Transformation of P. tricornutum by electroporation

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15 ECTS thesis submitted in partial fulfillment of a *Baccalaureus Scientiarum* degree in Biochemistry

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Abstract

The objective of this project was to transform the diatom *P. tricornutum* by electroporation in order to optimize the method. A test protocol was composed in order to incorporate zeocin resistant pPha-T1-GFP plasmid, linearized by ScaI-HF digestion. Transformation of *P. tricornutum* with electroporation is not entirely new nor is it common either due to poor yield of transformants. Electroporation is a method that offers simple solutions, especially if it's combined with a successful optimizing protocol. It´s commonly thought that the electroporation itself is a major factor in limited viability but other aspects have not really been pursued as preparation steps regarding the procedure might also be a limiting factor e.g. even the centrifuge speed could compromise the yield. As it turned out the centrifuge speed was limiting viable cells, either by killing them before electrophoresis or leaving them to week to survive the electroporation. After adjustments of the protocol, viable transformants emerged that were verified by their selective markers i.e. showing zeocin resistance and expression of eGFP.

Útdráttur

Markmið þessa verkefnis var að umbreyta kísilþörungnum *P. tricornutum* með rafgötun til að bæta heimtur. Tilraunaáætlun var sett saman til þess að koma inn zeocin þolnu pPha-T1-GFP plasmíði, klipptu með ScaI-HF skerðisensími. Umbreyting á *P. tricronutum* með rafgötun er ekki óþekkt aðferð en hún er almennt ekki talin hagkvæm þar sem hún gefur fremur litlar heimtur af lífvænlegum kísilþörungum. Rafgötun er í sjálfu sér einföld aðferð, sérstaklega ef mögulegt er að hámarka heimtur. Almennt er talið að rafgötunin sjálf leiði til lélegra heimta en fátt er um að leitað hafi verið eftir öðrum þáttum sem gætu haft neikvæð áhrif á heimturnar. Þar má nefna hraða skilvindu sem dæmi um takmarkandi þátt. Í reynd kom í ljós að harkalegur spuni í skilvindu takmarkaði lífvænlegar frumur, annaðhvort með því að drepa þær eða veikja þær of mikið til að þola rafgötunina. Breytingar voru gerðar á verklagi er leiddi til lífvænlegra, umbreyttra kísilþörunga sem síðan fékkst staðfest í gegnum sértæka merkingu m.ö.o. sýndu zeocin-þol og tjáningu á eGFP

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Abbreviations

P. tricornutum	Phaeodactylum tricornutum
eGFP	enhanced Green fluorescent protein

1 Introduction

1.1 P. tricornutum

These diatoms are relatively simple to grow and already have a history of biological exploitation as living biochemical systems from manipulations of their natural synthesis.

They are unicellular organisms that have a tightly regulated photosynthesis system and depend largely on the oceans seasonal changes in temperature and saline to access nutrition and sunlight which leads to an active biosynthesis for short period of time or commonly known as algae bloom.

They are opportunists that also play an important role in nature by promoting carbon binding in connection to the ocean's silica circle (Armbrust, 2009).

The diatom *P. tricornutum* has some interesting features besides being extremely easy to grow. Remarkably it can be grown without frustule if the growth media has no silicon, (a frustule is a porous silica shell made from silicic acid and coated with a layer of polysaccharides). Other features are difference in morphotypes that can be affected by changes in the environment (Vartanian, Desclés, Quinet, Doady, & Lopez, 2009).

Most diatoms tend to reproduce by binary fission which leads to smaller frustules, meaning that at some point forming of an auxospore. *P. tricornutum* on the other hand does not show any reduction and is considered interesting in its unusual properties of frustule formation (Vartanian, Desclés, Quinet, Doady, & Lopez, 2009). This diatom has also been connected to possible building blocks of nanotechnology or as a pollution indicator (Sendra, Yeste, Gatica, Morreno-Garrido, & Blasco, 2017).

As a photosynthetic organism it has the capability to produce pigments like fucoxanthin and because of high lipid content it has given reason for research as a source of biofuel etc.

1.2 Popular research subjects on *P. tricornutum*

By all accounts *P. tricornutum* has numerus interesting possibilities regarding biosynthesis and perhaps most notable is the environmental and mixotrophic factor. The diatom has numerous biotechnical possibilities as numerous research papers are revealing e.g.

- Mechanism of light absorption systems under various conditions as diatoms are a vital part of the ecosystem (Lepetit, et al., 2017).
- Photosynthetic pigments, particularly the carotenoid fucoxanthin and its antioxidative properties (Eilers, Bikoulis, Breitenbach, Büchel, & Sandmann, 2016).

- High lipid content as a source of biofuel (Caporgno, Torras, Salvadóa, Clavero, & Bengoa, 2016).
- Morphotypes and structure (Tommasi, Gielis, & Rogato, 2017).

1.3 Electroporation as a method

Electroporation has been used for decades to introduce foreign material into yeast, bacteria, plant and animal cells. Its major advantages are nondiscrimination between cell types and reduced biological risk compared to methods that depend on chemical or viral tools for transformation (Qin, Lin, & Jiang, 2012).

The method relies on an electric current that shifts the hydrophobic barrier of the cell for a short period i.e. punches holes into the cell membrane allowing foreign material to seep in. Important technical factors are based on having a successful mixture of wave form, field strength, buffer and temperature.

This method has been used in research on diatoms before, both as a multi pulse method (Miyahara, Aoi, Inoue-Kashino, & Kashino, 2013) and single pulse method (Zhang & Hua, 2014) and on cells with or without frustule regarding *P. tricornutum* (Niu, et al., 2012).

2 Project objective

The goal of this project was to transform *P. tricornutum* by electrophoresis and if successful to develop an outline for an optimizing research protocol based on the results.

3 Methods and materials

3.1 Diatoms

The diatom *P. tricornutum*, shown in figure 1, was purchased from the CCAP algae collection and grown in purified domestic seawater mixed with Guillard's f/2 medium recipe (Guillard, 1975), rather standard in cultivation of marine diatoms, ingredients are shown in table 1.

Figure 1. Phaeodactylum tricornutum Bohlin (1897). CCAP 1055/15. Isolator: De Martino & Ten-Hage (2003). Origin: Marine; Blackpool, England, UK. Culture: Medium f/2 + Si; Axenic; sub. Other: From original strain CCAP 1055/1 - treated with antibiotics (Culture Collection of Algae and Protozoa, 2019)

Table 1. Ingredients of Guillard's f/2 medium stock solution.

Materials	**Stock**	**Molar mass**	**Molar**
(f/2 + Si)	g / L	g / mol	mol/L
$NaNO_3$	75,00	84,99	8,82, E-04
$NaH_2PO_4.2H_2O$	5,00	137,99	3,62, E-05
Na_2SIO_3	25,78	122,06	2,11, E-04
$Na_2EDTA.2H_2O$	4,360	372,24	1,17, E-05
$FeCl_3.6H_2O$	3,150	270,30	1,17, E-05
$CuSO_4.5H_2O$	0,010	249,69	4,00, E-08
$ZnSO_4.7H_2O$	0,022	287,58	7,65, E-08
$CoCL_2.6H_2O$	0,010	237,93	4,20, E-08
$MnCl_2.4H_2O$	0,180	197,91	9,10, E-07
$Na_2MoO_4.2H_2O$	0,006	241,95	2,48, E-08
Cyanocobalamin B_{12}	5,0, E-04	1355,37	3,69, E-10
Thiamine HCl B_1	1,0, E-01	337,27	2,96, E-07
Biotin	5,0, E-04	244,31	2,05, E-09

3.2 Plasmid structure

The Zeocin resistant pPha-T1-GFP plasmid originated from the cloning of *P. tricornutum* transformation vector pPha-T1 (Zaslavskaia, Lippmeier, Kroth, Grossman, & Apt, 2000) and for eGFP (Chu, Ewe, Bártulos, Kroth, & Gruber, 2016) the EcoRV restriction site was used for gene insertion (Gruber, et al., 2007).

E. coli containing pPha-T1-eGFP plasmid was grown in LB media with 100 ug/ml ampicillin overnight, before extraction. Plasmid linearization was obtained through the usage of ScaI-HF digestion, shown in figure 3.1. After linearization the plasmid is purified with DNA cleaning kit. All quantity and quality estimates of the resulting plasmid were examined by Nanodrop and electrophoresis, respectively.

3.3 Electroporation

Cells (1 x 10^8) were collected in exponential phase by centrifugation at 700 g, 15 ℃ for 4 min and washed 3 times with 1 ~ 2 ml of 375 mM sorbitol, followed by addition of 5 µg linearized plasmid and 50 µg of salmon sperm DNA, and incubation on ice for 10 min (Zhang & Hua, 2014).

Electroporation was performed in a Bio-Rad Gene Pulser II Electroporation System. Samples were placed in 2 mm cuvettes and the equipment settings were as follows: Exponential decay mode, 25µF capacitance, 400 Ohm resistance at a field strength of 0.5kV.

Cells were then transferred to 5 ml f/2 media and incubated without shaking (under week light conditions) overnight.

Cell harvesting was performed by centrifugation at 700 g for 5 min at 15°C and then the resulting pellets were resuspended in about 0.2 ~ 0.4 ml f/2 medium. Finally, the cells were transferred onto selective media plates containing 100 µg/µl zeocin.

Solid culture was grown on 1% agar f/2 medium.

4 Results

4.1 Plasmid linearization

Plasmid linearization was obtained through the usage of ScaI-HF digestion, shown in figure 2. The restriction reaction was incubated for 2 hours at 37° C before electrophoresis.

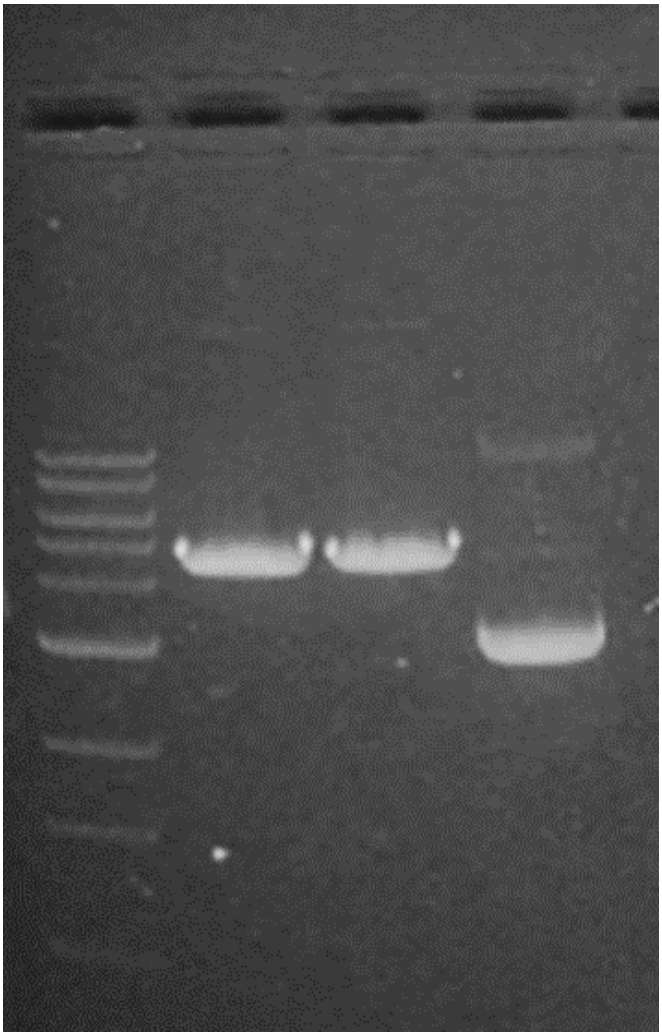

Figure 2. Example of ScaI-HF plasmid linearization. Starting from left: Lane 1 (ladder: 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb, 0.5 kb), lane 2. and 3. show a successful linearization as pPha-T1-egfp is slightly bigger than 4 kb and lane 4. shows a circular plasmid.

4.2 Viable Zeocin transformants

Four plates were prepared, three of them containing transformants and the fourth as a control, which were incubated at 20°C and constant light. After two to three weeks the colonies were ready, the result is shown in figure 3. Two more plates were then inoculated for the three strains of viable transformants and a wild type for comparison. The second plating shows that the transformants are resistant to zeocin, while the wild type is not, shown in figure 4.

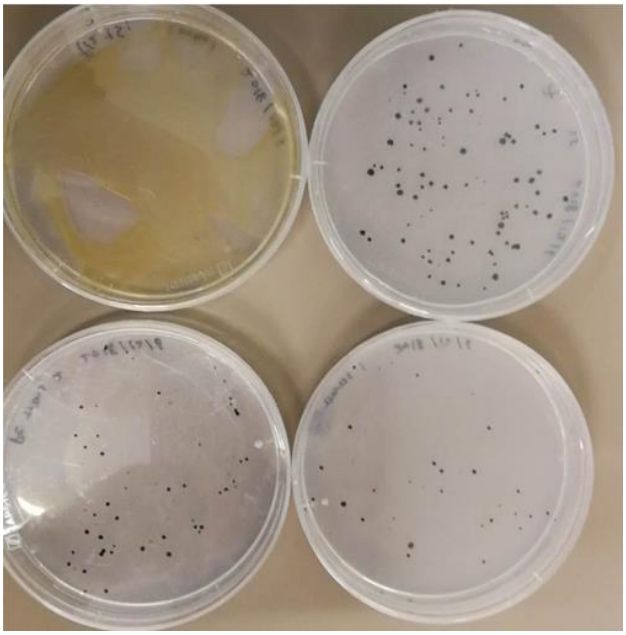

Figure 3. Viable colonies are visible as black spots on plates.

Figure 4. Plates show area of ¾ transformed diatoms and ¼ wild type.

4.3 GFP

To try to determine if the transformants express the eGFP an Olympus FV1200 Confocal microscope (Olympus, Tokyo, Japan), was used in order to capture the images in figure 5. The images confirmed expression of eGFP as it´s clearly visible and can be distinguished from chloroplasts. Both can be detected in the merged image of eGFP and the chloroplasts images and again in a bright field image.

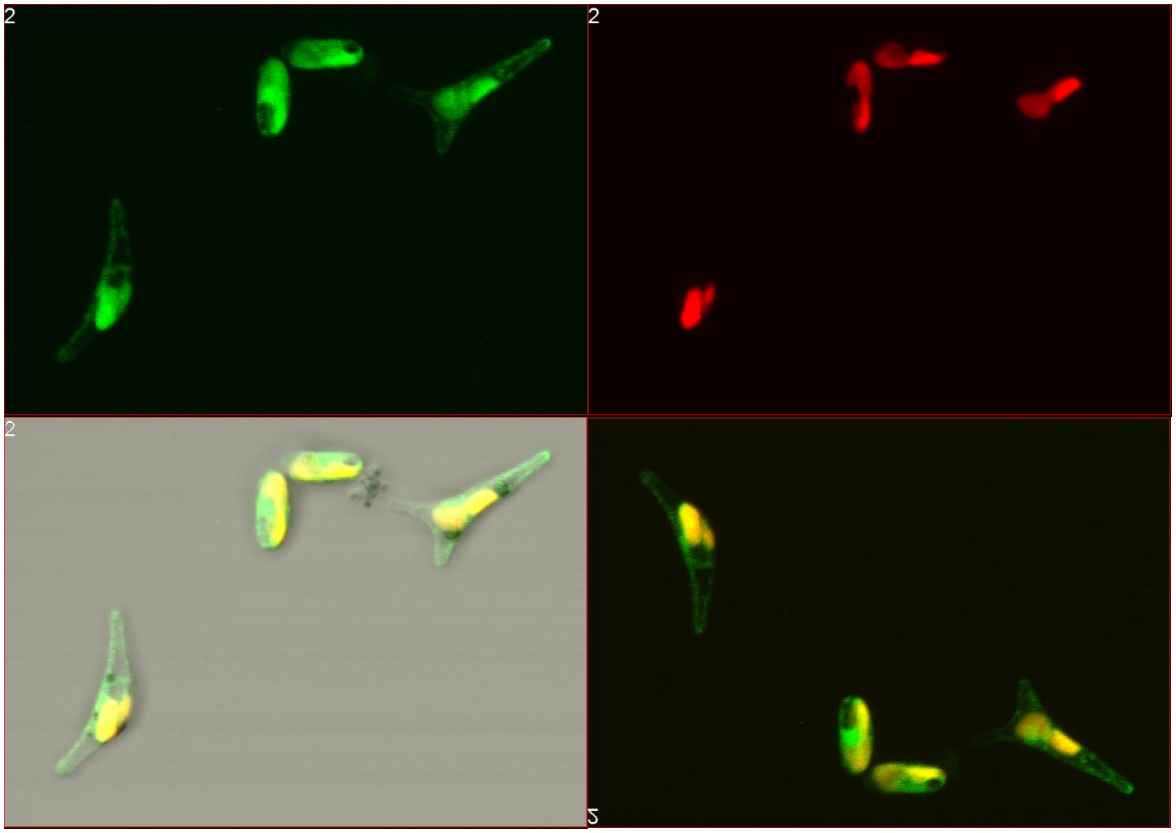

Figure 5. Shows GFP results from an Olympus FV1200 Confocal microscope (Olympus, Tokyo, Japan). Upper left the image shows eGFP, lower left a bright field image, upper right a chloroplast image and lower right shows a merged image.

5 Conclusions

From the results some success can probably be claimed for a few survivors, but the major issue regarding this method has been the poor yield of transformants. As the plates show in figure 3, of the three zeocin resistant plates two of them barely pass 30 colonies.

At first, no cells survived the protocol until a change was made regarding the centrifuge speed by slowing it down from over 1000 x g to 700 x g. It´s rather remarkable how much centrifuge speed varies in older data, from 1500 x g for 5 minutes (Niu, et al., 2012) or 1500 x g for 10 minutes (Zhang & Hua, 2014) down to 700 x g for 4 minutes (Miyahara, Aoi, Inoue-Kashino, & Kashino, 2013).

Studies have mainly suggested that viability is compromised during electroporation, measured by the yield of surviving colonies. The viable cells shown in results above are only viable because of a simple change in centrifuge speed leaving questions regarding optimal treatment before electroporation and further research, exploring viability before and after electroporation.

Other factors that might interfere with the electrophoresis are possibly connected to different strains and morphotypes of *P. tricornutum* e.g. shapes or lack of frustule and amounts of salts or purity, which in turn can increase electric conductivity. Technical factors which could also have effect on the cells are e.g. temperature, resting time after centrifuge, growth phase and the nutritional quality of f/2 media.

Results from GFP are a further confirmation of successful viable transformants as images in figure 5. clearly demonstrate the expression of eGFP. Confirming that a major part of the project's objective yielded some success. On the other hand, these results do not offer any chance for optimizing, without further studies as even if a small number of diatoms seem to be viable and transformed, there are still too many uncertain variables. Nevertheless, this might still include a positive find as it shifted the attention to other obviously important factors regarding the viability of *P. tricornutum*, before any attempts to preform electroporation are made.

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