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Manipulation of carbon flux into fatty acid biosynthesis pathway in *Dunaliella salina* using *AccD* and *ME* genes to enhance lipid content and to improve produced biodiesel quality

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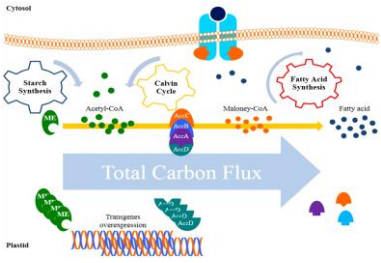
HIGHLIGHTS

- *Construction of a vector harboring ME and AccD genes act polycistronically.*
- *Stable integration of the cassette in transcriptionally silent region of chloroplast Zgenome.*
- *12% increase in total lipid content in transgenic microalgae.*
- *Improvement in prospective biodiesel quality especially less oxidation susceptibility.*

GRAPHICAL ABSTRACT



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ABSTRACT

Advanced generations of biofuels basically revolve around non-agricultural energy crops. Among those, microalgae owing to its unique characteristics i.e. natural tolerance to waste and saline water, sustainable biomass production and high lipid content (LC), is regarded by many as the ultimate choice for the production of various biofuels such as biodiesel. In the present study, manipulation of carbon flux into fatty acid biosynthesis pathway in *Dunaliella salina* was achieved using pGH plasmid harboring *AccD* and *ME* genes to enhance lipid content and to improve produced biodiesel quality. The stability of transformation was confirmed by PCR after several passages. Southern hybridization of *AccD* probe with genomic DNA revealed stable integration of the cassette in the specific positions in the chloroplast genome with no read through transcription by endogenous promoters. Comparison of the LC and fatty acid profile of the transformed algal cell line and the control revealed the over-expression of the *ME*/*AccD* genes in the transformants leading to 12% increase in total LC and significant improvements in biodiesel properties especially by increasing algal oil oxidation stability. The whole process successfully implemented herein for transforming algal cells by genes involved in lipid production pathway could be helpful for large scale biodiesel production from microalgae.

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1. Introduction

Unicellular microalgae have been at the center of attention of research efforts aimed at developing technologies for the renewable production of biofuels e.g. biodiesel. This is ascribed to the ability of algal cells to survive or proliferate over a wide range of environmental conditions, their remarkable diversity, and the ability to modify heir lipid metabolism efficiently through changing environmental conditions and genetic/metabolic engineering approaches. On the other hand and despite the remarkable attractions of algal fuels, economic and technical challenges are still faced and need to be addressed before algae could efficiently compete with the other biofuels feedstocks e.g. oil crops (Chisti Y, 2008).

Courchesne *et al.* reviewed three possible strategies/approaches for enhanced algal lipid production namely (Courchesne et al., 2009), biochemical engineering (BE), genetic engineering (GE), and transcription factor engineering (TFE). Through the GE strategies, generally one or more key genes such as Acetyl-CoA carboxylase (ACCase) and diacylglycerol acyl-transferase (DGAT) could be up-regulated (Klaus et al., 2004; Bouvier-Nave et al., 2000), while phosphoenolpyruvate carboxylase (PEPC) could be down-regulated (Song et al., 2008; Zhao et al., 2005) . However, GE of the lipid production pathway in the context of the whole cell rather than at a single step, has recently provoked widespread studies. These studies are aimed to channel carbon flux into lipid biosynthesis using multi-genes approach (Blatti et al., 2013) or to use the regulatory factors such as transcription factors (TFs) in order to control the abundance or activity of multiple enzymes relevant to the lipid production process (Huang et al., 2013; Huanget al., 2014).

ACCaseas is a key enzyme responsible of regulating the rate of de novo fatty acid (FA) biosynthesis in plant's plastid. The modulation of carbon flux is started by the formation of malonyl-CoA through ACCase`s activity. This is the first rate-limiting step in the FA biosynthesis pathway. Plastidic ACCase is composed of 4 subunits i.e. *BC*, *BCCP*, *AccA* and *AccD* (Sasaki et al., 2004). The first evidence on the positive effect of ACCase over-expression on FA synthesis was observed in transformed *Brassica napus* harboring a homomeric ACCase which showed a 5% increase in seed oil content (Roesler et al., 1997). Reports in some of the major peer-reviewed journals published reports suggest that *BC* and *BCCP* subunits are not the limiting factors to the ACCase activity (Shintani et al., 1997; Thelen et al., 2002) , while over-expression of the *AccD* subunit in a study conducted by Madoka *et al.* was shown to have boosted ACCase activity (Madoka et al., 2002).

Apart from the ACCase, GE of Malic enzyme (*ME*); an enzyme responsible for the conversion of malate to pyruvate (Chang et al., 2003), could also further promote carbon flux into lipid biosynthesis. *ME* also reduces $NADP^+$ to NADPH simultaneously and this byproduct is also utilized by the enzymes involved in FA and TAG syntheses which could potentially lead to enhanced lipid production. It was reported that *ME* over-expression led to the cytosolic NADPH increase and this extra reducing power could be used by lipogenic enzymes such as ACCase (Courchesne et al., 2009).

The present study was aimed to enhance lipid biosynthesis pathway both in quantity and quality in a strain of microalgae *Dunaliella salina* for biodiesel production. To achieve that, the FAS pathway was manipulated by transferring the pGH vector harboring the *ME* and *AccD* genes into the chloroplast genome of *D. salina* through the particle bombardment method. Then, lipid content, FA profile and the quality parameters of the resultant biodiesel were investigated in the transformed algal line.

2. Material and method

2.1. Strain cultivation

D. salina 19/18 was purchased from the Culture Collection of Algae and Protozoa (CCAP)(Sams Research, Scotland) and was cultivated in Johnson Medium (Johnson et al., 1968). The cells were kept at 20 ^{o}C and under a constant (24:0) 3klux photon flux of white and red LED lamps. Solid culture was prepared by implementation of 10% agar in the same medium.

2.2. Chloramphenicol Inhibition Test of D. salina

The cell culture of *D. salina* at the exponential phase was exposed to various concentrations of chloramphenicol (Sigma Aldrich, USA), ranging from 10 to 200 µg mL^{-1} liquid Johnson medium, to determine the optimum antibiotic concentration to screen the transformants. The cell concentration in the liquid medium was observed for 30 days. The chloramphenicol inhibition test was performed in triplicates for each concentration and LC_{50} value was determined based on OD_{620} and the cell count data were obtained using the Probit value Method (Ashton, 1972)

2.3. Genetic transformation of D. salina

2.3.1. Sample preparation

The exponential phase (OD_{620} = 0.5) of *D. salina* cultures was used as the time point for cell bombardment. Briefly, 20 mL of the *D. salina* culture was concentrated using a centrifuge at 1000×g for 10 minutes. The harvested biomass was dissolved in 1 ml Johnson medium and was streaked on a plate containing the same medium with agar. Before bombardment, the plates were kept under semi-dark condition in a phytotron for 24 h.

2.3.2. Plasmid Construction

The pGH-ME-AccD construct (10.3 kb) harboring 2 types of inducible and constitutive promoters in order to express the ME-AccD gene cassette was synthesized and used to enhance lipid production in *D. salina.* To facilitate cloning of the regulatory elements, all oligonucleotides were synthesized and cloned on the pGH vector by Genestar Co. (Shanghai, China). The sequence for 16S promoter (constitutive promoter) used in the current study was selected as suggested by Rasala *et al.* (2011). As for the inducible promoter (Nit), a partial sequence (636–1206) of the NIT1 gene (AF203033.1) coding nitrate reductase (EC1.7.1.1) in *Chlamydomonas reinhardtii* was used.

In order to regulate the expression of the ME-AccD gene cassette polycistronically, the 118-nucleotide upstream sequence of the rbcl gene was used as the 5' untranslated region (UTR) and the 280-nucleotide downstream sequence of the same gene was used as the 3'UTR.

To optimize the codon usage of *C. reinhardtii ME* nuclear gene (XM_001692632.1), the coding region of this gene was synthesized de novo (Shanghai Genestar Co., Ltd. China) preferringly with A or U at the third position of codons to enhance expression in green microalgae chloroplast. *AccD* was amplified by specific primers of the *AccD* gene of *Brassica napus*. Forward (5'TTTCATGTAAATAGAGGCCAGAAGC3') and reverse (5' CTGTTTTATTTGATTTCATTTTTGTTC3') primers were synthesized by CinnaGen Inc. (Tehran, Iran). The PCR product was directly cloned into pGEM vector using T easy vector systems (promega). *AccD* gene was subcloned from pGEM-AccD vector into pGH-ME vector by *Sal*I-*Nco*I digestion. In fact, the resultant pGH-ME-AccD vector was constructed allowing polycistronic expression of these two genes (Fig.1).

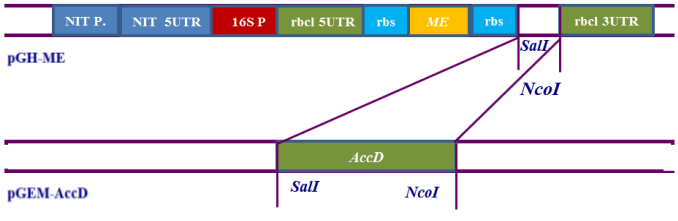

Fig.1. Construction of pGH-ME-AccD vector. Polycistronic expression of acetyl CoA carboxylase (*AccD*) and malic enzyme (*ME*) genes achieved by subcloning of *AccD* from pGEM-AccD vector into pGH-ME vector.

The *CAT* gene responsible for antibiotic resistance was isolated from an invitrogene gateway vector (AB752383.1) through PCR. The primers were designed with *Bam*HI and *XbaI* restriction site at 3' ends. PCR fragments were digested with *Bam*HI and *XbaI* and directly inserted into pGH-AccD-ME vector, downstream of atpB promoter as shown in Figure 2.

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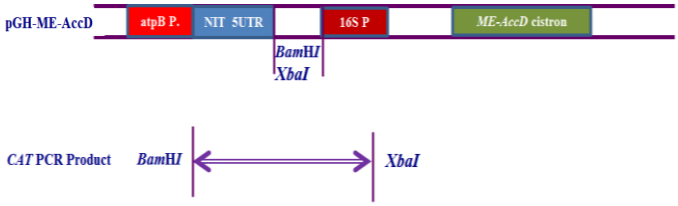

Fig.2. Addition of chloramphenicol resistance gene (*CAT*) into the pGH-AccD-ME vector. *CAT* PCR fragments were digested in the flanking restriction sites and were directly inserted into the pGH-AccD

A flanking region was included on both sides of the ME-AccD genes as well as the *CAT* gene. The 2000 bp transcriptionally - silent flanking region was synthesized de novo based on a unique intergenic region (i.e. rrnS-chlB) from *D. salina* strain CCAP 19/18 chloroplast genome (GQ250046.1) (Fig. 3). As a matter of fact, the lack of read through transcription in this intergenic region makes the pGH vector an ideal choice for studying transgene promoter activity.

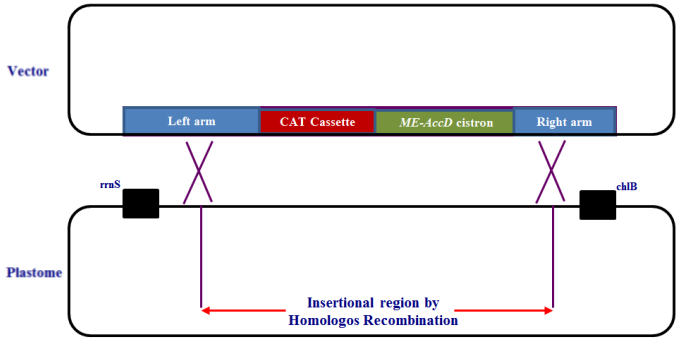

Fig.3. Homologues recombination. Two flanking regions (left and right arms) making possible the insertion of the ME-AccD gene cassette into a unique intergenic region (i.e. rrnS-chlB) in *D. salina* strain chloroplast genome.

Finally, *Escherichia coli* strain DH5α carrying the pGH-ME-AccD plasmids was cultured in LB medium containing 50 mg L^{-1} ampicillin and 30 mg mL^{-1} chloramphenicol and the plasmids were isolated using high pure plasmid isolation kit (Roche, Germany). The quantity and purity of the plasmids were determined by nanodrop (Thermo Scientific, Wilmington, DE) and the extracted plasmids were used for the cell bombardment experiment.

2.3.3. Micro particle bombardment

To coat the 0.6 µm diameter gold particles (Bio-Rad Laboratories, USA), with the pGH-ME-AccD vector, the method described by Talebi *et al.* (2013b) was used. Briefly, 60 mg of gold particles was weighed and washed twice by 100% ethanol and then the gold pellet was dissolved in 1 ml of dd water. Then, 60 µL of the resultant gold particles solution (60 mg mL^{-1}) was mixed with 5 µL of a pGH-ME-AccD vector (6 µg $µL^{-1}$), 50 µL of 2.5 M $CaCl_2$, and 20 µL of 0.1M spermidine. After that, the coated particles were washed in 60 µL of 100% ethanol twice. Finally, 10 µL of the vector-coated particles was layered on a macrocarrier and allowed to air dry at ambient temperature for bombardment.

Then, *D. salina* cells at their exponential phase were bombarded under vacuum with the vector-coated gold particle using a Bio-Rad PDS-1000/He Biolistic Particle Delivery System (Bio-Rad Laboratories, USA) at gas (helium) pressure of 900 psi at distances of 6 cm and 9 cm according to the method described by Jiang (Jiang et al., 2002). Each plate was bombarded for the second time after it was turned for 90 degrees. Prior to bombardment, screens were sterilized by autoclaving and the macrocarriers as well as the rupture disks were sterilized by washing in isopropanol for 15 minutes.

2.3.4. Selection of putative clones using chloramphenicol

After bombardment, the targeted cells were kept under semi dark condition for 24 h at 25 °C. Then, the bombarded and non-bombarded (control) *D. salina* cells were cultured in Johnson liquid medium enriched by 250 µg mL^{-1} of chloramphenicol. The recovered cells were streaked on selective agar plates containing the same medium plus 100 µg mL^{-1} of chloramphenicol. After 2 weeks of incubation at 25 °C, green-colored colonies, representing transformed cells, were visible on the plates. Single colonies were picked using a pipette tip and were inoculated in liquid Johnson medium containing 80 µg mL^{-1} of chloramphenicol. Both bombarded and non-bombarded cells were left to grow for two weeks in antibiotic-contained and antibiotic-free media, respectively. Harvested biomass was used for genetic and expression analyses. The cultures were maintained for further stability and expression analyses.

2.4. DNA extraction analysis of D. salina clones survived on the antibiotic enriched medium

2.4.1. DNA extraction

One hundred mg of *D. salina* cells (OD620 = 1) were harvested from the 20 d old cultures obtained from the single clones grown in the presence of chloramphenicol. This was achieved by centrifugation (10,000×g, 10 min, and ambient temperature) of about 50 ml of cell suspensions.

The total DNA was extracted by DNeasy Plant Mini Kit (QIAGEN, Germany). The quantity and purity of the genomic DNA were determined by a nanodrap at OD_{260} and OD_{280}. The quality and integrity of the DNA sample were also verified with 1.0 % (w/v) agarose gel electrophoresis in 1× TAE buffer at 90V for 45 min. The genomic bands were viewed and photographed using AlphaImager TM 2200 (Alpha Innotech Corporation, USA).

2.4.2. Polymerase chain reaction (PCR)

The PCR analysis for the detection of the integrated region of the vector into the *D. salina* chloroplast genome was conducted using specific primers of the *AccD* gene. PCR amplification of *AccD* gene was done in a BIO-RAD DNAEngine® thermocycler through 35 cycles of: 94 °C (1 min), 59°C (1 min) and 72 °C (1.50 min).

2.4.3. Southern blot analysis

To determine the integration of the gene cassette in the chloroplast genome of the transformed lines, 10 µg of the isolated DNA (transformed line and control) and 0.1 µg of plasmid, were digested with *EcoR*I restriction enzyme as recommended by the manufacturer (Fermentas, Vietnam). The digestion pattern was monitored by electrophoresis along with size markers on a 0.8% agarose gel in a 1X TBE buffer and then transferred to nitrocellulose membrane based on the procedure described by Maniatis (Maniatis et al., 1982). A 1467 bp fragment from plasmid pGH containing the entire coding region of *AccD* gene was amplified and labeled by incorporation of DIG-11-dUTP into DNA using PCR (DIG labeling Kit, Roche, Germany). The probe hybridization was carried out at 43 °C for 16 h and washing step was conducted at moderate stringency in 0.5 X SSC, 0.1 SDS. The probed blots were detected with an enzyme-linked immunoassay.

2.5. Physiologic studies

2.5.1. Total lipid content and free fatty acid profiling

By the time the cell growth phase reached the lag stationary phase, physiologic parameters were studied in the transformed and control cell lines. Total lipids content (Lc) and free FA profile were obtained in triplicates for the studied strains. Data comparison was then carried out using the ANOVA test. LC reported as percentage of the total biomass (%dwt), was determined based on the Bligh and Dyer method (Bligh et al., 1995). FA profile was investigated by using Gas Chromatography (GC) analysis based on the procedure described by Talebi *et al.* (2013a). The intracellular neutral lipid distribution in microalgal cells was examined by staining the cells by Nile Red fluorescent dye. Fluorescence-based quantification of the accumulated lipids was achieved following the protocol reported by Chen *et al.* (2009).

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The excitation and emission wavelengths of 522 and 628 nm, respectively, were selected based on a previous report by Talebi *et al.* (2014b).

2.5.2. Bioprospecting biodiesel quality parameters

The quality characteristics of produced biodiesel from any oil stocks could be predicted by investigating the FA profile of the oil used (Bigelow et al., 2011). A number of studies have previously reported empirical equations based on which all most of the biodiesel quality parameters such as Cetane number (CN), Cloud Point (CP) and the oxidation stability (OS), could be predicted (Ramos et al., 2009; Ramírez-Verduzco et al., 2012). Bioprospecting of biodiesel quality parameters for the oil samples obtained from the control and transformed algal cells was achieved using the BiodiselAnalyser ver. 1.1 software (available on http://www.brteam.ir/biodieselanalyzer) (Talebi et al., 2014a). The data obtained were used to evaluate the effect of transgenes overexpression on the biodiesel quality parameters in the transformed lines in comparison with the control.

3. Results and discussion

3.1. DNA analysis of D. salina transformed lines

The presence of the 1467 bp *AccD* gene in the algal cells bombarded with the pGH-ME-AccD vector was verified through PCR using specific primers (Fig. 4). The negative controls *i.e.* the non-transformed *D. salina* DNA and the reaction mixture containing no DNA template produced no bands.

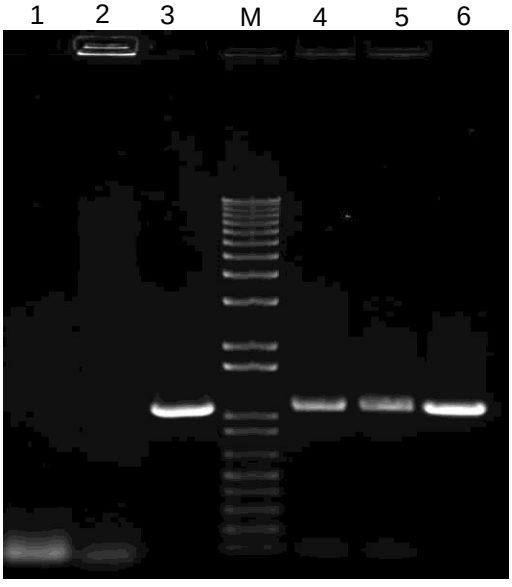

Fig.4. Agarose gel electrophoresis verification of the *AccD* PCR products (1467 bp). Lane 1: negative control without any DNA. Lane 2: negative control with untransformed DNA, lanes 3: positive control (pGH-ME-AccD vector), line 4-6: transformed *D. salina*, and M represents the 1 Kb DNA marker (Fermentas).

The insertion stability of the *AccD* gene in the transformed clones was also proved in the repetitive subcultures/generations via PCR analysis. However, the transformed cells lost their resistance to chloramphenicol after the 5th subculture (day 100). In a similar observation, Chow (Chow et al., 1999), reported shorter transient maintenance of plasmid in the algal cells, and that the trasformed lines gradually lost their hygromycin resistance.

Southern blot analysis performed with the genomic DNA and the labeled *AccD* probe showed no background formation and the specific hybridization of probe with the transformed DNA revealed stable integration of the flanking regions of the pHG-ME-AccD vector into the chloroplast genome (Fig. 5).

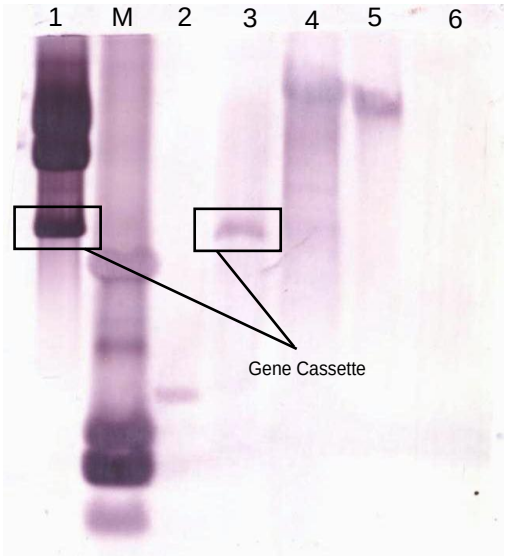

Fig.5. Integration of pGH-ME-AccD vector into the chloroplast genome of transformed *D. salina* by Southern analysis. Lane 1: positive control (pGH-ME-AccD vector); lanes 2 and 3: the transformed cells (digested DNA); lanes 4 and 5: the transformed cells (undigested DNA); lane 6: the untransformed cells; and lane M refers to the size marker.

More specifically, a similar band aligned in the patterns observed for the control and the transformed line (Figure 5, lanes 1 and 3, respectively), confirmed the successful insertion of the gene cassette in the targeted positions in the circular genome. Among the optimal transformation procedures reported for *D. salina* by Feng *et al.* (2009) *i.e.* glass beads, particle bombardment and electroporation, PDS1000/He micro-particle bombardment system has been frequently used to introduce different construct into the nuclear genome of *D. salina* (Liu et al., 2010; Tan et al., 2005). The Finding of the present study also further enforced the acceptable efficiency of the bombardment method to introduce a transgene into the *Dunaliella* cells.

3.2. Effect of transgenes activity on total lipid production

The effect of the presence of the *AccD-ME* transgenes in lipid metabolism was surveyed by LC measurement in the 35-day old transformed and the control cell lines. The mean value of LC for the transformed cell line showed a slight increase to 25% dwt. which was 12% higher than that of the control. Moreover, high-throughput fluorescence-based technique using a liposoluble fluorescence probe (i.e. Nile Red), was used to quantify neutral lipids in the cells. As shown in Table 1, the fluorescence intensity obtained showed 23% increase in the transformed cell lines in comparison with the control. The lipid measurement results obtained using the fluorometry technique was in line with the LC increase measured by the gravimetrical method.

Table 1.
Fluorescence intensity emitted by microalgae stained by Nile red.

Sample	Fluorescence intensity
Water	3011±188[a]
Non stained control cell	3566±242
Stained control cell	15933±3155
Transformed cell	19639±2668

In previous attempts, Dunahay (Dunahay et al., 1996) and Wang et al. (Wang et al., 2009) also used genetic manipulation of microalgae through overexpression of TAG biosynthesis pathways genes such as ACCase to improve algal lipid content but failed to achieve any significant increases in lipid accumulation. To the best of our knowledge, there is no report on

overexpression of other enzymes involved in lipid biosynthesis like *ME*, nor on blocking competing pathways such as β-oxidation in microalgae.

Plastidic ACCase is responsible for a major part of unsaturated FAs production (Sasaki et al., 2004). Having considered the impermeable nature of the plastid's membrane to the Acetyl-COA (Ke et al., 2000), it could be concluded that the plastidic ACCase plays an independent role in free FAs synthesis and is not related to the existing ACCase in the cytoplasm. The heteromeric plastidic ACCase is composed of four nuclear- and plastid-encoded subunits. The *BC* and *BCCP* subunits have been shown as non-limiting factors to the ACCase activity (Shintani et al., 1997; Thelen et al., 2002) , while over-expression of the *AccD* subunit has been proved to boost ACCase activity (Madoka et al., 2002). As Madoka *et al.* (2002) reported that the overexpression of *AccD* caused twice FA production per transformed tobacco in comparison with the wild-type plant. On the other hand, *ME* catalyzes the oxidative decarboxylation of L-malate, producing pyruvate, CO_2, and NAD(P)H (Chang et al., 2003) and could play a stimulating role in lipid biosynthesis by providing carbon skeletons and also reducing power i.e. NAD(P)H. This hypothesis was confirmed through a study by Zhang and coworkers (Zhang et al., 2007), in which the recombinant strains of *Mucor circinelloides* harboring *ME* showed an increase in biosynthesis of FAs and formation of unsaturated FAs. They argued that the unique role of *ME* in elimination of the rate-limiting step of FA biosynthesis through supplying NADPH was the cause.

Therefore in the present study, simultaneous overexpression of both *AccD* and *ME* was put to test which resulted in 12% increase in total lipid accumulation in the algal cells. In more details, overexpression of *AccD* and *ME* made LC reach to 25% in the transformed cell line in comparison with 22% in the control line. The introduction and overexpression of *AccD* in chloroplast genome of *D. salina* along with the *ME* gene into the operation site (Fig. 6), must have accelerated the carbon flux into the free FA synthesis leading to increased lipid production.

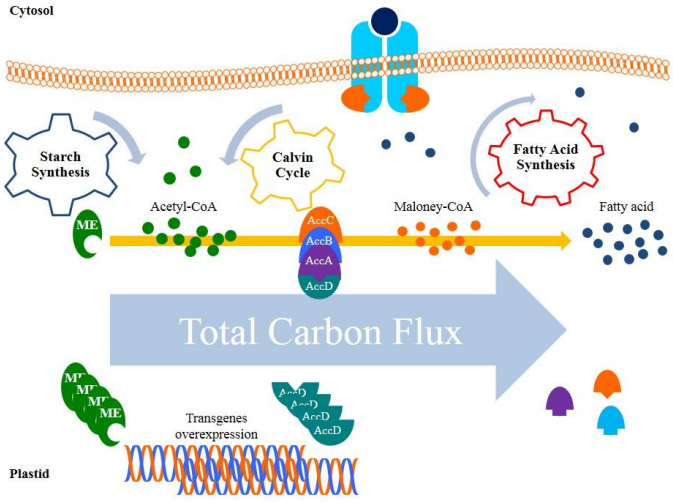

Fig.6. Over-expression of *AccD* and *ME* in chloroplast genome of *D. salina*. The carbon flux was switched from starch synthesis to FAs synthesis.

Concerning the regulatory elements used which are responsible for successful overexpression of *AccD* and *ME*, sequence analysis of NIT1 inducible promoter sequence from *C. reinhardtii* revealed a putative CCAAT box at -836, a TFII-I binding site at -664, a TFII-A binding site at -509, and a CCAAT box at -245, relative to the translational start site. The responsive sequence to nitrogen deprivation in the growth media which was used in this study was located -284 nucleotides upstream the start codon. 287 nucleotide of the beginning CDS was inserted to act as a 5' UTR. In other studies, transformation of some microalgae strains such as *Chlamydomonas*, *Chlorella*, *Dunaliella* with the Nit1 promoter is accomplished by electroporation (Champagne et al., 2009). Nit promoter previously was introduced into the *D. salina* genome as a inducible promoter to control expression of the bar and EGFP genes using the inexpensive inducer nitrate and repressor ammonium (Li et al., 2007; Li et al., 2008). On the other hand, the designed construct in the present study harbors 16S rRNA gene promoter as a constitutive promoter. This promoter resembles the bacterial type with typical -10 and -35 elements (Klein et al., 1992).

In result, the combination of nuclear gene promoter, NIT1, and chloroplastic gene promoter, rrnS, guaranteed the high expression of *AccD-ME* transgenes due to the co-presence of nuclear encoded RNA polymerase (NEP) and chloroplast encoded RNA polymerase (PEP) in the chloroplast (Sasaki et al., 2004) . This strategy was a mimetic behavior of the rRNA operon which also benefit two types of promoters in order to express in the chloroplast abundantly (Vera et al., 1995).

3.3. Study of transgenes activity on oil quality

3.3.1. Fatty acid profile

Fatty acid methyl ester (FAME) profiles of the transformed and control line of *D. salina* are summarized in Table 2. It has been frequently reported that 16-18 carbon chain FAs are dominant in FAME profile of microalgal species and that the FAs have major impact on oil quality as well (Talebi et al., 2013a).

Table 2.
Fatty acid composition and properties of the produced algal oils.

Strain	Fatty acid (%)							SFA/ USFA	Oil content (%)
	16:0	16:1	18:0	18:1	18:2	18:3	20:1		
D. salina (transformed)	22.60±0.4	1.60±0.2	18.60±0.8	16.36±0.6	5.86±0.5	30.80±1.4	2.66±0.2	72	25±2.8[B]
D. salina (control)	16.33±0.4	1.06±0.3	8.60±0.7	19.57±0.4	6.76±0.8	38.60±0.9	3.61±0.5	35	22±1.1[A]

As a whole, the results of this study showed significant differences between the FAME profile of the studied cell lines. In more details, overexpression of *AccD* and *ME* in the transformed cell line caused an obvious accumulation in the saturated FAs (SFA) like plametic acid (C16:0) and stearic acid (C18:0). On the contrary, this manipulation led to decreased percentages of mono unsaturated FAs (MUFA) i.e. palmitoleic acid (C16:1) and oleic acid (C18:1) and also poly unsaturated FAs (PUFA) i.e. Linolenic acid (C18:3). As a result, SFA/USFA parameter showed over 106% increase in the transformed line. This phenomenon could be explained by the fact that the beginning of the desaturation pathways involving delta-9 desaturase is a highly energy-consuming process and when cells are encouraged to produce and accumulate more lipids in their cytoplasm, they face energy and time scarcity to evolve new-born FAs to desaturated ones. Similar observations have been reported when lipid production is enhanced through biochemical engineering approaches. For instance, Talebi *et al.* (2014b) achieved a 50% increase in total lipid accumulation through myo-inositol supplementation, as a lipid inducer treatment in *D. salina.* while also reported 54% deacrease in USFA/FAME. Zhila *et al.* (2005) while tried to enhance lipid accumulation using N-starvation treatment on *Botryococcus braunii*, observed an increase in the SFAs content (up to 76.8%) and a decrease in the PUFA content (up to 6.8%).

3.3.2. Estimation of biodiesel properties

FAME profile of algal strains could be used as a tool to predict the characteristics of produced biodiesel (Talebi et al., 2013a). The degree of unsaturation (DU) was decreased by 20% in the transformed line which had major impacts on the BAPE and APE values as well as the oxidation stability (OS) of the produced biodiesel (Table 3). In fact, the APE and BAPE values were reduced by 19 and 20 % in the transformed line, respectively. The OS of the transformed and control lines were estimated at 5.81 and 5.19, respectively. This would mean that the obtained oil from the transformed algal cells was less susceptible to oxidation/rancidity at high temperatures over long-time storage periods. Slim improvements in the estimated CP

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values were also anticipated. Overall, it was shown that the overexpression of *AccD* and *ME* led to improved fuel properties (Table 3).

Table3.
Comparison of the estimated properties of algal biodiesel from the transformed and control cells of *D. salina*.

	Biodiesel properties									
Strains	**CN**	**CP**	**BAPE**	**APE**	**DU**	**CFPP**	**OS**	**HHV**	**V**	**p**
D. salina (transfomed)	48.05	6.90	67.46	89.62	93.88	19.84	5.81	38.78	1.28	0.86
D. salina (control)	43.15	3.58	83.90	110.20	115.46	2.15	5.19	37.42	1.18	0.83

4. Conclusion

The feasibility of biodiesel production from genetically modified micro-algae can be expedited if large-scale production facilities can be integrated with other processes, such as wastewater treatment and utilization of carbon dioxide from power plants. Screening of genetic variability between algal isolates could lighten the different potential of variant strains and the result could be used in prone strain selection for gene transformation. 26 years since the first stable introduction of a heterogene into the *Chlamydomonas* chloroplast by the Boynton and Gillham laboratory, we have shown the potential of chloroplast as a realistic platform for GE approaches towards the goal of establishing the microalgae in biofuel production worldwide. We have shown that the introduced transgenes at the insertion site are stable, and that there is no readthrough transcription from outside promoters. In this research we have gained of two promoters combination to reach the highest reported record of enhanced lipid production in a transformed microalgae. It was also demonstrated that GE approaches such as overproduction of multiple involved enzymes has the potential to solve two major obstacles in microalgal biodiesel production namely low lipid content and unfavorable fatty acid profile.

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