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RESEARCH PAPER

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Establishment of Stable Genetic Transformation Conditions for Novel Thermotolerant *Scenedesmus* sp. (*Chlorophyta*)

Dilan Akın[1], Çağla Sönmez[2,*], Meral Yücel[1,3], Hüseyin Avni Öktem[1,3]

[1]Middle East Technical University, Department of Biotechnology, Ankara, Türkiye.
[2]Konya Food and Agriculture University, Konya, Department of Molecular Biology and Genetics, Faculty of Agriculture and Natural Sciences, Türkiye.
[3]Middle East Technical University, Department of Biology, Faculty of Arts and Science, Ankara, Türkiye.

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Corresponding Author
Tel.: +90(332)2235453
E-mail:
cagla.sonmez@gidatarim.edu.tr

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Abstract

Microalgae became an attractive source of high value compounds such as fatty acids, carotenoids and other nutrients in recent years. In a previous study, the characterization of a novel thermo-tolerant freshwater microalga, *Scenedesmus* sp. ME02 with high phenolic content, particularly rich in flavonoids was done. Here, two different approaches are reported to increase the efficiency of the growth parameters of this strain. Firstly, the effects of phosphorus (P) depletion on biomass and lipid characteristics of *Scenedesmus* sp. ME02 were investigated. Although, P depletion resulted in a slight reduction in biomass productivity of microalgae and had a minimal effect on the lipid content and productivity; fatty acid methyl ester profile changed substantially. Particularly, percent oleic acid (18:1) increased by more than two-fold, whereas percent linolelaidic acid (C18:2n6t) and linolenic acid (C18:3n6) decreased significantly when cells were switched from P-replete (control) to P-deplete conditions. Overall, the polyunsaturated fatty acid content was significantly reduced. Secondly, stable genetic transformation conditions for *Scenedesmus* sp. ME02 via electroporation were optimized using two different transformation vectors. *GFP* gene expression was detected in colonies transformed with both vectors by RT-PCR. GFP protein expression was detected in the transgenic lines one year after initial transformation by confocal microscopy.

Introduction

Microalgae cultivation for high-value products including carbohydrates, lipids and secondary metabolites has gained considerable attention recently (Malavasi et al., 2020; Oslan et al., 2021; Bulut et al., 2023). Although several microalgal strains with a suitable fatty acid composition and high lipid content are utilized for production at industrial scales, the quest for identification of new strains, optimization of growth conditions and harvesting methods for greater lipid productivity continues in an attempt to lower the operational costs (Chisti, 2007; Mata et al., 2010; Chen et al., 2018; Goh et al., 2019). A common approach to increase the lipid content of a microalgal strain is to redirect the organism's metabolism towards elevated neutral lipid (e.g. TAG) accumulation under stress conditions such as nutrient limitation and temperature (Subhash et al., 2014; Goncalves et al., 2016). Nitrogen (N) depletion has been the most widely employed type of nutrient starvation strategy among members of the *Chlorophyta* with success (Goncalves, 2016).

Phosphorus (P) depletion is also a common method used with some mixed results (Hu et al., 2008).

Metabolic engineering of microalgae via genetic transformation of genes involved in lipid synthesis is an alternative means of microalgal improvement for higher production capacity (Gimpel et al., 2015). This approach is limited by the low efficiency of recombinant protein expression and the necessity to establish specifically optimized transformation methods for each species (Doron et al., 2016). Two *Scenedesmus* species, *S. obliquus* and industrial S*. almeriensis* were stably transformed via electroporation and *Agrobacterium tumefaciens-mediated* genetic transformation, respectively (Guo et al., 2013; Dautor et al., 2014). *S. obliquus* was further assessed for high lipid production via overexpression of the type 2 diacylglycerol acyltransferase gene (*DGTT1)* from *Chlamydomonas reinhardtii* and an increase in both the lipid content and biomass concentration was reported for the transgenic lines compared to non-transformants (Chen et al., 2016).

The microalgal strain, *Scenedesmus* sp. ME02 (C*hlorophyta*) used in this study is a novel strain that has been previously isolated and characterized in our laboratory (Onay et al., 2014). The strain was initially obtained from hot spring flora in Central Anatolia and found to be adaptable to growth in a wide temperature range of 10-50 °C (Onay et al., 2014; Sonmez et al., 2016). *Scenedesmus* sp. ME02 contained 18% palmitic acid (16:0), 6% palmitoleic acid (16:1), 17% oleic acid (18:1), and 25% linoleic acid (18:2) when grown in BG-11 growth medium at 25 °C (Onay et al., 2014). The fatty acid methyl ester (FAME) composition varied significantly depending on the type of growth medium and growth temperature used (Sonmez et al., 2016). Similarly, the lipid content was considerably different under the above-mentioned growth conditions. In our recent study, this strain was shown to have a high total phenolic content and to accumulate high levels of phenolics; gallic acid, 4-hydroxy benzoic acid and chlorogenic acid and flavonoids; quercetin and rutin (Bulut et al., 2019). The adaptability of this strain to different growth conditions, its desirable FAME profile and the ease of manipulation of its metabolic make-up under varying parameters prompted us to evaluate *Scenedesmus* sp. ME02 further for its potential industrial use.

The present study aimed to investigate the ways of improving *Scenedesmus* sp. ME02 particularly for high-value lipid production by 1) evaluating its lipid and biomass productivities and FAME profile under nutrient depletion; 2) establishing a stable genetic transformation protocol that can be further used for metabolic engineering of lipid synthesis or any other recombinant protein production in future studies. As a result, significant changes in the FAME profile under phosphorus (P) depletion were detected, although a notable increase in lipid content or productivity was not observed. In the second part, hygromycin B resistance gene, *aph7* and *GFP* were successfully transformed separately and together into *Scenedesmus* sp. ME02 via electroporation using two different transformation vectors and stable GFP expression was observed by confocal microscopy one year after the initial transformation of cells.

Materials and Methods

Culture and Growth Conditions

Scenedesmus sp. ME02 used in this study was isolated from the thermal springs of Haymana, Ankara (latitude 39.4° N, longitude 32.48° E) in Central Anatolia as previously reported (Onay et al., 2014). The composition of the growth medium, 1:1 TAP: BG-11 containing Tris-Acetate-Phosphate (TAP) and BG-11 media mixed in a 1:1 (v/v) ratio (1:1 TAP: BG-11), was previously described in Sonmez et al. (2016). For phosphate depletion 1:1 TAP: BG-11 medium (TAP: BG-11-P) K_2HPO_4/KH_2PO_4 was substituted with an equimolar amount of KCl. Cultures were either maintained in Petri plates containing 1.5% agar or in flasks with constant shaking at 150 rpm at 25 °C under 16:8 h of light/dark photoperiod with 54 µmol photons m^{-2} s^{-1} light intensity. The growth characteristics of this strain in TAP:BG-11 (1:1 v/v) growth medium were previously reported in Sonmez et al. (2016).

Long-term Maintenance of *Scenedesmus* sp. ME02 Cells via Cryopreservation

Scenedesmus sp. ME02 were grown in TAP: BG-11 (1:1) liquid medium and harvested when optical density (OD) at 680 nm reached 0.8. Filter sterilized DMSO (Merck, USA) was mixed with the liquid culture of cells in 2 ml cryotubes at a final concentration of 8% DMSO. Prepared samples were placed in a cold Mr. Frosty Freezing Container (Nalgene, USA) containing 250 ml isopropanol. The freezing container with the samples was kept at -80 °C for a minimum of 2 hours. 2 weeks, 1 month, 2 months and 6 months later, one tube of cryopreserved sample was thawed in a water bath set at 35 °C for 5 min. After complete thawing, tubes were centrifuged at 800 g at room temperature for 10 min. Pellet was suspended in 1.5 ml TAP: BG-11 medium and 200 µL of culture was spread on TAP agar plates to check the viability of the cells.

Dry Weight and Biomass Concentration Analysis

2-3 × 10^5 cells/ml *Scenedesmus* sp*.* ME02 was inoculated into 1 L TAP: BG-11 (1:1) medium and into 1 L (TAP: BG-11)-P medium (for P depletion studies) in 2 L Erlenmeyer flasks and grown for seven days (until cells reached late logarithmic phase). 5 mL culture samples were taken and centrifuged at 3600× g for 10 min at 4 °C. Pellet was washed twice and dried in glass test tubes at 80 °C for 24 hours and then weighed. Dry weight and

biomass concentrations were performed with three biological replicates and each biological replicate contained three technical measurements.

Lipid Extraction and FAME Analysis

Microalgae cultures were grown as indicated above. Lipid extraction experiments were performed using lyophilization assisted Bligh-Dyer method as described in Onay et al. (2016) and Sonmez et al. (2016) and lipid content (% w/w) was measured gravimetrically. The FAME profile of extracted oils was detected by Gas Chromatography (GC) and analyzed by Agilent HP GC 6890 as previously described in Onay et al. (2014). Averages of three biological replicates with the standard error were reported for all analyses.

Specific Growth Rate, Biomass Productivity and Lipid Productivity Analyses

The specific growth rate was calculated using the formula;

Specific growth rate (μ): $\ln(X_1-X_2)/(t_2-t_1)$

where X_1: biomass concentration at the end of the selected time interval in mg L^{-1};

X_2: biomass concentration at the beginning of the selected time interval in mg L^{-1};

t_2-t_1 time elapsed between the selected time points in days (d)

Biomass productivity (g $L^{-1}d^{-1}$) was described as biomass concentration produced in a specific time interval. Lipid productivity (mg $L^{-1}d^{-1}$) was calculated by multiplying biomass productivity by lipid content.

Hygromycin B Sensitivity of *Scenedesmus* sp. ME02 Cells

5×10^6 cells were spread on a solid TAP medium containing 0, 25, 50 and 75 mg L^{-1} hygromycin B (Sigma Aldrich, Germany). Plates were incubated at 25 °C under 16:8 h of light/dark photoperiod with 54 μmol photons m^{-2} s^{-1} light intensity for 10 days. The growth of *Scenedesmus* sp. ME02 was observed daily and photographed on day 10.

Optimization of transformation conditions

To optimize the transformation parameters, *Scenedesmus* sp. ME02 was transformed with the aminoglycoside phosphotransferase (*aph7*) gene that confers resistance to hygromycin B (Berthold et al., 2002). To amplify the region containing the entire *aph7* cassette in the *pHyg3* plasmid, *Aph7*-F "TCGATATCAAGCTTCTTTCTTGC" and *Aph7*-R "AAGCTTCCATGGGATGACG" were used as forward and reverse primers, respectively. PCR conditions were as follows; 98 °C for 30 sec, denaturation (98 °C for 10 sec), annealing (58 °C for 30 sec) and extension (72 °C for 30 sec) for 35 cycles, then a final extension at 72 °C for 5 min using proofreading enzyme Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific, USA). PCR products were purified with QiaQuick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified PCR products were identified on a 1.5% agarose gel and visualized.

To optimize the transformation conditions, the following transformation parameters were examined; pulse voltage, pulse capacitance, osmosis solution and DNA quantity. Mainly four different conditions were applied; 1 kV voltage, 25 μF capacitance, osmosis solution (0.2 M mannitol, 0.2 M sorbitol, 10% glycerol), one μg DNA; 1 kV voltage, 25 μF capacitance, osmosis solution, 2 μg DNA; 1kV voltage, 25 μF capacitance, Gene Art MAX Efficiency Transformation Reagent (Invitrogen, Life Technologies Inc., USA), 2 μg DNA and 2 kV voltage, 10 μF capacitance, osmosis solution, 2 μg DNA.

Approximately 1×10^8 cells ml^{-1} *Scenedesmus* sp. ME02 were precipitated by centrifugation at 1000× g for 10 min. Pellet was suspended in either cold osmosis solution (0.2 M mannitol, 0.2 M sorbitol, 10% glycerol) or Gene Art MAX Efficiency Transformation Reagent in 1.5 ml plastic tubes and incubated at 42 °C in a water bath for one minute and on ice for five minutes, immediately afterward. After the incubation, the 1.7 kb PCR product containing the *aph7* gene cassette was added to the suspension in indicated amounts. The tubes were further incubated on ice for five minutes. The solution was transferred to 4 mm electroporation cuvettes (Bio-Rad, USA) and electroporated at indicated voltage and capacitance values via Bio-Rad Gene Pulser II electroporation system (Bio-Rad, USA). Electroporated cells were taken into fresh 8 ml 1:1 TAP: BG-11 medium, incubated in the dark for 24 hours, then transferred to TAP agar plates with 75 mg/L hygromycin B, and incubated for 10 days. For the negative control, the same procedure was applied to *Scenedesmus* sp. ME02 cells without the addition of DNA.

Vector Construction and Transformation Experiments

pCAMBIA1302 vector (Abcam, ab275760) contains the *mgfp5* gene that encodes the green fluorescent protein (GFP) under the control of the CaMV 35S promoter. pCAMBIA1302 was linearized with *SacII* restriction enzyme (Thermo Fisher Scientific, USA) before the transformation.

mgfp5 (711 bp) was cut from pCAMBIA1302 with *XbaI* and *KpnI* restriction enzymes (Thermo Fisher Scientific, USA). The fragment was ligated into the multiple cloning site (MCS) of the pChlamy_3 vector (Invitrogen, Life Technologies Inc., USA) within the same restriction sites. The newly constructed vector (pChlamy_3-GFP) was linearized with *ScaI* restriction enzyme (Thermo Fisher Scientific, USA) before transformation (Figure 1).

Scenedesmus sp. ME02 cells were transformed with linearized pCAMBIA1302 or pChlamy_3-GFP using the optimized electroporation protocol described above. The electroporation parameters were 1 kV voltage, 25 µF capacitance, with Gene Art MAX Efficiency Transformation Reagent and 2 µg DNA.

Total DNA Isolation and PCR Analysis of Positive Transformants

Scenedesmus sp. ME02 cells were collected at the stationary phase and lyophilized. Total DNA was extracted from 200 mg freeze-dried microalgae using preheated Cetyltrimethyl Ammonium Bromide (CTAB) solution (5.4×10^{-4} M CTAB, 1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl) to 65 °C in a water bath. The homogenate was incubated with chloroform: isoamyl alcohol (24:1, v:v) and centrifuged. The liquid phase was mixed with 3 M sodium acetate (pH 5.2) and isopropanol and centrifuged for DNA precipitation. The pellet was washed with 70% ethanol and dissolved in 1 M Tris buffer. DNA was amplified using GFP-F "TCAAGGAGGACGGAAACATC"; GFP-R "GGGTCTTGAAGTTGGCTTTG" primers under the following PCR conditions; Initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 30 sec, annealing at 53 °C for 30 sec and extension at 72 °C for 30 sec for 32 cycles, then a final extension at 72 °C for 5 min. PCR products were separated on 1.5% agarose gel by electrophoresis and photographed.

Total RNA Isolation and Reverse Transcriptase PCR (RT-PCR) Analysis

For total RNA isolation from selected transformants, approximately 100 mg microalgae collected at late logarithmic phase were homogenized with liquid nitrogen. Samples were dissolved in RNA extraction buffer (100 mM Tris-HCl pH 8 containing 100 mM NaCl, 5 mM EDTA, 0.5% SDS (v/v), 0.7% 2-mercaptoethanol (v/v)). Phase separation was achieved by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1,v/v) followed by centrifugation at 10,000g for 10 min. The upper phase was taken to a separate tube, mixed with equal volume of isopropanol and 1:10 volume of 3 M sodium acetate (pH 5.2), and centrifuged. Pellet was washed with 70% ethanol and dissolved in diethylpyrocarbonate (DEPC) treated water. RNA was quantified via nanodrop (Bio Drop, England).

First-strand cDNA synthesis was performed with 1 µg total RNA and oligo dT primers using Maxima H Minus First Strand cDNA Synthesis with dsDNase kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RT-PCR was done using GFP-F and GFP-R and ITS2 Forward "GAGCATGTCTGCCTCAGC" and ITS2 Reverse "GGTAGCCTTGCCTGAGC" primers. RT-PCR conditions were the same as in the PCR analysis of transformants.

Imaging of GFP Fluorescence by Confocal Microscopy

Selected transformant cells were grown to a late logarithmic phase and a 10 µl sample was fixed on a slide with formaldehyde. The fluorescent signal in transformant cells was visualized via Leica DMI 4000 confocal microscope. Confocal microscope images were obtained at 482 nm and 583 nm excitation wavelengths for green and red channel, respectively under 40X magnification. The fluorescence of GFP was shown in green and auto-fluorescence from chloroplasts was shown in red.

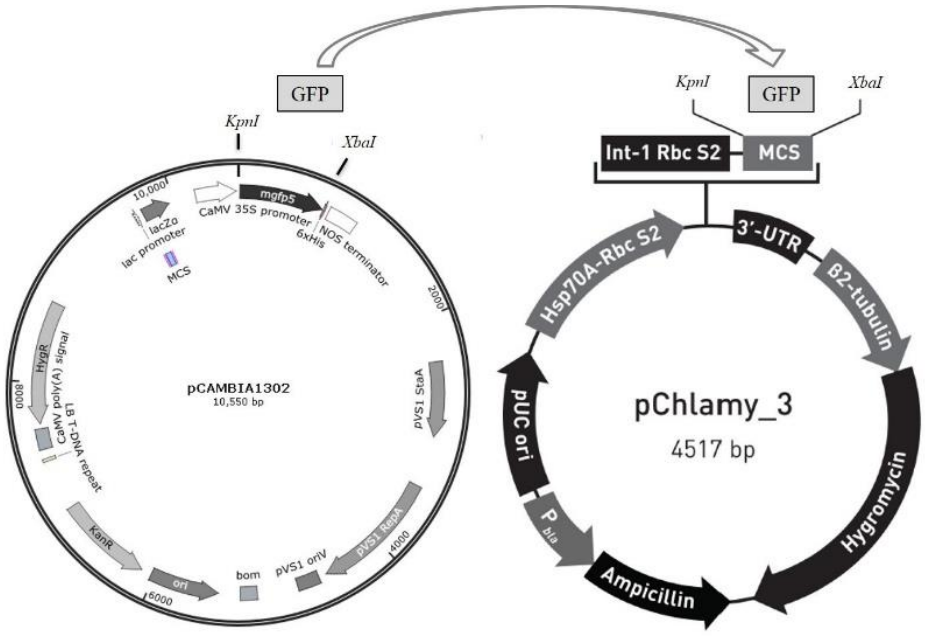

Figure 1. Maps of pCAMBIA1302 and pChlamy_3 vectors. See text for a detailed description of pChlamy_3-GFP vector construction.

Statistical Analysis

All experiments in this study were performed as biological triplicates (n=3) and results were expressed as mean ± standard error (SE). The statistical significance of mean values between the two treatments was evaluated using paired T-test analysis. A p-value<0.05 was considered statistically significant.

Results

Effect of P-Depletion on *Scenedesmus* sp. ME02

Biomass and Lipid Accumulation of *Scenedesmus* sp. ME02 Under P-deprivation

Initially, the growth characteristics of *Scenedesmus* sp. ME02 were investigated under Nitrogen (N) and Phosphorus (P) depletion. Cells grown in a liquid medium deprived of nitrogen (N-deplete) for seven days were pale yellow, whereas the culture medium deprived of P was uniform and green (data not shown). Therefore, further experiments and analyses were carried out under P-depletion—the growth rate, biomass and lipid accumulation and productivities of batch cultures of *Scenedesmus* sp. ME02 were assessed under complete P deprivation (P-deplete) compared to control (P-replete). The results are given in Table 1. There was a slight reduction in specific growth rate, biomass concentration and biomass productivity of samples grown in P-deplete media compared to control. Biomass productivity decreased from 44.44±1.59 mg $L^{-1}d^{-1}$ in P-replete to 38.42±1.27 mg $L^{-1}d^{-1}$ under P-deplete ($p<0.05$). There was no significant difference in lipid content and productivity between the two growth conditions ($p>0.05$).

FAME Profile of *Scenedesmus* sp. ME02 Under P-deprivation

Next, it was addressed whether P deprivation resulted in a significant change in the fatty acid methyl ester (FAME) profile of *Scenedesmus* cells. Although a full array of FAMEs was analyzed by gas chromatography, only the results of those above 1% of the total amount are listed (Table 2). The most striking increase was observed in percent oleic acid (18:1) by more than two-fold from 11.1±0.8% under P-replete (control) to 27.6±0.57% under P-deplete ($p<0.01$). Linolelaidic acid (C18:2n6t) decreased from 10.83 ± 0.55% to 3.3±0.29% and linolenic acid (C18:3n6) decreased from 35.3±0.39% to 19.1±0.23% when cells were switched from P-replete (control) to P-deplete conditions ($p=0.001$). Overall, polyunsaturated fatty acids (PUFAs) decreased by 23%; whereas saturated fatty acids and monounsaturated fatty acids (MUFAs) increased by 6% and 17%, respectively ($p<0.05$) (Table 3).

Table 1. Specific Growth Rate, Biomass Concentration, Lipid Content and Productivities of *Scenedesmus* sp. ME02 under P-replete (control) and P-deplete conditions

	P-replete (control)	P-deplete	*p*-value
Specific Growth Rate (d^{-1})	0.83 ± 0.01	0.81 ± 0.01	0.02
Biomass Concentration (g L^{-1})	0.34 ± 0.02	0.29 ± 0.02	0.02
Biomass Productivity (mg $L^{-1}d^{-1}$)	44.44 ± 1.59	38.42 ± 1.27	0.003
Lipid Content (% w/w)	16.51 ± 1.46	17.01 ± 0.50	0.76
Lipid Productivity (mg $L^{-1}d^{-1}$)	7.25 ± 0.91	6.51 ± 0.19	0.47

Table 2. Percent saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid compositions of *Scenedesmus* sp. ME02 under P-replete (control) and P-deplete conditions

	SFA	MUFA	PUFA
P-replete (control) (%)	20.4 ± 0.52	12.4 ± 0.3	67.1 ± 0.8
P-deplete (%)	26.5 ± 0.03	29.1 ± 0.3	44.4 ± 0.2
p-value	0.006	0.001	0.002

Table 3. Effects of different transformation solutions, DNA amount, voltage/capacitance values on the transformation efficiency (number of transformants per µg of DNA) of *Scenedesmus* sp. ME02

Solution	DNA amount	Voltage/Capacitance	Transformation Efficiency
Osmosis Solution	1 µg	1kV; 25µF	34±1
Osmosis Solution	2 µg	1kV; 25µF	14±3
Osmosis Solution	2 µg	2kV; 10µF	16±2
Osmosis Solution	2 µg	2kV; 25µF	7±1
MAX Efficiency Transformation Reagent	2 µg	1kV; 25µF	43±3

Genetic Transformation of *Scenedesmus* sp. ME02

Hygromycin B Sensitivity of *Scenedesmus* sp. ME02 cells

Before the optimization of transformation, the sensitivity of *Scenedesmus* sp. ME02 cells were tested on varying concentrations of hygromycin B as all the constructs used in the transformation experiments contained hygromycin resistance gene *aph7* as the selectable marker of choice. To determine the appropriate dose for transformation experiments, *Scenedesmus* sp. ME02 cells were spread on solidified TAP medium with 0, 25, 50 and 75 mg L^{-1} hygromycin B. Cells showed growth on 25 mg L^{-1} hygromycin, minimal growth on 50 mg L^{-1} and no growth on 75 mg L^{-1} hygromycin 10 days after culturing (Figure 2). Therefore, transformation experiments were carried out using 75 mg L^{-1} hygromycin B in selective TAP media.

Optimization of Transformation Conditions

Four different parameters including DNA amount, voltage, capacitance and use of osmosis solution or Gene Art MAX Efficiency Transformation Reagent (Invitrogen, USA) before transformation were tested in different combinations to establish the optimum transformation conditions for *Scenedesmus* sp. ME02 cells via electroporation (Figure 3). No significant difference in transformation efficiency in the DNA amount, voltage and capacitance was observed (Figures 3a and 3b). However, the use of Gene Art MAX Efficiency Transformation Reagent instead of the osmosis solution during electroporation increased the number of positive transformants more than two-fold (Figure 3c). The change was statistically significant (p=0.03). A plate containing positive transformant colonies grown on selective TAP media with 75 mg L^{-1} hygromycin B was photographed 10 days after transformation (Figure 3d).

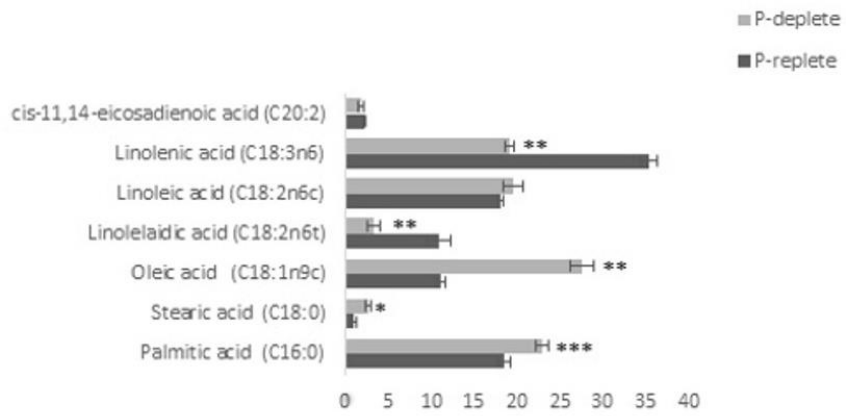

Figure 2. Change in percent FAME composition in P-deplete versus P-replete cells of *Scenedesmus* sp. ME02

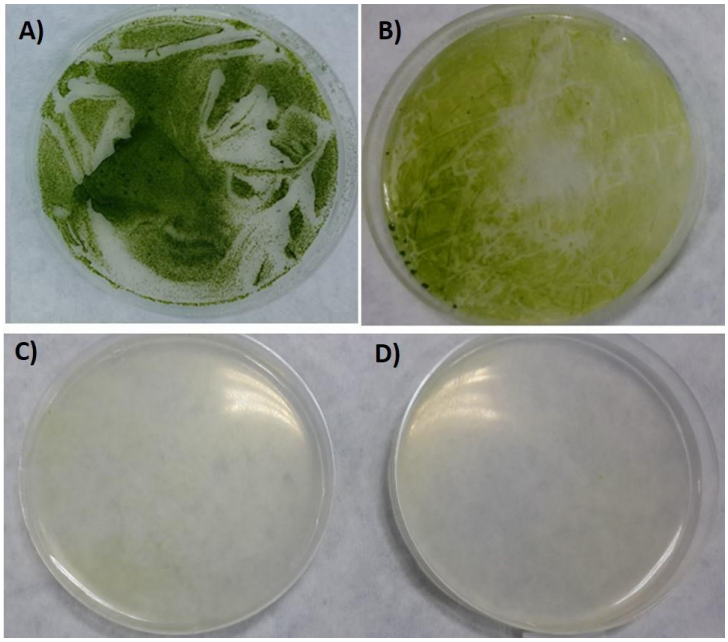

Figure 3. Hygromycin B sensitivity of *Scenedesmus* sp. ME02 cells are tested on solid TAP media containing A) 0 mg L-1; B) 25 mg L-1; C) 50 mg L-1; D) 75 mg L-1 hygromycin B.

Transformation of *Scenedesmus* sp. ME02 with pCAMBIA1302 and pChlamy_3-GFP constructs

Using the optimum transformation conditions established above, *Scenedesmus* sp. ME02 cells were transformed with two different constructs: linearized pCAMBIA1302 vector harboring the plant-optimized *GFP* coding sequence driven by the 35S Cauliflower Mosaic Virus promoter and the same *GFP* cDNA being inserted into the pChlamy_3 vector designed for transformation of *Chlamydomonas reinhardtii*. Stable integration of constructs was verified by DNA isolation from transformants followed by PCR (Figure 4a). A positive band of the expected size of 114 bp was detected in transformants pChlamy_3#5, pCAMBIA#3 and pCAMBIA#12 but not in the non-transformed (wild type) cells.

Verification of GFP mRNA Expression by Reverse transcriptase-PCR (RT-PCR)

GFP expression was assessed at the transcript level by RT-PCR. A positive band of 114 bp was detected in cells transformed with pChlamy_3 (pChlamy_3#5) and pCAMBIA1302 (pCAMBIA#3 and pCAMBIA#12) constructs but not in the wild type cells. A 246 bp band corresponding to the *ITS2* amplicon used as an internal control was detected in all cells (Figure 4b).

Verification of GFP Protein Expression by Confocal Microscopy

GFP protein expression in transformants was visualized by confocal microscopy. Two selected transgenic colonies (pCAMBIA#3 and pCAMBIA#12) transformed with the pCAMBIA1302 vector showed GFP expression, whereas no GFP expression was displayed in two other colonies transformed with the pChlamy_3 construct (pChlamy_3#5 and pChlamy_3#8) (Figure 5). GFP expression in pCAMBIA#12 was still visible one year after the transformation.

Discussion

Choosing a microalgal strain that consorts to high standards simultaneously for biodiesel and other high-value products such as secondary metabolites is of utmost importance for an economically sustainable system (Markou and Nerantzis, 2013). The present study aimed to improve the novel thermo-tolerant microalgal

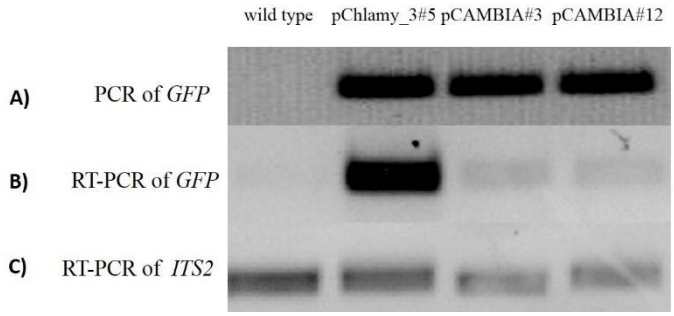

Figure 4. Stable transformation of GFP was verified by detection of a 114 bp amplicon via A) PCR; B) RT-PCR amplification in wild type cells and pChlamy_3#5, pCAMBIA#3 and pCAMBIA#12 transformant colonies. C) A positive band of 246 bp ITS2 was used as an internal control in RT-PCR and was detected in all cell types.

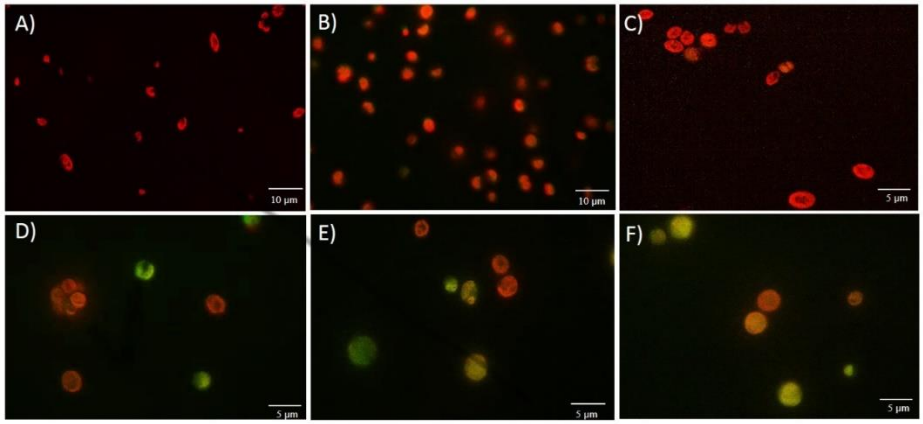

Figure 5. Confocal microscopy imaging of A) wild type Scenedesmus sp. ME02, B) pChlamy_3#5, C) pChlamy_3#17, D) pCAMBIA#3, E) pCAMBIA#12, F) pCAMBIA#12 one year after transformation. The green color represents GFP expression and the red color is the chloroplast auto-fluorescence.

strain *Scenedesmus* sp. ME02 for potential future use at an industrial scale by employing two approaches: 1) assessing the change in the lipid content, composition and productivity under phosphorus depletion, 2) establishing a stable genetic transformation protocol for *Scenedesmus* sp. ME02. This strain was previously characterized as having a suitable fatty acid composition for biodiesel production. However, the lipid and biomass productivities may further be improved to lower operational costs for large-scale utilization (Onay et al., 2014; Sonmez et al., 2016). Additionally, it was recently presented that the same strain exerts potential use in the food or feed industry with a remarkable antioxidant capacity and phenolic content including high amounts of gallic acid, 4-hydroxy benzoic acid, chlorogenic acid, caffeic acid, vanillic acid and quercetin and rutin (Bulut et al., 2019).

Nutrient limitation or starvation is an approach that has been successfully applied in numerous studies to improve the lipid content of microalgae (Hu et al., 2008). It is considered to be advantageous at two levels: Firstly, the operational cost for the cultivation of microalgae can be lowered by eliminating the continuous supply of non-renewable nutrients such as nitrogen or phosphorus in the growth media (Norsker et al., 2011; Wu et al., 2012). It is expected that the costs of elements such as phosphorus will gradually increase with increasing biofuel production as the supply from natural resources is predicted to last for another hundred years (Mayers et al., 2014; Wu et al., 2015). Secondly, the nutrient stress forces microalgal cells to accumulate TAGs as a survival strategy under nutrient limitation which leads to an increase in the lipid content (Xin et al., 2010). Mainly, nitrogen starvation has been successfully applied to increase the TAG content of microalgae (Hu et al., 2008). In a study by Breuer et al., nine microalgal strains including freshwater microalgae *Chlorella vulgaris, Chlorella zofingiensis*, *Nannochloris* sp., *Neochloris oleoabundans* and *Scenedesmus obliquus* were evaluated for changes in biomass accumulation and lipid content and composition under Nitrogen replete (control) and deplete conditions (Breuer et al., 2012). *Scenedesmus obliquus* strain was found to be particularly promising in high amounts of fatty acid and TAG accumulation under nitrogen depletion with minimal loss in biomass productivity compared to nitrogen replete. Similarly, Griffiths et al. (2012) determined that two freshwater microalgae including *Chlorella vulgaris* and *Scenedesmus* sp., among eleven other freshwater and marine microalgal species had the highest biomass productivity and most significant increase in lipid content under nitrogen limitation. In our previous study, thermo-tolerant freshwater microalgae *Micractinium* sp. ME05 was cultivated under 25% and 50% nitrogen, 50% phosphorus and complete phosphorus depletion and evaluated for changes in biomass and lipid productivities compared to control (replete) (Sonmez et al., 2016). There was no significant change in either biomass or lipid productivities under nitrogen limitation; however, lipid productivity increased under phosphorus depletion despite a slight decrease in biomass productivity.

Phosphorus is an essential component of nucleic acids and phospholipid molecules and has a central role in energy transfer in all living cells. Tillberg and Rowley (1989) observed an increase in cell size and shape and cell wall thickness and an increase in the number and size of starch granules and lipid bodies in *Scenedesmus* cells within 96 hours of phosphorus starvation. In the present study, *Scenedesmus* sp. ME02 showed no growth under nitrogen depletion and no change in either biomass or lipid productivities under P depletion compared to control conditions. In a previous study by Xin et al. (2010), *Scenedesmus* sp. LX1 showed a substantial increase in its lipid content under a low concentration of initial P compared to a high initial concentration. However, lipid productivity did not change due to the low yield of algal biomass under low P. In yet another study, lipid and triacylglycerol (TAG) contents of the same *Scenedesmus* strain increased by 35% and 60%, respectively under P deprivation (Wu et al., 2015).

Although changes in biomass and lipid productivities in freshwater microalgae under phosphorus deprivation have been reported in numerous studies, there is relatively limited information on the changes in fatty acid profiles of these microorganisms under P-depletion. In the current study, a significant shift in fatty acid composition towards saturated and monounsaturated fatty acids (SFAs and MUFAs) was observed under P depletion with more than 20% decrease in polyunsaturated fatty acid (PUFA) content, which can be explained by a decrease in desaturase activity in the cells leading to slower/decreased synthesis of PUFA (Granger et al., 1993; Reitan et al., 1994). This hypothesis is further supported by a recent transcriptomic analysis which revealed a significant downregulation in transcript levels of three desaturase genes involved in fatty acid biosynthesis in *Scenedesmus* sp. upon P-limitation (Yang et al., 2018). Similarly, *Micractinium* sp. was previously reported to show an increase in MUFAs and a decrease in PUFAs under P depletion compared to the control (Sonmez et al., 2016). However, other reports demonstrate that the relative changes in saturated/unsaturated fatty acid content upon nutrient limitation may be species-specific (Liu et al., 2016). In this study, oleic acid (18:1) increased more than two-fold, while linolelaidic acid (C18:2n6t) and linolenic acid (C18:3n6) contents showed a sharp decline. In a previous study, *Chlamydomonas reinhardtii* cells showed an increase in oleic acid and linoleic acid and a decrease in linolenic acid contents under P-deplete compared to the control (Çakmak et al., 2014).

As stated earlier, a combination of different approaches is necessary to maximize the efficiency of microalgae cultivation at the industrial scale for use as feedstock for biorefinery and bioproducts. For this

purpose, in this study, stable genetic transformation conditions of *Scenedesmus* sp. ME02 cells were optimized via electroporation. Different genetic transformation methods including biolistics, glass bead, electroporation and Agrobacterium-mediated gene delivery have been established in microalgae. The success of the transformation method is reported to be species-specific. For instance, electroporation but not the glass bead method proved successful for the efficient transformation of a transgene into *Scenedesmus obliquus* cells; however, in yet another *Scenedesmus* sp., *S. almeriensis*, researchers reported no success with electroporation but obtained transgenic microalgae using Agrobacterium-mediated transformation (Guo et al., 2013; Dautor et al., 2014).

Stable genetic transformation into *C. reinhardtii* was previously established using electroporation in our laboratory (unpublished results). Therefore, electroporation was the initial method for genetic transformation into *Scenedesmus* sp. ME02 cells in this study. Out of four different parameters that were tested including DNA amount, voltage and capacitance and osmosis solution, only the choice of the osmosis solution (0.2 M mannitol, 0.2 M sorbitol, 10% glycerol *vs.* Gene Art MAX Efficiency Transformation Reagent) had a significant effect on the transformation efficiency ($p=0.03$).

The choice of vector harboring the appropriate selective marker gene and promoter is also crucial for a successful transformation. In this study, the Hygromycin resistance gene, *aph7* was used in the initial phases of transformation optimization studies of *Scenedesmus* sp. ME02 as a PCR product and during the transformation of *GFP* gene within two different vectors, pChlamy_3 and pCAMBIA1302, as a selective marker. pChlamy_3 is a commercially available transformation vector designed for recombinant protein production in *Chlamydomonas reinhardtii*. The plasmid contains a chimeric constitutive promoter of Heat shock protein 70A and Ribulose Bisphosphate Carboxylase/Oxygenase Small Subunit 2 (HSp70A/RbcS2) and intron1 of RbcS2 upstream of the multiple cloning site, which is reported to aid in high transgene expression by facilitating the transport of processed mRNA through the nucleopores (Doron et al., 2016). However, RT-PCR results revealed high transcript levels of *GFP* in *Scenedesmus* sp. ME02 cells transformed with pChlamy_3, GFP expression was not visible via confocal microscopy. This conflicting result may be attributed to differences in the splicing mechanism of introns in *Scenedesmus* sp. versus *C. reinhardtii* which subsequently resulted in inefficient splicing of intron1 of RbcS2 before translation in transgenic lines.

The second vector used in transformation experiments, pCAMBIA1302 contains a 35S CaMV promoter upstream of the multiple cloning site. Stable GFP expression was detected in transgenic *Scenedesmus* sp. ME02 via confocal microscopy using this vector construct. Guo et al. also reported high levels of GFP expression driven by 35S promoter using the same pCAMBIA1302 vector, whereas Dautor et al. attributed low GUS expression to the same promoter in transgenic *Scenedesmus almeries* (Guo et al., 2013; Dautor et al., 2014). Hence, not only the choice of transformation method but also the choice of vector and promoter must be individually tailored for each microalgal species.

A successful example of implementation of genetic engineering for enhanced lipid synthesis in *C. reinhardtii* and *S. obliquus* has been the overexpression of a diacylglycerol acyltransferase (*DGAT*) gene, which codes for the critical terminal enzyme in TAG synthesis and accumulation in microalgae (Ahmad et al., 2015; Chen et al., 2016). In *C. reinhardtii*, heterologous overexpression of the rapeseed *Brassica napus DGAT2* gene resulted in a two-fold increase in neutral lipids and altered fatty acid composition towards higher accumulation of PUFA (Ahmad et al., 2015). Similarly, overexpression of the *Chlamydomonas reinhardtii* diacylglycerol acyltransferase type two 1 (*DGTT1*) gene significantly enhanced both lipid and biomass contents of recombinant *S. obliquus* cells with only a minor change in fatty acid composition (Chen et al., 2016). Iwai et al. (2014) integrated metabolic and genetic engineering of microalgae for improved oil accumulation in a single study by designing a chimeric construct for overexpression of *DGTT2-4* gene driven by the sulphoquinovosyltransferase (*SQD2*) gene promoter induced upon phosphorus starvation. The results revealed that recombinant cells overexpressing the *DGTT2-4* gene cassette accumulated significantly higher amounts of TAG under P-depletion compared to the wild type.

Finally, in a recent study, *Scenedesmus* sp. CPC2 strain was genetically engineered to overexpress a synthetic construct of the phytoene synthase gene and recombinant microalgal cells showed elevated ß-carotene production (Chen et al., 2017). This and other emerging examples of engineering microalgae for the cultivation of high-value products prove to be a promising strategy for harnessing these microorganisms as cell factories.

Conclusion

Utilizing microalgae as a biorefinery simultaneously for different high-value compounds is an economically feasible strategy. A microalgal strain with high lipid content and suitable lipid composition, which accumulates excessive amounts of high-value compounds is an ideal candidate for this purpose. It is also possible to improve microalgae through metabolic engineering to lower the operational costs of cultivation. Previously identified thermo-tolerant strain, *Scenedesmus* sp. ME02 which has a suitable FAME profile and high phenolic content was used in the present study. The effect of P deprivation on biomass and lipid productivities of *Scenedesmus* sp. ME02 and FAME composition was explored. Biomass productivity

was slightly reduced, and lipid productivity was not significantly changed. A substantial change in the FAME profile was noted; the percent oleic acid amount more than doubled and PUFA content decreased by 23% under P deprivation compared to control.

Additionally, stable genetic transformation conditions to *Scenedesmus* sp. ME02 were optimized using two different expression vectors; pChlamy_3 and pCAMBIA1302. Stable recombinant GFP expression was detected in pCAMBIA1302 lines one year after the initial transformation. A protocol that can be used in future studies to improve this novel thermo-tolerant strain for higher biomass, lipid production, or other high-value compounds was successfully established.

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Author Contribution

ÇS, MY and HAO contributed to the conception and design of the experiments, DA and ÇS performed the experiments and the statistical analyses and wrote the article, ÇS, MY and HAO drafted the article, DA, ÇS, MY and HAO collected, analysed and interpreted the data. All authors performed the critical revision of the article for important intellectual content and approve of the final version of the article.

Conflict of Interest

No conflicts, informed consent, or human or animal rights are applicable to this study.

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