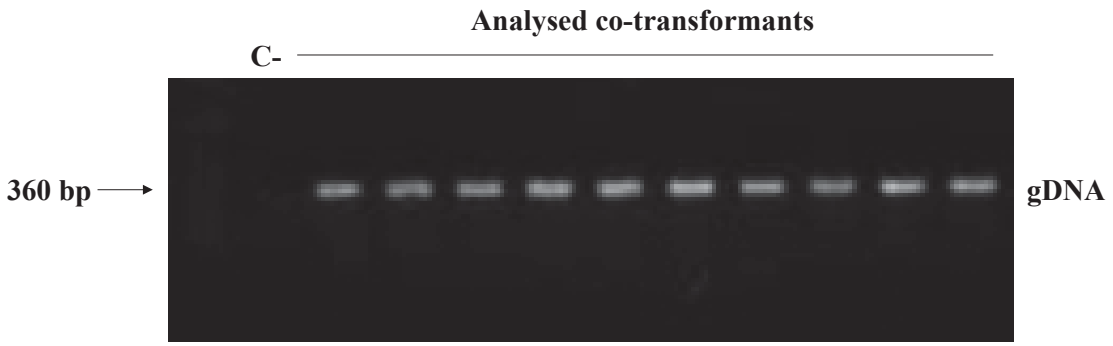

Fig. 1. PCR analysis of genomic DNA in the analysed transformants using *APHVIII* primer pair. Line C- is a negative control. The subsequent lines show a 360 bp fragment corresponding to the *APHVIII* gene into the genome of the co-transformants.

In contrast, only in the 25% of these co-transformants, a band with the expected size, about 3.2 kb, was found using the primer pair for *FLO5* gene amplification (Fig. 2).

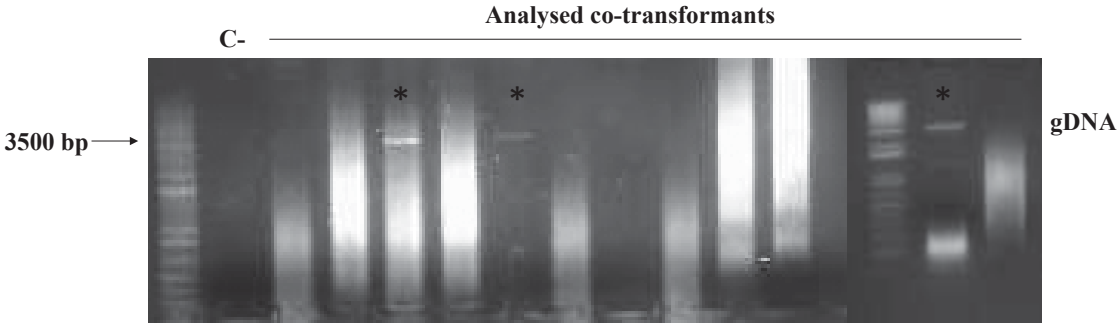

Fig. 2. PCR analysis of genomic DNA in the analysed transformants using *FLO5* primer pair. Line C- is a negative control. The subsequent lines show a 3500 bp fragment corresponding to the *FLO5* gene into the genome of the transformants.

Both gel bands were sequenced to confirm the identity of the genes, obtaining positive results in both of them. These results established that the 100% of the transformants had successfully incorporated the paromomycin resistance gene and instead, only ¼ of these transformants had successfully integrated the flocculin gene into the genome. As is described in bibliography, can be exist a high variability in the transformation efficiencies for different genes and it seems be strongly species-genes-transformation methods dependent (Radakovits et al., 2010). In this case also this variability could be due to the size differences between both genes co-transformed, being easier the genetic integration for shorter exogenes. The transformants which had incorporated both genes were selected and subjected to expression analyses. Two mL of each culture were harvested by centrifugation and the total RNA was isolated as indicated in Materials and Methods section. Single strand cDNA was synthesized from the total RNA according to the SuperScript II RNaseH-reverse transcriptase manual (Invitrogen) and used as substrate for PCR. The

electrophoresis gel showed the presence of the expected fragment corresponding to the *APHVIII* in all analysed transformants but only in three of them the expected fragment for *FLO5* was found (Fig. 3).

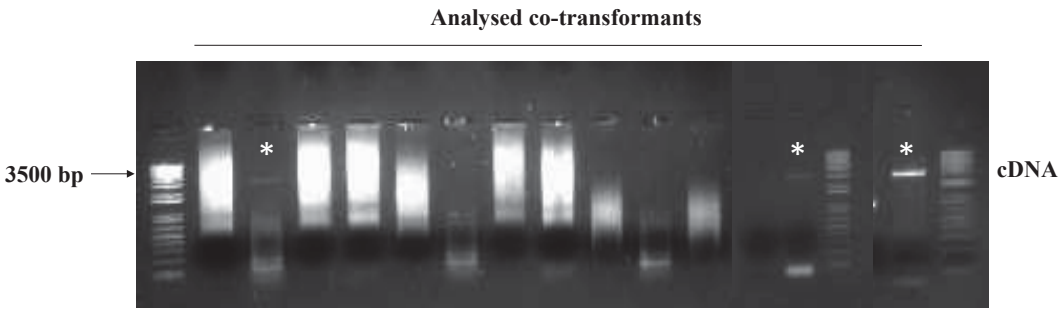

Fig. 3. PCR analysis of complementary DNA in the analysed co-transformants using *FLO5* primer pair. Lines (*) show a 3500 bp fragment, corresponding to *FLO5* gene, indicating its expressions only in those transformants.

These data confirmed that the flocculin gene is not expressed in all transformants in which the gene is integrated into the genome, indicating that probably in these transformants the *FLO5* gene was integrated in a non-coding region, under the influence of any promoter or that the integration was not stable. According to Eichler-Stahlberg et al. (2009), the stability of expression can be improved through proper codon usage, the use of strong endogenous promoters, and inclusion of species-specific 5′, 3′, and intron sequences.

The three transformants which showed expression for the *APHVIII* and *FLO5* genes simultaneity, called CrFLO511, CrFLO513 and CrFLO520, were selected and subjected to subsequent physiological analysis to check if the integration into the genome of the microalgae and the further expression of the *FLO5* gene resulted in self-flocculating phenotypes.

3.3. Self-flocculation assays of Chlamydomonas reinhardtii CrFLO511, CrFLO513 and CrFLO520

The selected *Chlamydomonas* co-transformants, CrFLO511, CrFLO513 and CrFLO520 were subjected to a self-flocculation activity test, in glass tubes, during a total settling period of 360 min as described in Material and Methods. A wild type strain of *Chlamydomonas reinhardtii* was used in parallel as a control. The kinetic of sedimentation was followed to an optical density of 660 nm in the top part of the tubes and the self-flocculation percentage could be calculated according to the equation 1, developed in Material and

Methods section. The obtained results were summarized in figure 4, in which could be observed a higher diminution of the OD_{660nm} during the settling period (Fig. 4A), for the three *Chlamydomonas* co-transformants in comparison with the wild type strain and thus, after application of the self-flocculation percentage equation, a higher increment of this percentage for the co-transformants (Fig. 4B).

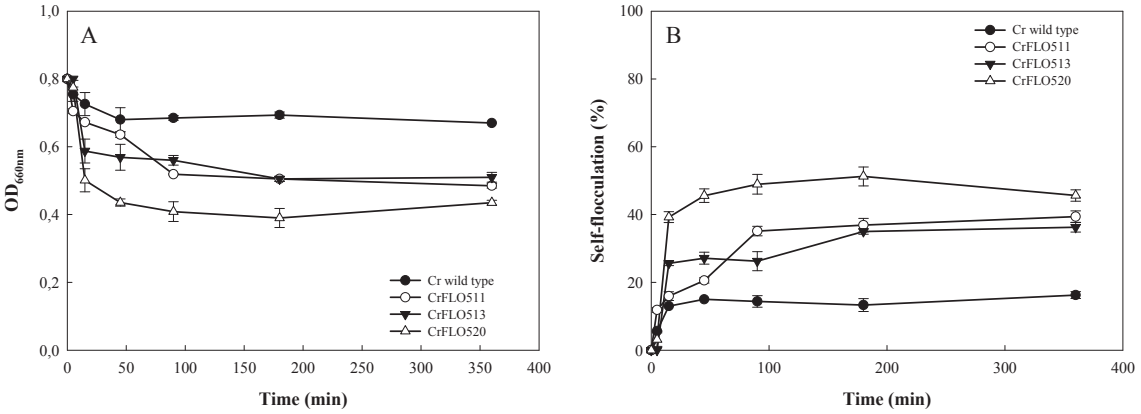

Fig. 4. Analysis of the sedimentation kinetic followed at OD660nm (A) and Self-flocculation percentage (B) in *Chlamydomonas* wild type (Cr wild type) and the co-transformants (CrFLO511, CrFlo513 and CrFLO520).

While the *C. reinhardtii* wild type exhibited a minimal ability of spontaneous flocculation, not reaching the 15%, CrFLO511, CrFLO513 and CrFLO520 showed values of self-flocculation between 36% and 52%, indicating an improvement of the self-flocculation ability of 2-fold and 3.5-fold respectively regarding the wild type and confirming that the insertion of the *FLO5* gene into the genome of these *Chlamydomonas reinhardtii* co-transformants generates self-flocculation phenotypes. Although to date in microalgae studies in which a flocculin gene from yeasts is integrated in their genome do not exist, in yeasts, is described that the insertion of the *FLO5* gene and others dominant homologous genes such as *FLO1* or *FLO8* from flocculating yeasts into the genome of non-flocculating strains, induces flocculating phenotypes (Bidard et al., 1994; Kobayashi et al., 1996). Comparing the self-flocculation percentage obtained for the three selected transformants CrFLO511, CrFLO513 and CrFLO520 at the end of the experiments (360 min), the results showed the maximum value for CrFLO520, 52%, followed by CrFLO511 and CrFLO513 with values of 40% and 36% respectively (Fig 5). Although the ability of self-

flocculation is acquired by the three of *C. reinhardtii* transformants, small differences are observed among their percentage values, being it probably due to differences in the expression degree of the flocculin gene integrated into their genome.

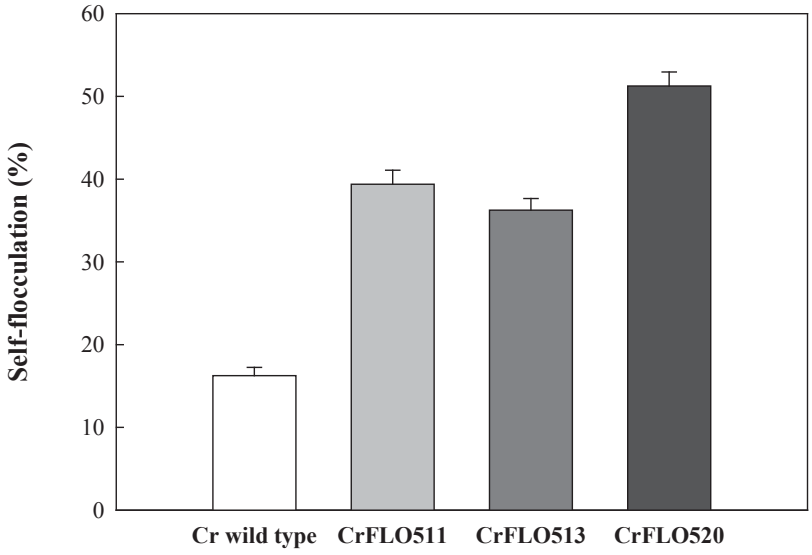

Fig. 5. Self-flocculation percentage in *Chlamydomonas* wild type (Cr wild type) and the co-transformants (CrFLO511, CrFlo513 and CrFLO520) at the end of the settling period (360 min).

Nowadays, the studies about microalgae with spontaneous flocculation, such as *Tetraselmis suecica*, *Ettlia texensis*, *Scenedesmus obliquus* AS-6-1 or *Chlorella vulgaris* JSC-7, their involved factors and their uses for efficient microalgal biomass harvesting, in co-culture with non-flocculating microalgae, are increasing (Salim et al., 2011; Gou et al., 2013; Alam et al., 2014; Salim et al., 2014). In contrast, genetic studies about the genes coding the factors involved in microalgae with self-flocculation phenotypes are not found. The results obtained in the current experiments with the model microalga *Chlamydomonas reinhardtii* could be a good example for the understanding of the genetics involved in spontaneous flocculation phenotypes in microalgae, may be similar to genetics of *FLO* genes family in yeasts.

3.4. Microscopic analysis of the flocs formation

At the end of the self-flocculation experiments, microscopic pictures of an aliquot from the bottom part of the assay tubes were taken, for the transformants CrFLO511, CrFLO513 and CrFLO520 and the *C. reinhardtii* wild type strain. While large flocs were observed for CrFLO511, small and more disperse flocs were observed for CrFLO513 and CrFLO520 and, individual cells were appeared in the sample of *C. reinhardtii* wild type (Fig. 6A). Zooning in the flocculating area (Fig. 6B), could be confirmed how the cells were arranged forming flocs in the three cases. The differences observed in the formation flocs among the three transformants could be due to the variability existing in the flocculation processes, although studies about this topic are scanty in literature. In 1972 Tilton et al. described a cationic adsorption mechanism to explain the bioflocculation induced by microorganisms and most recently, Salim et al. in 2011 proposed the mechanisms bridging and patching to explain the mechanism behind the flocs formation by polymers for different self-flocculation microalgae.

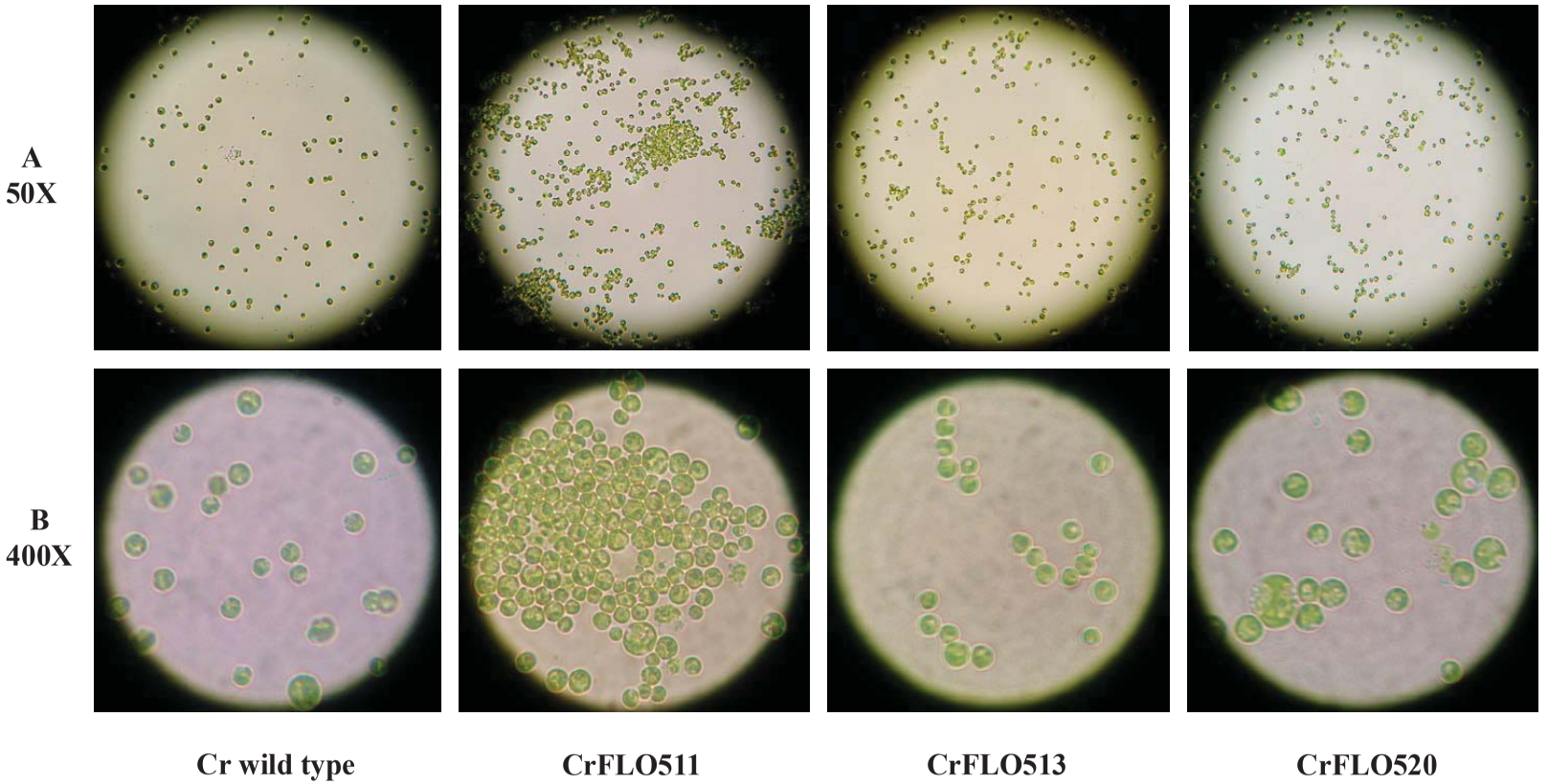

Fig. 6. Microscopic pictures of *Chlamydomonas* wild type cells and the con-transformants CrFLO511, CrFLO513 and CrFLO520 and the flocs formation at 50X (A) and in more detail, zooning at 400X (B).

4. Conclusions

According to the results obtained in the current study could be concluded that the nuclear insertion of a flocculin dominant gene (*FLO5*) from a strongly flocculating yeast, *Saccharomyces bayanus var. uvarum*, into the genome of the non-flocculation microalgae *Chlamydomonas reinhardtii*, induces self-flocculating phenotypes.

The development of a collection of self-flocculating microalgae could contribute greatly to improve the understanding of the molecular bases in self-flocculation processes, to facilitate the harvesting processes of microalgal biomass and to improve the economic costs for a feasible microalgal industry.

To our knowledge, this is the first report in which the induction of self-flocculation in microalgae by nuclear genetic insertion of a flocculin gene from a highly flocculating microorganism, is induced.

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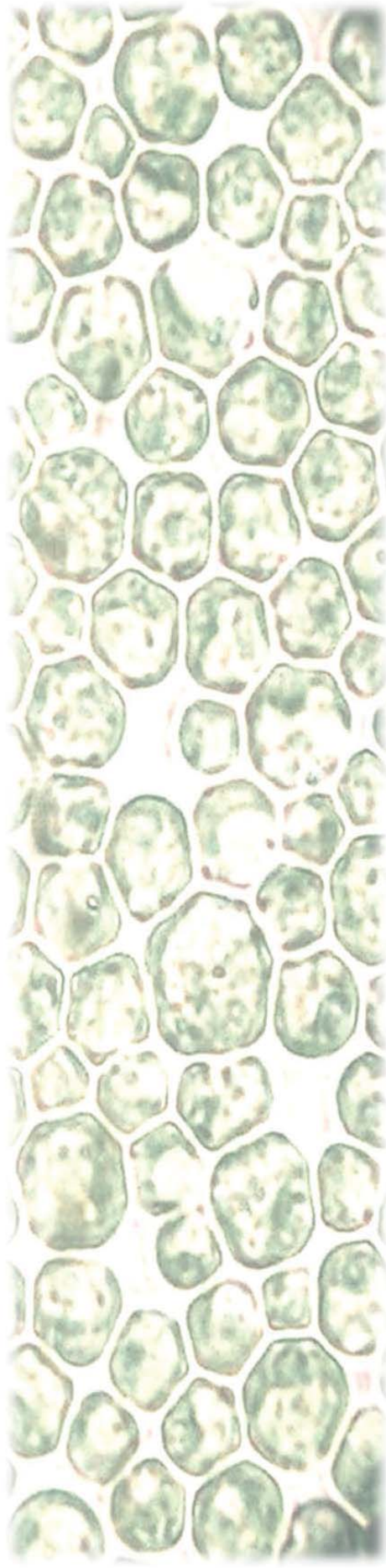
Conclusions



According to the objectives proposed for the present doctoral thesis and the results obtained along the different studies performed in each chapter, the following conclusions can be exposed:

CHAPTER 2

- In *Chlamydomonas reinhardtii*, the endogenous chimeric promoter *HSP70A/RBCS2* shows slightly higher values of transformation efficiencies and *APHVIII* gene expression than the cauliflower mosaic virus 35S promoter (*CaMV 35S*) and the nopaline synthase promoter from *Agrobacterium tumefaciens* (*NOS*), although both heterologous promoters can successfully conduct the *APHVIII* gene expression.

- In *Chlamydomonas reinhardtii*, among the different heterologous promoters tested, the highest transformation efficiencies and the highest levels of *APHVIII* gene expression are found using the nopaline synthase promoter from *Agrobacterium tumefaciens* (*NOS*).

- The rarely used nopaline synthase promoter (*NOS*) could be an efficient system for the genetic manipulation of microalgae.

CHAPTER 3

- In *Chlamydomonas reinhardtii*, the transformation with the promoterless *APHVIII* gene from *Streptomyces rimosus*, which encodes for an aminoglycoside 3′-phosphotransferase and provides resistance to the antibiotic paromomycin, and subsequent isolation of the most robust transformants, enables easy identification of novel promoter sequences by the promoter trapping technique.

- Promoter trapping can allow the selection of endogenous promoters and can be applied to many chlorophytes sensitive to paromomycin.

CHAPTER 4

- The electroporation system is a feasible and stable method for genetic transformation of *Chlorella sorokiniana*, using 2.5 kV of electric field strength and 3 electric pulses as optimal parameters.

- In *Chlorella sorokiniana*, among the different heterologous promoters tested, the highest transformation efficiencies and the highest levels of *APHVIII* gene expression are found using the cauliflower mosaic virus 35S promoter (*CaMV 35S*).

- The use of heterologous promoters for genetic manipulation of microalgae is a specie-dependent system.

CHAPTER 5

- The highly self-flocculating yeast *Saccharomyces bayanus var. uvarum* induces bioflocculation in the microalgae *Chlamydomonas reinhardtii* and *Picochlorum sp.* HM1, reaching recovery efficiency values of 90% and 60% respectively.

- The flocculating protein factors released into the culture medium by S*accharomyces bayanus var. uvarum* during the anaerobic fermentative phase of growth, induce bioflocculation in *Chlamydomonas reinhardtii* and *Picochlorum sp.* HM1 cells, reaching recovery efficiency values of 95% and 75% respectively.

- The plant lectin Concanavalin A from *Canavalia ensiformis* induce bioflocculation in *Chlamydomonas reinhardtii* cells, reaching recovery efficiency values of 90%. And, this ability is not found for *Picochlorum sp.* HM1 cells.

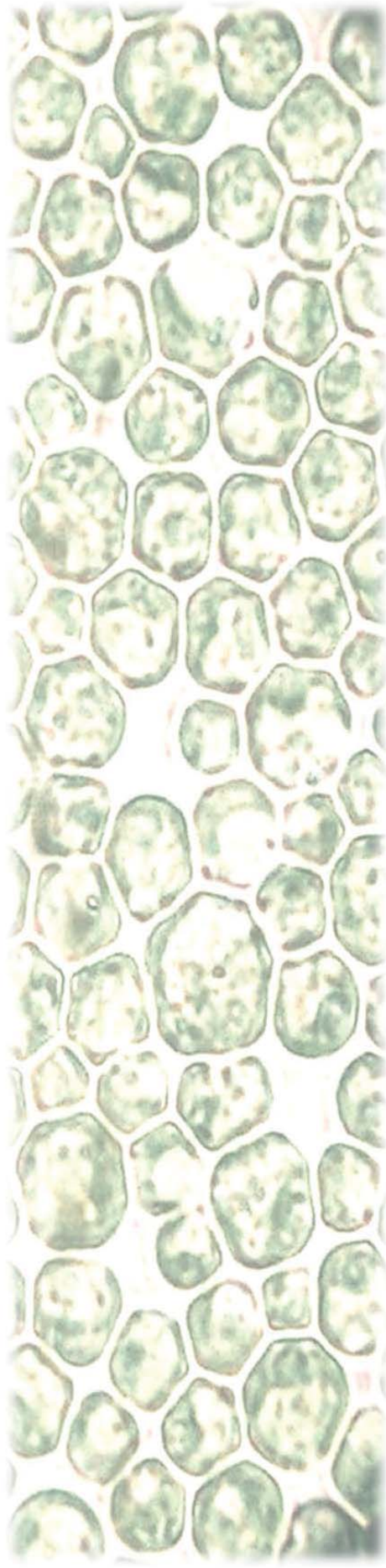
- The recovery efficiency values are higher in *Chlamydomonas reinhardtii* than in *Picochlorum sp.* HM1, due to the first one is a deficient cell wall strain,

concluding that the bioflocculation mechanisms like-lectins are specie-dependent and strongly composition-cellular surface-dependent.

CHAPTER 6

- The nuclear integration of the flocculin dominant gene *FLO5* from the self-flocculating yeast *Saccharomyces bayanus var. uvarum* into the genome of *Chlamydomonas reinhardtii* cells, induce self-flocculating phenotypes in this chlorophyte, exhibiting the transformants self-flocculating percentages up to 3.5-fold more than the wild type strains.

Conclusiones



Teniendo en cuenta los objetivos iniciales propuestos para el desarrollo de la presente tesis doctoral y los estudios llevados a cabo en cada uno de los capítulos de los que consta la misma, se pueden exponer las siguientes conclusiones:

CAPÍTULO 2

- En *Chlamydomonas reinhardtii*, el promotor quimérico endógeno *HSP70A/RBCS2* muestra valores ligeramente superiores de eficiencia de transformación y de expresión del gen de resistencia a pramomicina, *APVHIII*, en relación a los promotores heterólogos utilizados, el promotor del virus del mosaico de la coliflor (*CaMV 35S*) y el de la nopalina sintasa de *Agrobacterium tumefaciens* (*NOS*), aunque éstos últimos muestran capacidad para conducir la expresión de manera eficiente del gen *APHVIII*.

- Entre los diferentes promotores heterólogos analizados, en *Chlamydomonas reinhardtii*, las mayores eficiencias de transformación y los mayores niveles de expresión del gen *APHVIII* son encontrados para el promotor de la nopalina sintasa de *Agrobacterium tumefaciens* (*NOS*).

- El promotor de la nopaline sintasa (*NOS*), un promotor raramente utilizado, podría ser un sistema eficiente para la manipulación genética de microalgas.

CAPÍTULO 3

- En *Chlamydomonas reinhardtii*, la transformación genetica con el gen *APHVIII* carente de promotor, que codifica para una aminoglicosido 3′-fosfotransperasa y confiere resistencia a paramomicina y el posterior aislamiento de los transformants más robustos, hace possible una fácil identificación de nuevas secuencias promotras usando la técnica conocida como promoter trapping.

- La técnica del promoter trapping puede permitir la selección de promotores endógenos en muchas clorofitas sensibles al antibiótico paramomicina.

CAPÍTULO 4

- El sistema de electroporación es un método adecuado y estable para la transformación genética de la microalga *Chlorella sorokiniana*, aplicando como parámetros óptimos 2.5 kV de intensidad de campo eléctrico y 3 pulsos eléctricos.

- En *Chlorella sorokiniana*, de entre los diferentes promotores heterólogos analizados, las mayores eficiencias de transformación y los más elevados niveles de expresión del gen *APHVIII* son encontrados para el promotor del virus del mosaico de la coliflor (*CaMV 35S*).

- El uso de promotores heterólogos como sistema para la manipulación genética de microalgas parece ser fuertemente dependiente de cada especie.

CAPÍTULO 5

- La levadura altamente floculante *Saccharomyces bayanus var. uvarum* induce biofloculación en las microalgas *Chlamydomonas reinhardtii* y *Picochlorum sp.* HM1, alcanzándose valores de eficiencias de recuperación del 90% y 60% respectivamente.

- Los factores proteínicos floculantes liberados al medio de cultivo por S*accharomyces bayanus var. uvarum* durante la fase de crecimiento anaeróbica fermentativa, inducen biofloculación en células de *Chlamydomonas reinhardtii* y *Picochlorum sp.* HM1 alcanzándose valores de eficiencias de recuperación del 95% y 75% respectivamente.

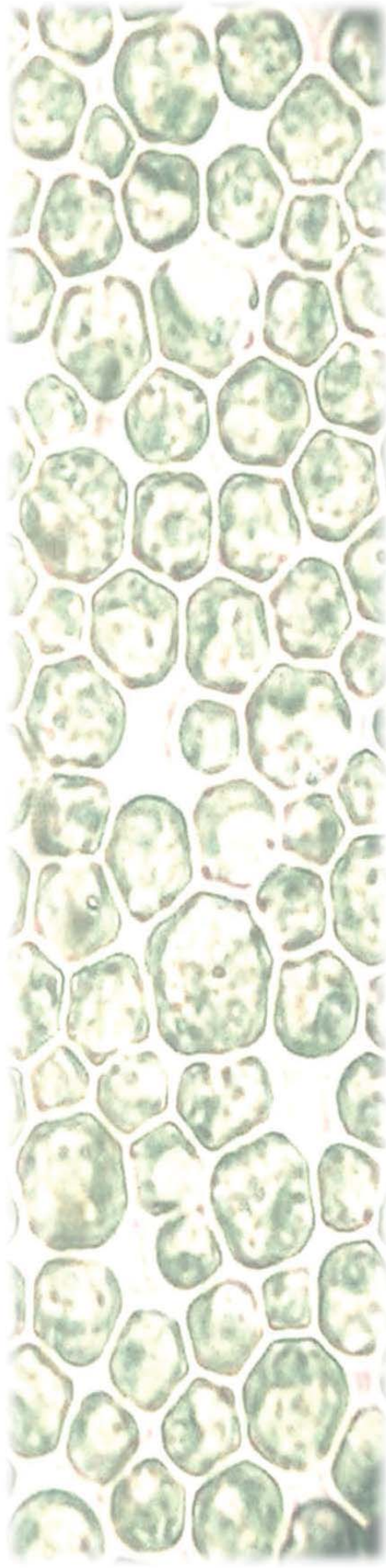
- La lectina de planta Concanavalin A de *Canavalia ensiformis* induce biofloculación en células de *Chlamydomonas reinhardtii*, alcanzándose valores de eficiencias de recuperación del 90%, no encontrándose esta habilidad para células de *Picochlorum sp.* HM1.

- Los valores de eficiencias de recuperación son más elevados en *Chlamydomonas reinhardtii* que en *Picochlorum sp.* HM1 debido en primera estancia a que la primera es una estirpe microalgal carente de pared celular y por consiguiente la composición de la superficie celular en contacto con los agentes biofloculantes es más adecuada, facilitando los procesos de adhesión célula-célula, concluyéndose que los mecanismos de bioflocculación mediados por interacciones lectínicas son dependientes tanto de especie como de composición de la superficie celular en contacto.

CHAPTER 6

- La integración nuclear en el genoma de células de *Chlamydomonas reinhardtii*, del gen dominante *FLO5*, que codifica para una proteína floculina, procedente de la levadura autofloculante *Saccharomyces bayanus var. uvarum*, induce fenotipos de autofloculación en esta clorofita, exibiéndose porcentajes de autofloculación en los transformantes de hasta 3.5 veces superiores a los encontrados en las estirpes silvestres.

Nomenclature

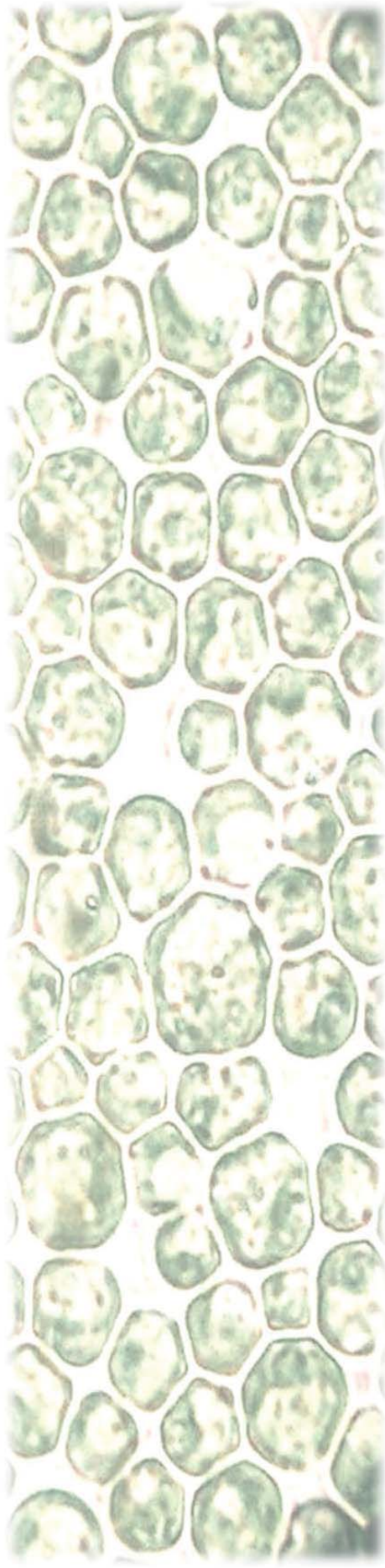


AadA	Spectinomycin and Streptomycin resistance gene
Acc1	Acetyl CoA carboxylase
ADP	Adenosine diphosphate
AP	Phosphatase alkaline
APHVIII	Aminoglycoside 3'-phosphotransferase
Arg	Arginine
Ars	Arylsulphatase
BLAST	Basic local alignment search tool
Ble	Bleomicin
BSA	Bovine serum albumin
C-	Negative control
C.I	Identification coefficient
C+	Positive control
CabII-1	Chlorophyll-ab binding
CaMV 35S	Cauliflower mosaic virus 35S promoter
Cat	Chloramphenicol acetyltransferase
CBB	Coomassie Brilliant Blue
CON A	Concanavalin A
Cop	Chlamyopsin
Cry1-1	Cryptopleurine and emetine resistance gene
Ct	Treshold cycle
DBA	*Dolichos biflorus* agglutinin
DMSO	Dimetysulphoside
EDTA	Ethylenediaminetetraacetic acid
FLO	flocculin gene

For	Forward
Gfp	Green fluorescent protein
GUS	B-Glucoronidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
His	Histidine
Hpt	Hygromycin phosphotransferase
HSP70S/RBCS2	Chimeric promoter heat shock protein/rubisco
Hup1	Hexose uptake protein
IBVF	Instituto de Bioquímica Vegetal y Fotosíntesis
IFAPA	Instituto de Investigación y Formación Agraria y Pesquera
IPTG	Isopropil-β-D-1-tiogalactopiranósido
Luc	Luciferase
mt	mutant
mut	mutant
NCBI	National Center for Biotechnology Information
Nia1	Nitrate reductase
Ni-NTA	Nickel-nitrilotriacetic acid
Nit1	Nitrate reductase
NOS	Nopaline synthase promoter
NptII	Neomycin phosphotransferase
NR	Nitrate reductase
p	promoter
p	pulse
PEG	Polyethylene glycol
PNA	Peanut agglutinin

pro	promoter
PsaD	Photosystem I complex protein
PVDF	Polifluoruro de vinilideno
qPCR	quantitative PCR
RCA I	*Ricinus communis* agglutinin I
RESDA-PCR	Restriction enzyme site-directed amplification PCR
RT	Retrotranscription
RT-PCR	Real time PCR
Rev	Reverse
RNAase	Ribonuclease
SBA	Soybean agglutinin
SV	Simian Virus
tag	tagged
TAP	Tris acetate phosphate medium
TBS	Tris buffered saline
TBS-D	Dilution TBS
TBS-L	Washing TBS
ter	terminator
Ubiq	Ubiquitin gene
UBQL	Ubiquitin ligase gene
UEA I	*Ulex europeaus* agglutinin I
UTR	Untranslated region
WGA	Wheat germ agglutinin
YPD	Yeast extract peptone dextrose
ΔΔCt	Comparative Ct method

Journal Impact Factor Report



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CHAPTER 2: *Biotechnology Progress*, 1999-2014 John Wiley & Sons, Inc. **Impact Factor 1.883**. ISI Journal Citation Reports © Ranking: 2013: 40/123 (Food Science & Technology); 90/165 (Biotechnology & Applied Microbiology).

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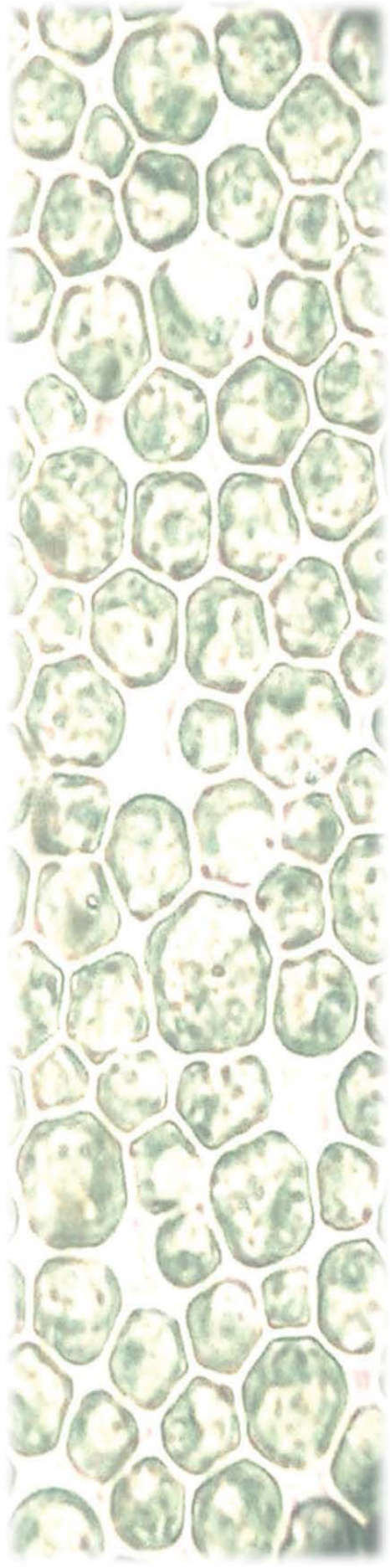

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