GREEN ALGAE SELECTION USEFUL FOR PHYCOREMEDIATION OF OLIVE-MILL WASTEWATERS AND INCREASE OF THEIR LIPID CONTENT BY GENETIC ENGINEERING

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Ai miei genitori

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SUMMARY

The main aim of this work was the selection and the characterization of algal strains useful for phycoremediation of olive-mill wastewaters (OMW). 100 algal strains from the ACUF collection of the Department of Biology of the University of Naples were screened for their capability to release enzymes with phenol-oxidase activity in the culture broth. The presence of enzymes with phenol-oxidase activity was revealed by ABTS enzymatic assay in 16 of the 100 analysed culture broths. Further enzymatic assays were carried out in order to: 1) evaluate the enzymatic activity of the culture broths in presence of ABTS and different phenolic compounds, as 2,6-DMP and syringaldazine; 2) evaluate the capability of the culture broths to decolourizate the azo dye RBBR. On the base of the results achieved with the enzymatic assays, two algal strains were selected in order to evaluate their capability to grow in, and to degrade the phenolic component of, OMW: *Chlamydomonas pitschmannii* and *Scenedesmus vacuolatus*. The two algae were grown for 21 days in OMW diluted with growth medium and the cell concentration and the total phenols amount were measured weakly. *S. vacuolatus* showed a capability to grow in presence of OMW, and to reduce the phenolic content, higher than *C. pitschmannii*.
The second part of the PhD work was carried out at the Algae Biotechnology & Bioenergy research group of the University of Bielefeld; during this period the research was focused on the characterization of genes encoding for putative enzymes involved into the lipid biosynthetic pathway in the green alga *Chlamydomonas reinhardtii*. 5 genes encoding for putative diacylglycerol acyltransferases (*Cr*DGAT1-5) were cloned into expression vector pGenD and used for the genetic manipulation of *Chlamydomonas* in order to obtain mutants able to overexpress these genes. For each gene, 1 to 3 mutants were able to over-express the new *CrDGAT* construct. The mutant with the highest expression level was selected by quantitative real time PCR analyses for phenotype characterization during the standard growth condition and during nitrogen and sulphur depletion. Number of cells, optical density and total amount of chlorophyll were evaluated in order to verify if the over-expression of these genes could affect the cell phenotype. The total lipid amount was measured by Nile Red fluorescence assay and compared with the wild type. During the normal growth condition no differences were noticed between mutants and wild type strain, demonstrating that the over-expression of these genes doesn't affect the cell phenotype and suggesting the presence of a second bottleneck in the lipid biosynthetic pathway. When cultivated in nitrogen depletion, all cultures showed the highest lipid accumulation, however without differences between mutants and wild type strains. During the sulphur starvation, mutants D3-18 and D4-66 showed an increase in the lipid accumulation in average 2.0 and 1.8 times higher than the wild type.

RIASSUNTO

Uno dei maggiori problemi connessi con lo sviluppo industriale e tecnologico nelle società avanzate è rappresentato dall'inquinamento ambientale collegato alle attività umane che, in alcuni casi, ha ampiamente superato i livelli di accettabilità per il benessere dei biomi nella loro interezza. Molte sostanze tossiche, prodotte dall'uomo e rilasciate nella biosfera, sono entrate ormai in forma stabile nelle catene alimentari ed attualmente costituiscono un concreto pericolo per la salvaguardia della biodiversità e per il benessere non solo dell'uomo (Stefanidou et al., 2009). Esse contribuiscono a rendere sempre meno possibile ed attuabile uno sviluppo tecnologico ed industriale eco compatibile.
L'inquinamento del suolo e delle acque può essere dovuto a sostanze di tipo inorganico, come i metalli pesanti ed i radioisotopi, o di tipo organico, come gli idrocarburi. Tra le sostanze organiche, i composti fenolici e gli idrocarburi aromatici policiclici (IPA) hanno particolare importanza per il loro impatto sull'ambiente (Meagher et al., 2000). Questi composti si dividono in IPA a basso peso molecolare, se contengono da 1 a 3 anelli benzenici, e in IPA ad alto peso molecolare, se contengono più di 3 anelli aromatici. La loro tossicità è strettamente correlata al loro numero di anelli aromatici che aumentando incrementano anche l'idrofobicità della molecola. Queste sostanze sono spesso fortemente rappresentate tra i prodotti di scarto dell'industria conciaria e agro-alimentare, tra cui quelli derivanti dai frantoi oleari. Questi reflui suscitano particolare interesse in quanto presentano una forte carica inquinante a causa della loro acidità, della ricchezza in sali minerali e soprattutto dell'elevato contenuto in sostanza organica, costituita per la maggior parte da polifenoli (Morillo et al., 2009). Il loro smaltimento costituisce oggi un grosso problema per l'ambiente, in quanto essi sono spesso scaricati direttamente sui terreni agricoli oppure immesse nelle fogne o nei corsi d'acqua. Tali comportamenti mettono in luce chiaramente la problematica rappresentata dallo smaltimento delle acque di vegetazioni dei frantoi oleari, in particolar modo nei paesi del bacino del Mediterraneo.
L'impiego di organismi fotosintetici rappresenta oggi una valida ed ecocompatibile alternativa ai metodi di purificazione per il trattamento delle acque di scarico finora impiegati, basati sulla rimozione o sull'interramento degli agenti inquinanti. Le microalghe sono da tempo utilizzate per il trattamento terziario dei reflui urbani in quanto riescono ad aumentare il tasso di rimozione di nutrienti, metalli pesanti e patogeni da tali fonti di inquinamento, fornendo nello stesso tempo O_2 per i batteri eterotrofi aerobi in grado di mineralizzare inquinanti organici e abbattendo la CO_2 emessa durante la respirazione di tali organismi(Muñoz e Guieyesse, 2006). La conoscenza di tali meccanismi fa sì che l'aerazione foto sintetica sia oggi ritenuta un'alternativa più sicura ed economica ai meccanismi di aerazione meccanica, durante i quali spesso ci si imbatte in fenomeni di volatilizzazione degli inquinanti. Le alghe unicellulari sono già state utilizzate con successo anche per il trattamento dei reflui dell'industria della carte e della cellulosa (Tarlan et al., 2002) e altri studi hanno dimostrato la capacità di alcuni ceppi di alghe verdi unicellulari, presenti presso la collezione del Real Orto Botanico di Napoli, di trasformare diverse sostanze fenoliche presenti nelle acque di vegetazione (Pinto et al., 2003). Malgrado la ricerca di ceppi in grado di produrre sostanze ad alto valore aggiunto spinge sempre di più i gruppi di ricerca ad effettuare screening di intere collezioni, la grande biodiversità presente in tale collezione non è stata ancora analizzata a fondo.

A tal fine è nata la collaborazione con il Prof. Dr. Antonino Pollio e il Prof. Dr. Gabriele Pinto del Dipartimento di Scienze Biologiche dell'Università Federico II di Napoli presso il Real Orto Botanico. Tale dipartimento è custode di una delle più grandi collezioni algali presenti in Italia. Tale collezione è una fonte non ancora del tutto esplorata di microrganismi appartenenti a differenti generi e classi isolati dai più svariati ambienti. La collezione vanta più di 400 accessioni, tra cui alcuni ceppi algali autoctoni di particolare interesse in quanto isolati da zone di origine vulcanica e in grado di crescere in condizioni estreme di temperatura e pH. Il presente progetto di dottorato ha avuto come primo obiettivo quello di individuare, tra le centinaia di specie algali presenti nella collezione, quelle in grado di produrre fenol-ossidasi extracellulari. Tale *screening* è stato effettuato analizzando i brodi di coltura di 100 differenti ceppi algali; la capacità delle alghe di rilasciare enzimi ad attività fenol-ossidasica nel mezzo di coltura è stata valutata mediante saggio biochimico in presenza di 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonico) (ABTS), un substrato specifico per laccasi. Il primo screening ha portato alla selezione di 16 accessioni, appartenenti ai generi *Chlamydomonas*, *Chlorella* e *Scenedesmus*. Successivamente, tali accessioni sono state oggetto di analisi più approfondite e mirate; è stata infatti valutata l'attività enzimatica dei brodi di coltura in presenza di ABTS e dei composti fenolici 2,6-dimetossifenolo (2,6-DMP) e N,N'-*bis*-(3,5-dimetossi-4-idrossi benzilidene) idrazina (siringaldazina) e la loro la capacità di decolorare il prodotto di sintesi industriale Remazol Brilliant Blue R (RBBR). Tali analisi hanno portato alla selezione di due ceppi algali, sui quali sono stati condotti ulteriori studi al fine di valutare la loro possibile capacità fitorisanatrice in presenza di acque di vegetazione. Le alghe selezionate sono state: *Chlamydomonas pitschmannii*, che ha mostrato la più alta attività fenol-ossidasica in presenza dei composti 2,6-DMP (4,84 ± 0,35 U.E. ml^{-1}) e siringaldazina (0,28 ± 0,01 U.E. ml^{-1}), e *Scenedesmus vacuolatus*, che ha mostrato la più alta capacità di decolorazione dell'RBBR (38,6% ± 6,0) e la più alta attività fenol-ossidasica in presenza di ABTS (1.598,4 ± 340,7 U.E. ml^{-1}). I due ceppi in questione sono stati allevati per 21 giorni in condizioni di luce e di buio in acque di vegetazione filtro sterilizzate diluite con mezzo di coltura (1:20 v/v) e i risultati paragonati a quelli ottenuti coltivando nelle stesse condizioni l'alga modello *Chlamydomonas reinhardtii*. *C. pitschmannii* è risultata essere meno efficace nel trasformare la componente fenolica delle acque di vegetazione (35,7% e 25,5% rispettivamente in condizioni di luce e di buio) con un incremento in 21 giorni del numero di cellule al buio e alla luce di 1,6 e 4,5 volte rispetto all'inoculo di partenza (circa 500.000 cellule per ml). *C. reinhardtii* ha mostrato valori leggermente superiori, con una degradazione della componente fenolica delle acque di vegetazione del 42,5% in condizioni di luce e del 33,3% in condizioni di buio, e con un incremento della concentrazione cellulare dopo 21 giorni di 2,2 e 13,5 volte, rispettivamente al buio e alla luce. *S. vacuolatus* ha mostrato in entrambe le condizioni di crescita risultati più promettenti sia per quanto riguarda la capacità di rimuovere la componente fenolica nelle acque di vegetazione (60,0% e 52,3% rispettivamente alla luce e al buio) sia per quanto riguarda la sua capacità di crescere in presenza di questi reflui, incrementando in 21 giorni il numero di cellule di 30 volte al buio e di 85 volte alla luce.

Il secondo obiettivo di questo progetto di dottorato è nato dalla necessità di individuare il miglior impiego della biomassa ottenuta in seguito al trattamento delle acque di vegetazione. Tra le varie alternative, la scelta è ricaduta sulla possibilità di adoperare tale biomassa come materia prima per la produzione di biocarburanti. È ben noto che un'altra sostanziale conseguenza dello sviluppo industriale, in questo

caso strettamente legata al largo impiego di carburanti fossili, consiste nell'inquinamento atmosferico. Tra gli inquinanti più rappresentativi vi si ritrova l'anidride carbonica, i cui devastanti effetti sono ben percepibili con il continuo riscaldamento globale. Le emissioni di questo gas, una volta bilanciate dalla rimozione da parte delle piante tramite il ciclo fotosintetico, hanno raggiunto il limite massimo negli ultimi decenni. Per raggiungere la sostenibilità ambientale ed economica quindi c'è necessità che i processi di produzione di carburante non solo siano rinnovabili, ma anche a impatto di CO_2 pari a zero (Hu et al., 2008). Sistemi di produzione basati su microalghe di seconda generazione possono fornire la biomassa necessaria per la produzione di diversi tipi di carburanti rinnovabili, tra cui biometano bioetanolo, bioidrogeno e biodiesel. Il principale vantaggio di questi sistemi di seconda generazione è l'impiego di microalghe con più alta efficienza fotosintetica (con conseguente aumento della produzione di biomassa), in grado di crescere ad alte concentrazioni di salinità o direttamente in acque reflue ed in grado di abbassare l'impatto di CO_2 tramite processi di *carbon sequestration* (Hu et al., 2008).

Numerosi lavori riportano *screening* di microalghe al fine di identificare le specie e i ceppi in grado di produrre notevoli quantità di lipidi da poter convertire in biodiesel (Hu et al., 2008). Altrettanti studi hanno dimostrato che condizioni di stress, come carenza di nutrienti, temperatura, cambio di pH o di salinità dei mezzi di coltura, portano ad un incremento della componente lipidica in questi organismi. Tuttavia, pur essendo note le vie metaboliche che portano alla sintesi dei lipidi, non sono presenti lavori di ingegneria metabolica riguardanti la sovra espressione in condizioni di crescita normali, sia autotrofiche che mixotrofiche, di geni coinvolti in questi *pathway* (Hu et al., 2008). A tal fine è nata la collaborazione con il gruppo di ricerca Algae Biotechnology & Bioenergy (Università di Bielefeld – Germania), presso il quale è stato svolto il terzo anno del seguente dottorato di ricerca, del Prof. Dr. Olaf Kruse, il quale vanta numerose pubblicazioni che riguardano studi di ingegneria metabolica sull'alga modello *C. reinhardtii* finalizzati all'impiego di quest'organismo come fonte alternativa di energia. Durante il corso del terzo anno sono stati eseguiti lavori riguardanti lo studio di geni codificanti enzimi putativamente coinvolti nei *pathway* biosintetici dei trigliceridi in *C. reinhardtii* e la loro sovra espressione nell'alga stessa al fine di aumentare la capacità di accumulo dei lipidi.

L'enzima diacilglicerolo aciltransferasi (DGAT) è un enzima chiave nella sintesi dei trigliceridi, in quanto catalizza la formazione di triacilglicerolo a partire da diacilglicerolo e acil-CoA. La sovra espressione in diversi organismi di geni codificanti questi enzimi hanno spesso portato ad un conseguente incremento nella capacità di accumulo dei lipidi. Le analisi condotte su banche dati hanno mostrato la presenza in *C. reinhardtii* di 5 geni codificanti putative DGAT. I 5 geni sono stati clonati e inseriti nel vettore di espressione pGenD sotto il controllo del promotore forte del gene codificante la subunità PsaD del fotosistema I dell'alga stessa. Tali vettori sono stati impiegati in concomitanza al vettore pHyg3, che conferisce la resistenza all'antibiotico higromicina B, per la trasformazione genetica di *C. reinhardtii* mediante tecnica dei *glass beads*, al fine di ottenere mutanti in grado di sovra esprimere tali geni. La selezione dei cloni positivi è avvenuta mediante analisi PCR del DNA estratto dalle colonie in grado di crescere su substrato selettivo e l'analisi della frequenza di trasformazione, riportata come percentuale di colonie positive sul totale di colonie analizzate, ha mostrato che sono stati trovati, per ciascun gene, cloni positivi con una frequenza di trasformazione che va dal 2,4% per il gene *DGAT1* al 7,9% per il gene *DGAT4*. I cloni risultati positivi alle analisi di amplificazione del DNA

genomico sono stati sottoposti ad analisi di retro trascrizione degli RNA totali al fine di verificare la corretta espressione del transgene. Per ciascun *DGAT* sono stati ottenuti da 1 a 5 mutanti in grado di esprimere correttamente il nuovo costrutto. Ulteriori analisi di PCR quantitativa in tempo reale (qRT-PCR) hanno permesso di valutare il grado di espressione dei transgeni rispetto al ceppo *wild type* e di selezionare i mutanti con la più alta espressione per valutarne la capacità di produzione dei lipidi. I cloni selezionati hanno mostrato una capacità di sovra espressione dei geni analizzati, rispetto al *wild type*, superiore di 30 volte per i cloni D1-160 e D5-36, di 16 volte per il clone D4-66 e di 4 volte per il clone D3-18. È attualmente in corso la valutazione del grado di espressione per l'unico mutante ottenuto per il gene DGAT2.
Per ogni *DGAT*, il clone con il più alto livello di espressione è stato sottoposto ad analisi fenotipiche al fine di valutare gli effetti della sovra espressione dei geni sovra citati e la capacità di accumulare più lipidi rispetto al ceppo *wild type*. I mutanti sono stati allevati per 8 giorni in condizioni ottimali di crescita, durante i quali sono stati valutati parametri come densità ottica, numero di cellule e contenuto totale di clorofilla. La quantità di lipidi apolari è stata valutata direttamente su cellule tramite saggio in fluorescenza con Nile Red (Chen et al., 2009) e i valori riportati sono stati espressi in unità arbitrarie di fluorescenza per milioni di cellule. Tutti i risultati sono stati confrontati con il ceppo *wild type*, allevato nelle stesse condizioni di crescita. Nelle normali condizioni di crescita, per tutti i mutanti non sono state riscontrate differenze statisticamente significative ne per quanto riguarda i parametri di crescita ne per quanto riguarda il contenuto lipidico. Alla base di questi risultati è possibile dedurre che la sovra espressione di questi geni non influenza il fenotipo dei mutanti che rimane identico al ceppo wild type. In più, questi risultati suggeriscono che la sola sovra espressione di questi geni non è sufficiente ad ottenere un maggior accumulo di lipidi, probabilmente a causa della presenza di altri step limitanti nel *pathway* biosintetico dei lipidi.
Diversi lavori riportano varie condizioni di stress, tra cui shock fisici (temperatura) o chimici (cambio di pH, rimozione di nutrienti dal mezzo di coltura), durante le quali molte alghe sono in grado di accumulare più lipidi. Timmins et al. (2008) hanno riportato che *C. reinhardtii* è in grado di aumentare la capacità di accumulo di trigliceridi nell'arco di 24 ore dal suo trasferimento in terreno carente di zolfo. È stato inoltre dimostrato che, quando cresciuta in carenza di azoto o fosforo, la quantità di trigliceridi in *Chlamydomonas* corrisponde rispettivamente al 30,4% e al 64,7% del contenuto totale di lipidi, valore che si aggira intorno al 2,2% quando coltivata nelle normali condizioni di crescita (Weers and Gulati, 1997).
I mutanti sono stati allevati in normali condizioni di crescita fino al raggiungimento della fase stazionaria (~ 20 milioni di cellule per ml), dopo la quale sono stati trasferiti in terreni mancanti di fonte di azoto (TAP -N) o fonte di zolfo (TAP -S). Le cellule sono state allevate per 4 giorni in deficit di nutrienti, durante i quali sono stati valutati parametri come densità ottica, numero di cellule, quantità totale di clorofilla e contenuto lipidico.
In entrambe le condizioni di stress, la densità ottica delle colture, sia per i mutanti che per il ceppo wild type, è raddoppiata dopo 24 ore. Questo valore è rimasto pressoché constante per tutto il corso dell'esperimento per quanto riguarda le colture allevate in TAP -N, mentre per le colture allevate in TAP -S ha subito una leggera diminuzione nell'arco delle 96 ore. In carenza di azoto non c'è stata nessuna sostanziale differenza tra la densità ottica delle colture di mutanti e del wild type,

mentre in carenza di zolfo la coltura del mutante D3-18 ha mostrato una densità ottica a 72 e 96 ore superiore rispetto al wild type ed agli altri mutanti.
L'incremento nella densità ottica non dipende dall'aumento del numero di cellule, valore in entrambe le condizioni di stress pressoché costante per tutto il corso dell'esperimento. Come ripostato da Zhang et al. (2002), durante la crescita in carenza di zolfo le cellule tendono a cambiare la loro morfologia, passando dalla tipica forma ellissoidale ad una forma più ampia e sferoidale. Questo cambiamento morfologico è stato dimostrato essere correlato con l'accumulo di amido come riserva di energia durante la carenza di zolfo o di azoto.
Per quanto riguarda il contenuto totale di clorofilla, nessuna differenza è stata riscontrata tra i mutanti e il ceppo wild type in entrambe le condizioni di stress.
In linea generale, in carenza di azoto le cellule sono state in grado di accumulare fino a due volte più lipidi rispetto alle cellule cresciute in assenza di zolfo. Tuttavia, durante la crescita in TAP -N, non sono state riscontrate differenze significative sul contenuto lipidico tra mutanti e wild type, differentemente da quanto accaduto in carenza di zolfo, dove i mutanti D3-18 e D4-66 hanno mostrato una capacità di accumulo di lipidi in media 2,0 e 1,8 volte maggiore rispetto al ceppo wild type. Nessuna differenza è stata riscontrata invece per i mutanti D1-160 e D5-36.

1. INTRODUCTION

1.1 Environmental pollution by polycyclic aromatic compounds

The first effect of the technological and industrial envelopment in the modern society is represented by the consequent environmental pollution. A large number of toxic substances released in the biosphere are now integrated part of the food chain, being a concrete risk for the biodiversity and for the human health. Persistent pesticides, chemical solvents and other substances tend to invade slowly the environment, to bioaccumulate in the food chain and to have long half-lives in animals and humans (Stefanidou et al., 2009).

Heavy metals and hydrocarbons are the most popular pollutants present in the waters. Phenols and derivates like nitrophenols and chlorophenols are known to be toxic to fish and other aquatic organism at concentrations of 5 mg l^{-1} to 25 mg l^{-1} (Khan et al., 1981; Haghighi Podeh et al., 1995; Shigeoka et al., 1988). Some of these phenolic pollutants are suspected to be endocrine disrupter and have adverse effects on human and other organisms health at concentration lower than the authorized emission for phenols (Hulzebos et al., 1993; Schafer et al., 1999; Staples et al., 1998).

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of hazardous organic chemicals consisting of two or more fused benzene rings in linear (e.g. anthracene), angular (e.g. phenanthrene) and cluster (e.g. pyrene) arrangements (Figure 1.1). The physico-chemical properties of the PAHs largely determine their environmental behaviour; low molecular weight (LMW) PAHs, containing two or three fused rings, are more water soluble and volatile, and hence more available, than high molecular weight (HMW). HMW PAHs are hydrophobic compounds and their persistence within ecosystems is due chiefly to their low water solubility. HMW PAHs rapidly become associated with sediments (Bergknut, 2006), where they may become buried and persist until degraded, resuspended, bioaccumulated, or removed by dredging. The lipophilicity, environmental persistence and genotoxicity increase as the molecular size of PAHs increases up to 4 or 5 fused benzene rings and toxicological concern shifts towards chronic toxicity, primarily carcinogenesis (Jacob et al., 1986; Miller and Miller 1981).

PAHs enter the environment from a multiplicity of sources which include: direct aerial fallout, chronic leakage of industrial or sewage effluents, accidental discharges during the transport, use and disposal of petroleum products, or from natural sources such as oil seeps and surface water run-off from forest and prairie fire sites (Giger & Blumer 1974; LaFlamme & Hites 1978). More specifically, industrial effluents from coal gasification and liquefaction processes, waste incineration, coke, carbon black, and other petroleum-derived products release high quantities of PAHs into the environment. Exposure to PAHs constitutes a significant health risk for people living in industrialized areas of the world (Thakker et al. 1985; Dipple et al. 1990). PAHs are on the United States Environmental Protection Agency's priority pollutant list since some are known carcinogens and mutagens (Table 1.1, Mackay et al., 1992). The possible fates of PAHs in the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation, adsorption to soil particles, leaching and microbial degradation. However, their degradation by microorganism in aqueous environmental is often limited by their low water solubility allowing the accumulation in soil, plants, fishes and invertebrate.

1.2 Olive-mill wastewaters

The olive oil industry generates large amounts of by-products that are harmful to the environment. According to the Food and Agriculture Organisation of the United Nations (FAOSTAT 2006) 2.7 millions of tonnes of olive oil are produced annually worldwide, 76% of which are produced in Europe, with Spain (35.2%), Italy (23.1%) and Greece (16.1%) being the highest olive oil producers. Other olive oil producers are Africa (12.5%), Asia (10.5%) and America (0.9%). Olive oil production is a very important economic activity, particularly for Spain, Italy and Greece (where combined exports are valued at more than 5,500 million US$); worldwide, there has been an increase in production of about 30% in the last 15 years (FAOSTAT 2006). Moreover, olive oil production is no longer restricted to the Mediterranean basin, and new producers such as Australia, USA and South America will also have to face the environmental problems posed by olive-mill wastes (Morillo et al. 2009).
The chemical composition of olives, which is the raw material for olive oil extraction, is very variable and depends on factors such as the olive variety, soil type and climatic conditions, but in general it consists of 18–28% oil, 40–50% vegetation water and pit and 30–35% of olive pulp (Niaounakis and Halvadakis 2004). Following olive oil extraction, mainly by mechanical procedures in olive mills, a large quantity of liquid and solid residues is produced, with a high organic load, the nature of which depends on the extraction system employed. Three systems are used worldwide for the industrial-scale extraction of oil from olives: the traditional press-cake system, the three-phase decanter system and the modern two-phase centrifugation system (Figure 1.2). Nowadays, two-phase and three-phase centrifugation systems are most commonly used (Morillo et al, 2009).
The three-phase system, introduced in the 1970s to improve extraction yield, produces three streams: pure olive oil, olive-mill wastewaters (OMW) and a solid cake-like by-product called olive cake. From an environmental point of view, OMW is considered the most critical waste emitted by olive mills in terms of both quantity and quality (Niaounakis and Halvadakis 2004). The olive cake, which is composed of a mixture of olive pulp and olive pits, is transferred to central seed oil extraction plants where the residual olive oil can be extracted. The two-phase centrifugation system was introduced in the 1990s in Spain as an ecological approach for olive oil production since it drastically reduces the water consumption during the process. This system generates olive oil plus a semi-solid waste, known as the two-phase olive-mill waste (TPOMW) (Morillo et al. 2009).
The problems arising from OMW are derived from its high organic load and its chemical composition (Table 1.2) which renders it resistant to degradation. The OMW contains a majority of the water-soluble chemical species present in the olive fruit, a high organic load and high C/N ratio (chemical oxygen demand (COD) values up to 200 g l^{-1}) and has an acidic pH of between 4 and 6. The organic fraction contains large amounts of proteins, lipids and polysaccharides, but unfortunately OMW also contains phytotoxic components that inhibit microbial growth (Capasso et al. 1995; Ramos-Cormenzana et al. 1996) as well as the germination and vegetative growth of plants (Linares et al. 2003). Olive oil phenolic compounds are the main determinants of antimicrobial and phytotoxic actions of olive-mill wastes. These compounds are either originally synthesised by the olive plants as a defence against a remarkable variety of pathogens (Bianco et al. 1999) or formed during the olive oil extraction process (Pannelli et al. 1991). Once in the olive oil, olive oil phenols show a range of antioxidant, functional, nutritional and sensory properties (Saija and

Uccella 2000). Because olive oil phenols are amphiphilic, only a fraction of the phenolics enters the oil phase, and a large proportion (>98%) is lost with the waste stream during processing (Rodis et al. 2002). It is estimated that the toxic load of OMW in terms of phenolic compounds is up to a thousand times larger than that of domestic sewage (Niaounakis and Halvadakis 2004). The phenolic profile of OMW is complex and variable. The occurrence of specific phenolic compounds depends on the fruit (e.g. maturity, cultivar), climatic conditions and storage time, in addition to the processing technique. The phenolic fraction is divided into low molecular weight (caffeic acid, tyrosol, hydroxytirosol, p-cumaric acid, ferulic acid, syringic acid, protocatechuic acid, etc.) and high molecular weight compounds (tannins, anthocianins, etc.) as well as catechol-melaninic polymers (Davies et al. 2004). Due to their instability, OMW phenols tend to polymerise during storage into condensed high-molecular-weight polymers that are particularly difficult to degrade (Ayed et al. 2005; Crognale et al. 2006). For these reasons, the uncontrolled disposal of OMW has traditionally become a great problem in Mediterranean countries because of their polluting effects on soil and water (Sierra et al. 2001; Piotrowska et al. 2006).
Besides traditional decantation, several disposal methods have been proposed for olive-mill wastes, such as thermal processes (combustion and pyrolysis), physico-chemical treatments (e.g. precipitation/flocculation, ultrafiltration and reverse osmosis, adsorption, chemical oxidation processes and ion exchange), extraction of valuable compounds (e.g. antioxidants, residual oil, sugars), agronomic applications (e.g. land spreading), animal-breeding methods (e.g. direct utilisation as animal feed or following protein enrichment) and biological treatments (Niaounakis and Halvadakis 2004). Among the different options, biological treatments are considered the most environmentally compatible and the least expensive of wastewater treatments methods (Mantzavinos and Kalogerakis 2005). These processes use microorganisms to break down the chemicals present in olive-mill wastes and/or to valorise the residues by the production of added-value compounds such as a diverse range of microbial-derived substances including biopolymers and biofuels. The actual type of microorganism that is involved depends on the conditions under which the olive-mill waste is treated, particularly whether it is aerobic or anaerobic. Aerobic processes are applied to waste streams with low organic loads and/or concentration of nutrients or as a polishing step to further remove residual organic matter and nutrients from olive-mill wastewaters, whereas anaerobic processes are applied to waste streams with high organic loads (Niaounakis and Halvadakis 2004). In any case, high organic loads, presence of some classes of antimicrobial or biostatic compounds such phenols and lipids, low pH values, low water activity and unbalanced composition of nutrients represent barriers that should be overcome to achieve an optimal biological process (Morillo et al. 2009).

1.3 Bioconversion of olive-mill wastes to high added-value products

Many applications have been attributed to OMW, with or without further treatment, to obtain added-value products (Figure 1.3). For example, OMW have been used as substrate for the culture of certain microorganisms in order to obtain a potentially useful microbial biomass or to induce a partial bioremediation of the residues. Such applications have a long history, and already 50 years ago Fiestas Ros de Ursinos (1961) reported the production of yeast biomass using OMW in a chemostat for use in industrial applications. A few edible fungi, especially species of *Pleurotus*, can also

be grown using OMW as the source of nutrients by the application of different strategies (Kalmis et al. 2008; Sanjust et al. 1991; Zervakis et al. 1996). These authors also reported biochemical changes and detoxification of the substrates due to the active excretion of ligninolytic enzymes and partial consumption/adsorption of the organic fraction by fungi. Kalmis et al. (2008) recently suggested the cultivation of the oyster mushroom (*Pleurotus ostreatus*) on wheat straw substrate containing a mixture of tap water and OMW (25% OMW, v/v) as an environmentally friendly solution for the purpose of commercial mushroom production. Similar approaches have been exploited by several small companies for more than a decade (Ramos-Cormenzana et al. 1995). Mention should also be made of the possibility of using the microbial biomass produced from OMW fermentations either as an additive to animal feed or to improve its agronomic use. For example, Laconi et al. (2007) achieved an intense degradation of most polluting substances of OMW and the production of biomass that could be used as an animal feed integrator using a chemical–biological method (alkaline-oxidative treatment to decrease the polyphenolic content followed by fermentation with a fungal mixture). As far as agronomic use of the waste is concerned, the idea of re-using microbially treated OMW as fertilizer has been also proposed (Vassilev et al. 1997). To this end, a strain of the acidogenic fungus *Aspergillus niger* was grown in either free or immobilised form on OMW with rock phosphate added in order to solubilise it. After fermentation, the phosphorus-enriched OMW was tested as fertilizer on *Trifolium repens* L. and *Triticum durum* systems, resulting in enhancement of plant growth (Cereti et al. 2004).

In recent years, considerable attention has been directed towards the production of energy from lignocellulosic wastes. As mentioned above, anaerobic digestion is a practical alternative for the treatment of TPOMW, which produces biogas (Borja et al. 2006; Antizar-Ladislao and Turrion-Gomez 2008). As it has been reported for other agroindustrial residues, such as potato pulp (Zhu et al. 2008) or cattle manure (Güngör-Demirci and Demirer 2004), anaerobic processes applied to OMW, whether in one or two stages, must be selected according to the C/N ratio of the residues in order to obtain a satisfactory anaerobic degradation. The TPOMW is biodegradable by anaerobic digestion at mesophilic temperatures in stirred tank reactors, with COD removal efficiencies in the range of 72–89% and an average methane yield coefficient of 0.31 dm^3 CH_4 per gramme COD removed (Borja et al. 2006). Similar production of hydrogen and methane has been reported using thermophilic reactors at 55°C (Gavala et al. 2005) and in mesophilic anaerobic treatment of TPOMW in continuous and batch experiments, in which approximately 0.28 dm^3 CH_4 per gramme COD was removed, and hydrogen production was coupled with a subsequent step for methane production, giving the potential for production of 1.6 mmol H_2 per gramme of TPOMW (dry matter; Borja et al. 2006).

Additionally, the high content of organic matter makes OMW an interesting alternative resource to produce ethanol as a biofuel (Li et al. 2007). Even if the content of free reducing sugar in these wastes is low, different kinds of polysaccharides can be converted to ethanol via different reactions that occur in two separate steps: first an enzymatic hydrolysis using commercial enzymes followed by the conversion of reducing sugars to ethanol performed by yeasts (alcoholic fermentation; Zanichelli et al. 2007). Amongst the many parameters that can affect the process of alcoholic fermentation, the presence of inhibiting compounds in OMW is critical. Bambalov et al. (1989), using a collection of several yeast strains of different species, confirmed that fresh OMW was unfavorable to yeast growth and ethanol production. The removal of the phenolic fraction using an

adsorption/desorption technique seems to be a necessary procedure for efficient ethanol production from OMW (Zanichelli et al. 2007).
The selection of microorganisms to use as alternative fuel source and able to grow on wastewaters is a continuing challenge for scientists. In the last years, microalgae seem to be a favorite candidate for this purpose, due for their easily cultivation and the favourable possibility to use them as an alternative biomass for bioenergy production.

1.4 Phycoremediation

Organic pollutants in the aquatic environment are subject to biodegradation by a range of naturally occurring microorganisms. Studies have concentrated almost exclusively on the role of bacteria (Dagley, 1978) and fungi (Middlehoven, 1993). In spite of their ubiquitous distribution, their central role in the fixation and turnover of carbon and other nutrient elements and recognition of their heterotrophic abilities, algae have been considered as good candidate for bioremediation only in the recent years (Semple et al., 1999). However, information on the relationship between algal heterotrophy and the biodegradation of xenobiotic compounds is far less than the information accumulated concerning bacteria and fungi.
Microalgae usually play an important role during the tertiary treatment of domestic wastewaters in maturation ponds or the treatment of small/middle scale municipal wastewater in facultative or aerobic ponds (Aziz and Ng, 1993; Abeliovich, 1986; Mara and Pearson, 1986; Oswald, 1988, 1995). They enhance the removal of nutrients, heavy metals and pathogens and furnish O_2 to heterotrophic aerobic bacteria to mineralize organic pollutants, using in turn the CO_2 released from bacterial respiration (Figure 1.4) (Muñoz and Guieyesse, 2006). Photosynthetic aeration is therefore especially interesting to reduce operation costs and limit the risks for pollutant volatilization under mechanical aeration and recent studied have shown that microalgae can indeed support the aerobic degradation of various hazardous contaminants (Muñoz et al., 2004; Safonova et al., 2004). The mechanisms involved in microalgal nutrient removal from industrial wastewaters are similar than that from domestic wastewaters treatment. Microalgae capability to biodegrade hazardous organic pollutants is well known and *Chlorella*, *Ankistrodesmus* and *Scenedesmus* species have been already successfully used for the treatment of olive oil mill wastewaters and paper industry wastewaters (Abeliovich and Weisman, 1978; Narro, 1987; Pinto et al., 2002, 2003; Tarlan et al., 2002). Thus, phycoremediation can be define as the use of marco and microalgae for: a) nutrient removal from municipal wastewater and effluents rich in organic matter; b) nutrient and xenobiotic compounds removal with the aid of algae-based biosorbents; c) treatment of acidic and metal wastewaters; d) CO_2 sequestration; e) transformation and degradation of xenobiotics; and (f) detection of toxic compounds with the aid of algae-based biosensors (Olguin, 2003).
One way to investigate the capability of algae to biodegrade organic pollutants is to encourage the cells to grow in the presence of the pollutant. For this purpose, Walker et al. (1975) performed experiments with the achlorophyllous alga *Prototheca zopfii*, which was found to degrade petroleum hydrocarbons found in Louisiana crude and motor oils. Interestingly, in the crude oil, 38-60% of the saturated aliphatic hydrocarbons and 12-41% of the aromatic compounds were degraded, whereas in the motor oil, 10-23% of the saturated aliphatic hydrocarbons and 10-26% of the

aromatic compounds were degraded. This suggested that the alga was capable of degrading different oils to varying levels. Jacobson and Alexander (1981) observed that cultures of *Chlamydomonas* species, grown in the light and in the dark on acetate, were able to dehalogenate 4-chloro-3,5-dinitrobenzoic acid and produce a metabolite which was identified as 2-hydroxymuconic semialdehyde, indicating *meta* cleavage. Jinqi and Houtian (1992) investigated the degradation of azo dyes by *Chlorella vulgaris* and *Chlorella pyrenoidosa* and found that certain dyes, such as Eriochrome blueSE and blackT, could be decolorized and actually used as carbon and nitrogen sources. The degradation was found to be an inducible catabolic process. They also found that the algae degraded aniline, a potential degradation product of the azo dye breakdown. In another study, *Ochromonas danica*, a nutritionally versatile chrysophyte, grew heterotrophically on phenol or *p*-cresol as the sole source of carbon up to concentrations of 4 mM (Semple, 1997).

PAHs are known to produce carcinogenic and mutagenic effects and are therefore considered to be priority pollutants in the environment. Their modes of degradation have been studied in some detail and have been reviewed by Cerniglia (1992). Several works reported the use of algae for the removal of these compounds. Naphthalene has proved interesting to algal researchers, who found that this compound was toxic to *Chlamydomonas angulosa*: 60-98% of cells were killed (lethality was determined by monitoring mobility) in open and closed flasks containing saturated aqueous solutions of naphthalene within 1 day in the light (Soto et al., 1975a; 1975b). However, after a lag phase of up to 3 days, the alga began to multiply at similar rates to that of the control (no naphthalene), with final growth yields being similar between the test and control incubations. It was noted that the algal cells were able to remove naphthalene from the growth medium by accumulation within the cells, but were unable to metabolize the pollutant. Cerniglia et al. (1979; 1980a; 1980b) showed that both cyanobacteria (blue-green algae) and eukaryotic microalgae were capable of biotransforming naphthalene to four major metabolites, 1-naphthol, 4-hydrox-4-tetralone, *cis*-naphthalene dihydrodiol and *trans*-naphthalene dihydrodiol at concentrations which were non-toxic. The formation of *cis*-naphthalene dihydrodiol was the first demonstration in a eukaryotic cell. However, the total degradation of naphthalene was not great, ranging from 1 to 1.9%. In another study, Cerniglia et al. (1982) characterized the initial degradation of naphthalene in diatoms. Once again, 1-naphthol was found to be a major metabolite, but only 0.7-1.4% of the aromatic compound was degraded. Liebe and Fock (1992) found that *Chlamydomonas reinhardtii*, after having been suitably adapted, was able to remove some of the iso-octane-extracted PAHs from diesel particulate exhaust. The extent of removal ranged from 4 to 95%, although the mechanisms for this are unclear. However, there were no significant differences in growth between adapted cells in fresh media with or without the extract. It is therefore unlikely that the PAH derivatives were consumed as nutrients. Luther and Soeder (1987) and Luther (1990) were able to confirm that *Scenedesmus obliquus* was able to utilize naphthalene sulfonic acids as a source of sulphur for the production of biomass, releasing the desulfonated carbon ring into the medium. Furthermore, Luther (1990) found that the chlorophyte alga also utilized nitro and amino substituents from aminonaphthalenes and aminoand nitrobenzoates as nitrogen sources. Luther (1990) noted that bacteria then metabolized the products of desulfonation. Bacteria require long periods of adaption to degrade naphthalene monosulfonic acids (Luther and Soeder, 1987); however, *S. obliquus* can desulfonate these compounds in a matter of hours in sulphur-deficient medium. This suggests that an algal-bacterial

consortium may accelerate the degradation of these compounds. Diaryl ethers such as halogenated diphenyl ethers are important environmental pollutants. Therefore, the bacterial degradation of these and structurally related compounds attracted considerable interest (Schmidt et al., 1993; Bünz and Schmidt, 1997). However, by using the diphenyl ether herbicide Diclofop methyl, Wolfaardt et al. (1994) demonstrated the significant contribution of a *Chlorococcum* sp. present in an algal-bacterial consortium to the rate of $^{14}CO_2$ production from ^{14}C-labelled Diclofop methyl. Pinto et al. (2003) showed the capability of the two green algae *Ankistrodesmus braunii* and *Scenedesmus quadricauda* to remove over 50% of the low molecular weight phenols contained in OMW when grown in dark condition. Lima et al. (2003) reported *p*-nitrophenol removal of 50 mg l^{-1} d^{-1} by a consortium of *C. vulgaris* and *C. pyrenoidosa* under not optimized conditions, which was closed to the 100 mg l^{-1} d^{-1} achieved with *Pseudomonas* species by Kulkarni and Chaudhari (2006). Extensive works have been conducted to explore the feasibility of using microalgae for wastewater treatment, especially for the removal of nitrogen and phosphorus from effluents (Mallick, 2002; Aslan et al., 2006; Hernandez et al., 2006; Abdel Hameed, 2007; Lebeau and Robert, 2003), which would otherwise result in eutriphication if dumped into lakes and rivers (Galvez-Cloutier et al., 2006). Ironically enough, it is algae in the lakes and rivers that cause this problem. It is simply a matter of allowing the consumption of nitrogen and phosphorus by microalgae in a controlled manner that benefits rather than deteriorates the environment. Levels of several contaminant heavy metals have also been shown to be reduced by the cultivation of microalgae, which is a subject discussed extensively by Muñoz and Guieysse (2006).
These examples demonstrate that algae are indeed capable of contributing to the degradation of environmental pollutants, either by directly transforming the pollutant in question or by enhancing the degradation potential of the microbial community present. The biomass resulting from the treatment of wastewaters can be easily converted in added value products. Depending by the species used for this purpose, the resulting biomass can be applied for different aims, including the use as additives for animal feed, the extraction of added value products like carotenoids or other bio-molecules or the production of biofuel. In addition to the apparent benefit of combining microalgal biomass, and therefore biofuel, production and wastewater treatment, successful implementation of this strategy would allow the minimizing of the use of freshwater, another precious resource especially for dry or populous countries, for biofuel production. The screening of the numerous algal collection has as first aim the investigation of the wide biodiversity accumulated in the years in order to characterize species and strains and to furnish information about their possible employment. For example, a major concern associated with using wastewater for microalgae cultivation is contamination (De la Noüe and De Pauw, 1988; Muñoz and Guieysse, 2006). This can be managed by using appropriate pretreatment technologies to remove sediment and to sterilize the wastewater (Tamer et al., 2006) or, even better, by selecting extremophilic algal strains able to grow in condition not favorable for other non-desiderate organisms.

1.5 Biofuel from microalgae

The development of CO_2-neutral fuels is one of the most urgent challenges facing our society. Global surface temperatures have increased by 0.8°C since the late

nineteenth century and 11 out of the 12 warmest years on record have occurred since 1995 (IPCC 2007). Earth's mean temperature is projected to increase by 1.5-5.8°C during the twenty-first century (IPCC 2001). The rate of increase in global temperature has been 0.15°C per decade since 1975. In addition to the sea-level rise of 15-23 cm during the twentieth century (IPCC 2007) there have been notable shifts in ecosystems (Greene and Pershing, 2007) and frequency and intensity of occurrence of wild fires (Running, 2006; Westerling et al., 2006). These and other observed climate changes are reportedly caused by emission of greenhouse gases through anthropogenic activities including land-use change, deforestation, biomass burning, draining of wetlands, soil cultivation and fossil fuel combustion. Consequently, the concentration of atmospheric greenhouse gases and their radiative forcing have progressively increased with increase of the human population, but especially since the onset of industrial revolution around 1850 (Lal, 2008). The concentration of carbon dioxide has increased by 31% from 280 parts per million by volume (ppmv) in 1850 to 380 ppmv in 2005 and is presently increasing at 1.7 ppmv yr^{-1} or 0.46 % yr^{-1} (WMO 2006, IPCC 2007).

Between 1850 and 1998, anthropogenic emissions are estimated at 270 ± 30 billion tonnes of carbon (Pg C) by fossil fuel combustion and at 136 ± 30 Pg C by land-use change, deforestation and soil cultivation (IPCC 2001). Actually, approximately 7 Pg C yr^{-1} is emitted by fossil fuel combustion (Pacala and Socolow, 2004) and 1.6 Pg C yr^{-1} by deforestation, land-use changing and soil cultivation. Of the total 8.6 Pg C yr^{-1}, 3.5 Pg C yr^{-1} is absorbed by the atmosphere, 2.3 Pg C yr^{-1} by the ocean and the remainder by an unidentified terrestrial sink probably in the Northern Hemisphere (Tans et al., 1990; Fan et al., 1998). There is a strong interest in stabilizing the atmospheric abundance of CO_2 and others greenhouse gases to mitigate the risk of global warming (Kerr, 2007; Kintish, 2007). Three strategies of lowering CO_2 emission have been proposed to mitigate climate change (Shrang, 2007): a) reducing the global energy use; b) developing low or no-carbon fuels; and c) sequestering CO_2 from point source or atmosphere through natural and engineering techniques.

A variety of biomasses from different sources, including forestry, agricultural, and aquatic sources have been investigated as the feedstock for the production of different biofuels including biodiesel, bio-ethanol, bio-hydrogen, bio-oil, and bio-gas. Techno-economic assessments indicated that cost-effectiveness of biofuel production is achievable (Bridgwater et al., 2002). However, burning fuels derived from existing biomass has an environmental impact similar to the combustion of fossil fuels in terms of its impact to the carbon cycle (carbon balance), i.e., conversion of fixed carbon into CO_2. In addition, depletion of certain existing biomasses (e.g., wood) without appropriate compensation (e.g., replanting) may result in massive biomass deficit, resulting in serious environmental problems (e.g., deforestation) (Li et al., 2008). To overcome these problems, a range of second generation biofuel-production systems, with better net energy balance, higher water-efficiency and less arable land request are now under development (Schenk et al., 2008). Among these, biofuel production system based on microalgae is one of the most promising.

Conventional terrestrial plants are not very efficient in capturing solar energy. It was estimated that switchgrass, the fastest-growing terrestrial crop, can convert solar energy to biomass energy at a yearly rate of no more than 1 W m_2^{-1}, less than 0.5% of the solar energy received at a typical mid-latitude region (200-300 W m_2^{-1}) (Li et al., 2008). Kruse et al. (2005) reported that the green algae have a solar light conversion efficiency in biomass around 9%. Furthermore, the capability of some microalgae species to tolerate high CO_2 content in feeding air streams (Chang and

Yang, 2003), allowing efficient capturing of CO_2 from high-CO_2 streams such as flue gases and flaring gases (CO_2 content 5-15%) (Hsueh et al., 2007). In comparison to terrestrial plants, which typical absorb CO_2 from the atmosphere containing only 0.03-0.06% CO_2, the benefit of microalgae is evident in terms of CO_2 mitigation. It was reported that using a outdoor cultivation of *Chlorella* sp. in a 55 m^2 culture area photobioreactor, flue gas containing 6-8% of CO_2, 10-50% CO_2 mitigation (flue gas decarbonization) was achievable and the residual NO_2 and NO in the flue gas was found not to affect algal growth (Doucha et al., 2005). Depending on the microalgal species and condition used in the facilities, algal biomass produced could be further processed for biodiesel, bio-oil, and bio-gas production. Microalgae are reported to have the potential to produce 15-300 times more oil for biodiesel production than traditional crops on an area basis (table 1.3). Within addition, conventional crop plants are usually harvested once or twice a year whereas microalgae have a very short harvesting cycle (1-10 days depending from the process), allowing multiple or continuous harvest with significantly increased yields (Schenk et al., 2008). The cultivation of microalgae for biofuel production can potentially be carried out on marginal or non-arable land, reducing the competition for arable land and open up new economic opportunities for arid, drought or salinity-affected regions. In addition, while traditional biofuel crop require substantial amounts of fresh water, algal cultivation in closed bioreactor systems with minimal evaporation can lead to considerable savings of net water use. The employment of closed bioreactor systems also allows the disposal of biological GMO waste in an environmentally sensitive way (Schenk et al., 2008).

Another interesting aspect of second generation microalgal systems resides in the highly innovative biotechnology approaches that have the potential for rapid improvements of algal strains. Gene technology could provide the opportunity to increases yields by metabolic engineering, for example by increasing the photosynthetic efficiency, the tolerance to grow in saline or wastewaters streams or the over-expression of enzymes involved in specific biosynthetic pathways.

The use of seawater and wastewater offers clear advantage because it decreases the pressure on freshwater resources, as water scarcity could become problematic in the face of climate change and population growth. However, wastewaters quality can vary dramatically from one source to another and also can be fluctuating over time. Wastewaters can contain valuable nutrients such as nitrogen and phosphorous, but also can contain heavy metals and other contaminants that could lead to down regulation of the lipid biosynthesis (Gillet et al., 2006).

Algae collection worldwide contain thousands of different algae strains that, combined with recent advances in genetic engineering and material science, provide a good starting point for further development of microalgal biofuel production systems based on wastewaters treatment. Strain selection and characterization, as well as the breeding and adaption of strains with desirable phenotypes that allow the use of water resources of varying water quality, also play important roles in the perspective of using microalgae for biofuel production. Future algal strain improvement will utilize methodologies such as lipidomics, genomics, proteomics and metabolomics to screen for and develop new strains that exhibit high growth and lipid biosynthesis rates, broad environmental tolerances and the ability to produce high value by-products.

1.6 Diacylglycerol acyltransferase: a key-enzyme of the triacylglycerol biosynthetic pathway

Biodiesel is produced by a mono-alcoholic trans-esterification process, in which triacylglycerol (TAG) reacts with a mono-alcohol (most commonly methanol or ethanol) with the catalysis of alkali, acids, or enzymes (Demirbas, 2007, Meher et al., 2006). TAGs are synthesized in two major pathway elucidated in the 1950s and 1960s: the glycerol phosphate, or Kennedy pathway, and the monoacylglycerol (MAG) pathway (Lehner and Kuksis, 1996; Coleman and Lee, 2004). Both pathways use fatty acyl-CoAs, the activated forms of fatty acids synthesized by intracellular acyl-CoA synthases, as acyl donors (Coleman et al., 2002). The glycerol phosphate pathway is present in most cells whereas the MAG pathway is found in specific cell types, such as enterocytes, hepatocytes, and adipocytes, where it may participate in the re-esterification of hydrolyzed TAG (Xia et al., 1993). The MAG pathway is the dominant mode of TAG synthesis in human small intestine, where TAGs are synthesized from components of hydrolyzed dietary fats (Kayden et al., 1967; Mansbach II and Gorelick, 2007).

Triacylglycerol biosynthesis in algae has been proposed to occur via the direct Kennedy pathway (Ratledge, 1988). Fatty acids produced in the chloroplast are sequentially transferred from acyl-CoAs to positions 1 and 2 of glycerol-3-phosphate, resulting in formation of the central metabolite phosphatidic acid (PA) (Ohlrogge and Browse, 1995). Dephosphorylation of PA catalyzed by a specific phosphatase releases diacylglycerol (DAG). In the final reaction of both pathways, a fatty acyl-CoA and diacylglycerol (DAG) molecule are covalently joined to form TAG. This reaction (Figure 1.5) is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT, E.C. 2.3.1.20) an enzymatic reaction that is unique to TAG biosynthesis.

TAG biosynthesis is believed to occur mainly at the endoplasmic reticulum (Weiss et al., 1960). Newly synthesized TAGs are thought to be released into the associated lipid bilayer, where they are channeled into cytosolic lipid droplets or, in cells that secrete TAG, nascent lipoproteins (Figure 1.6). Although several models have been proposed (Walther and Farese, 2008), the precise mechanism by which TAGs are deposited into lipid droplets is unknown.

DGAT activity was first reported in 1956 (Weiss et al., 1960). Although there was much interest subsequently in the biochemistry of TAG synthesis, the purification of a DGAT proved to be difficult. Only in the last decade DGAT genes have been cloned, and the molecular tools for studying TAG synthesis become available. At least two DGAT enzymes exist in a wide variety of eukaryotes and their over-expression in different organisms provide an increment in the lipid accumulation. Although cloning and over-expression of DGAT genes in microalgae could be an alternative way to increase the lipid content of these organisms for biodiesel production, no works reporting the study of these enzymes in algae were published until now.

2. MATERIALS AND METHODS

2.1 Growth media

Substrates have been prepared using deionized water from a reverse osmosis system (Millipore) with a resistivity of 18.2 MΩ cm^{-1} at 25°C. The sterilization was conducted in an autoclave for 20 minutes at 121°C and 0.12 Mpa. All thermolabile compounds have been previously filtersterilized using nitrocellulose filters 0.22 µm pore size (Millipore) and then added at the autoclaved media after cooling down to ca. 40°C. The solid media were prepared with 1.5% (w/v) agar and dispensed in 10 cm Petri dishes (20 ml for capsule). The substrates used in this work were:

- Luria Bertani broth (LB; Miller, 1972)
- SOC medium (Sambrook et al., 1989)
- Bold basal medium (BBM; Nichols and Bold, 1965)
- Tris acetate phosphate Medium (TAP; Gorman and Levin, 1965)

All cultivations were performed sterile in a horizontal laminar flow hood which was sterilized before use with UV light for 20 minutes and cleaned with 70% ethanol. All non-sterile metal instruments have been sterilized with a Bunsen burner.

2.2 Algal and bacterial strains and their growing condition

The phenol-oxidase biochemical screening was carried out on 100 algal accessions which belong mainly to the *Chlamydomonas*, *Chlorella* and *Scenedesmus* species. All accessions were obtained from the Algal Collection at University Federico II (ACUF, http://www.biologiavegetale.unina.it/acuf.html) present at the Biology Department of the University of Naples Federico II.
The algae were grown for 10 days in liquid BBM, starting with an optical density value of 0.10 ± 0.02 at 550 nm. A Beckman spectrophotometer was used for the OD_{550} measurements. The cultures were grown in 100 ml Pyrex glass flasks containing 50 ml of BBM and shaken on a planetary shaker at 100 rotations per minute (rpm) under continuous light conditions at 24 ± 1°C. The algal growth has been evaluated using the Growth Index (G.I.) expressed as:

$$\frac{(OD_{550}\ t_{10}) - (OD_{550}\ t_0)}{OD_{550}\ t_0}$$

where $OD_{550}\ t_0$ was the culture absorbance at the inoculum time and $OD_{550}\ t_{10}$ was the culture absorbance 10 days after the inoculation.
Chlamydomonas reinhardtii strain CC3491 was used for DGAT over-expression. It was grown in TAP medium, complemented with 10 mg l^{-1} hygromycin B for positive clone selection. The growth has been evaluated by measuring the optical density at 750 nm with a Genesys20 spectrophotometer (ThermoSpectronic) and the cell numbers were counted in a *Bürker* chamber (Marienfeld). Chlorophyll *a* and *b* levels were determined spectrophotometrically by measuring of the optical density at 663 and 645 nm after extracion with 80% (v/v) acetone solution (Harris, 1989).
Escherichia coli DH5α strain has been cultivated at 37°C for 16 hours on LB, supplemented with 100 mg l^{-1} ampicillin when necessary.

2.3 Biochemical screening

2.3.1 Preparation of samples for enzymatic assays and definition of Efficiency Index

All enzymatic assays have been carried out on supernatants obtained from 10 days old cultures. 25 ml of cultures have been centrifuged at 3,000 x g for 10 minutes at 4°C. The supernatants were transferred in 2 ml Eppendorf tubes and centrifuged at 13,000 x g for 10 minutes at 4°C in order to remove remaining cell fragments. The supernatant was transferred in new 2 ml Eppendorf tubes and preserved at -80°C or kept on ice when used immediately for enzymatic assays.
All buffers were prepared using deionized water. The presence of enzymes with phenol-oxidase activity in the culture broths has been detected following the oxidation of the substrate 2,2'-azino-*bis*-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). ABTS assay was performed on 20 µl of supernatant in 200 µl of 1 mM ABTS and 100 mM sodium citrate buffer, pH 3.0 (Piscitelli *et al*., 2005). The reaction was carried out at 25°C for 10 minutes, measuring the absorbance at 420 nm every minute using a Wallac Victor Multireader (Perkin Elmer). The ABTS oxidation capability of supernatants was evaluated using an Efficiency Index (E.I.):

$$\frac{OD_{420}\, t_{10} - OD_{420}\, t_0}{G.I.}$$

The E.I. is expressed as the ratio between the ABTS absorbance variation, detected at the end of the reaction, and the Growth Index. The algal accessions showing an E.I. higher than 0.1 were chosen for further experiment in order to evaluate the enzymatic activity in presence of ABTS and others phenolic compounds.

2.3.2 Evaluation of enzymatic activity

The enzymatic activity of culture broths has been evaluated in the presence of ABTS and with the phenolic compounds 2,6-dimethoxyphenol (2,6-DMP) and N,N'-*bis*-(3,5-dimethoxy-4-hydroxybenzylidene) hydrazine (syringaldazine). The enzymatic activity was calculated using a Beckman spectrophotometer in kinetic mode and was expressed as enzymatic units per ml of culture, whereas the enzymatic unit is the amount of protein that catalyzes 1 µmol of substrate per minute at 25°C.
ABTS oxidation assay was performed on 1 ml solution containing 200 µl supernatant, 1 mM ABTS and 100 mM sodium citrate buffer, pH 3.0. ABTS oxidation was monitored by following the absorbance increase at 420 nm (ε_{420} = 36 mM^{-1} cm^{-1}; Piscitelli *et al*., 2005).
2,6-DMP oxidation assay was performed on 1 ml solution containing 500 µl supernatant, 10 mM 2,6-DMP and 100 mM sodium tartrate buffer, pH 5.0. 2,6-DMP oxidation was monitored by following the absorbance increase at 469 nm (ε_{469} = 27.5 mM^{-1} $cm^{-}1$; Zouari-Mechichi *et al*., 2006).
Syringaldazine oxidation assay was performed on 1 ml solution containing 500 µl supernatant, 10 µM syringaldazine and 25 mM phosphate buffer, pH 6.5. Syringaldazine oxidation was monitored by following the absorbance increase at 525 nm (ε_{525} = 65 mM^{-1} cm^{-1}; Laufer *et al*., 2006).

2.4 RBBR decolourisation assay

The supernatants capability to decolorize recalcitrant dyes was investigated evaluating the decolourisation percent of the anthraquinone dye Remazol Brilliant Blue R (RBBR). RBBR decolourisation assay was performed on 1 ml solution containing 750 µl supernatant in 100 µM RBBR dissolved in 20 mM sodium acetate buffer pH 4.5 (Palmieri *et al*., 2005). The mixture reaction was incubate at 25°C and the decolourisation was followed for 6 hours at 592 nm using a Beckman spectrophotometer.

2.5 Algal cultivation in presence of olive-mill wastewaters

2.5.1 Olive-mill wastewaters sterilization and phenols quantification

The OMW were collected from an oil olive mill in Campania region and sterilized using the following protocol. In order to remove the corpuscular fraction, the OMW were centrifuged at 3,000 x g for 15 minutes. The liquid phase was diluted with BBM (1:10 v/v) and filtered with 0.44 µm filter using a vacuum pump system. The OMW were made axenic by filtersterilization with 0.22 µm nitrocellulose filter (Millipore) under laminar flow clean bench.
The OMW were characterized for the total phenols amount by Folin-Ciocalteau assay, using catechol for the calibration curve, as reported by Jenning (1981).

2.5.2 Growth condition in olive-mill wastewaters

In order to evaluate the capability to grow in presence of OMW and to remove the phenolic component, selected algae were cultivated in BBM added with OMW (OMW:BBM, 1:20 v/v). The cultures have been started with a concentration of 0.5×10^{-6} cells ml^{-1}, in 50 ml OMW:BBM. The flasks were incubated in light and dark condition for 21 days. All data were collected at the inoculation-time t_0 and after 7, 14 and 21 days. For each time point the growth was assessed by cells counting and the total phenols amount was measured by Folin-Ciocalteau assay.

2.6 In silico analyses

In silico analyses were carried out using bioinformatics software and database available on the internet:

- Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) used for protein and nucleotide alignment;
- Clustalw (http://www.ebi.ac.uk/Tools/clustalw2/index.html) used for phylogenetic tree analysis;
- One-Way ANOVA (http://faculty.vassar.edu/lowry//anova1u.html) used for statistical analysis of variance for independent or correlate samples;
- Primer3 (http://frodo.wi.mit.edu/primer3/) used to draw all primers;
- DOE JGI (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html) for nucleotide and protein database of *C. reinhardtii*.

2.7 Employed vectors

The vectors used were:

- pGem-T Easy (Promega);
- pGenD (Fisher and Rochaix, 2001) derived from pKS (Stratagene). The vector contains the light induced promoter and the 3' untranslated region of the Photosystem I complex subunit PsaD. At the 3' of the promoter and the 5' of the termination region are present the two unique restriction sites NdeI and EcoRI that allow the cloning of the gene;
- pHyg3 (Berthold et al., 2002) derived from pUCBM20 (Boehringer). The vector contains the *Streptomyces hygroscopicus* aminoglygoside phosphotransferase gene (*aph7"*). Its expression is controlled by *C. reinhardtii* regulatory elements, namely the ß2-tubilin gene promoter in combination with the first intron and the 3' untranslated region of the small subunit of ribulose bisphosphate carboxylase, rbcS2.

2.8 *DGAT* cloning and expression vectors construction

All diacylglycerol acyltransferase genes (*CrDGAT1-5*) have been amplified by PCR from *C. reinhardtii* nuclear genomic DNA using specific primer derived from the sequences found at the DOE JGI *Chlamydomonas* database (Table 2.1). The selected primer pairs allow the introduction of NdeI and EcoRI restriction sites at 5' and 3' UTR regions of amplified genes, respectively. *CrDGAT1* and *CrDGAT3* amplifications were performed using the SynergyN™ DNA Polymerase according to the protocol suggested by Genecraft. SynergyN™ is a mix of thermostable polymerases possessing 5'-3' DNA polymerase activity that is optimized to amplify longer GC-rich fragments (KlenThermN™) and 3'-5' proof-reading activity (AccuTherm™). This mixture provides more robust synthesis of longer GC-rich amplification products. *CrDGAT2*, *3* and *5* were cloned using PCR Extender System (5Prime) following the protocol High Fidelity PCR. The PCR Extender Polymerase Mix is a blend of thermostable DNA polymerases with a processivity-enhancing factor providing both an extremely high extension rate and maximal proofreading assisted fidelity. For all reactions 10x PCR Enhancer System (Promega) was used. All products were checked by 1% agarose gel electrophoresis (40 minutes, 100 V) and purified with the peqGOLD Gel Extraction kit (peqlab).
All sequences were cloned into pGem T-Easy vectors (3:1 insert:vector molar ratio) by ligation with T4 DNA ligase enzyme (Promega) for 24 hours at 4°C. 0.5 µl ligation mixture was used directly to transform the competent *E. coli* DH5α cells by freeze-thaw protocol (Ausubel et al., 1987). Positive clones were resistant to ampicillin and were grown for 16 hours in 5 ml of liquid LB medium. The vectors were analyzed by restriction analyses after plasmid DNA minipreparation by alkaline lysis with SDS (Sambrook et al., 1989).
To facilitate the removal of the EcoRI restriction site present in the third intron of DGAT2 gene, a point mutation was performed using the QuickChange® II Site-Directed Mutageneis kit (Stratagene) according to the kit protocol.
The pGem T-Easy::*CrDGAT* construct was incubated for 1 hour at 37°C with the restriction enzymes EcoRI and NdeI (Biolabs) in order to isolate the cloned genes. The genes were separated from the vector by 1% agarose gel electrophoresis (40 minutes, 100 V) and purified with the peqGOLD Gel Extraction kit. The purified genes

were cloned into the vector pGenD by ligation with T4 DNA ligase enzyme for 24 hours at 4°C and 0.5 µl ligation mixture were used to transform the competent *E. coli* DH5α cells by freeze-thaw protocol. Positive clones were inoculated and the vectors analyzed by restriction analyses after miniprep extraction. Positive constructs were checked by sequencing.
The plasmid purification for sequencing and nuclear genomic transformation was performed using the peqGOLD Plasmid Miniprep kit (peqlab) according to the kit protocol. All constructs were sequenced from Seqlab (www.seqlab.de) and analyzed with the software Chromas Lite.

2.9 Nuclear transformation of *Chlamydomonas reinhardtii*

The co-transformation for the production of random DNA insertion mutants of *C. reinhardtii* strain CC3491 was carried out by glass beads method, how described by Kindle (1990) with some modifications. The mutants selection was carried out used TAP plates (1.5% w/v Agar) supplemented with hygromycin B (10 mg l^{-1}). *Chlamydomonas* cells were grown in 100 ml TAP medium until the cell density reached 0.4 OD_{750}. The culture was divided in two 50 ml Falcon tubes and centrifuged for 10 minutes at 1,000 × g. The pellets were washed with 2 ml TAP medium and centrifuged again, then resuspended in 1.5 ml TAP medium. 300 µl cell suspension were transferred into sterile glass tubes with 0.3 g of acid washed and baked glass beads (Ø: 212-300 µm – Sigma-Aldrich) and 2 µg of pGenD::*CrDGAT* and pHyg3. The suspension was then vortexed for 15 seconds. After glass beads sedimentation, the cells were carefully transferred to the plates with sterile Pasteur tubes and incubated at 23°C under continuous light (40 µmol m^{-2} s^{-1}) until colonies appeared (10-14 days).

2.10 Amplification analysis of genomic DNA

The genomic DNA extraction was carried out on the basis of Doyle and Doyle protocol (1990) with some modifications. 20 ml of cell culture were centrifuged for 10 minutes at 3,000 × g and the pellet was resuspended in 1 ml sterile water. The culture was transferred into 2 ml Eppendorf tubes and centrifuged for 5 minutes at 13000 × g. The supernatant was discarded and the cell pellet resuspended in 350 µl of CTAB buffer (2% w/v CTAB, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1.4 mM NaCl) and mixed with 100 µl Proteinase K solution (2 mg ml^{-1} in 50 mM Tris-HCl pH 8.0, 50% w/v glycerol, 5 mM $CaCl_2$) and 50 µl 20% SDS solution. The cell suspension was incubated in a water bath for 2 hours at 55°C. The samples were briefly cooled down in ice and 50 µl 5 M Potassium acetate were added. The samples were inverted briefly and incubated for 30 minutes on ice. The cell fragments were then centrifuged for 15 minutes at 13,000 × g. The supernatant was transferred into a new sterile 1.5 ml Eppendorf tube and extracted with an equal volume of phenol/chloroform/isoamylic alcohol solution (Roti®). The solution was inverted briefly and centrifuged at 16,000 × g for 5 minutes at room temperature to separate the aqueous phase. The aqueous phase was transferred into new sterile 1.5 ml Eppendorf tube and washed with 1 ml of -20°C cold 100% ethanol. Solutions were mixed by inversion and the DNA was precipitated by centrifugation at 16,000 × g for 5 minutes at room temperature. The DNA pellet was washed with 500 µl 70%

ethanol and centrifuged for 1 minute at 16,000 × g. After the complete ethanol evaporation, the dried pellet was resuspended in 50 µl sterile H_2O and incubated with 1 µl RNase (1 mg ml^{-1}) for 30 minutes at 37°C. DNA was quantified using a Nanodrop spectrophotometer (peqlab).
The amplification of nucleic acids was performed with a Biometra thermocycler using specific primer pairs (Table 2.2). Each reaction was conducted in 25 µl reaction mix with 100 ng of genomic DNA and 2.5 µl primers (10 µM), 2.5 µl 10× amplification buffer with 15 mM $MgCl_2$ (GeneCraft), 0.2 mM dNTPs and 1 unit of BioTherm™ Taq DNA Polymerase (GeneCraft).
The amplification products were loaded on a 1% agarose gel and separated by electrophoresis (100 V per 40 minutes) in TAE buffer 1x (Tris acetate 4 mM; EDTA 1 mM). Gels were stained in an ethidium bromide solution (50 ppm w/v) for 5 minutes and visualized under UV light and photographed with a Biometra image analyzer. The size of amplified products were estimated by comparison with 2 Log ladder (Sigma).

2.11 Reverse Transcription PCR analysis

The total RNA extraction was performed using a protocol based on guanidine isothiocyanate extracting solution (Chomeczynski e Sacchi, 1987). To reduce RNA degradation, all solutions were prepared using diethyl phyrocarbonate (DEPC) treated water and cleaning of work bench and pipettes were cleaned with 10% (w/v) SDS solution. Two day old cultures (20 ml) were centrifuged for 5 minutes at 3,000 × g and the pellet lysed with 900 µl denaturing solution (4 M guanidine isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% (w/v) N-lauroyl sarcosine, 0.1 M ß-mercapoethanol). Lysate was added rapidly with 90 µl 2 M sodium acetate solution, 900 µl aqua-phenol solution (Roti®) and 180 µl chloroform. Between each addition, the sample was mixed by vortexing. The mixture was transferred into a 2 ml Eppendorf tube, incubated on ice for 15 minutes and centrifuged at 16,000 × g for 20 minutes at 4°C. 800 µl of the upper aqueous phase were transferred into a new 2 ml Eppendorf tube, filled up with isopropyl alcohol and mixed by inversion. The sample was incubated for 60 minutes at -20°C to allow RNA precipitation and then centrifuged for 10 minutes at 16,000 × g. The supernatant was discarded and the pellet resuspended in 300 µl of denaturing solution. The RNA was precipitated by adding 300 µl of isopropyl alcohol, incubation for 30 minutes at -20°C and centrifugation for 10 minutes at 10,000 × g. The pellet was washed with 500 µl of 70% ethanol, incubated at room temperature for 15 minutes and centrifuged for 5 minutes at 10,000 × g. Residual ethanol was removed by pipetting and finally the pellet was resuspended in 20 µl DEPC water. Total RNA amount and purity were evaluated by measurements with a Nanodrop spectrophotometer (peqlab). RNA was used if the 260 nm/280 nm ratio was between 1.8-2.0, indicating a low protein contamination.
Possible genomic DNA contaminations were removed from RNA samples by digestion with DNase I (RQ1 RNase-free DNase; Promega). 5 µg of RNA were incubated with 1 µl 10× RQ1 DNase reaction buffer, 1 µl of RQ1 DNase and 10 units of RNase inhibitor (RNasin Plus RNase Inhibitor; Promega) for 25 minutes at room temperature. RQ1 DNase deactivation was carried out by adding 1 µl of RQ1 stop solution and incubation of the mixture at 70°C for 20 minutes. The reverse transcription of interesting mRNAs was performed using the Moloney Murine

Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega) with a specific reverse primer for the 3′ UTR region of PsaD ("PsaD 3′ UTR"; Table 2.3). 5 µg DNase treated RNA were incubated with 30 pmol of "PsaD 3′ UTR" primer for 10 minutes at 70°C to allow the annealing of the primer and then kept on ice for 5 minutes to avoid the formation of secondary structures. A mastermix solution composed of 6 µl M-MLV 5× reaction buffer, 10 nmol dNTPs and 10 units of RNase inhibitor was added at the RNA-primer solution and incubated for 10 minutes at 40°C. The cDNA synthesis was performed by adding 200 units of M-MLV and incubaton of the reaction mixture for 60 minutes at 42°C and for 15 minutes at 70°C.
Amplification of cDNA was performed as described above for the genomic DNA PCR analysis, using the primer pairs presented in table 2.3.

2.12 Quantitative Real Time PCR analysis

Quantitative Real Time PCR (qRT-PCR) is a technique which allows to amplify and quantify cDNA molecules of interest, simultaneously. Reverse transcription of expressed genes is performed and housekeeping genes (e.g. actin) are used as references against which the expression level of a gene of interest can be normalized.
RNA extraction and DNase I treatment were performed in the same way described above for the reverse transcriptase PCR analysis. Preliminary experiments were carried out to evaluate the amplification efficiency for each primer pair (table 2.4) and to find the RNA dilution that gives a Ct value ranging between 25 and 35 cycles. The Ct value is the cycle number at which the fluorescence starts to increase rapidly and the sigmoid amplification curve starts the exonential phase. A comparison between Ct values from reactions with different RNA dilutions allowed the determination of the best concentration of nucleic acid for quantification.
Each reaction was performed using 12.5 µl of 2x SensiMix One Step solution (Quantace), containing the reverse transcriptase for the cDNA synthesis, a heat-activated DNA polymerase for the cDNA amplification, dNTPs, 6 mM $MgCl_2$, an internal references and reaction stabilizers. 50 pmol of each primer, 500 ng of DNase treated RNA, 0.5 µl 50x SYBR® Green I solution (Quantace) and 5 units of RNase inhibitor (Quantace) were added to this solution. The reaction was carried out using a DNA Engine Opticon™ Real Time detection system composed of a PTC-200 DNA Engine Cycler and CFD-3200 Opticon Detector (MJ Research). The program used for the reaction was: 30 minutes at 42°C for the retro transcriptase step, 10 minutes at 95°C for the DNA polymerase activation step and then 15 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C for 35 cycles.
Mutant *DGAT* expression level was analysed with the Opticon monitor software and compared with the wild type *CrDGAT* expression level, set to 100%.

2.13 Nile Red fluorescence assay

Total neutral lipids amount was evaluated by a Nile Red fluorescence assay and are presented as arbitrary fluorescence units (A.F.U.). The Nile Red fluorescence assay was performed as described by Chen *et al.* (2009) after optimization for *Chlamydomonas* cells. The samples were diluted to the concentration of 3×10^5 cells ml^{-1} in 2 ml 20% (v/v) DMSO solution. The cells were stained with 4 µl of Nile Red

(250 µg ml^{-1} in acetone) and vortexed for 30 seconds. Fluorescence was measured with a Infinite M200 spectrofluorometer (Tecan) using the excitation and emission wavelengths of 530 and 580 nm.

2.14 Mutant phenotype analysis

For each *CrDGAT*, the clone showing the highest expression level was chosen for phenotypic analyses. For each mutant, 3 biological replica were cultivated for 168 hours in 150 ml TAP medium starting from the same optical density (0,05 OD_{750}). Samples were collected from each replica after 16 hours (t_1), 24 hours (t_2), 48 hours (t_3), 72 hours (t_4), 96 hours (t_5) and 168 hours (t_6). The parameters measured for each time point were: a) number of cells, b) optical density at 750 nm, c) total chlorophyll amount and d) lipid fluorescence unit per number of cells.

2.15 Algal cultivation during nutrient starvation

Stress condition experiments were carried out by cultivation of the cells under nutrient starvation. The media used for this experiment was TAP without nitrogen source (TAP -N) and TAP medium without sulphur (TAP -S).
Mutants were cultivated in TAP medium and harvested when they reached the early stationary phase. Then, cells were harvested, washed twice and resuspended to the same optical density of the harvesting time point (~ 20×10^6 cells ml^{-1}) with the appropriate starvation medium. Cells were grown for 4 days under nutrient starvation and optical density, number of cells, total amount of chlorophyll and lipid fluorescence were measured every 24 hours.

3. RESULTS

3.1 Screening of algal collection by ABTS assay

After 10 days cultivation all 100 algal accessions showed an OD_{550} values higher of the starting point (0,1 OD_{550}) ranging from a minimal of 0.14 OD_{550} to the maximal value of 1.56 OD_{550}. For each accession the cultures growth rate expressed as G.I. value is reported in figure 3.1. The accessions showed a wide range of growth rates, which values ranged between 0.16 for the algal accession #35 to 9.77 for the accession #92, suggesting that all of them have performed cell division.
The screening of algal strains able to release phenol-oxidase in the growth medium was evaluated using the chromogenic substrate ABTS. Most of the tested algae were not able to release in the medium an amount of phenol-oxidase relievable by ABTS assay. The presence of enzyme was related at the growth rate through the E.I. coefficient. Based on the this evaluation, 84 algae showed values lesser than 0.08. The remaining 16 accessions showed an E.I. ranging from 0.10 to 1.19. The best results were achieved by the accessions #2, #78 and #42 respectively with E.I. of 0.46, 0.78 and 1.19 (Figure 3.2). The algae showing an E.I. higher of the threshold 0.10 were selected for the evaluation of enzymatic activity in presence of phenolic compounds (Figure 3.3). In table 3.1 the list of the 16 selected microalgal species and related accession number is reported.

3.2 Evaluation of phenol-oxidase activity of supernatants using natural phenolic compounds

The capability of selected supernatants to oxidise the ABTS, and the two phenolic compound 2,6-DMP and syringaldazine, was quantified by kinetic assay determining the enzymatic units for ml of culture (E.U. ml^{-1}).
In Figure 3.4 the E.U. of the 16 supernatants in presence of ABTS are reported. The E.U. ml^{-1} values ranged from 36.2 ± 16.0 E.U. ml^{-1} to 1,598.4 ± 340.7 E.U. ml^{-1}, respectively for the accessions #59 and #25. Based on the statistical difference of these data ($p<0.05$), the accessions were classified in 6 different groups. The first group is represented by the accession #59, that showed the lowest value. To the second group belonged the accessions #23, #28 and #82 with values ranging between 98.6 and 110.7 E.U. ml^{-1}; the third one was composed by the accessions #79, #78 and #42 (204.8 – 313.8 E.U. ml^{-1}); the fourth one by the accessions #98, #104 and #144 (433.9 – 481.2 E.U. ml^{-1}); the fifth one, with values twice bigger than the fourth, by the accessions #93, #26, #95, #103 and #2 (964.0 – 1,147.5 E.U. ml^{-1}). The last group was represented by only the accession #25, that showed an activity (1,598.4 ± 340.6 E.U. ml^{-1}) 30% more than the fifth group.
The E.U. estimation in presence of phenolic substrates showed an higher specificity of supernatants for 2,6-DMP instead of syringaldazine. In Figure 3.5 were reported the E.U. achieved by reaction with 2,6-DMP. Except the supernatant isolated from the accession #23, all the others were able to oxidise the phenolic compound. The values ranged from 0.27 ± 0.10 E.U. ml^{-1} to 4.84 ± 0.35 E.U. ml^{-1}, respectively for the accessions #82 and #42. Based on the statistical difference of these data ($p<0.05$), the 16 accessions were classified in 5 different groups. The accession #23, which did not show any reaction in presence of 2,6-DMP, belonged to the first group. The

second group was represented by the accessions #82, #28, #144, #98 and #104, with values ranging from 0.27 to 0.65 E.U. ml^{-1}; the third group, showing an E.U. three times higher than the previous, was composed by the accessions #26, #93, #103, #95, #2 and 59 (1.31 – 1.88 E.U. ml^{-1}); the fourth group was represented by only the accession #25, that showed a value of 2.56 ± 0.37 E.U. ml^{-1}; to the last group, showing an E.U. two times higher than the previous, belonged the accessions #79, #79 and #42 (4.29 – 4.84 E.U. ml^{-1}).
The syringaldazine oxidation was not revealed in most of the analyzed supernatants (Figure 3.6). Only the accessions #79, #78, #42 and #59 were able to oxidise this compound, with values ranging from 0.12 to 0.28 E.U. ml^{-1}. Moreover, these values were much lower compared to the values obtained with the 2,6-DMP. The highest value showed by the accession #78 (0.28 ± 0.01 E.U. ml^{-1}) was almost the same of the lowest value obtained in the 2,6-DMP assay (0.27 ± 0.09 E.U. ml^{-1}). Based on the statistical difference of these data ($p<0.05$), the accessions were classified in 3 different groups. The first group was represented by the supernatants that did not show any reaction; to the second group belonged only the accession #79, showing an activity of 0.12 ± 0.02 E.U. ml^{-1}; to the third group, belonged the accessions #42, #59 and #78, with values ranging from 0.25 to 0.28 E.U. ml^{-1}. This latter showed an average value of activity two fold higher than to the accession listed in the second group.

3.3 Evaluation of RBBR decolourisation capability of microalgae supernatants

All 16 supernatants were tested for the capability to decolorize the azo dye RBBR. Figure 3.17 shows the percentage of RBBR decoloured after 6 hours of treatment. Based on the statistical difference of these data ($p<0.05$), the accessions were grouped in 3 different clusters. The accessions belonging to the first group (#79, #59, #42, #78, #28, #82 and #23) showed the lowest removal capability with decolourisation values ranging from 0% to 6.0%. At this group belonged all accessions with the highest activity in presence of 2,6-DMP and syringaldazine (#79, #59, #42 and #78). The second group was composed by accessions #144, #104 and #98 able to decolorize RBBR solution from 16.6% to 19.0%. These three accessions did not show activity in presence of syringaldazine. However, they belonged at the same accession group of the 2,6-DMP oxidation experiment. The third group (#93, #25, #26, #103, #95 and #2) contained accessions able to decolorize RBBR from 33.0% to 38.8% in 6 hours only. These accessions were also unable to oxidise the substrate syringaldazine; moreover, all of them, except #25, belonged to the same group showing an enzymatic activity in presence of 2,6-DMP higher than the previous group (#144, #104 and #98). The accession #25 showed the second best result in presence of 2,6-DMP.

3.4 Algal cultivation in presence of olive-mill wastewaters

In order to evaluate the growth capability in presence of diluted OMW and the ability to reduce the phenolic content of this reflue, two accessions, showing the best results in the previous enzymatic assays, were chosen. The algal species selected were *C. pitschmanni*, which showed the highest enzymatic activity in presence of 2,6-DMP and syringaldazine, and *S. vacuolatus*, which gave the best result with RBBR and

ABTS. The experiments were carried out under light or dark condition and the results compared with the model alga *C. reinhardtii*.
In both condition the two *Chlamydomonas* species showed a cell concentration lower than *S. vacuolatus*. Figure 3.8 and 3.9 show the culture concentration, expressed as number of cells, during algal cultivation under dark and light condition, respectively. Under dark condition *C. pitschmanni e C. reinhardtii* increased the number of cells of 1.1 and 1.5, after the first week, and 1.3 and 2.0, after the second week, respectively. *S. vacuolatus* was able to increase the number of cells of 16.2 and 22.7 times after the first and the second week, respectively. After three weeks, *S. vacuolatus* increased the number of cells of 29 times (from 0.52 to 14.9 × 10^6 cells ml^{-1}), whereas *C. pitschmanni* and *C. reinhardtii* increased the number of cells of 1.6 (from 0.91 to 1.43 × 10^6 cells ml-1) and 2.2 (from 0.28 × 10^6 to 0.62 × 10^6 cells ml^{-1}) times, respectively.
The cultures grown under light condition showed a higher concentration than dark condition. *C. pitschmanni e C. reinhardtii* increased the number of cells of 1.5 and 7.9, after the first week, and 2.0 and 19.6, after the second week, respectively. *S. vacuolatus* was able to increase the number of cells of 20.0 and 39.2 times after the first and the second week, respectively. Again the best result was achieved with *S. vacuolatus*, which increased the number of cells near 85 times in 21 days (from 0.62 to 53.00 × 10^6 cells ml^{-1}). After three weeks *C. pitschmanni* increased the number of cells of 4.5 times (from 0.73 to 3.25 × 10^6 cells ml^{-1}) whereas *C. reinhardtii* cells increased of 13.5 times (from 0.33 to 4.38 × 10^6 cells ml^{-1}).
The capability of the selected algae to reduce the phenolic content in the modified growth medium was also investigated. Figure 3.10 and 3.11 show the total phenol content during algal cultivation under light and dark condition, respectively.
Under light condition, in 7 days *S. vacuolatus* was already able to reduce the phenol content of 43.1%, whereas the percentage of phenol removal in darkness was of 24.3%. The values recorder after 14 days showed a slowing down in the phenol removal rate during the second cultivation week, with a removal percent of the total phenol amount of 2.1% and 5.2%, respectively under dark and light condition. However, during the third week, *S. vacuolatus* showed a resumption of the phenol-oxidase activity with a removal rate of the total phenol amount of 25.6% in the darkness and 11.5% in the light, for a total removal percentage of 59.8% in light and 52.3% in dark condition.
In both the conditions, the capability to reduce the phenol content in the medium of the two *Chlamydomonas* species was lower than *S. vacuolatus*. After 7 days, *C. reinhardtii* showed a percentage of phenol removal of 13.7% and 2.6% under light and dark condition, respectively. During the second week, *C. reinhardtii* showed a decrease in the phenol removal capability under the light condition, with a removal rate of 2.1% of the total phenol amount. However, the higher capability of phenol removal for this alga was measured after the third week (34.6%). Under dark condition cells were able to remove almost the same amount of phenols from the medium, with value of 14.8% after 14 days and 16.1% after 21 days.
C. pitschmannii showed the lowest capability to remove phenols from the OMW. After the first week, the phenol removal rate was of 2.3% and 6.7% under light and dark condition, respectively. Under light condition, the phenols were reduced of 17.8% and 15.6% during the second and the third week, respectively; however in dark condition cells seem to need for more time before to start to degrade phenols from the medium. During the second week, *C. pitschmannii* showed a removal rate of 2.7%,

similar to the removal rate reported for the first week. Only after 21 days cells were able to degrade up to 22.2% of phenols from the medium.

3.5 In silico search for potential *DGAT* in *C. reinhardtii*

Database analyses showed that *C. reinhardtii* contains five genes classified as putative *CrDGAT* candidates. The corresponding proteins were designated *Cr*DGAT1-5 and correspond to the following NCBI Accession Numbers: *Cr*DGAT1 (XP_001693189), *Cr*DGAT2 (XP_001702848), *Cr*DGAT3 (XP_001691447), *Cr*DGAT4 (XP_001694904) and *Cr*DGAT5 (XP_001701667). NCBI's Conserved Domain Database showed that all 5 proteins belong at the acyltransferase superfamily (Figure 3.12). Conserved regions analysis showed that *Cr*DGAT2 protein contains the highly conserved sequence of amino acids HPHG, typical of DGAT2 family. As expected from *C. reinhardtii*, the G/C content of these genes ranged between 60-65%.
Figure 3.13 shows the phylogenetic correlation of five *Cr*DGAT with DGAT1 and DGAT2 protein sequences of plants, animals and fungi. The phylogenetic tree shows that all *Cr*DGATs are not correlated with DGAT1 from plant and animals. All *Cr*DGATs seem to belong at the class of DGAT2. *Cr*DGAT2 is more close to the DGAT2 protein sequences of animals and fungi whereas *Cr*DGAT1, 3, 4 and 5 are more similar to the DGAT2 from plants.

3.6 *DGAT* cloning and expression vectors construction

All five nucleotide sequences of *CrDGAT* genes were obtained by amplification of genomic DNA of *C. reinhardtii* CC3491 with the primer pairs reported in table 2.1. The molecular size of all amplified DNA fragments corresponded to the expected size: *CrDGAT1*: 2,813 bp, *CrDGAT2*: 3,017 bp, *CrDGAT3*: 2,543 bp, *CrDGAT4*: 4,486 bp and *CrDGAT5*: 2,864 bp (Figure 3.14). The amplification products were purified from agarose gels and cloned into the vector pGem T-Easy to transform competent *E. coli* DH5α cells. Figure 3.15 shows the products of pGemT-Easy constructs, isolated from *E. coli* positive clones, after cutting with restriction enzymes EcoRI and NdeI. For all constructs, excepted for pGemT-Easy::*CrDGAT2*, two DNA fragments were detected, representing the vector (3,015 bp) and the gene. The presence of an EcoRI restriction site in the third intron of *CrDGAT2* gene led to formation of three products for this construct. The restriction site was removed by point mutagenesis using the specific primer pair "DGAT2_Mut_F" and "DGAT2_Mut_R". The primer pair created a point mutation in the *CrDGAT2* gene that reverted the C residue present in the EcoRI restriction site (GAATTC) to a G residue (GAATTG). The successful point mutation was confirmed with restriction analysis; because after cutting with EcoRI and NdeI two product of the same size are produced, SspI restriction enzyme was added to cut the pGemT-Easy in 2 parts (Figure 3.16).
Before continuing with the cloning, all *CrDGAT* genes were checked by sequencing in order to confirm the absence of mutation or stop codons in exon regions. All genes showed the correct sequences.
After sequencing, all genes were cloned in the unique EcoRI and NdeI restriction site of expression vector pGenD. In this vector, the gene of interest is expressed by using

the promoter and the 3′ UTR region of *Chlamydomonas PsaD* gene. The genes were cut from the cloning vector pGem T-Easy, purified by agarose gel electrophoresis and ligated into the vector pGenD. *E. coli* DH5α competent cells were transformed with the pGenD::*CrDGAT* constructs and the positive clone selection was performed by restriction analyses of extracted vectors. Figure 3.17 shows the products of the pGenD constructs, isolated from *E. coli* positive clones, after cutting with restriction enzymes EcoRI and NdeI. All vectors showed a product for the pGenD backbone (4.3 kbp) and one for each *CrDGAT* genes.

3.7 Nuclear transformation of *Chlamydomonas reinhardtii*

The nuclear transformation of *Chlamy*domonas was carried out by applying the glass beads method (Kindle, 1990) with the constructs pGenD::*CrDGAT1-5* and pHyg3, as described in section 2.9. For each construct, around 230 to 300 colonies able to grow in presence of hygromicine B were obtained, with an efficiency of transformation for the *aph7″* gene of $6.5 – 8.5 \times 10^{-6}$.

3.8 PCR analysis of the putative transgenic algae

To facilitate the selection of positive DGAT mutants, amplification analyses of nuclear genomic DNA, extracted from colonies grown on selective plates, were carried out using specific primer pairs for each construct. The percentage of colonies which integrated the *CrDGAT* expression cassette was evaluated by comparing the number of PCR-positive DGAT clones with the total number or analyzed colonies.
Table 3.2 shows the transformation rates for each construct; the number of colonies analysed ranged between 102 to 208, with a percentage of positives of 2.4 to 7.8.
The *CrDGATs* PCR amplification confirmed the integration of the *CrDGAT* expression boxes in the genomic DNA of *Chlamydomonas* cells after the transformation (data not shown).

3.9 Analysis of transgene transcription

The gene expression was evaluated by RT-PCR of total mRNA extracted from PCR-positive clones. To distinguish the exogenous *DGAT* expression from the endogenous expression, cDNAs were obtained by RNA reverse-transcription with a specific reverse primer for the 3′ UTR of *PsaD*.
Figure 3.18 shows the cDNA amplification products with the forward primer “PsaD_cDNA” and the specific DGAT reverse primers. Because of the presence of traces of the reverse primer “PsaD_3′ UTR” in the cDNA samples, all clones and the wild type strain showed also the expression product of the endogenous *PsaD* gene (~ 700 bp). However, not all clones were able to express the new *CrDGAT* construct. For *CrDGAT1* and *CrDGAT3* the amplification product was found in 3 of 5 analyzed clones (Figure 3.18a and 3.18b). For *CrDGAT4* and *CrDGAT5*, two colonies able to transcribe the new construct (Figure 3.18c and 3.18d) whereas only 1 clone was able to express the *CrDGAT2* construct (Figure 3.18e). All positive clones showed an amplification product of the expected size (*CrDGAT1*: 351 bp; *CrDGAT2*: 455 bp;

CrDGAT3: 432; *CrDGAT4*: 341; *CrDGAT5*: 341), which differed from the product expected from the genomic DNA.

3.10 Evaluation of *DGAT* expression level by qRT-PCR analysis

Quantitative Real Time PCR analyses were performed in order to find the mutant with the higher expression level for each DGAT clone. The RNA was extracted 48 hours after the cell inoculation to facilitate the analysis of *DGAT* expression during the exponential growth phase.
The expression analyses of positive clones for the construct pGenD::*CrDGAT1*, *CrDGAT3*, *CrDGAT4* and *CrDGAT5* were reported in Figure 3.19. The results of the *CrDGAT2* mutants are not available yet.
The mutant expression profile were correlated with the wild type, which was set to 100 in the figures. Compared with the wild type, all DGAT1 mutants showed a strongly increased expression level, 15 times higher for the mutant D1-158 and 30 times higher for mutants D1-160 and D1-167 (Figure 3.19a). On the other hand, the DGAT3 mutants showed a lower over-expression level, with an increase of 4 times for the mutant D3-18 and only of 3.5 and 1.7 for the mutants D3-52 and D3-133 (Figure 3.19b). Figure 3.19c shows the expression level of the two positive clones for DGAT4. Both clones showed an increased cDNA amount two times (D4-24) and sixteen times (D4-66) higher than the wild type. For DGAT5 clone D5-28 showed an expression level which was 6 times higher, while the clone D5-36 showed a very high expression level of around 30 times the wild type level (Figure 3.19d).
Based on these results, the clones showing the highest expression for each *DGAT* gene, were selected for phenotypic characterization.

3.11 Establishment of a Nile Red lipid assay with *Chlamydomonas reinhardtii*

Nile Red assay was established in order to find the right condition for lipid quantification in *Chlamydomonas*. A correlation curve between different cell concentrations and their relative fluorescence units was performed to investigate in which range the correlation was linear. The effect of cell concentration on the fluorescence of neutral lipids in *C. reinhardtii* was presented in figure 3.20. As shown, the range in which cell concentration correlates in a linear relationship with fluorescence intensity was between 1.0×10^5 cells ml^{-1} and 2.5×10^6 cells ml^{-1}, with a R^2 coefficient value of 0.99. Therefore, the concentration of circa 3×10^5 cells ml^{-1} (equal to $OD_{750} = 0.05$) was selected for all lipid measurements.
Several pre-treatment approaches were tested for *C. reinhardtii* cells and *Chlorella vulgaris* cells. Prior Nile to Red staining, following pre-treatments were tested:

a) resuspension in water
b) resuspension in 20% (v/v) DMSO solution;
c) vortexed for 30 seconds with glass beads;
d) frozen in liquid nitrogen.

Figure 3.21 shows fluorescence emissions of the two algal species after pre-treatment. The values obtained with *C. vulgaris* fitted with the data reported by Chen et al. (2009) in which the highest fluorescence intensity was obtained after pre-treatment with 20% DMSO solution. The untreated samples resulted in the lowest fluorescence value. Like *Chlorella*, the best fluorescence emission for *C. reinhardtii*

was obtained after pre-treatment with 20% DMSO. However the lowest value was obtained after freezing with liquid nitrogen and not after resuspension in water.

3.12 Mutant phenotype characterization during standard growth condition

Clones showing the highest expression levels were chosen in order to evaluate the effects of *CrDGATs* over-expression on the cell phenotype. The mutants were grown for 8 days during which total neutral lipid amount and growth parameters, such optical density, cells number and total amount of chlorophyll, were monitored. All measurements were compared to the wild type strain CC3491 and statistical analysis of variance was done with One-Way ANOVA software.
Figure 3.22a and 3.22b show the optical density values (OD_{750}) measured during the experiment for the four mutants D1-160, D3-18, D4-66 and D5-36. All mutants showed a growth pattern similar to each other and without significant differences to the wild type.
The cell concentration (millions of cells per ml) was reported in figure 3.24a and 3.24b. All mutants showed no differences compared to the wild type strain.
The total chlorophyll amount values were reported as the average of three different measurements for each biological replica. The chlorophyll values (mg l^{-1}) measured at different time points for all mutants and wild type were shown in figure 3.23a and 3.23b. Mutants D1-160, D4-66 and D5-36 did not show any significant differences compared to the wild type strain. However, the mutant D3-18 seemed to produce more chlorophyll during the stationary phase after 96 and 168 hours, showing values significantly different to the wild type per $p<0.05$.
The lipid amount was determined using a spectrofluorometer with multireader capacity and was expressed as arbitrary fluorescence unit (A.F.U.) per millions of cells. The results were reported as the average of 8 measurements for each biological replica (three). All mutants showed a lipid pattern similar to the wild type (Figure 3.25a and 3.25b).

3.13 Mutant phenotype characterization during nutrient starvation

In order to evaluate if *Chlamydomonas* cells can produce more lipids under stress conditions, mutants were cultivated for 4 days in TAP medium without nitrogen or sulphur source. The experiment was carried out in three different biological replicas. Mutants were grown in normal TAP until the early stationary phase and harvested to be transferred into the fresh starvation medium. The starting optical density was of circa 0.8 OD_{750} and the cell number around 20×10^{-6} cells ml^{-1}. In both starvation conditions, the optical density values doubled in 24 hours and stayed constant for 96 hours during nitrogen depletion (Figure 3.26); during the sulphur depletion, the optical density of the culture started to decrease slowly after 48 hours (Figure 3.27). In contrast to the other mutants, which showed similar pattern as the wild type, D3-18 optical density decreased slower, shifting from 1.56 to 1.35 OD_{750} whereas the other mutants decreased from circa 1.5 to circa 1.0 OD_{750}.
Despite the change of the optical density, cells seemed not to be able to duplicate during nutrient starvation, showing a concentration of 20-30 $\times 10^{-6}$ cells ml^{-1} throughout the experiment (Figure 3.28; 3.29). In addition, no difference were noticed between mutants and wild type for the total amount of chlorophyll (Figure 3.30; 3.31).

Figure 3.32 shows the lipid fluorescence of the mutants during the sulphur starvation. Mutants D1-160 and D5-36 did not show significant differences in the lipid amount compared to the wild type, reaching the highest lipid accumulation after 96 hours with 7,854 and 7,277 A.F.U. per millions of cells, respectively, compared to the 7,359 A.F.U. per millions of cells measured for the wild type. In contrast, mutants D3-18 and D4-66 showed an increased lipid content compared to the wild type already after 48 hours. For D4-66 the maximum lipid accumulation was measured after 48 hours, which remained constant around at 13,000 A.F.U. per millions of cells. D3-18 showed a peak in the lipid accumulation after 72 hours, with 18,486 A.F.U. per millions of cells; however, after 96 hours the lipid content decreased and reached 14,581 A.F.U. per millions of cells. After 96 hours, D3-18 therefore produced 98.1% more lipids compared to the wild type, while the increase for mutant D4-66 was 70.9%. The highest lipid production for the two mutants was achieved after 48 hours for D4-66, showing 116.7% more lipid than wild type, and after 72 hours for D3-18 with a A.F.U. 123.9% higher than wild type.
Figure 3.33 shows the lipid fluorescence for each culture during the nitrogen starvation experiment. Although no significant differences were detected between mutants and wild type, the increase in the lipid fluorescence is generally higher compared to the values measured during the sulphur starvation. After 96 hours all cultures showed A.F.U. per millions of cells ranging between 12,950 and 15,993, values two times higher than A.F.U. measured for the wild type and the mutants D1-160 and D5-36 during the sulphur depletion. On the other hand, no significant differences were noticed between the lipid fluorescence detected during sulphur and nitrogen depletion for the mutants D3-18 and D4-66, with the only exception for the mutant D3-18 that showed after 72 hours of sulphur depletion a lipid fluorescence 46% higher than the value detected during nitrogen starvation.

4. DISCUSSIONS

4.1 Screening of algal collection and growing of algae in presence of olive-mill wastewaters

Bioremediation by microorganisms would be the best choice to combat the pollution caused by these hazardous materials. Many studies have proved the efficiency of fungi in degrading phenolic compounds from wastewaters and decolorizing synthetic dyes, and this ability was found out to be due to the production of enzymes, especially laccases (EC 1.10.3.2) and polyphenol oxidases (EC 1.10.3.1), by fungi (Eggert et al. 1996; Soares et al. 2001; Olivieri et al., 2006). Both laccases and polyphenol oxidases are copper-containing oxidases with structural difference in their copper-binding sites (McGuirl and Dooley 1999), and are distinguished by their catalytic actions; laccase catalyzes the oxidation of both *ortho*- and *para*-diphenols, whereas polyphenol oxidase reacts only with *ortho*-diphenols (Carunchio et al. 2001). In fungi, the activities of laccases and polyphenol oxidases were found to increase in response to copper and aromatic and phenolic substances (Koroljova-Skorobogatko et al. 1998). Bacterial laccases were only discovered 10 years ago and their actual functions are hardly understood so far. The role of bacterial blue multicopper proteins in different cellular processes, like sporulation (Kim et al. 2001) (*Bacillus*), resistance to copper (Wesenberg et al. 2003) (*Escherichia coli*) and laccase activity (Hullo et al. 2001; Solano et al. 2001; Kim et al. 2001) were reported only recently.
The presence of laccases in superior photosynthetic organism is due to participation of these enzymes in the radical-based mechanisms of lignin polymer formation (Boudet, 2000), whereas in fungi laccases probably have more roles including morphogenesis, fungal plant-pathogen/host interaction, stress defence and lignin degradation (Thurston, 1994). Laufen et al. (2006) reported the tyrosinase and laccase oxidative activity in lichens, organism phylogenetically strictly correlated with algae and fungi. Recently was also reported the capability of some cyanobacteria to produce enzymes with laccase and polyphenol-oxidase acitivty (2009). However, this activity was detected on total protein extract and not in the broth culture, whereas in fungi these phenol-oxidase enzymes are usually extracellular. Secreted enzymes present the big advantage to have a direct access to the contaminants (Uchida et al., 2005). The extracellular production of enzyme is known to occur also in plant and bacterial kingdom. However, no study reporting the capability of algae to produce extracellular enzymes and, in the specific case, phenol-oxidase or laccase.
The first aim of this work was to select algae showing phenol-oxidase activity useful for phycoremediation of OMW. For this purpose we started a collaboration with Prof. Dr. Antonino Pollio and Prof. Dr. Gabriele Pinto, responsibles of the ACUF algal collection present at the Biology Department of the University of Naples Federico II. This collection presents more than 400 accessions belong at the division *Cyanophita*, *Chlorophyta*, *Rhodophyta* and *Bacillariophyceae*. For the wide presence of more than 180 extremophilic strains, collected from thermal and acidophilic environment worldwide, and for the presence of all the known species of *Cyanidiophyceae* order, this collection can be considered one of the collection with the highest number of extremophilic algae.
The screening was carried out on 100 strains belong to the division *Chlorophyta*, selected randomly. The first part of the screening was performed using the ABTS as substrate for revelation of enzyme with phenol-oxidase activity in the broth culture.

ABTS, a synthetic nitrogen-substituted aromatic compound, is the most extensively investigated laccase mediator which allows the oxidation of nonphenolic lignin model compounds (Bourbonnais et al., 1990) and the delignification of kraft pulp (Bourbonnais and Paice., 1992) by laccase. In the presence of this compound, laccase can also catalyze the oxidation of PAHs (Collins et al., 1996; Johannes et al., 1996), chemical synthesis (Potthast et al., 1996), and textile dye bleaching (Schneider and Pedersen, 1995). ABTS is oxidized by laccase to its corresponding cation radical. In the case of ABTS, the radical ($ABTS^{\cdot+}$) is highly stable, and it was suggested that it may act as a diffusible oxidant of the enzyme (Bourbonnais et al., 1997). However, although the redox chemistry of ABTS (Hünig et al., 1964) and its radical was characterized, the mechanisms by which it interacts with laccase to "mediate" lignin oxidation are still unknown. Potthast et al. (1995) have found evidence suggesting that ABTS acts as an activator or cooxidant of the enzyme. The observation that the laccase/ABTS couple can oxidize the nonphenolic veratryl alcohol, while $ABTS^{\cdot+}$ alone cannot (Bourbonnais et al., 1990), provides a further indication of this activator role for ABTS.

The presence of enzyme with phenol-oxidase activity in the cultures broths was evaluated using an E.I. defined by us. This index correlates the difference in the optical density measured during the ABTS oxidation with the G.I., that shows how much the culture has grown. This means that for the same ABTS ΔOD_{420}, algae showing an higher G.I. present an E.I. lesser than algae with lower G.I. values. The choice of the accessions was carried out selecting only the algae showing an E.I. higher than the threshold set to 0.1. On the basis of this evaluation system, 84 of the tested accessions showed E.I. values, in presence of ABTS, ranging between 0 and 0.08, whereas the others 16 showed values ranging between 0.10 and 1.20. A taxonomic analysis showed that *Chlorella* is the more representative genus in the 16 selected accessions group. In this group we found also other two genera: *Scenedesmus*, represent by 4 accessions, and *Chlamydomonas*, represent by 3 accessions. *Chlorella* and *Scenedesmus* genera were represented respectively by four and two species (*C. vulgaris*, *emersonii*, *soronikiana* and *luteoviridis*; *S. vacuolatus* and *ovalternus*) whereas the genus *Chlamydomonas* was represented by 3 different strains of the same species, *C. pitschmannii*.

In order to evaluate the phenol-oxidase enzymatic activity of selected algae broth medium, further experiments were carried out in presence of phenolic compounds syringaldazine and 2,6-DMP. Syringaldazine is a compound that belongs at the subunit S of lignin molecule and is considered to be a unique laccase substrate (Harkin et al, 1974). Monophenols like 2,6-DMP or guaiacol are often used as laccase substrates, although the class of enzyme able to oxidize them are called diphenol oxidase (Baldrian, 2006).

Quantification of the protein in the broth medium by Bradford assay showed values not detectable with the spectrophotometer (data not shown). For this reason in this work the phenol oxidase activity was reported as enzymatic unit per ml of culture.

Chlorella luteoviridis, *Chlorella sorokiniana* and *Chlorella vulgaris* #82 showed the lower enzymatic activity with all tested compounds. That could depend by two factors: the weak presence of enzymes with phenol-oxidase activity in the broth or the aspecifity of the secreted enzyme for the analyzed compounds. The first point could be reasonable if we compare the ABTS oxidation result with the one from the other *Chlorella* accessions showing enzymatic unit values 4 to 16 times higher. On the other hand, a low reaction with ABTS does not mean exclusively less enzyme or less phenol-oxidase activity. For example, *S. ovalternus*, which has showed the

lowest activity in presence of ABTS, showed a good activity in presence of 2,6-DMP; moreover, it was and was the unique accession able to oxidize the syringaldazine, excepted *C. pitschmannii*. The three *C. pitschmannii* strains showed to be a few more active than *S. ovalternus*, *C. luteoviridis*, *C. sorokiniana* and *C. vulgaris* #82 in oxidize ABTS, whereas they were less efficient than the others accessions of *S. vacuolatus*, *C. emersonii* and *C. vulgaris*. However, all *C. pitschmannii* accessions performed the best in presence of 2,6-DMP and syringaldazine.
Excluding the accession #82, also *C. vulgaris*, *C. emersonii* and *S. vacuolatus* were represented by three strains. For these accessions a relation between ABTS and 2,6-DMP oxidation activity, and ABTS oxidation activity and RBBR decolourisation , was observed. The *C. vulgaris* accessions 98 and 104 and the *C. emersonii* accession 24 belonged at the same group, that was statistically different from the others in terms of oxidation activity of all compounds described above. This group showed an oxidation activity in presence of ABTS and 2,6-DMP, and a RBBR decolourisation capability, lower than the one reported for the other accessions of *C. vulgaris*, *C. emersonii* and *S. vacuolatus*, belonging to the same group and able to decolourizate the higher quantity of RBBR. For the accessions showing the highest RBBR decolourisation capability were also detected the highest activity in presence of ABTS; however, in presence of 2,6-DMP they showed an enzymatic activity comparable with the one reported for *S. ovalternus*, excepted for the accession #25 of *S. vacuolatus* showing a value slightly higher.
The direct relation between ABTS oxidation and RBBR decolourisation resulted valid also for *C. luteoviridis*, *C. sorokiniana*, *C. vulgaris* #82, *S. ovalternus* and *C. pitschmannii*. All these accessions, showing a really low phenol-oxidase activity in presence of ABTS, belonged to the same group that was not able to degrade RBBR. However, if this direct relation between ABTS and 2,6-DMP oxidation is still valid for *C. luteoviridis*, *C. sorokiniana* and *C. vulgaris* #82, the accessions of *S. ovalternus* and *C. pitschmannii* did not show the same behavior. This behavior should be explained if all accessions produced a common enzyme able to oxidize the RBBR and ABTS, whereas for syringaldazine and 2,6-DMP are involved different enzymes produced by only few microalgae species are involved.
In order to evaluate the capability of algae to grow in presence of OMW we selected two species that expressed the best oxidation results: *C. pitschmannii*, able to oxidize syringaldazine and 2,6-DMP, and *S. vacuolatus*, able to oxidize 2,6-DMP and RBBR. As control in the experiments of OMW oxidation the model microalgae *C. reinhardtii* was assayed.
The OMW were diluted with the microalgae culture medium BBM. This medium does not contain any kind of carbon source. The capacity of algae to grow in dark in the modified medium depends exclusively on their capability to use as carbon source the organic matter present in the OMW as a carbon source. Pinto et al. (2003) already reported the capability of *Ankistrodesmus braunii* C202.7a and *Scenedesmus quadricauda* T76 to grow and remove the phenolic component in the OMW after 5 days, starting from different cell concentration. The OMW, with a starting total phenol concentration of 1.5 g l^{-1}, were diluted 1:10 (v/v) with BBM and the algae were grown in the dark. Starting from an inocula of 1×10^6 cells ml^{-1}, they reported after 5 days a phenolic removal of 5% for *S. quadricauda* and 6% for *A. braunii* with an increase in the cell number of 0.18 and 0.21 times per day. In our condition, algae were grown in OMW, with a starting total phenol concentration of 2.3 g l^{-1}, diluted 1:20 (v/v) in BBM starting with an initial inocula of 0.5×10^6 cells ml^{-1}. By comparison with our results, *C. pitschmannii* and *C. reinhardtii* showed after 7 days of cultivation in darkness

condition an increase in the cell number of 0.16 and 0.22 per day with a removal of OMW phenols of 2.3% and 2.6%. On the other hand, *S. vacuolatus* showed an increase in the cell number of 2.3 times per day with a phenol removal of 24.3%.
Pinto et al. (2003) reported that the experiments carried out in darkness were repeated also at the light condition, getting comparable, although slightly lower, phenol removal values (data not shown). Moreover, no information about the growth in light condition were given. Compared to the dark condition, the accessions analyzed in this work showed an higher growth rate when cultivated in light condition, with an increase of the cell number after 7 days of 1.1 times per day for *C. reinhardtii*, 0.22 for *C. pitschmannii* and 2.9 for *S. vacuolatus*. Also for the phenol removal the best results were achieved during the incubation in light condition. The phenol removal values after 7 days were 43.1% for *S. vacuolatus*, 13.7% for *C. reinhardtii* and 6.7% for *C. pitschmannii*.
The better results achieved for all three species under the light condition can be explained by the higher oxygen concentration in the medium, due to the photosynthetic activity of algae: it is worthy to note that oxygen is necessary for phenols oxidation (Hoegger *et al*., 2006). Further experiments are necessary to know the fate of the phenols of the OMW, in order to evaluate if they are degraded, converted in intermediates or traslocated inside the cells.
The growth in the dark condition depends mainly from the capability of algae to use the organic matter in the OMW as carbon source. Some species of algae are capable of heterotrophic growth on organic carbon sources (Nelson and Lewin, 1974). It is also well known that *C. reinhardtii* can use acetate as carbon source, whereas other intermediates in the citric acid cycle do not support growth in the dark, nor do various pentose or hexose sugars, ethanol, glycerol, or other organic compounds (Harris, 2001). Moreover, the growth in light either with or without acetate is faster than growth in darkness.
Although after 21 days of cultures the cell number increased only two times, *C. reinhardtii* was able to survive in dark condition in presence of OMW. This can be explained since small traces of different organic acid, including acetate, are present in the OMW (Balice et al., 1982). Then, the growth in light condition was slightly higher, increasing the cell number of 19 times after 14 days. During the third week the cell number started to decrease passing from 6.4 millions of cells recorded after 14 days to 4.4 after 21 days. During the third week, *C. reinhardtii* showed the higher phenol removal activity; maybe the cells mortality can be correlated with the transformation of some compound presents in the OMW in toxic intermediates. However, this hypothesis is not comparable with the results achieved in the experiments carried out under darkness, where cells grew slower, but steadily, increasing their number during all the three weeks.
For *C. pitschmannii* it seemed that the phenol removal was strictly related to the cell number increasing. This hypothesis can be justified if we considering that in the light condition the cells start to remove more phenols after the second week, whereas for the dark condition it occurred only after three weeks. *C. pitschmannii* in dark condition was not able to grow well, increasing the cell number only of 1.5 times in 3 weeks. In the light condition, cells number duplicate after 2 weeks and then again during the third week.
The capability of *Scenedesmus vacuolatus* to remove phenolic compounds was already known. Tsuji et al. (2003) reported the use of this microalga to remove 2,4-dichlorophenol. However, no reports have been already published on its capability to grow directly in presence of OMW. On the basis of our results, *S. vacuolatus* seemed

to be the best microalgal species among the accession analyzed, both for growing on OMW and for phenol degradation rate. On the basis of the results achieved about the percentage of phenol removal and the growth, the best results were performed by the two *Chlamydomonas* species. This results may be explained if the enzymes involved in the syringaldazine oxidation were more specific in oxidizing the phenols present in the OMW. On the other hand, if we think about a microalgal species able to give a good biomass in presence of OMW, *S. vacuolatus* resulted the best candidate.

In general, available scientific information shows that fungi are the most effective organisms useful for degrading both simple phenols and the more complex phenolic compounds present in olive-mill wastes. The reason for this lies in the structure of the aromatic compounds present in OMW; they are analogous to that of many lignin monomers, and only a few microorganisms, mainly wood-rotting fungi, are able to efficiently degrade lignin by producing ligninolytic enzymes such as lignin peroxidases, manganese peroxidases and laccases (Hattaka 1994). Consequently, the bioremediation of OMWs using specific strains of fungi (some of them isolated directly from olive-mill wastes), primarily filamentous fungi, white rot fungi and yeasts, has been extensively investigated. There are a considerable number of reports on the application of fungi to reduce the organic load and phenolic content of OMW. The capability of fungi to remove phenols from OMW is strictly related to the species and way in which they are cultivated. For instance, several species of the genus *Pleurotus* were found to be very effective in the degradation of the phenolic substances present in OMW, removing more than 90% of the phenols (Martirani et al., 1996; Sanjust et al., 1991). However, the use of filamentous fungi for OMW treatment in large-scale processes is considered problematic due to the difficulty of achieving a continuous culture because of the formation of fungal pellets and other aggregations (Niaounakis and Halvadakis 2004). To overcome this limitation, the use of microalgae in bioreactors could be a way forward. The phenol removal capability of the microalgae selected in this work is comparable to the phenol removal capability of fungi as *Aspergillus* and *Penicillium*, which are able to remove from 32% to 76% of phenols when grown in flasks in presence of OMW (García García et al., 2000; Fadil et al., 2003; Robles et al., 2000) and to the phenol removal capability of some yeasts as *Geotrichum* sp. and *Candida tropicalis*, able to reduce the phenolic content of OMW of 46.6% and 51.7%, respectively (Fadil et al. 2003). Furthermore, the algal biomass coming out after the OMW treatment can be easily converted in high added values product, including biodiesel (Morillo et al., 2009).

4.2 Overxpression of endogenous diacylglycerol acyltransferase genes in *Chlamydomonas reinhardtii* and mutant phenotype characterization

Although biotechnological processes based on transgenic microalgae are still in their infancy, researchers and companies are considering the potential of microalgae as green cell-factories to produce value-added metabolites and heterologous proteins for pharmaceutical applications (León-Bañares et al., 2004). The commercial application of algal transgenics is beginning to be realized and algal biotechnology companies are being established. It was predicted that microalgae, due to the numerous advantages they present, could offer a powerful tool for the production of commercial molecules in a near future (Cadoret et al., 2008).

The fast growing interests in the use of transgenic microalgae for industrial applications is powered by the rapid developments in microalgal biotechnology.

Complete genome sequences from the red alga *Cyanidioschyzon merolae* (Nozaki et al., 2007), the diatoms *Thalassiosira pseudonana* (Armbrust et al., 2004) and *Phaeodactylum tricornutum* (Bowler et al., 2008) and the unicellular green algae *Ostreococcus tauri* (Derelle et al., 2006) and *C. reinhardtii* (Merchant et al., 2007) have been completed. Nuclear transformation of various microalgal species is now a routine, chloroplast transformation has been achieved for green, red, and euglenoid algae, and further success in organelle transformation is likely as the number of sequenced plastid, mitochondrial, and nucleomorph genomes continues to grow (Walker et al., 2005). Various genetic transformation systems have been developed in green algae such as *Chlamydomonas reinhardtti* and *Volvox carteri* (Walker et al., 2005). The feasibility of microalgae to be genetically modified and express heterologous genes opens up the possibility of enhancing the productivity of traditional algal compounds and producing new bioactive products for industrial and pharmaceutical applications through metabolic engineering (León-Bañares et al., 2004). Actually, the employment of transgenic microalgae in the biofuel production, one of the most interesting field in the biotechnological research, is not yet properly investigated. Numerous studies have been carried out using the genetic engineering strategy to enhance the lipid accumulation in different organisms. However, to our knowledge, this is the first work that reports the over-expression of putative genes involved in the lipid biosynthetic pathway in green algae.

In the past, several genes have been studied and overexpressed to enhance the lipid content of the host organism. The key enzymes studied for this purpose were acetyl-CoA carboxylase (AAC), fatty acid synthetase (FAS), lysophosphatidate acyl-transferase (LPAT) and diacylglycerol acyltransferase (DGAT). AAC is involved in the first reaction of lipid biosynthesis; this enzyme catalyzes the biotin-dependant carboxylation of acetyl-CoA to form malonyl-CoA. Since the successful demonstration by Page et al. (1994) that AAC exerts a strong control on the metabolic flux of fatty acid synthesis in plants, this enzyme has been overexpressed in different species for enhanced lipid production. For instance, the cytosolic ACC from *Arabidopsis* was overexpressed in *Brassica napus* plastids which resulted in a 1–2-fold increase of plastid ACC activity (Roesler et al., 1997). However, the fatty acid content of the recombinant was only 6% higher than the control, suggesting the presence of a secondary limiting step in the fatty acid synthesis pathway, emerged as a result of the removal of the primary bottleneck. Davis et al. (2000) cloned four ACC genes from *E. coli* BL21 and overexpressed them in the same strain. ACC subunits were produced in equimolar quantities. This caused an increase of the intracellular malonyl-CoA pool as a result of the enhanced ACC enzymatic activity. A 6-fold increase in the rate of fatty acid synthesis was observed, confirming that the ACC catalyzed committing step was indeed the rate-limiting step for fatty acid biosynthesis in this strain. However, the lack of lipid production enhancement seemed to suggest that again a secondary limiting step after fatty acid formation prevented the efficient conversion of fatty acids to lipids in *E. coli*. ACC was also isolated from microalgae (Roessler, 1990) and successfully over-expressed in the diatoms *Cyclotella cryptica* and *Navicula saprophila* with the enzyme activity enhanced to 2-3-folds (Dunahay et al., 1995, 1996). However, no significant increase of lipid accumulation was observed in the transgenic diatoms. These experiments demonstrated that, although ACC could be transformed efficiently into microalgae, the over-expression of this enzyme alone is not sufficient to enhance the whole lipid biosynthesis pathway (Sheehan et al., 1998). Nevertheless, Klaus et al. (2004) achieved an increase in fatty acid synthesis and a more than 5-fold increase

in the amount of TAG in *Solanum tuberosum* (potato) by over-expressing the ACC from *Arabidopsis* in the amyloplasts of potato tubers.
FAS is a multi-enzymatic complex that catalyzes fatty acids elongation by condensing malonyl-CoA molecules and acetyl-CoA (Subrahmanyam and Cronan, 1998). Over-expression of the KAS subunit of FAS in *E. coli* was carried out to facilitate the C2 concatenation. However, this manipulation was found extremely toxic for the cell (Subrahmanyamand Cronan, 1998). In another experiment, an *E. coli* KAS III was over-expressed in rapeseed (Verwoert et al., 1995), which caused a major change in the fatty acidcomposition profile with the increase of short-chain fatty acids (14:0) and a decrease of 18:1 fatty acids. This modification caused a response to stress, which significantly affected the growth of the plant cells. Similarly, KAS III from *Spinacia oleracea* was over-expressed by Dehesh et al. (2001) in *Nicotiana tabacum*, *Arabidopsis* and rapeseed, with the consequent results in a reduction of the rate of lipid synthesis and an accumulation of 16:0 fatty acids.
Transformation of rapeseed with a putative *sn*-2-acyl-transferase gene from the yeast *Saccharomyces cerevisiae* was carried out by Zou et al. (1997), leading to over-expression of seed LPAT activity. This enzyme is involved in TAG formation and its over-expression led to increases from 8% to 48% seed oil content on the seed dry weight basis. However, it was assumed that the steady-state level of diacylglycerol could be perturbed by an increase of LPAT activity in developing seeds.
As discussed previously, DGAT catalyzes the last step of TAG formation to form triacylgycerol from diacylglycerol and fatty acyl CoA. The DGAT enzymes are encoded by two classes of genes in eukaryotic cells. The first type 1 class DGAT enzymes (DGAT1) were identified in mouse and human by their similarity to the sequences of mammalian acyl-CoA:cholesterol acyltransferase (ACAT) enzymes (Chang et al., 1993; Cases et al., 1998a; Anderson et al., 1998; Cases et al., 1998b; Oelkers et al., 1998; Buhman et al., 2001) and were shown to encode proteins that possess DGAT activity (Cases et al., 1998b). Since then, orthologs have been identified in many species of eukaryotes, including yeast, fungi, plants, and invertebrates. DGAT1, like ACAT enzymes, is part of a large family of membrane-bound O-acyltransferases (MBOAT, NCBI Conserved Domains Database accession number: pfam03062) (Hofmann, 2000). Other MBOAT family members catalyze reactions that add fatty acyl chains to proteins (Zhai et al., 2004; Kadowaki et al., 1996; Chamon et al., 2001; Yang et al., 2008). This family of membrane-associated enzymes catalyzes O-acylation reactions, transferring fatty acyl moieties onto the hydroxyl or thiol groups of lipid and protein acceptors, and its members are involved in lipid metabolism, signal transduction, and hormone processing.
Since the first and simultaneous identification of a plant *DGAT1* gene in *Arabidopsis* by three independent laboratories (Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999), homologous *DGAT1* genes from several other plants have been cloned (tobacco, Bouvier-Nave *et al* ., 2000; canola, Nykiforuk *et al*., 2002; castor bean, He *et al*., 2004; burning bush, Milcamps *et al*., 2005; soybean, Wang *et al*., 2006; peanut, Saha *et al*., 2006; tung tree, Shockey *et al*., 2006). Most of these genes have been characterized and their function has been demonstrated with complementation in mutants or with the over-expression in homologues and heterologues systems. Hobbs et al. (1999) reported the first cloning and functional expression of a cDNA encoding DGAT from a plant in Sf21 insect cells. The cDNA, cloned from *Arabidopsis thaliana*, encoded a 520 amino acid protein with a 38% of sequence identity with DGAT from mouse. Insect cells able to express this putative DGAT cDNA were able to synthesise more TAG than the not transformed cells (Hobbs et al., 1999).

Jako et al. (2001) demonstrated that the expression of wild type DGAT cDNA in *A. thaliana* mutant can complement the fatty acid and the reduced oil phenotype in mutants lacking this gene. Furthermore, they reported that a seed-specific over-expression of the DGAT cDNA led to an enhanced oil deposition in seeds of wild type *Arabidopsis* (Jako et al., 2001). Recently, Xu et al. (2008) reported an increase in oil content (ranging from 11% to 30%), in *A. thaliana* and in *Brassica napus* after over-expressing a cDNA encoding a putative DGAT1 from *Tropaeolum majus*.

The existence of a type 2 class of DGAT enzymes (DGAT2) was predicted from the finding that mice lacking DGAT1 have abundant TAG in tissues (Smith et al., 2000). In 2001, a second DGAT enzyme was purified from the fungus *Mortierella ramanniana* (Lardizabal et al., 2001). This led to the cloning of mammalian DGAT2 and the identification of a seven-member gene family (Lardizabal et al., 2001; Cases e al., 2001) (DAGAT, NCBI Conserved Domain Database accession number: pfam 03982). In addition to DGAT2, this family includes acyl-CoA:monoacylglycerol acyltransferase-1 (MGAT1) (Yen et al., 2002), MGAT2 (Cao et al., 2003; Yen et al., 2003), MGAT3 (Cheng et al., 2003), and wax monoester synthases (Cheng et al., 2004; Turkish et al., 2005; Yen et al., 2005). DGAT2 and its family members share 40-45% amino acid identity throughout the entire lengths of the proteins. The most conserved regions, probably containing the catalytic domains of the proteins, are found in the C termini. All DGAT2 family members, from yeast to human, contain the highly conserved sequence of amino acids HPHG (Cases et al., 2001; Stone et al., 2006), and mutations of this sequence in mouse DGAT2 markedly reduce total DGAT activity in vitro (Stone et al., 2006). Thus, these amino acids may be part of the active site of DGAT2. Also in this case, the over-expression of gene encoding for DGAT2 in other organisms leads them to accumulate more lipids. Lardizabal et al. (2001) reported that the expression of two cDNA sequences isolated from *Mortiriella ramanniana* in insect cells led an increase in the cell lipid accumulation (Lardizabal et al., 2001).

The several successes achieved with DGAT over-expression could be explained by the fact that the substrate of DGAT, diacylglycerol, can be allocated to either phospholipid biosynthesis or TAG formation. Over-expression of DGAT would commit more diacylglycerol to TAG formation rather than phospholipid formation. Furthermore, studies with plants have revealed that increasing the rate of TAG synthesis by over-expressing DGAT also stimulated the formation of fatty acid (Galili and Hofgen, 2002). All these results seem to suggest that the reaction catalyzed by DGAT is an important rate-limiting step in lipid biosynthesis. However, no reports regarding the over-expression of this enzyme in microalgae are available (Chourchesne et al., 2009).

The green alga *C. reinhardtii* is often used in the laboratory as a genetic model system for several studies because of the ease of cultivation and of the presence of well known genetic and metabolic tools. However, for the lipid production studies, most of the attention is focused on other species. *Chlamydomonas* can be a good candidate to study the pathway and genes involved in lipid biosynthesis in algae. For this purpose, the research during the third year of my PhD was focused on the study of DGAT enzymes in *C. reinhardtii*. The research was carried out at the University of Bielefeld (Germany) in collaboration with the Algae Biotechnology & Bioenergy research group of the Professor Dr. Olaf Kruse.

Chlamydomonas has 5 genes encoding for putative DGAT enzymes and no information is available on any of these genes in literature. Analysis of their protein sequences showed that there is no correlation between *Cr*DGATs and type-1 DGAT

of plants and mammalians. These proteins seem to be more similar to the type-2 DGAT of mammalian and fungi (*Cr*DGAT2) and plants (*Cr*DGAT1, 3, 4 and 5). This can depend on the fact that DGAT2 enzymes, in contrast to DGAT1 enzymes, are more involved in the TAG synthesis starting from newly synthesized fatty acids, whereas DGAT1 is more involved in the conversion of fatty acids coming out from alimentation (Yen et al., 2008).
The decision to over-express the five genes in *Chlamydomonas* cells depends on the high GC content (up to 60%) and the codon usage of the genome of this alga that could affect the expression of these genes in other organisms. Furthermore, the availability of specific strong promoter for the expression in this alga allowed us to increase the expression of these endogenous genes easily. The nuclear manipulation of *Chlamydomonas* was performed by applying the glass beads method (Kindle, 1990). Since the first experiment of genomic manipulation on this alga (Debuchy et al., 1989), several alternative way have been developed to improve the efficiency of transformation (Kindle, 1990; Brown et al., 1991; Dunahay, 1993; Kumar et al., 2004). However, today the glass beads method still is the easiest way to obtain stable transgenic clones of this alga (Griesbeck et al., 2006).
The transformation rate of colonies that integrated the gene *aph7''* from the expression vector pHyg3 ranged between 6.5 - 8.5 × 10^{-6}. Molecular analysis performed by amplification of genomic DNA extracted from colonies able to growth in presence of hygromicine B allowed us to select the clones that integrated the *PsaD*::*CrDGAT* expression box. The percentage of positive clones found after PCR analysis was between 2.4% - 7.9%. Both the transformation rate than the percentage of co-transformed colonies are comparable with the data of *Chlamydomonas* co-transformation with pHyg3 reported by Berthold et al. (2002).
RNA expression analysis confirmed the correct transcription of *DGAT* genes. However, the transcription was not detected in all the putative positive clones. This phenomenon can be explained by the position at which the construct was integrated in the genome of *Chlamydomonas* (so called positional effect). The level of transcription in the positive clones was evaluated by quantitative real time PCR. The qRT-PCR experiments were carried out on RNA extracted during the exponential growth phase. During this phase cells are predominantly expressing genes involved in the primary metabolism, necessary for the growth and the duplication, and not the storage macromolecules. In this way, any detectable increasing in the DGAT expression in the mutant depends substantially on the over-expression of the gene under the strong *PsaD* promoter and not on the normal lipid biosynthetic pathway during stress condition. Compared to the mutants D1-160, D4-66 and D5-36, showing an expression level 15 to 30 times higher than the wild type strain, the expression of the gene DGAT3 in all the positive mutants was not higher than 4 times more than the wild type expression. This can explained in two ways: the integration of the expression box in the genome of *Chlamydomonas* did not take place in a favourable region; the endogenous expression of the DGAT3 gene is already quite strong and therefore the additional expression by the introduced vector does not lead to a difference as big as observed for the other DGAT construncts. It is worth noticing that during the exponential phase, all 5 genes are expressed in the wild type strain.
Phenotypical differences caused by the DGAT over-expression were investigated in the clones showing the highest expression. The integration of the expression box occurs randomly within the genomic DNA of Chlamydomonas. Therefore the possibility must be considered that some genes involved in the primary metabolism could be affected. Also the over-expression of these enzymes might slow down the

cell growth because of a flux of energy canalized for the lipid accumulation. Growth and photosynthetic parameters were investigated and compared to the wild type strain. When cultivated under standard growth condition, mutants did notshow any significant difference compared to the wild type strain. Optical density, number of cells and total amount of chlorophyll showed the same pattern throughout the experiment as the untransformed cells. The only statistically significant difference was detected for the mutant D3-18 that showed an increase in the total chlorophyll amount after 96 hours. However, is not clear how the TAG production can be correlated with the increasing in the chlorophyll amount. In all cases, these results showed that the over-expression of these genes doesn't affect the growth of the cells.

Total lipid amount was investigated by fluorimetric assay with Nile Red. Nile Red is a lipid soluble fluorescence dye, which has already been used to evaluate the lipid content of mammalian cells (Genicot et al., 2005), bacteria (Izard and Limberger, 2003), yeasts (Evans et al., 1985, Kimura et al., 2004), zooplankton (Kamisaka et al., 1999) and microalgae (McGinnis et al., 1997; Eltgroth et al., 2005; Elsey et al., 2007). However, despite the intensive use and the simplicity of the method, this fluorimetric assay provides only a qualitative or a semi-quantitative analysis of the lipids (Chen et al., 2009). In our case, this method was sufficient to evaluate the lipid content in the cells during different growing phase and to compare the lipid amount in mutants and wild type. No information about the quantification of lipids (e.g. % of lipids per mg of dry biomass) could be achieved with this assay.

During standard growth experiments, no differences in the lipid fluorescence between mutants and wild type were detected. Two main hypotheses can explain the achieved results: 1) the genes over-expressed are not involved in the triacylglycerol biosynthetic pathway; 2) over-expression of the DGAT genes alone is not sufficient to induce lipid accumulation during normal growth conditions.

The lack of antibodies against these protein did not allow us to study the DGAT expression at the protein level. Some kind of posttranslational regulation of these protein during the normal growth conditions might lead to an inactivated form. On the other hand, a secondary bottleneck in the lipid accumulation in the cells is the likely reason that no lipid accumulation was observed. *Chlamydomonas* can easily grow in the presence of acetate, which is used as carbon source. In favourable growth condition the cells don't spend energy to accumulate lipids, but channel all the energy into cell growth. Newly synthesized TAGs are released into the associated lipid bilayer, where they are channelled into cytosolic lipid droplets (Yen et al., 2008). The precise mechanism by which TAGs are deposited into lipid droplets is yet unknown, but probably enzymes involved in the storage of TAG in lipid droplets might not be strongly expressed during the normal growing condition. It is well know that Nile Red fluorescence is associated only with lipid droplets and maybe the down-regulation of these enzymes doesn't allow the detection of the free TAG by the Nile Red.

4.3 Lipid accumulation in mutants during nutrient starvation

Although the extent to which TAGs are produced appear to be species/strain-specific, and ultimately controlled by the genetic background of the individual organisms, algae produce only small quantities of TAG during optimal growth or favorable environmental conditions (Hu, 2004). Synthesis and accumulation of large

amounts of TAGs accompanied by considerable alterations in lipid and fatty acid composition occur when algae are placed under stress conditions, for example by chemical or physical environmental stimuli (e.g., temperature, pH, nutrient starvation and salinity). This strategy, also known as biochemical engineering approach, is the most common way to enhance lipid production of microalgae. This approach is based on the control of the nutritional or cultivation conditions to channel metabolic flux generated in photosynthesis into lipid biosynthesis. Nutrient-starvation has so far been the most commonly employed approach for directing metabolic fluxes to lipid biosynthesis of microalgae. In this scenario, microalgae accumulate lipids as a means of storage during nutrient limitation when energy source (i.e., light) and carbon source (i.e., CO_2) are abundantly available and when the cellular mechanisms for the photosynthesis are active.

Many of the commonly used limiting nutrients, like nitrogen, phosphorus and sulphur, are essential for photosynthesis of microalgae and their depletion may severely slow down the photosynthetic pathway. For instance, it was observed in *Neochloris oleoabundans* that chlorophyll, the essential pigment for light capturing in the biosynthesis of green algae, was consumed for cell growth when nitrogen was exhausted from the medium, resulting in a sharp drop of chlorophyll cell content (Li et al., 2008). Phosphorus is essential for the cellular processes related to energy bio-conversion (e.g., photophosphorylation). In particular, photosynthesis requires large amounts of proteins and proteins are synthesized by phosphorus-rich ribosomes (Wang et al., 2008). Sulphur, primarily assimilated by the cells as the sulfate anion SO_4^{2-}, plays an essential role in all organisms, as it is present in proteins, lipids and carbohydrates and several other metabolites (Pollock et al., 2005). In *C. reinhardtii* sulphur depletion limits the production of the D1 protein of the photosystem II, leading the cells to switch to an anaerobic mode. As a result, channeling metabolic flux to lipid biosynthesis by the means of phosphate and sulphur starvation may have a severe impact on photosynthesis. Thus, a disadvantage of the biochemical engineering strategy seems to be the association of the nutrient-starvation, required for accumulating high lipid content in cells, with the subsequently reduction in the cell division (Ratledge, 2002). However, a commonly suggested countermeasure is used to overcome this problem: the use of a two stage cultivation strategy, in which the first stage is dedicated for cell growth/division in nutrient-sufficient medium and the second stage for lipid accumulation under nutrient-starvation or other physiological stress. A hybrid closed photobioreactor/open pond microalgal cultivation system was suggested to be potentially the appropriate engineering solution accommodating the two-stage strategy with the photobioreactors dedicated to nutrient-rich inoculum build-up and the open ponds to low-nutrient lipid accumulation (Huntley and Redalje, 2007). It was also pointed out that employment of low-nutrient media in open ponds is not only beneficial for lipid accumulation and contamination control, but also environmentally friendly (Courchesne et al., 2009).

Although the depletion of several nutrients were reported as being able to cause cell growth cessation and channel metabolic flux to lipid/fatty acid biosynthesis, nitrogen is the most commonly reported nutritional limiting factor triggering lipid accumulation in microalgae (Courchesne et al., 2009). Nitrogen-starvation was observed to lead to lipid accumulation in a number of microalgal species. For instance, it was observed by Illman et al. (2000) that *C. emersonii*, *C. minutissima*, *C. vulgaris*, and *C. pyrenoidosa* could accumulate lipids of up to 63%, 57%, 40%, and 23% of their cell biomass on a dry weight basis, respectively, in low-N medium. *N. oleoabundans* was reported to be able to accumulate 35–54% lipids of its cell dry weight under nitrogen

deficient conditions and TAGs comprised 80% of the total lipids (Kawata et al., 1998; Tornabene et al., 1983). A 2.2-fold accumulation of TAGs was observed in *Nannochloris* sp. during nitrogen limitation (Yamaberi et al., 1998).
However, phosphate limitation was also observed to cause enhancement of lipid accumulation of *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006). The cellular total lipid content of starved cells increased with decreasing phosphate availability, mainly due to the drastic increase of TAG levels. In the absence of phosphate, the proportion of phospholipids was reduced from 8.3% to 1.4% of total lipids, and the proportion of TAG increased from 6.5% up to 39.3% of total lipids.
Furthermore, iron deficiency has also been reported to stimulate lipid accumulation in microalgae such as *Chlorella vulgaris*, which accumulated up to 56.6% of the dry biomass (Liu et al., 2008). In diatoms, silicon is an equally important nutrient which affects cellular lipid metabolism. For example, silicon-deficient *Cyclotella cryptica* cells had higher levels of neutral lipids and higher proportions of saturated and mono-unsaturated fatty acids than silicon-replete cells (Roessler, 1988). Other studies have also shown that sulphur deprivation enhanced the total lipid content in the green algae *Chlorella* sp. (Otsuka, 1961) and *C. reinhardtii* (Sato et al., 2000).
Even though the effects of biochemical engineering approach are well studied in a wide range of microalgae, only few works report this strategy to increase non-polar lipids in *Chlamydomonas* cells. Weers and Gulati (1997) reported that under nitrogen and phosphorus starvation *Chlamydomonas* can accumulate TAGs up to 30.4% and 64.7% of total lipids, respectively, whereas under normal condition the percent of TAGs detected was only the 2.2% of total lipids. Timmins et al. (2009) showed that during sulphur depletion triacylglycerol abundance increased substantially after 24 hours and remaining at high levels throughout the time course of the experiment. On the basis of these works, we decide to combine the genetic and the biochemical engineering approaches by stressing *C. reinhardtii* DGAT mutants with sulphur and nitrogen starvation, in order to activate the lipid biosynthetic pathway and to determine which DGAT candidate is involved in lipid accumulation.
In both the cases, cultures started to accumulate lipids immediately after 24 hours. Nitrogen depletion seemed to stimulate the lipid accumulation better than sulphur depletion. After 96 hours *Chlamydomonas* wild type strain showed 14,166 fluorescence units per million of cells compared to the 7,359 fluorescence units during the sulphur starvation. However, no significant differences were detected between mutants and wild type, suggesting that none of the over-expressed DGAT had an effect in the lipid accumulation during nitrogen starvation. Other enzymes might be involved in this pathway. On the other hand, after 48 hours of sulphur starvation, mutants D3-18 and D4-66 showed an increase of the lipid fluorescence compared to the wild type. This increase in the lipid content was in average 2.0 and 1.8 times higher for D3-18 and D4-66, respectively. Therefore, *Cr*DGAT3 seemed to be more involved in the lipid accumulation than *Cr*DGAT4. The over-expression of *Cr*DGAT3 in D3-18 was only 4 times higher than wild type, whereas in D4-66 the over-expression of *Cr*DGAT4 was 16 times higher on the mRNA level. It is possible that both the enzymes are involved in the lipid accumulation but at different levels. Analysis of the protein sequences for these two enzymes showed a sequence identity up to 52%, a value much higher than for the others *Cr*DGATs. This high similarity might explain the results achieved by the over-expression of these two genes among the others, which could be activated during different stress conditions.
It is not clear which mechanism are involved in the activation of these enzymes.

It is interesting to notice that during different nutrient starvation different enzymes are involved in the lipid accumulation. Timmins et al. (2009) reported that during the first 24 hours of sulphur depletion, cells accumulate energy stores in the form of starch and TAG. This is probably due to lack of sulphur for synthesis of amino acids and other cell components required for growth, in which circumstances carbon is stored in a form that is readily available when sulphur becomes available again. During nitrogen depletion, *C. reinhardtii* cells start to destroy the majority of their cytoplasmic and chloroplast ribosomes (Martin et al., 1976) and to degrade the bulk of their chloroplast membranes (Martin et al., 1975). Probably part of the lipids coming out after the chloroplast membrane degradation are converted in TAGs via metabolic pathways in which DGATs are not involved.
As described by Timmins et al. (2009), cells were grown in TAP until the early stationary phase, and subsequently transferred into fresh starvation medium (TAP -S and TAP -N) to induce the stress condition phase. As expected, cells were not able to duplicate anymore, keeping the same concentration for the entire experiment. The increasing of the optical density after 24 hours was a consequence of a morphological change of the cells during the nutrient starvation. Zhang et al. (2002) showed that during sulphur deprivation *C. reinhardtii* cells are subject to a morphological change, in which ellipsoid-shaped cells become mostly larger and spherical. This change was demonstrated to coincide with the accumulation of starch in the cells. Microscopic images of cells seemed to suggest that cells are subject to this morphological change also during the nitrogen depletion (data not shown). However, no difference in the form and the size have been detected between wild type and mutants during the normal growth condition and under different nutrient starvation. In both the starvation condition, the cultures showed a doubling in the optical density after 24 hours. During the nitrogen depletion all cultures kept the same optical density until the end of the experiment, without any differences between mutants and wild type. Differently, during the sulphur starvation the values started to decrease slowly after 48 hours, excepted for the mutant D3-18 that showed values a little bit higher than wild type. The higher values measured for this culture could be a consequence of the higher number of lipid droplets present in the mutant compared to the wild type. However, although the mutant D4-66 was able to accumulate more lipids that the wild type, is not clear why no differences were noticed in the optical density for this culture.
Since Nile Red method does not allow to quantify or qualify the lipid in the cells, further analyses are necessary to investigate the lipid composition of the two mutants. A complete lipid profile could give more information about the activity of the two DGATs and step of the lipid biosynthesis in which they are involved. At this aim, HPLC analyses are necessary to separate the different classes of lipids and to know their abundance in cells. Probably the presence of two enzymes with the same function is due to the different specificity that they have for fatty acids. Fatty acids analyses performed with GS/MS would be the best way to obtain information about the different kind of acyl-chain present into the lipids.
Chlamydomonas is not the most popular alga regarding the lipid production for biodiesel. However, several studies reported the successful use of this alga for hydrogen production. To induce hydrogen production *Chlamydomonas* cells have to be in an anaerobic state. The common way to induce anaerobiosis in *Chlamydomonas* is to grow cells into sulphur depleted medium. The results achieved with the over-expression of different *DGAT* genes open up a new scenario for biofuel production. The capability of mutants D3-18 and D4-66 to increase the lipid amount

during sulphur depletion to the extent of lipid accumulation during nitrogen starvation is a step towards a two-stage production cycle to produce hydrogen and biodiesel simultaneously. The exploitation of the biomass coming out from the hydrogen production allow to have a second generation biofuel production system with more than one added value product.

5. CONCLUSIONS

The main aim of this work was to select algal strains able to grow in presence of OMW with the future intention to use the consequent biomass as alternative source of biofuel. In this respect, the overexpression of genes involved in lipid biosynthesis was also pursued, during a stage at the University of Bielefeld (Germany).
For this purpose, 100 algal accessions of the Department of Biology of the University of Naples, belong to the division *Chlorophyta*, were screened for their capability to release enzymes with phenol-oxidase activity in the culture broth. After a screening based on the capability of microalgae supernatants to oxidise ABTS, the sixteen microalgae accessions best performer were selected and tested for their capability to oxidise phenolic compounds such as 2,6-DMP and syringaldazine and to decolorize the azo dye RBBR. Depending on the belonging specie, selected algae showed different results with the tested compounds. Two algae were selected to investigate their capability to growth in presence of OMW: *C. pitschmannii*, which gave the best results with 2,6-DMP and syringaldazine, and *S. vacuolatus*, which gave the best results with ABTS and RBBR. Both microalgae species were able to growth in presence of OMW and to reduce the phenol component during 21 days-cultivation, both under light and dark growth conditions. However, the best results were obtained with *S. vacuolatus*, which was able to remove up to 59.8% of the phenol component of the OMW and to increase the number of cells up to 85 times in 21 days. This is the first time in which *S. vacuolatus* was successful used for the OMW treatment.
The strategy to increase the lipids content in microalgae was pursued by the over-expression of endogenous diacylglycerol acyltransferase genes in the model alga *Chlamydomonas reinhardtii*. Five *Chlamydomonas* genes, encoding putative diacylglycerol acyltransferase, were cloned in the expression vector pGenD and used to obtain mutants able to over-express these genes. This is the first work in which is reported the successful over-expression of *CrDGAT* genes in algae and it is the first time in which the lipid accumulation in algal cells is enhanced by genetic engineering. Quantitative real time PCR analyses showed that *CrDGATs* over-expression in mutants was 4 to 30 times higher than wild type with an increase of the lipid content, during the sulphur starvation, of 2.0 and 1.8 times for the mutants D3-18 and D4-66, respectively. No increase of the lipid amount was found for the mutants D1-160 and D5-36 that showed values not statistically different from the wild type. Phenotypic analyses of the mutants showed that the over-expression of these genes do not affect the cell phenotype during the standard growth condition and during nitrogen starvation.

6. TABLES AND FIGURES

Table 1.1: US EPA's priority-pollutant PAHs and selected physical-chemical properties. US EPA has classified PAHs in italics as probable human carcinogens. (Mackay et al, 1992).

Polycyclic aromatic hydrocarbon	Structure (number of rings)	Molecular weight (g $mole^{-1}$)	Aqueous solubility (mg l^{-1})	Vapour pressure (mm Hg)
Naphtalene	2	128	31	8.89×10^{-2}
Acenaphthylene	3	152	16	2.90×10^{-2}
Acenaphthene	3	154	3.8	3.75×10^{-3}
Fluorene	3	166	1.9	3.24×10^{-3}
Phenanthrene	3	178	1.1	6.80×10^{-4}
Anthracene	3	178	0.045	2.55×10^{-5}
Pyrene	4	202	0.13	4.25×10^{-6}
Fluoranthene	4	202	0.26	8.13×10^{-2}
Benzo[a]anthracene	4	228	0.011	1.54×10^{-7}
Chrysene	4	228	0.006	7.80×10^{-9}
Benzo[b]fluoranthene	5	252	0.0015	8.06×10^{-8}
Benzo[k]fluoranthene	5	252	0.0008	9.59×10^{-11}
Benzo[a]pyrene	5	252	0.0038	4.89×10^{-9}
Dibenz[a,h]anthracene	5	278	0.0006	2.10×10^{-11}
Indeno[1,2,3-cd]pyrene	6	276	0.00019	1.40×10^{-10}
Benzo[*g,h,i*]perylene	6	276	0.00026	1.00×10^{-10}

Table 1.2: chemical composition of OMW. Data were calculated by Morillo et al. (2009) from eight independent studies.

Parameter	Mean	Range
Dry matter (%)	6.72	6.33 – 7.19
pH	4.84	4.2 – 5.17
EC (dS m^{-1})	8.36	5.5 – 12
Organic matter (g l^{-1})	55.80	46.5 – 62.1
TOC (g l^{-1})	37.00	34.2 – 39.8
TN (g l^{-1})	0.96	0.62 – 2.1
C/N	53.32	52.3 – 54.3
P_2O_5 (g l^{-1})	0.57	0.31 – 0.7
K_2O (g l^{-1})	4.81	2.37 – 10.8
Na (g l^{-1})	0.26	0.11 – 0.42
Ca (g l^{-1})	0.35	0.2 – 0.64
Mg (g l^{-1})	121.25	44 – 220
Fe (g l^{-1})	81.70	18.3 – 120
Cu (g l^{-1})	3.15	1.5 – 6
Mn (g l^{-1})	5.15	1.1 – 12
Zn (g l^{-1})	6.13	2.4 – 12
Density (g cm^{-3})	1.04	1.02 – 1.048
Lipids (g l^{-1})	6.39	1.64 – 12.2
Phenols (g l^{-1})	4.98	0.98 – 10.7
Carbohydrates (g l^{-1})	7.16	1.4 – 16.1
COD (g l^{-1})	124.67	67 – 178
BOD5 (g l^{-1})	65.00	46 – 94

Table 1.3: comparison of crop-dependent biodiesel production efficiencies from plant oils (Schenk et al., 2008)

Plant source	Biodiesel ($l\ ha^{-1}\ yr^{-1}$)	Area to produce global oil demand (hectares × 10^6)	Area required as percent global land mass	Area as percent global arable land
Cotton	325	15002	100.7	756.9
Soybean	446	10932	73.4	551.6
Mustard seed	572	8524	57.2	430.1
Sunflower	952	5121	34.4	258.4
Rapeseed/canola	1190	4097	27.5	206.7
Jatropha	1892	2577	17.3	130 (0[a])
Oil palm	5950	819	5.5	41.3
Algae (10 g $m^{-2}\ day^{-1}$ at 30% TAG)	12000	406	2.7	20.5 (0[a])
Algae (10 g $m^{-2}\ day^{-1}$ at 50% TAG)	98500	49	0.3	2.5 (0[a])

[a] if algal ponds and bioreactors are situated on non-arable land; jatropha is mainly grown on marginal land.

Table 2.1: codes and 5′-3′ sequences of primers used to clone the 5 DGAT genes in *Chlamydomonas reinhardtii* and to allow the point mutation in *CrDGAT2*. Bold letters show the EcoRI and NdeI restriction sites in the cloning genes and the mutation point in the primers “DGAT2_Mut_F” and “DGAT2_Mut_R”.

Primer	Sequence
DGAT_1F	CGAACG**CATATG**CCGCTCGCAAAGC
DGAT_1R	C**GAATTC**CTACATTATGACCAGCTCCTCGC
DGAT_2F	CGAAGT**CATATG**CAAAGTAAGCGTTGTGC
DGAT_2R	A**GAATTC**TCATTGCACGATGGCCAG
DGAT_3F	CGGAAG**CATATG**GCAGGTGGAAAGTCAAAC
DGAT_3R	T**GAATTC**CTACTCGATGGACAGCGGG
DGAT_4F	GCAATTC**CATATG**GCGATTGATAAAGCACC
DGAT_4R	CATG**GAATTC**TCAGCTGATGACCAGCGG
DGAT_5F	GCAATTC**CATATG**ACCCCGCGGGATC
DGAT_5R	CATG**GAATTC**TCAGCACACCTCCAGCG
DGAT2_Mut_F	CGTTACTATGAATT**G**TTTGAGTGCGCGTGCCGTA
DGAT2_Mut_R	TACGGCACGCGCACTCAAA**C**AATTCATAGTAACG

Table 2.2: codes and 5′-3′ sequences of primers used to select, after nuclear transformation, positive *C. reinhardtii* colonies by amplification of genomic DNA.

Primer	Sequence
PsaD_promoter_F	TAGTGGATCCCACACACCTG
PsaD_terminator_R	GATTGCACAGTCACGCTGTC
DGAT1_SR1	CGTACTCCAGCACCACGTT
DGAT1_SF1	GCTGTACGACAAGCACAAGG
DGAT2_SR1	CAGCTCTGCACAACGCTTAC
DGAT2_SF1	ACAAGTACGGCAAGGGTGTC
DGAT3_SR1	CCAGCTTTCAGGGTCAAGTG
DGAT3_SF1	GAGTTCGACAAGGAGGTGGA
DGAT4_SR1	ACCAGCGATGAGAAGATGCT
DGAT4_SF1	TACGACCGCCACAAGACC
DGAT5_SR1	CCAGCAGGCGATACGG
DGAT5_SF1	CAGTACGGCTGCGGTTG

Table 2.3: codes and 5′-3′ sequences of primers used to verify the RNA expression of positive clones selected by genomic DNA amplification. “PsaD 3′ UTR” was used to retrotranscriptase the RNA extracted from all clones.

Primer	Sequence
PsaD cDNA	CCCCACTGCTACTCACAACA
PsaD 3′ UTR	CCCGTATCAATCAGCGAAAT
DGAT1_cDNA_R	GCAGAATCCGAACAAGTAGC
DGAT2_ cDNA_R	AGGATGTACTCACGCAAACC
DGAT3_ cDNA_R	AAAGATGTAGCGCTTGTTGG
RT-D4 R1 Gen	TCCAGCACCTCATCAAACAC
RT-D5 R1 Gen	GCGTAGCTGTAGTTGAAGTAGGC

Table 2.4: codes and 5′-3′ sequences of primers used to quantify by qRT-PCR the expression level of *DGAT* in mutants.

Primer	Sequence
Actin_F	ATGGGCCAGAAGGACTCGTAC
Actin_R	TCGTTGAAGAAGGTGTGGTG
qRT_D1_F6	TCGTGCAGTTCAGTGTGG
DGAT1_cDNA_R	GCAGAATCCGAACAAGTAGC
qRT_D2_F	ATGCTAGCGCTGCAGGTG
qRT_D2_R	ACACCCTTGCCGTACTTGTC
qRT_D3_F	GCTACCTCACGGGATGGAT
qRT_D3_R2	TAGCTTAATGCGCTCCTTCT
qRT_D4_F	TTCCTGTACGGAGTGGCCTA
qRT_D4_R	CAGCCGTACTCGGTCTTGTG
qRT_D5_F	AGCCGCTCATGATGTGTGT
qRT_D5_R	TACTGCACGCGGTACCTGTT

Table 3.1: accession numbers of algae that showed an E.I. higher than 0.1 and corresponding species.

Accession number	Specie
2	*Chlorella emersonii v. globosa*
23	*Chlorella soronikiana*
24	*Chlorella emersonii*
25	*Scenedesmus vacuolatus*
26	*Scenedesmus vacuolatus*
28	*Chlorella luteoviridis*
42	*Chlamydomonas pitschmannii*
59	*Scenedesmus ovalternus*
78	*Chlamydomonas pitschmannii*
79	*Chlamydomonas pitschmannii*
82	*Chlorella vulgaris*
93	*Chlorella vulgaris*
95	*Scenedesmus vacuolatus*
98	*Chlorella vulgaris*
103	*Chlorella emersonii*
104	*Chlorella vulgaris*

Table 3.2: transformation rate (%) of colonies that integrated the construct *PsaD::CrDGAT1-5*.

	Number of colonies	Positive colonies	Transformation rate (%)
CrDGAT1	208	5	2.4
CrDGAT2	130	7	5.4
CrDGAT3	156	7	4.5
CrDGAT4	102	8	7.8
CrDGAT5	102	5	4.9

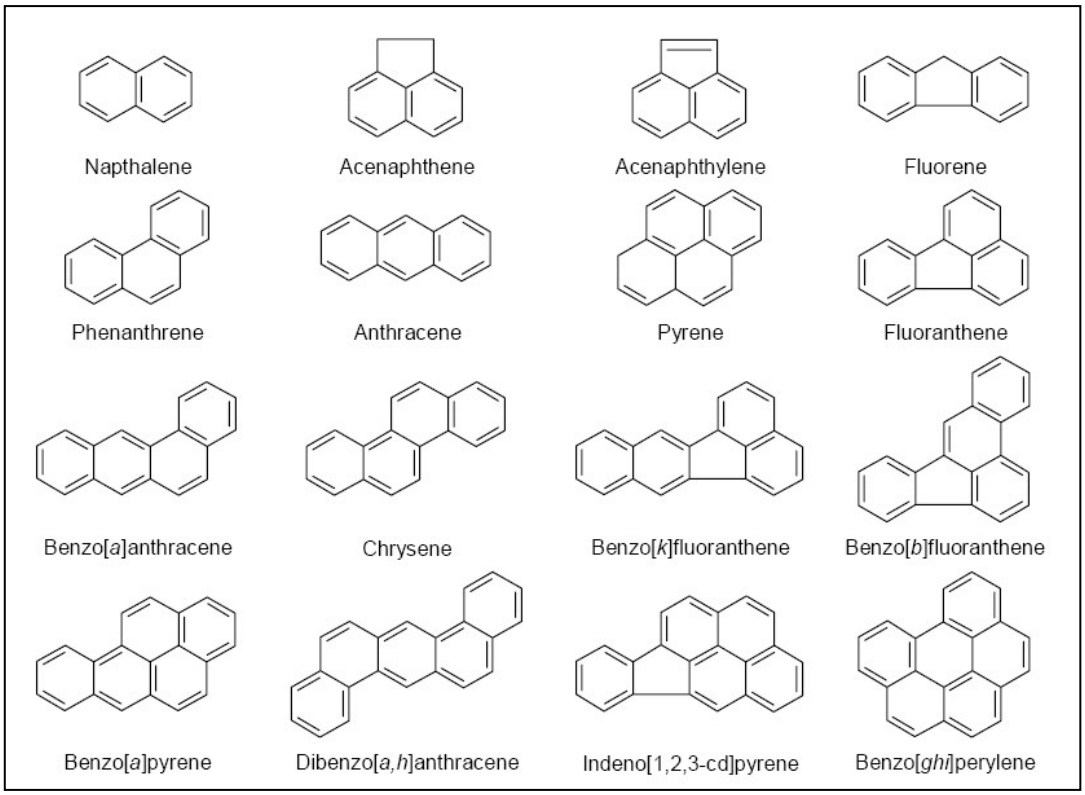


Figure 1.1: structure of the 16 US-EPA PAHs.

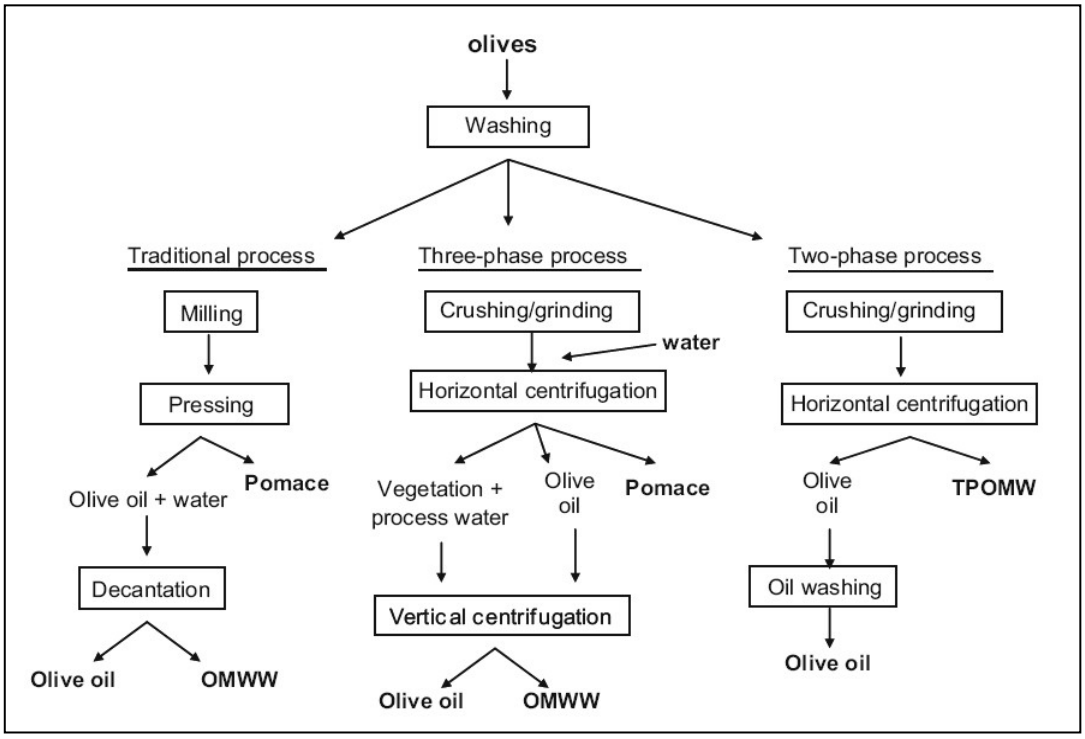


Figure 1.2: Simplified flow chart of industrial-scale olive oil extraction processes: traditional press-cake system, three phase decanter system and two-phase centrifugation system (Morillo et al., 2009).

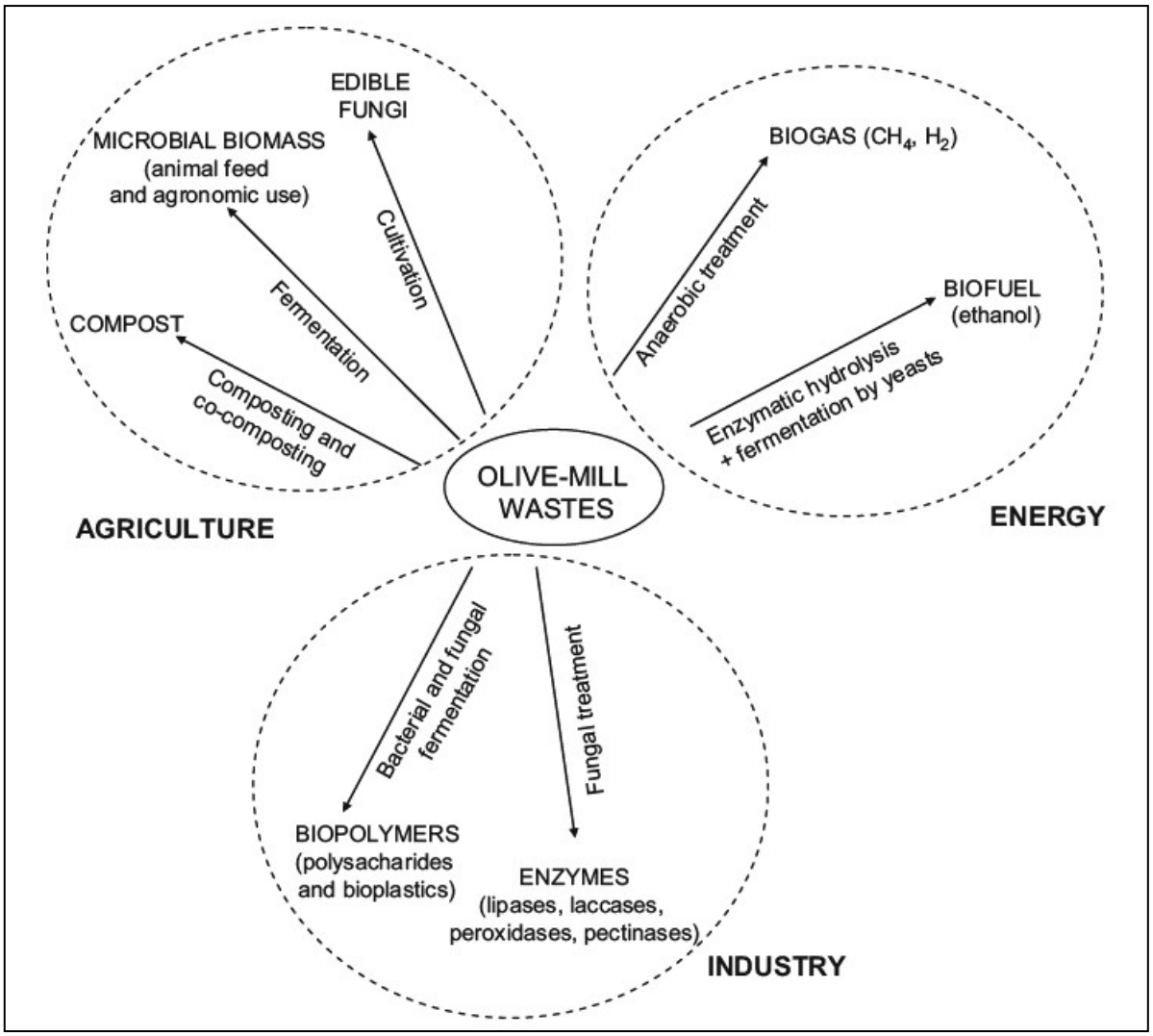


Figure 1.3: Potential uses and microbial valorisation of olive-mill wastes (Morillo et al., 2009).

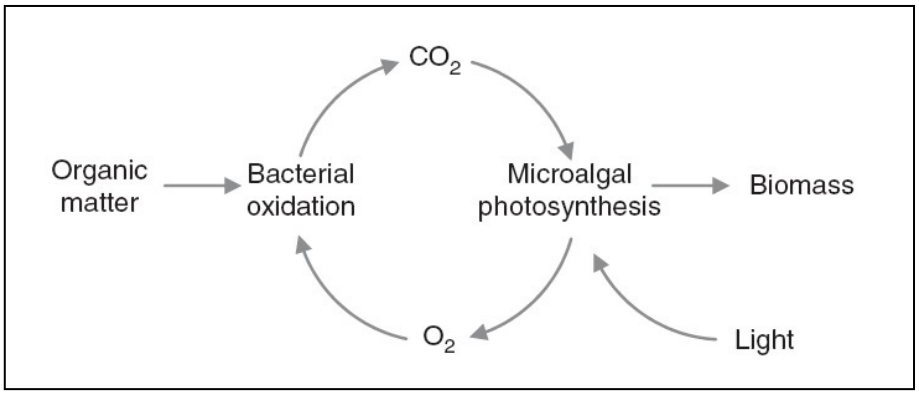


Figure 1.4: Principle of photosynthetic oxygenation in BOD removal process (Muñoz and Guieysse, 2006).

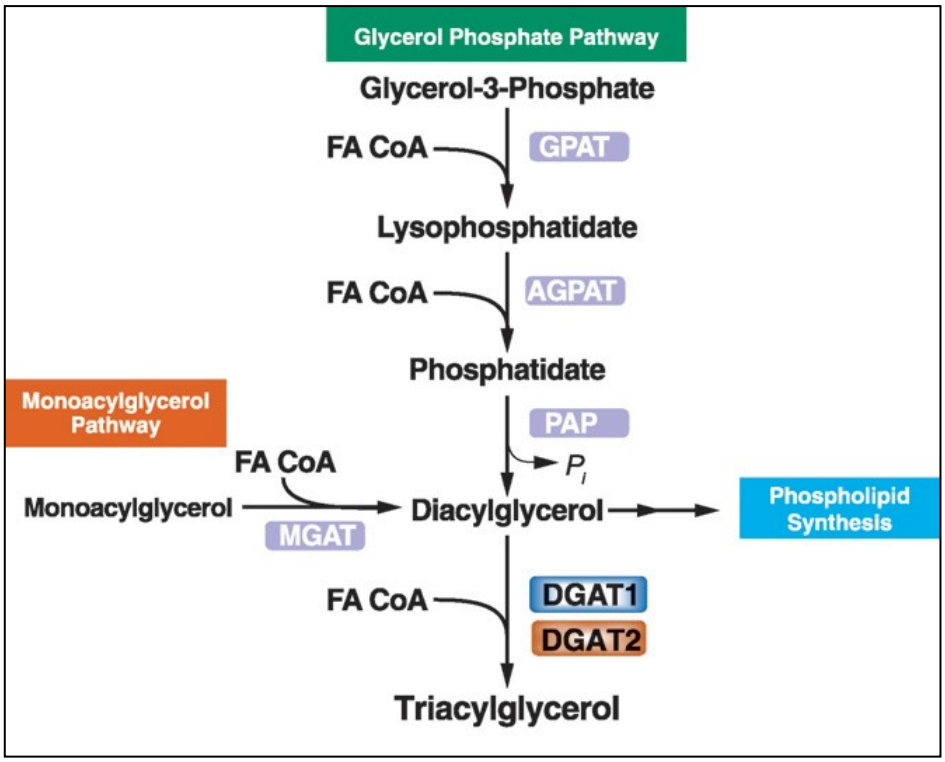


Figure 1.5: TAG biosynthesis. TAG are the end product of a multi-step pathway in which DGAT1 and DGAT2 catalyzes the final reaction using DAG coming out from the Glycerol Phospate and/or Monoacylglycerol pathways. (Yen et al., 2008).

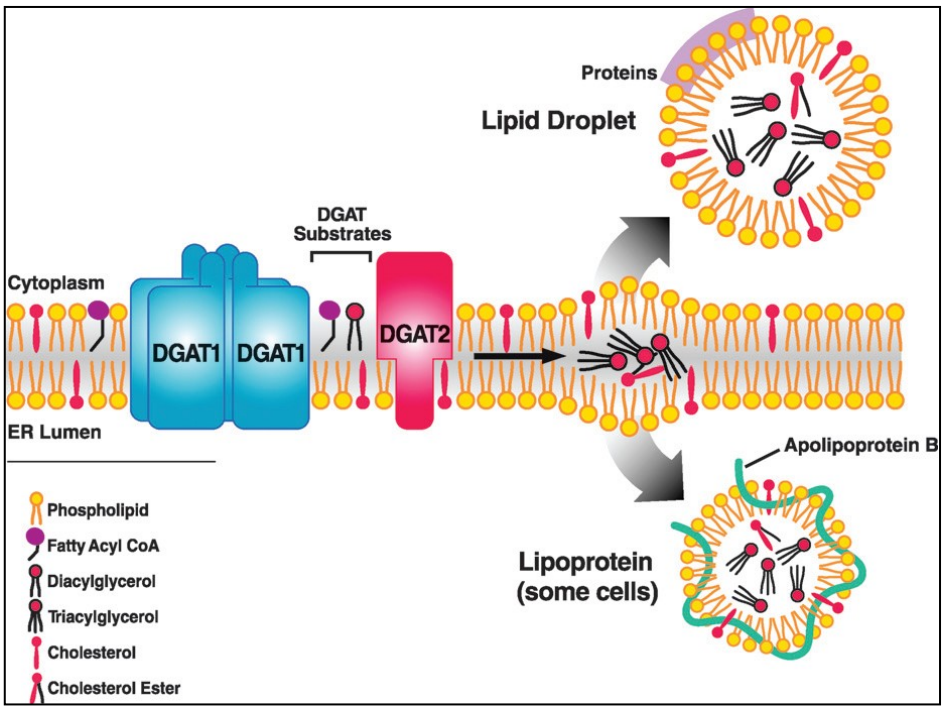


Figure 1.6: hypothetical model illustrating the role of DGAT enzymes in TAG synthesis in the ER. TAG products of the DGAT reaction may be channelled into the cores of cytosolic lipid droplets or TAG-rich lipoproteins for secretion in cells such as enterocytes and hepatocytes. (Yen et al., 2008).

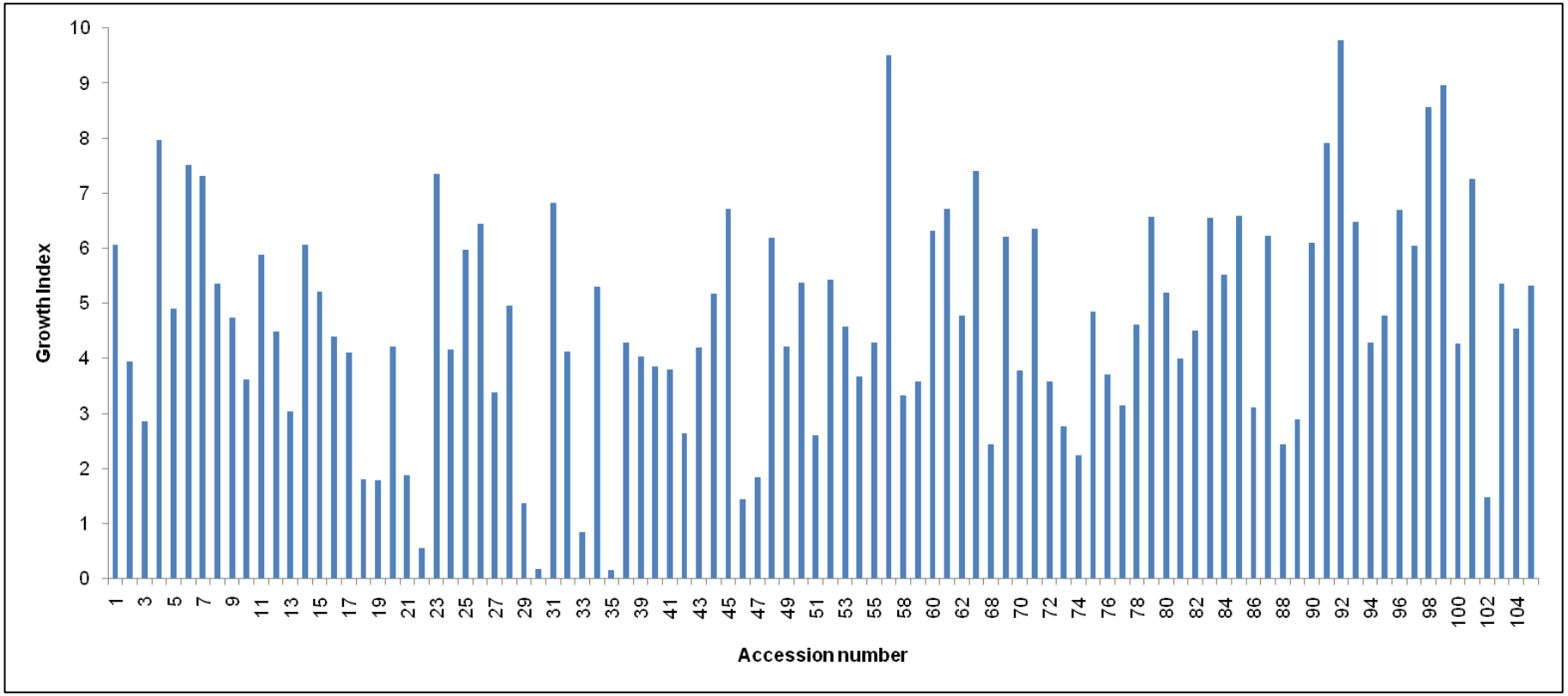


Figure 3.1. Growth Index of the 100 microalgae tested accessions after 10 days of culture.

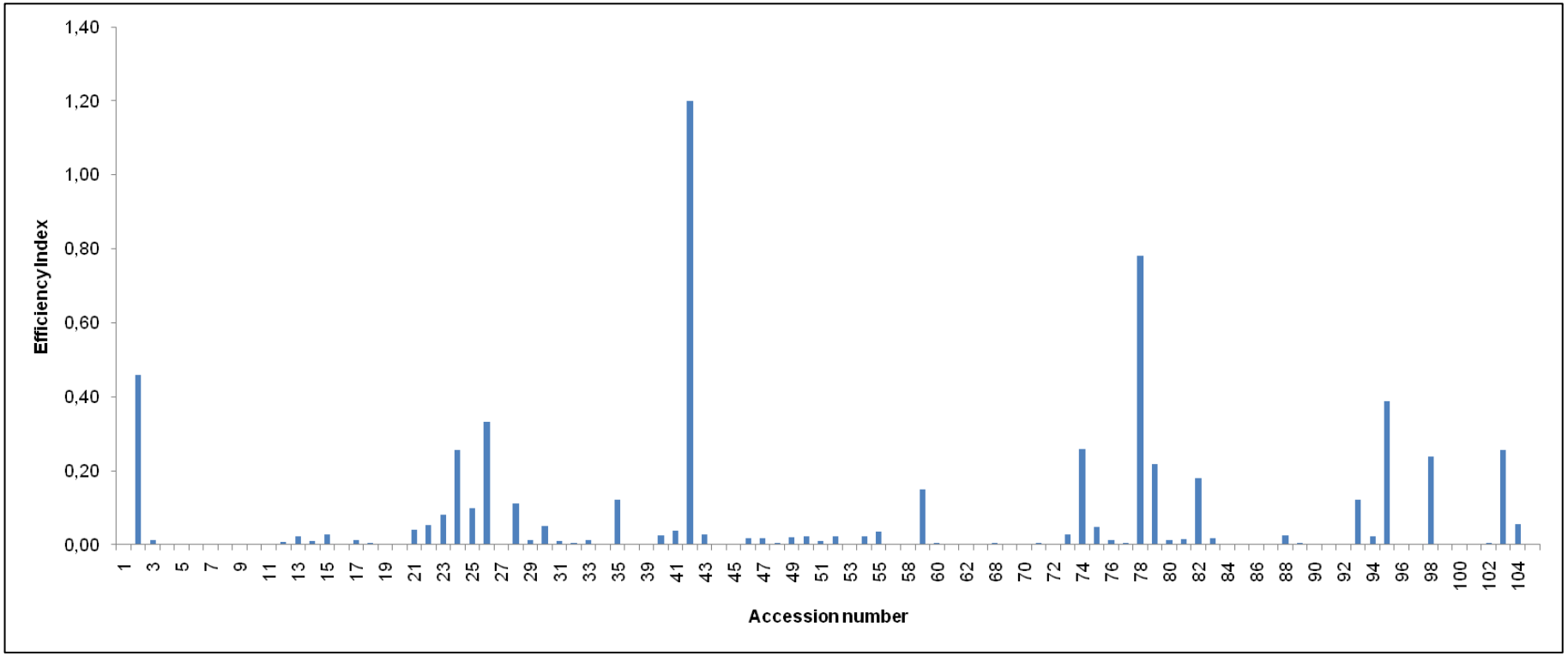


Figure 3.2. Evaluation of enzymes with phenol-oxidase activity in 100 culture broths by ABTS assay. The values are expressed as Efficiency Index. Only 16 out 100 tested accessions showed an Efficiency Index higher than 0.1.

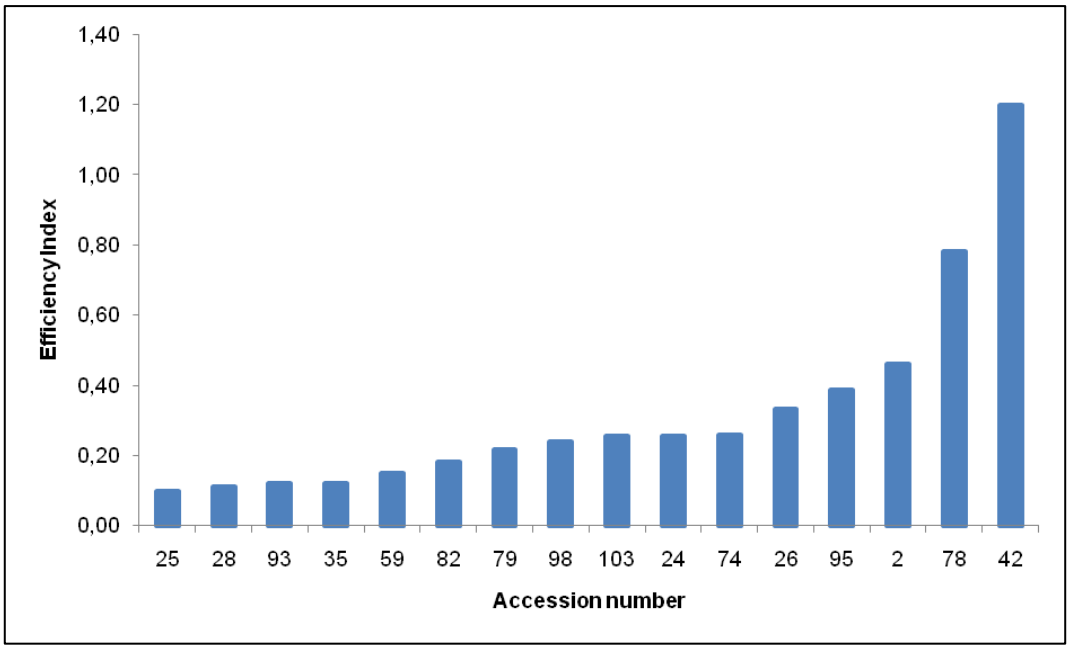


Figure 3.3. Efficiency Index of selected microalgae accessions.

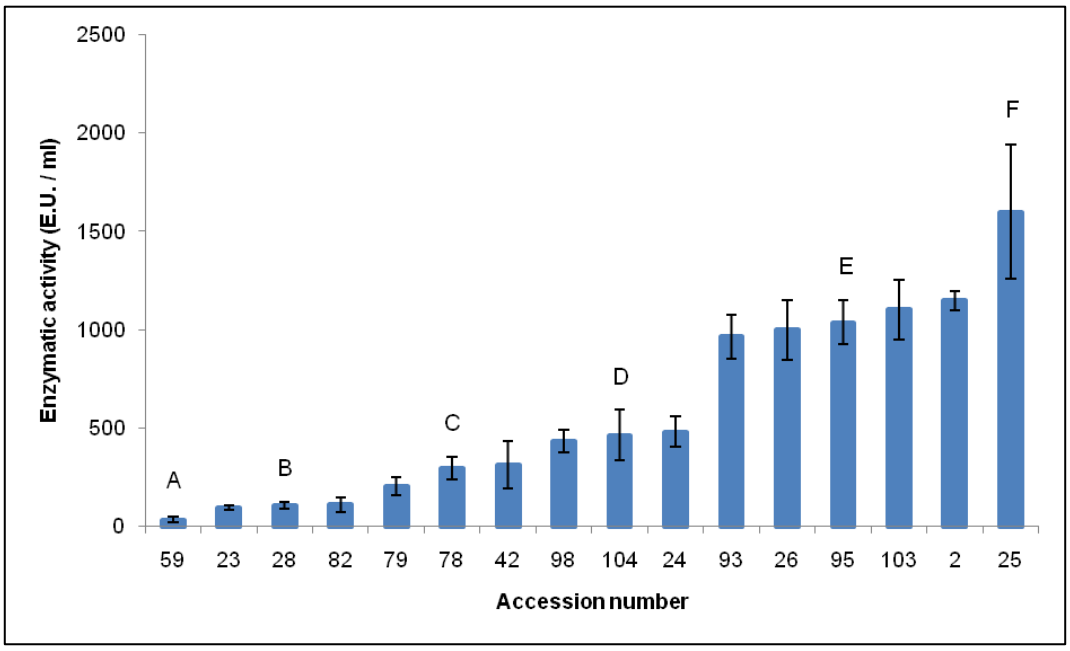


Figure 3.4. Enzymatic activity of culture broths of 16 selected microalgae accessions in presence of ABTS. Mean values and standard deviations of three different replica are reported. Accessions were clustered into six groups: **A)** 59; **B)** 23, 28, 82; **C)** 79, 78, 42; **D)** 98, 104, 24; **E)** 93, 26, 95, 103, 2; **F)** 25. Different letters mean significant difference among the different groups ($p<0.05$).

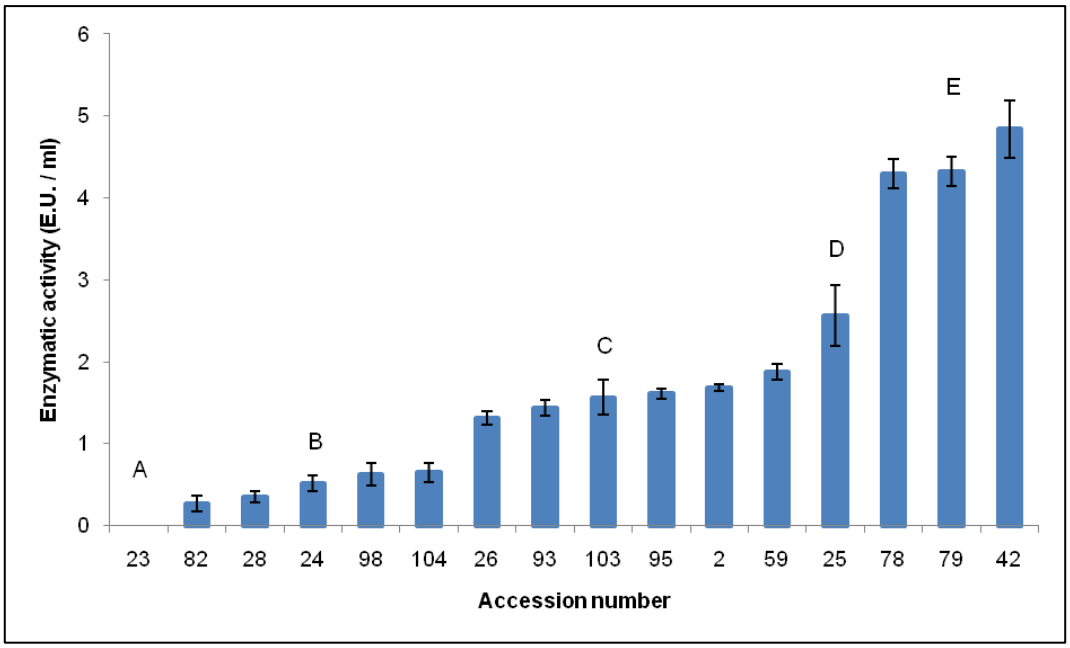


Figure 3.5. Enzymatic activity of culture broths of 16 selected microalgae accession in presence of 2,6-DMP. Mean values and standard deviations of three different replica are reported. Accessions were clustered into five groups: **A)** 23; **B)** 82, 28, 24, 98, 104; **C)** 26, 93, 103, 95, 2, 59; **D)** 25; **E)** 78, 79, 42. Different letters mean significant difference among the different groups ($p<0.05$).

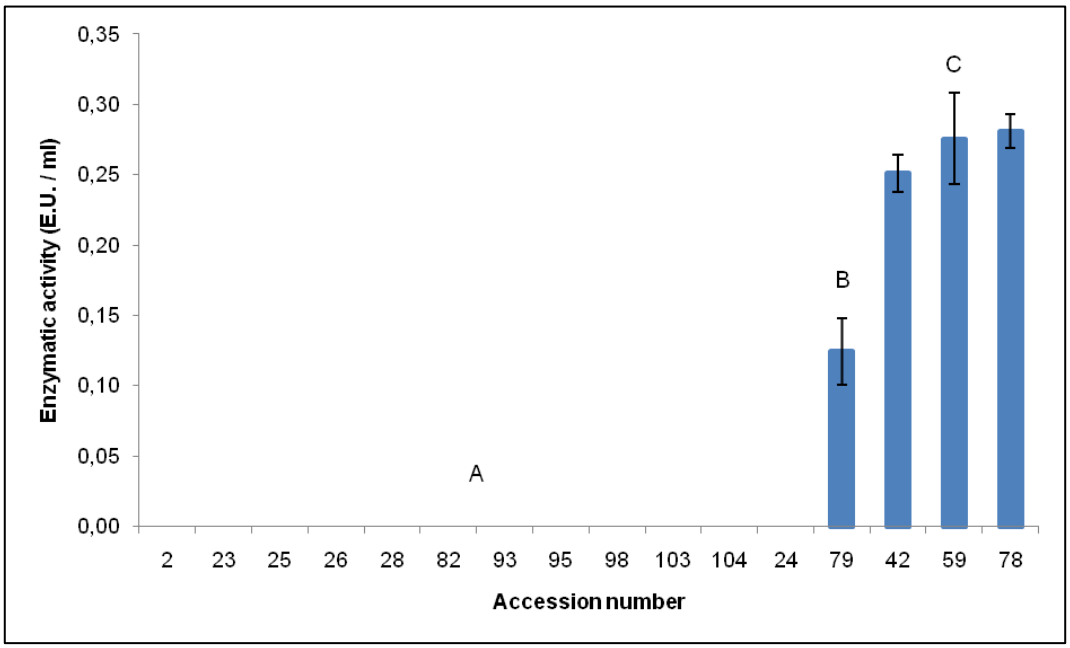


Figure 3.6. Enzymatic activity of culture broths of 16 selected accession in presence of syringaldazine. Mean values and standard deviations of three different replica. Accessions were clustered into three groups: **A)** 2, 23, 25, 26, 28, 82, 93, 95, 98, 103, 104, 24; **B)** 79; **C)** 42, 59, 78. Different letters mean significant difference among the different groups ($p<0.05$).

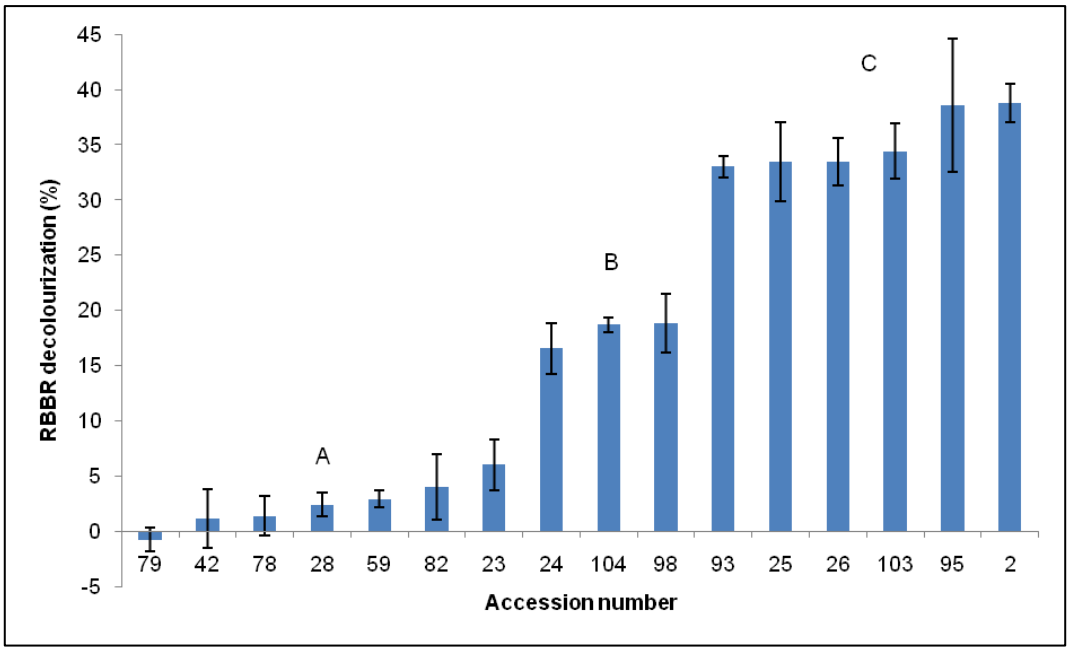


Figure 3.7. RBBR decolourisation after 6 hours incubation with microalgae culture supernatants of selected accessions. Data were expressed as percentage of decolourisation. Mean values and standard deviations of three different replica are reported. Accessions were clustered into groups: **A)** 79, 42, 78, 28, 59, 82, 23; **B)** 24, 104, 98; **C)** 93, 25, 26, 103, 95, 2. Different letters mean significant difference among the different groups ($p<0.05$).

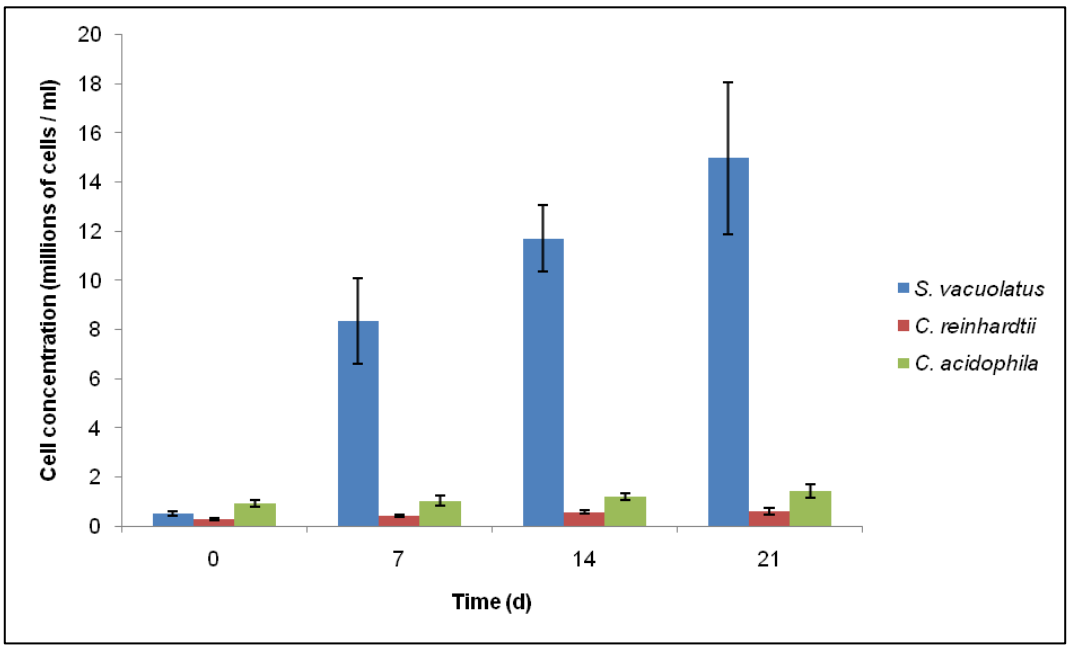


Figure 3.8. *S. vacuolatus*, *C. reinhardtii* and *C. acidophila* cell concentration (cells × 10^6 ml^{-1}) after 21 days of culture in OMW:BBM (1:20, v/v) under dark condition. Mean values and standard deviations of three different replica are reported.

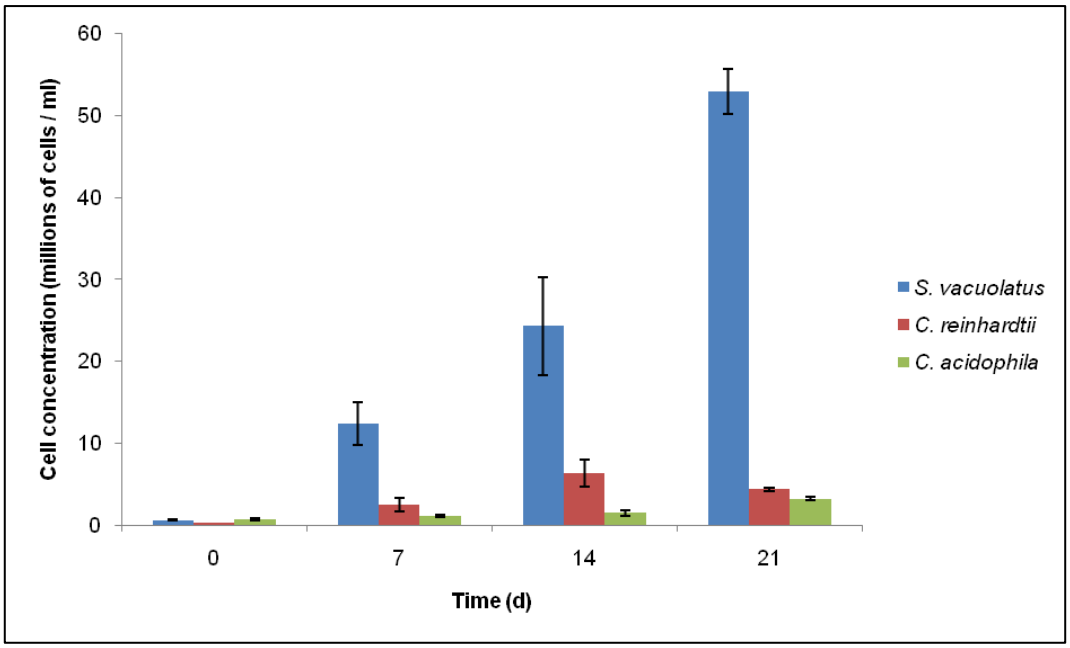


Figure 3.9. *S. vacuolatus*, *C. reinhardtii* and *C. acidophila* cell concentration (cells × 10^6 ml^{-1}) after 21 days of culture in OMW:BBM (1:20, v/v) under light condition. Mean values and standard deviations of three different replica are reported.

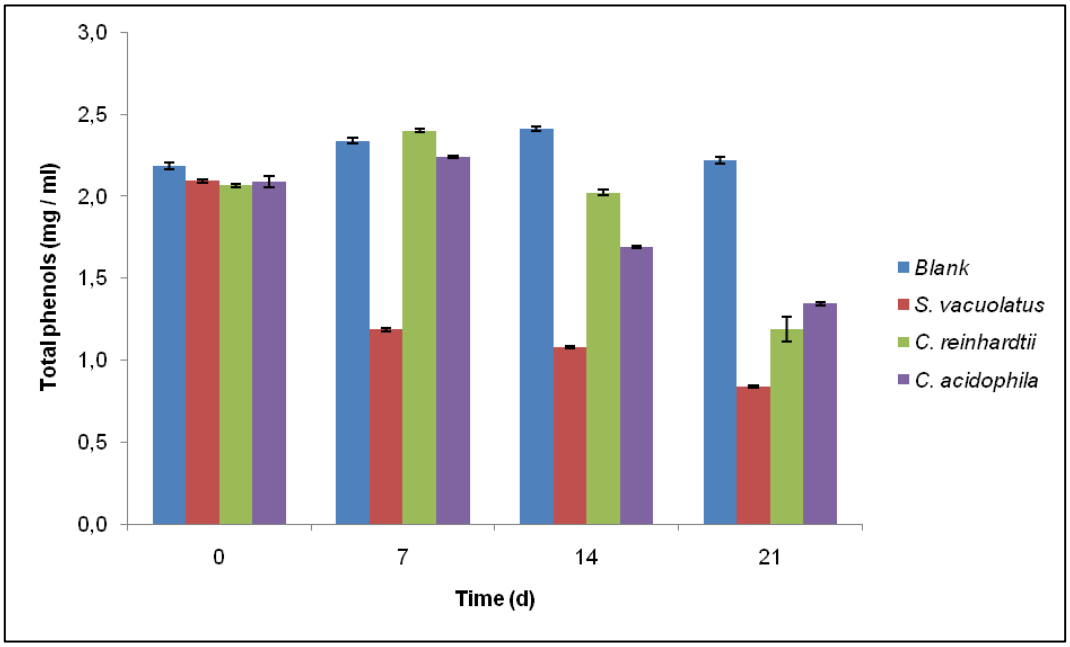


Figure 3.10. Total phenol concentration (mg ml^{-1}) in the OMW:BBM after 7, 14 and 21 days of microalgae culture under light condition. Mean values and standard deviations of three different replica are reported.

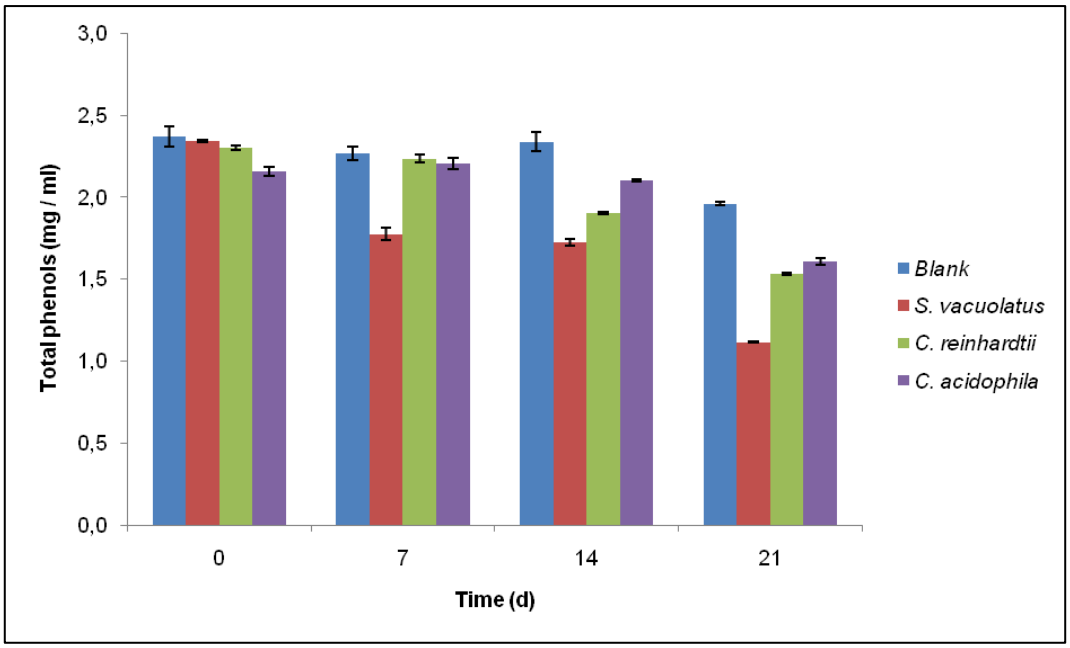


Figure 3.11. Total phenol concentration (mg ml^{-1}) in the OMW:BBM after 7, 14 and 21 days of microalgae culture under dark condition. Mean values and standard deviations of three different replica are reported.

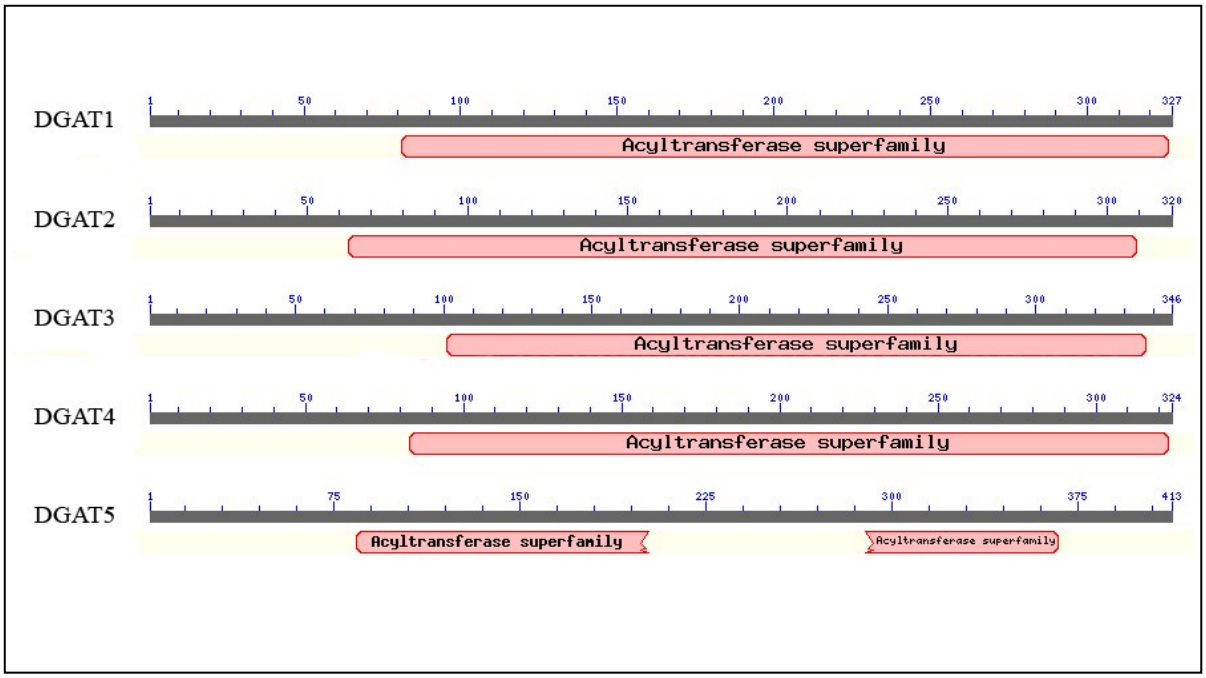


Figure 3.12. Putative conserved domain of *Cr*DGAT1-5 proteins in *C. reinhardtii* detected by NCBI's Conserved Domain Database.

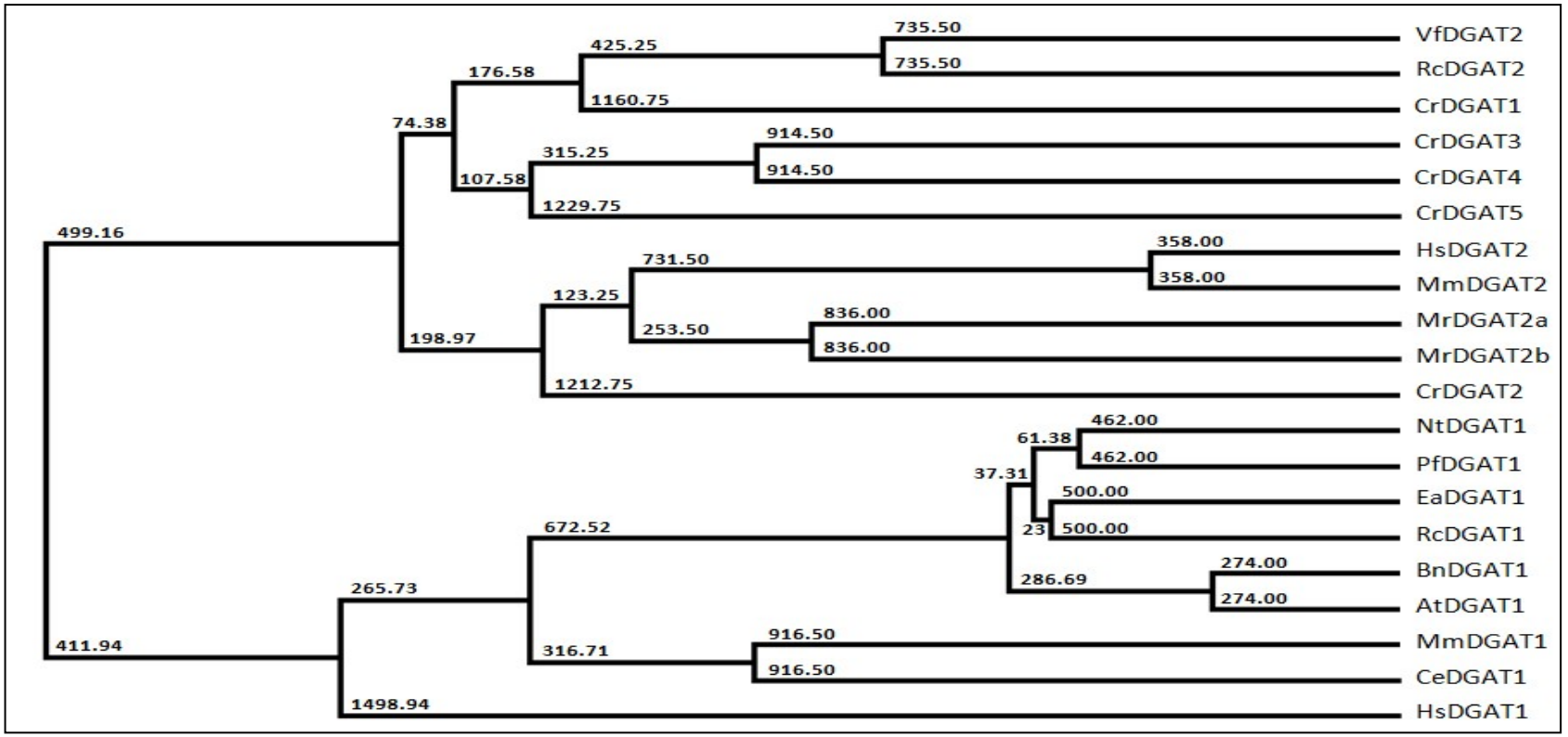


Figure 3.13. Comparison of amino acid sequences from different type-1 and type-2 DGATs. Alignment and phylogenetic tree were performed with ClustalV. GenBank accession number are shown in parentheses: *Vf*DGAT2, *V. fordii* (DQ356682); *Rc*DGAT2, *R. comunis* (XM_002528485); *Cr*DGAT1, *C. reinhardtii* (XM_001693137); *Cr*DGAT3, *C. reinhardtii* (XM_001691395); *Cr*DGAT4, *C. reinhardtii* (XM_001694852); *Cr*DGAT5, *C. reinhardtii* (XM_001701615); *Hs*DGAT2, *H. sapiens* (AF384161); *Mm*DGAT2, *M. musculus* (AF384160); *Mr*DGAT2a, *M. ramanniana* (AF391089); *Mr*DGAT2b, *M. ramanniana* (AF391090); *Cr*DGAT2, *C. reinhardtii* (XM_001702796); *Nt*DGAT1, *N. tabacum* (AF129003); *Pf*DGAT1, *P. frutescens* (AF298815); *Ea*DGAT1, *E. alatus* (AY751297); *Rc*DGAT1, *R. comunis* (AY366496); *Bn*DGAT1, *B. napus* (AF164434); *At*DGAT1, *A. thaliana* (AF051849); *Mm*DGAT1, *M. musculus* (AF078752); *Ce*DGAT1, *C. elegans* (AF221132); *Hs*DGAT1, *H. sapiens* (L21934).

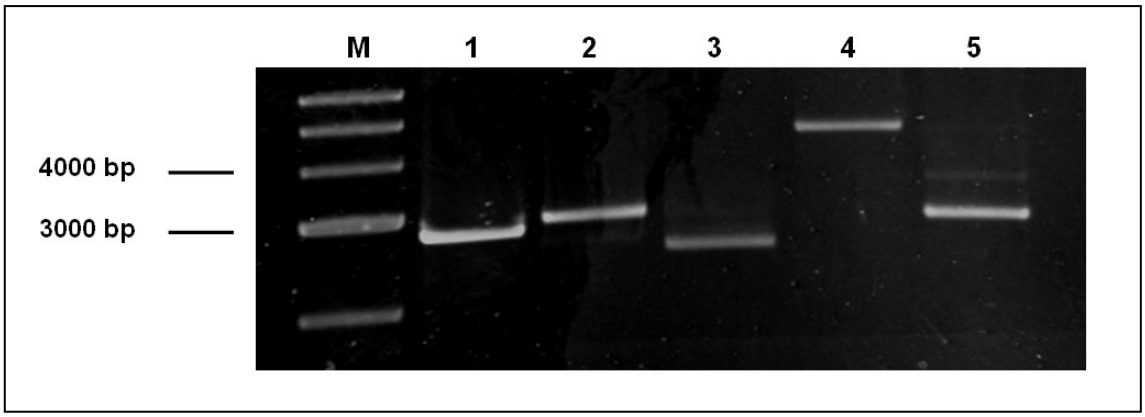


Figure 3.14. *C. reinhardtii* CC3491 genomic DNA amplification with cloning primer specific for each DGAT gene. **1)** *DGAT1* (2813 bp); **2)** *DGAT2* (3017 bp); **3)** *DGAT3* (2543 bp); **4)** *DGAT4* (4486 bp); **5)** *DGAT5* (2864 bp); **M)** Log-2 ladder.

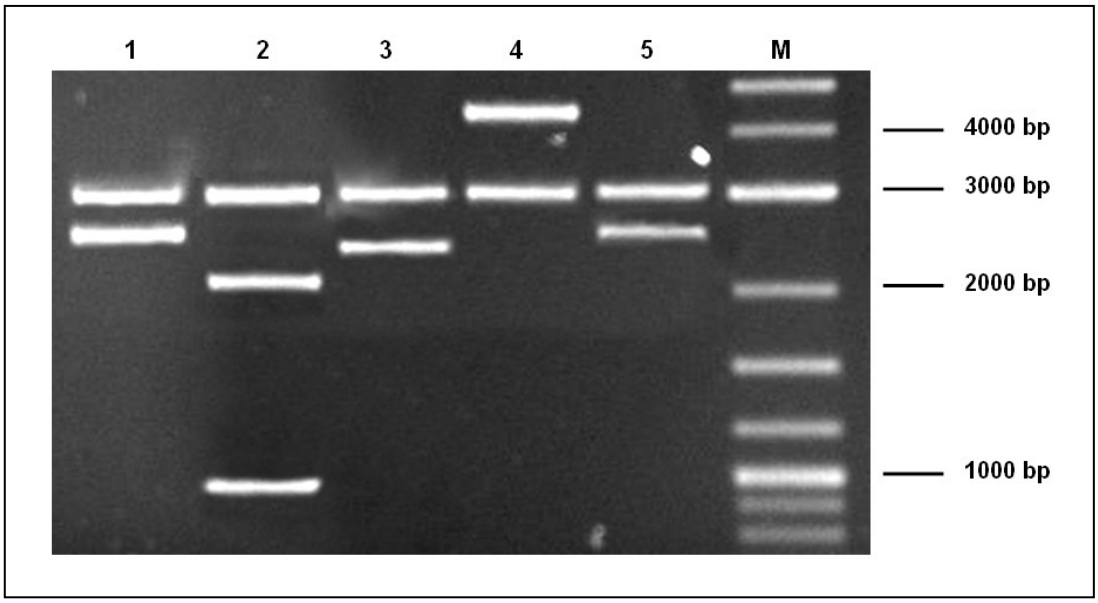


Figure 3.15. Restriction analyses of pGemT-Easy::*DGAT1-5* constructs cut with the restriction enzymes EcoRI and NdeI. **1)** pGemT-Easy::*DGAT1*; **2)** pGemT-Easy::*DGAT2*; **3)** pGemT-Easy::*DGAT3*; **4)** pGemT-Easy::*DGAT4*; **5)** pGemT-Easy::*DGAT5*; **M)** Log-2 ladder. All constructs show a common product of 3000 bp (pGemT-Easy) and the *DGAT* gene. The construct pGemT-Easy::*DGAT2* shows three products because of EcoRI restriction site present in the third intron.

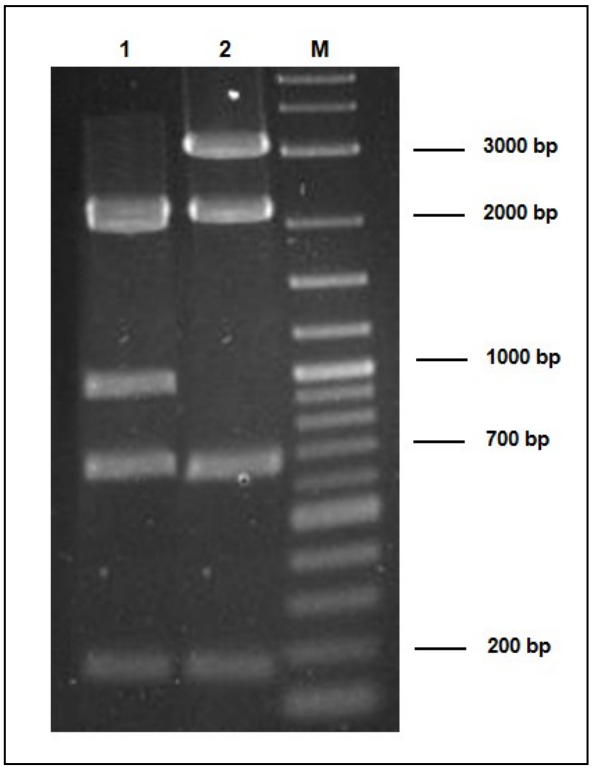


Figure 3.16. Restriction analyses of pGemT-Easy::*DGAT2* constructs cut with the restriction enzymes EcoRI, NdeI and SspI. **1)** pGemT-Easy::*DGAT2*; **2)** pGemT-Easy::*DGAT2* after point mutation; **M)** Log-2 ladder. The presence of a product of 3,000 bp confirm the successful of the point mutation.

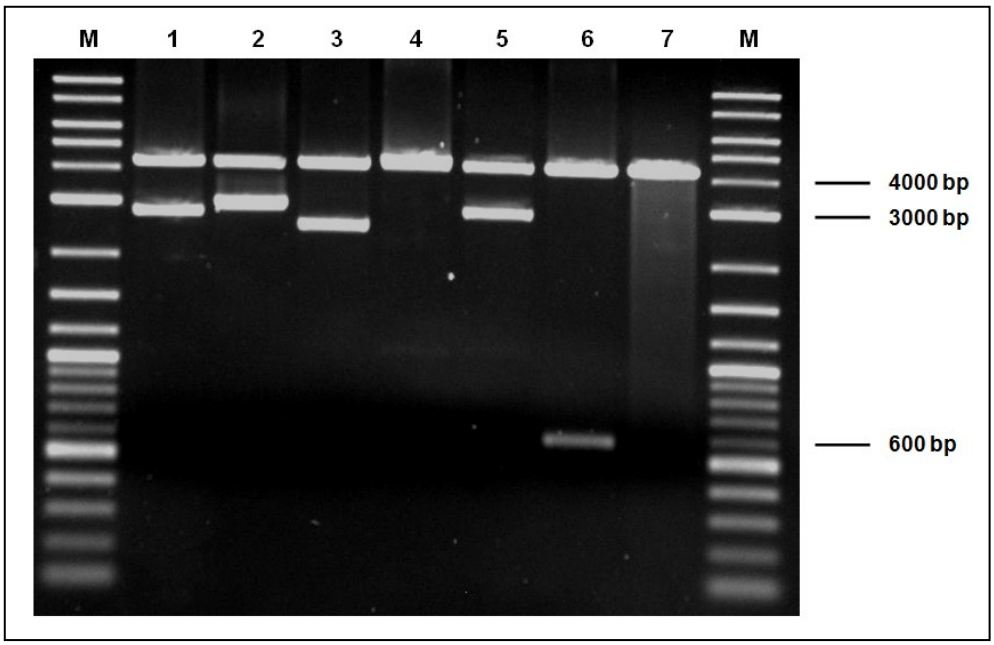


Figure 3.17. Restriction analyses of pGenD::*DGAT1-5* constructs cut with the restriction enzymes EcoRI and NdeI. **1)** pGenD::*DGAT1*; **2)** pGenD::*DGAT2*; **3)** pGenD::*DGAT3*; **4)** pGenD::*DGAT4*; **5)** pGenD::*DGAT5*; **6)** pGenD::*PsaD*; **7)** pGenD; **M)** Log-2 ladder.

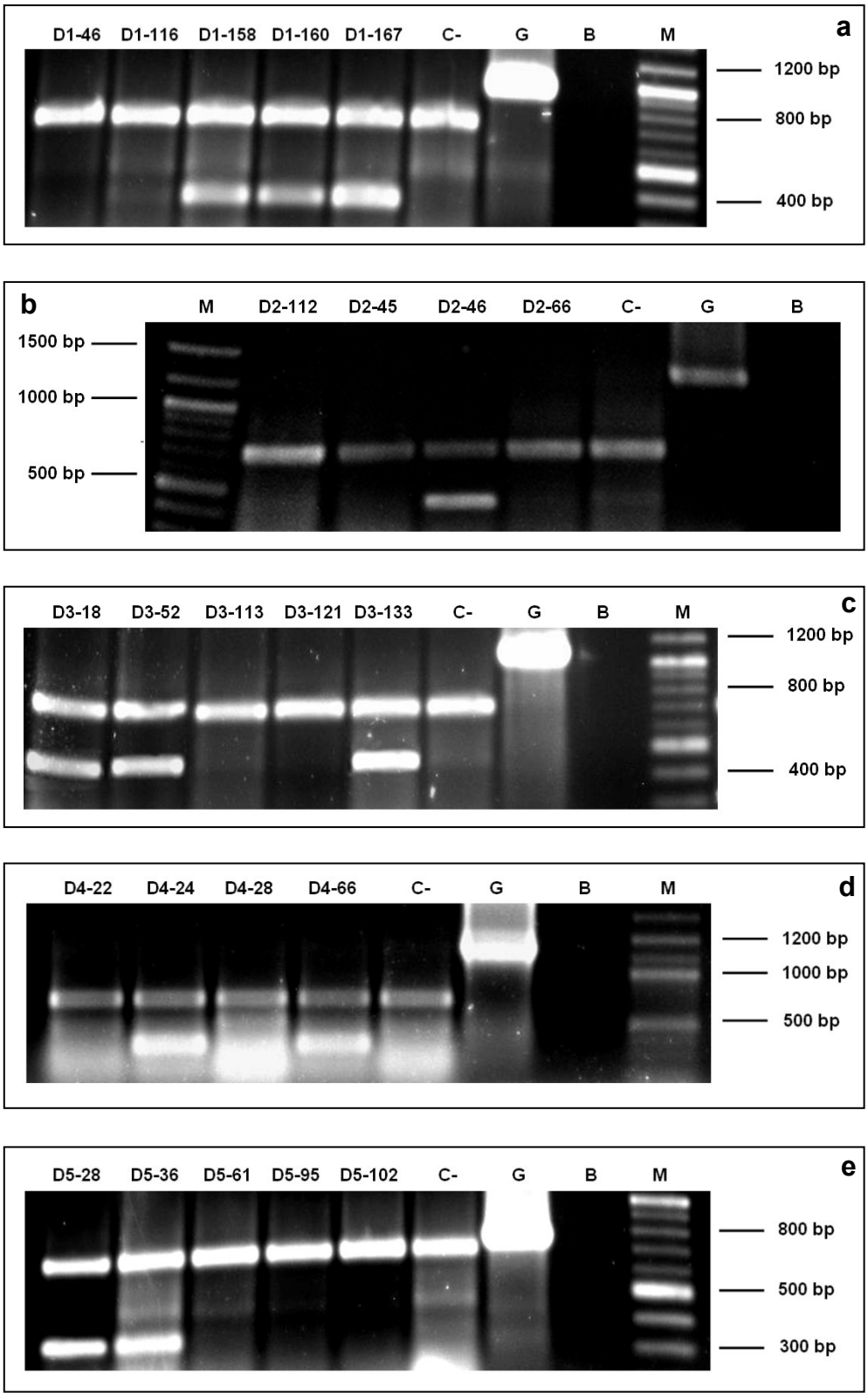


Figure 3.18. RT-PCR analyses carried out with specific primers for each DGAT on cDNA obtained by retrotranscription of total RNA, extracted from DGATs PCR positive clones, with the primer "PsaD 3′ UTR". **C-)** negative control (cDNA from *C. reinhardtii* CC3491 wild type strain); **G)** pGenD::*DGAT1-5*; **B)** blank; **M)** Log-2 ladder.

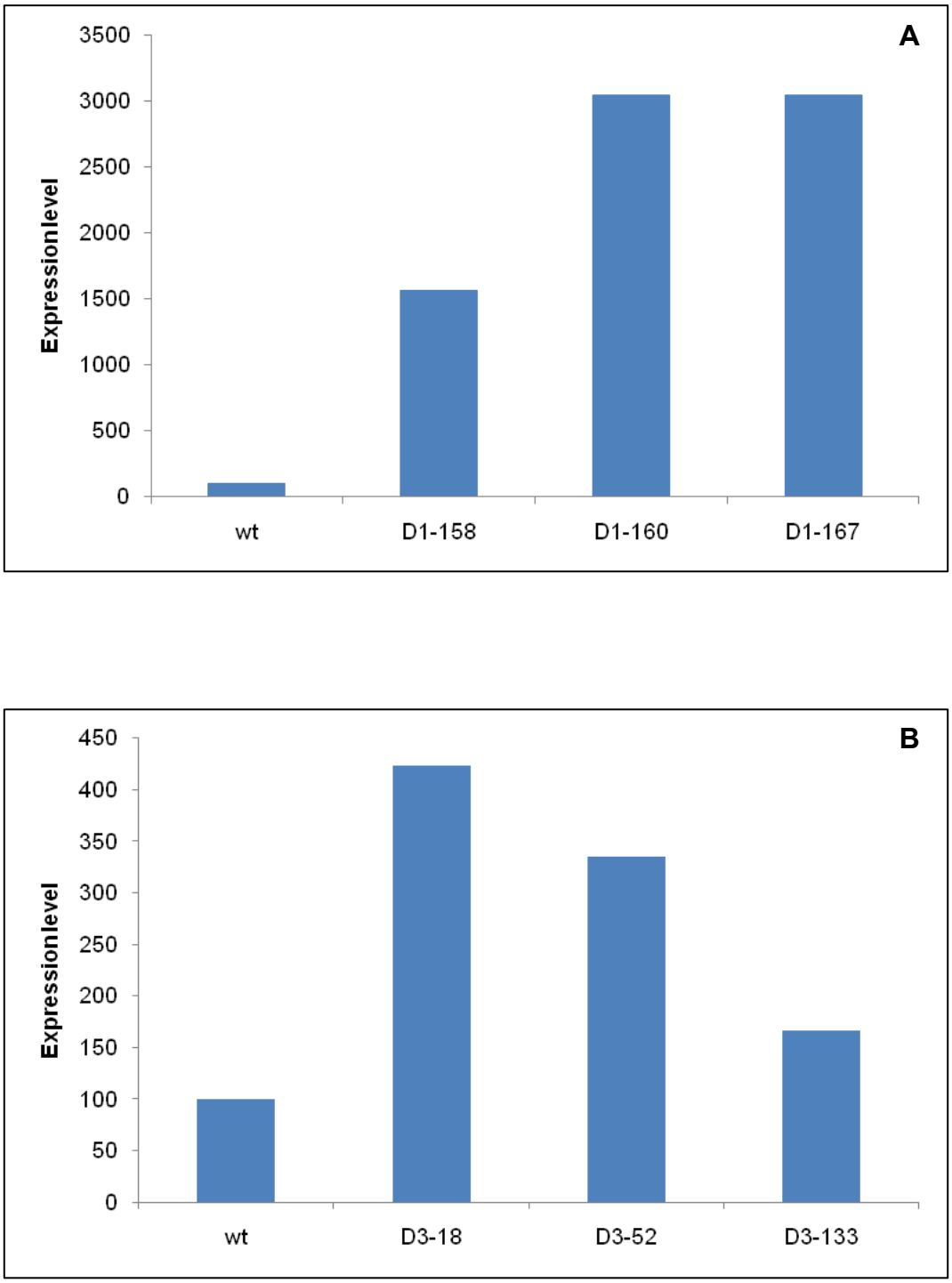
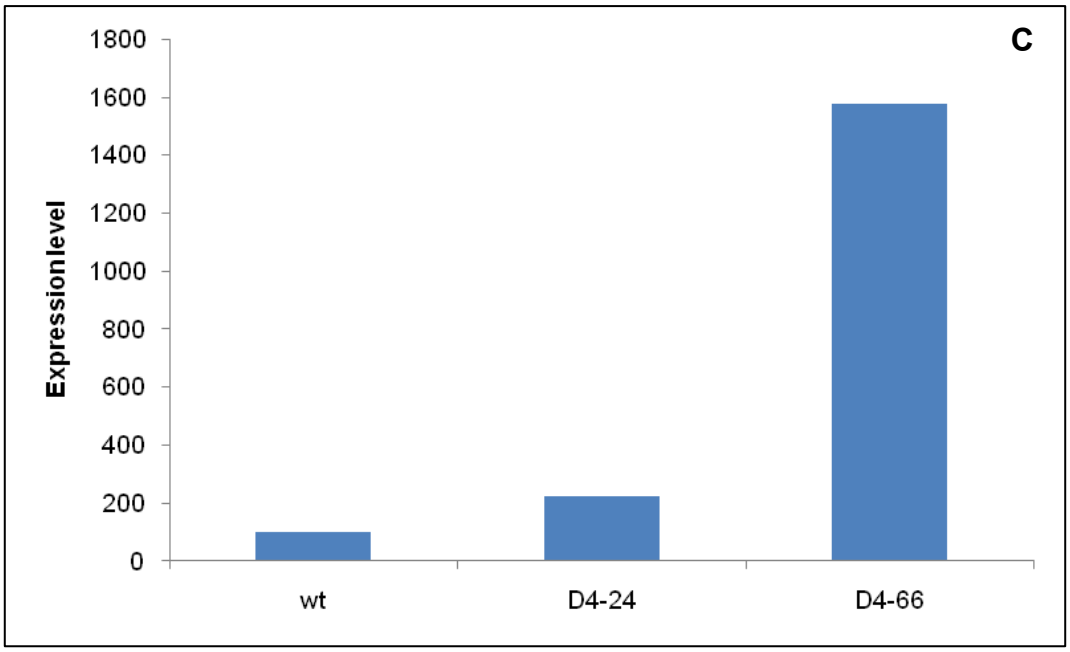


Figure 3.19. Expression level of *CrDGAT* in mutants during exponential growth phase. Data were the mean values of two replicates. **A)** *CrDGAT1* mutants; **B)** *CrDGAT3* mutants.



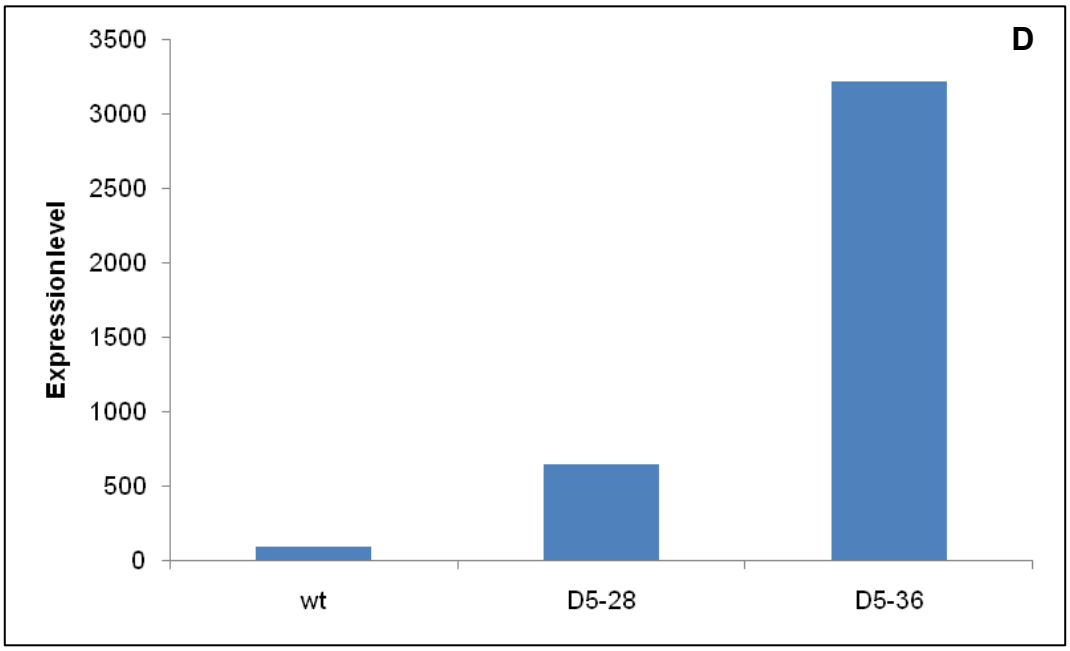


Figure 3.19. Expression level of *CrDGAT* in mutants during exponential growth phase. Data were the mean values of two replicates. **C)** *CrDGAT4* mutants; **D)** *CrDGAT5* mutants.

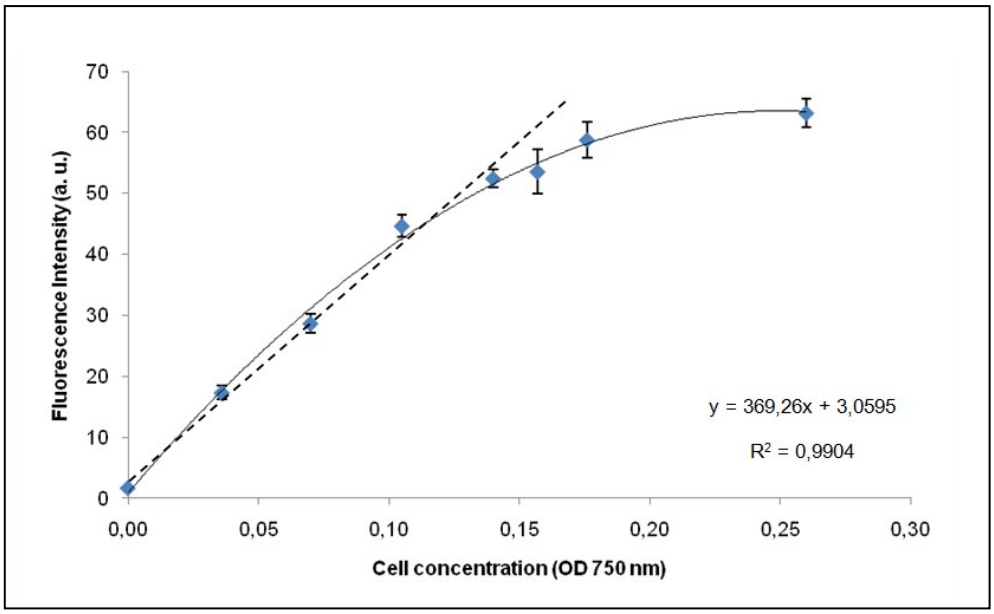


Figure 3.20. Effect of cell concentration on the fluorescence intensity of the green alga *C. reinhardtii* stained with Nile Red dye. The excitation and the emission wavelength used for fluorescence determination were 530 and 580 nm, respectively. Data were expressed as the mean value and standard deviation of three replicates.

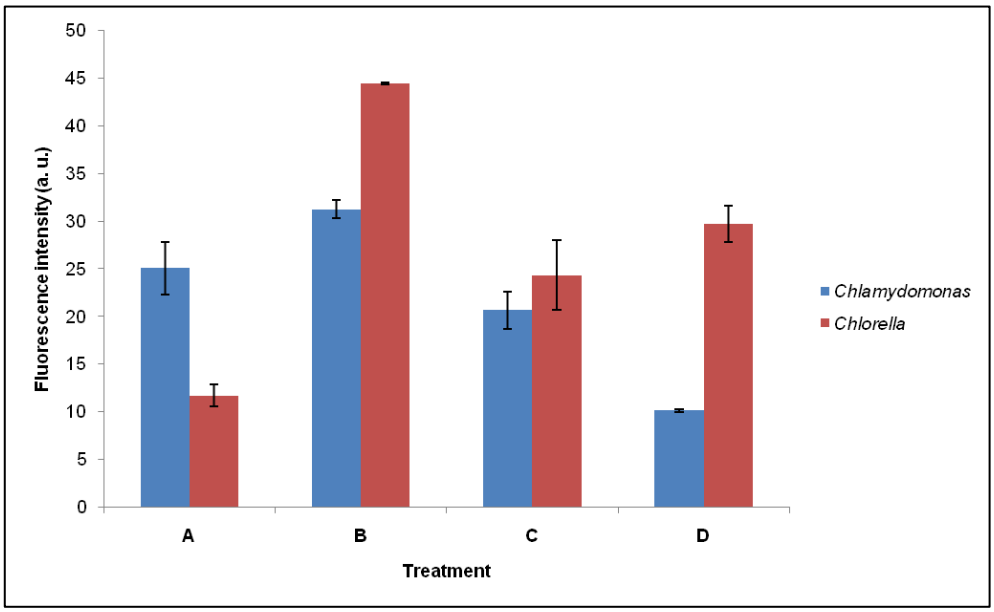


Figure 3.21. Fluorescence emission of the green algae *C. reinhardtii* and *C. vulgaris* cells subjected to different treatments prior to Nile Red staining: **A)** cell suspensions were dissolved in water; **B)** cell suspensions were treated with 20% DMSO (v/v); **C)** cells were vortexed 30 seconds with glass beads; **D)** cells were grinded in liquid nitrogen. Cell concentration for analysis was 0,05 OD_{750}. Data were the mean values and standard deviation of three replicates.

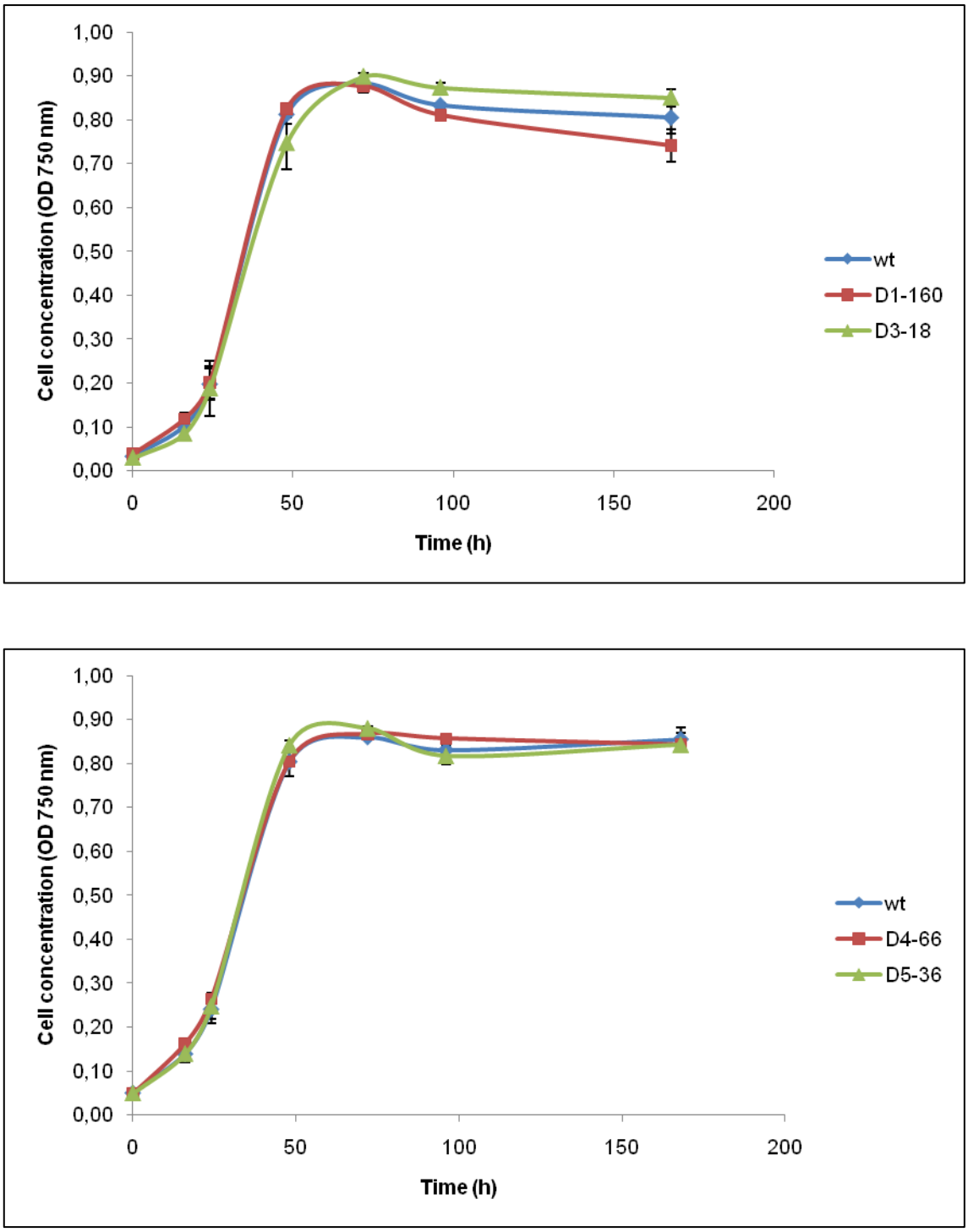


Figure 3.22. Cell concentration, expressed as optical density (OD_{750}), of cultures grown for 168 hours in standard condition. Data were the mean values and standard deviations of three replicates.

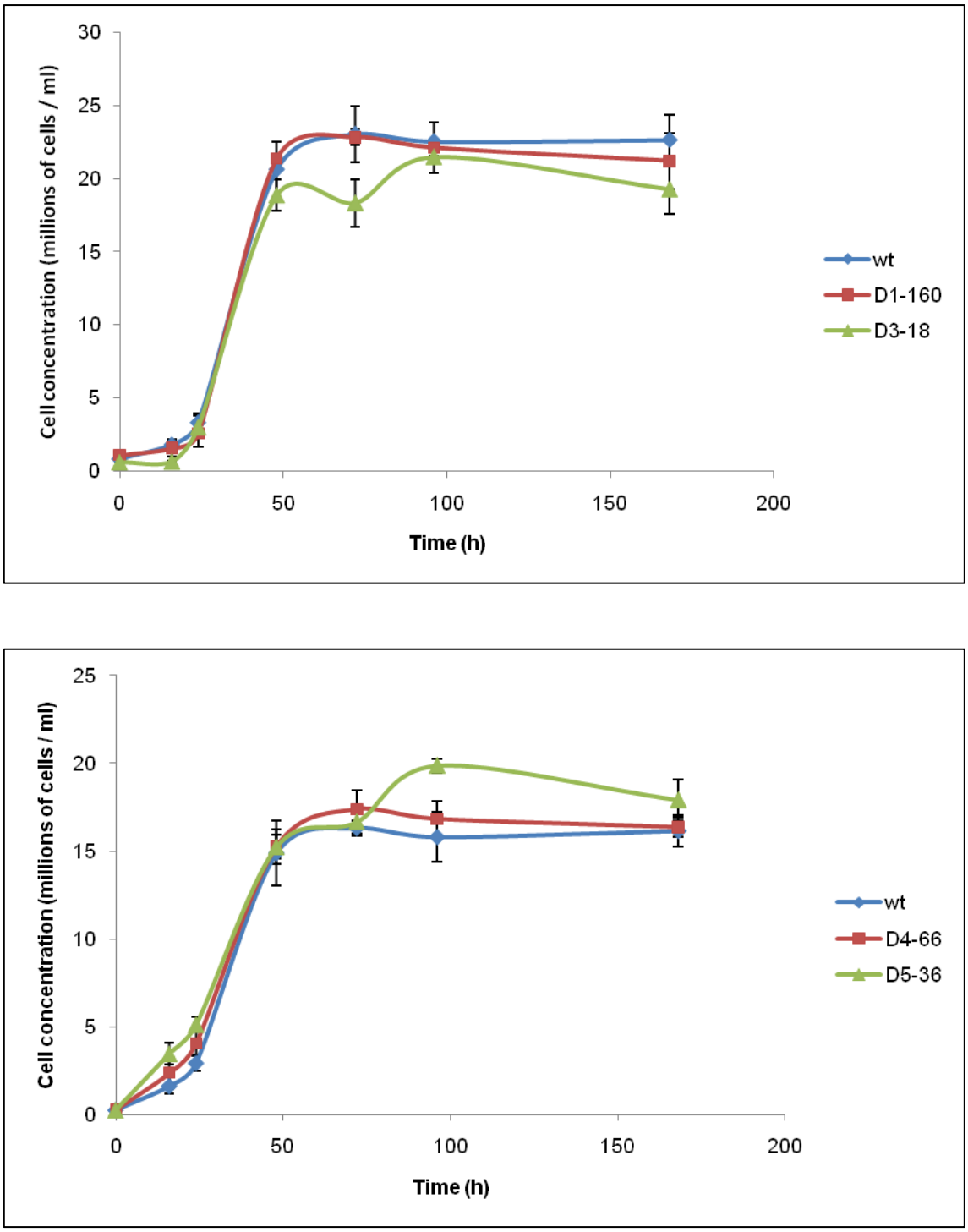


Figure 3.23. Cell concentration, expressed as millions of cells per ml^{-1}, of cultures grown for 168 hours in standard condition. Data were the mean values and standard deviations of three replicates.

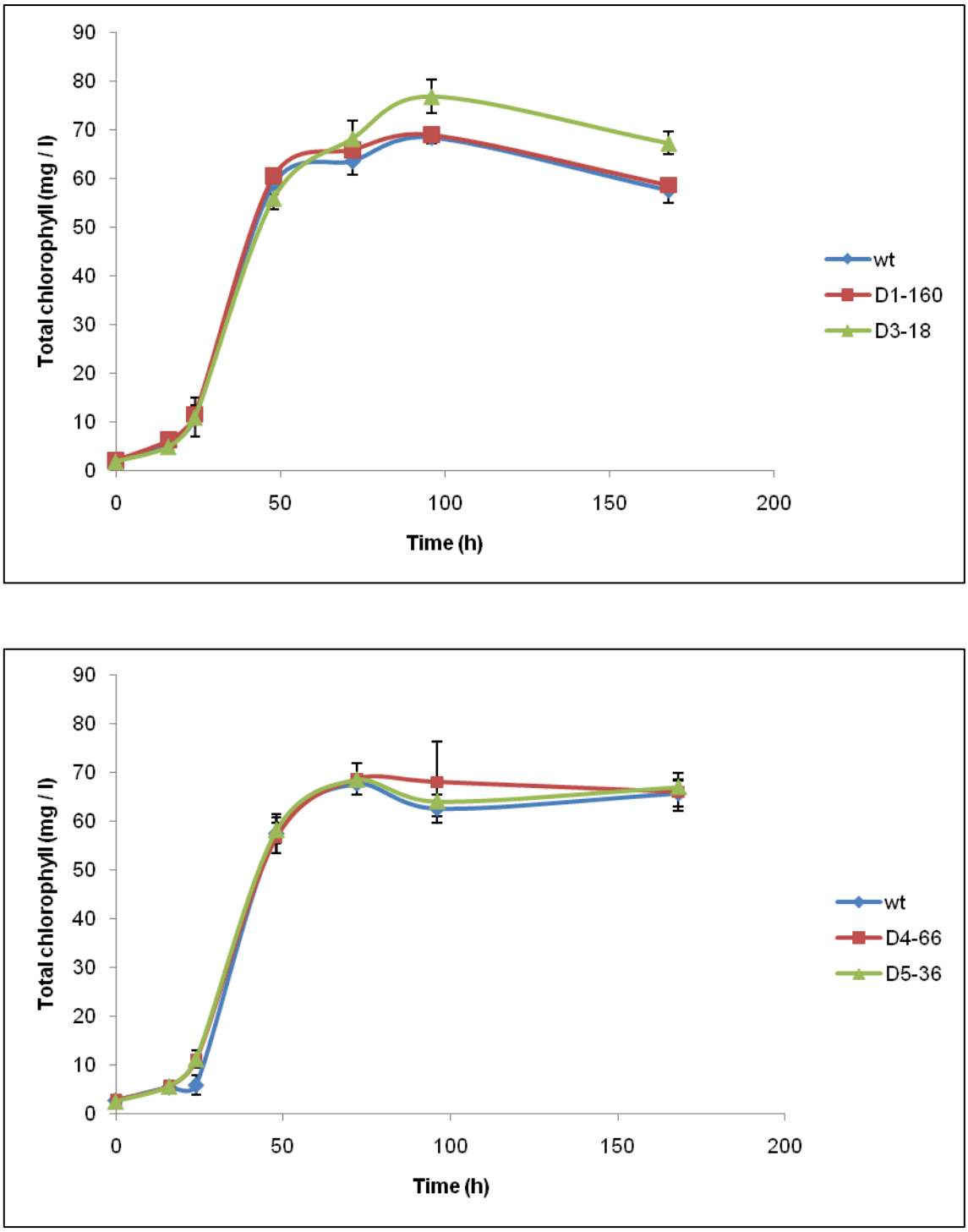


Figure 3.24. Total amount of chlorophyll, expressed as mg per l^{-1}, of cultures grown for 168 hours in standard condition. Data were the mean values and standard deviations of three replicates.

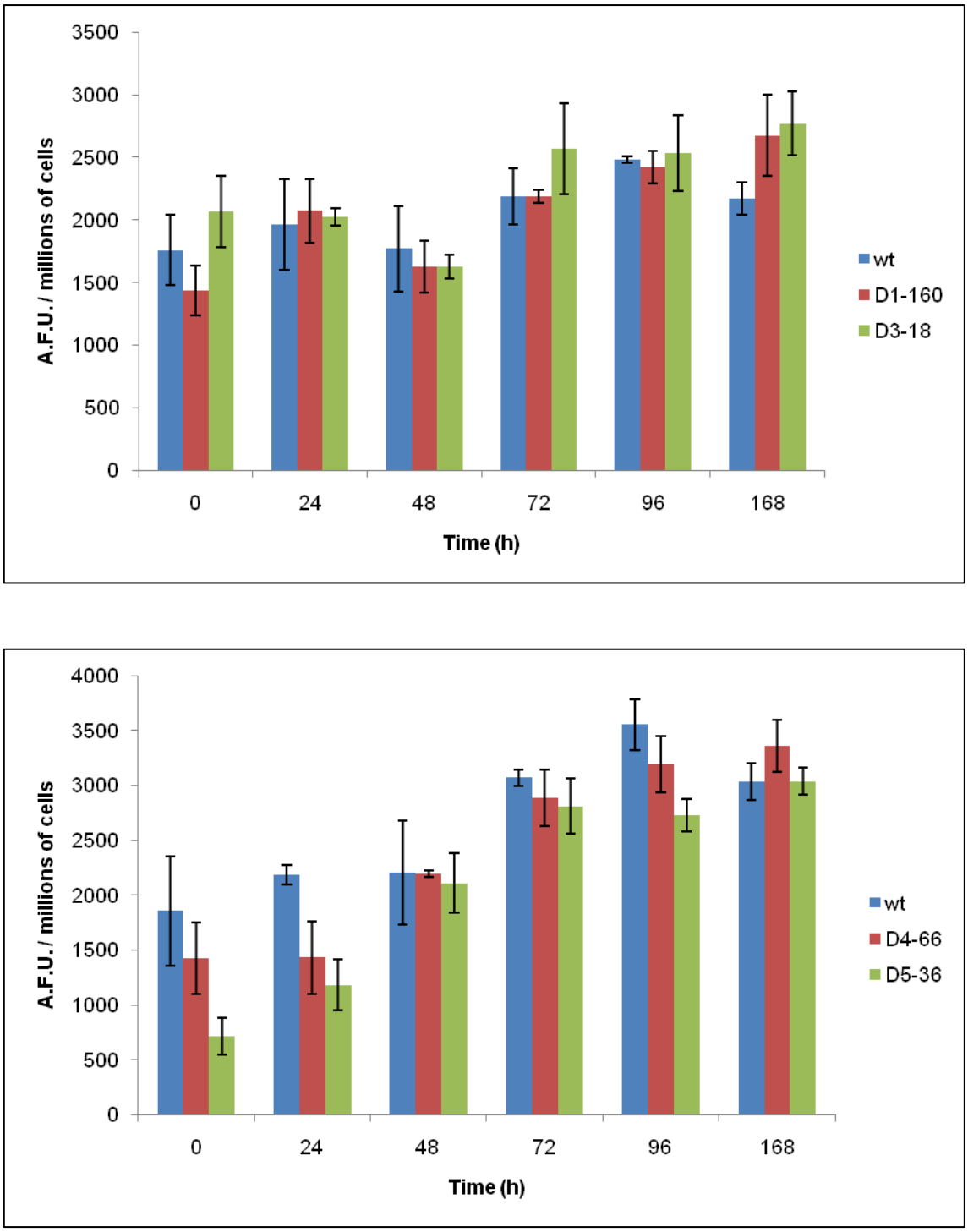


Figure 3.25. Fluorescence emission of Nile Red-stained cells grown for 168 hours in standard condition. Data were the mean values and standard deviations of three replicates.

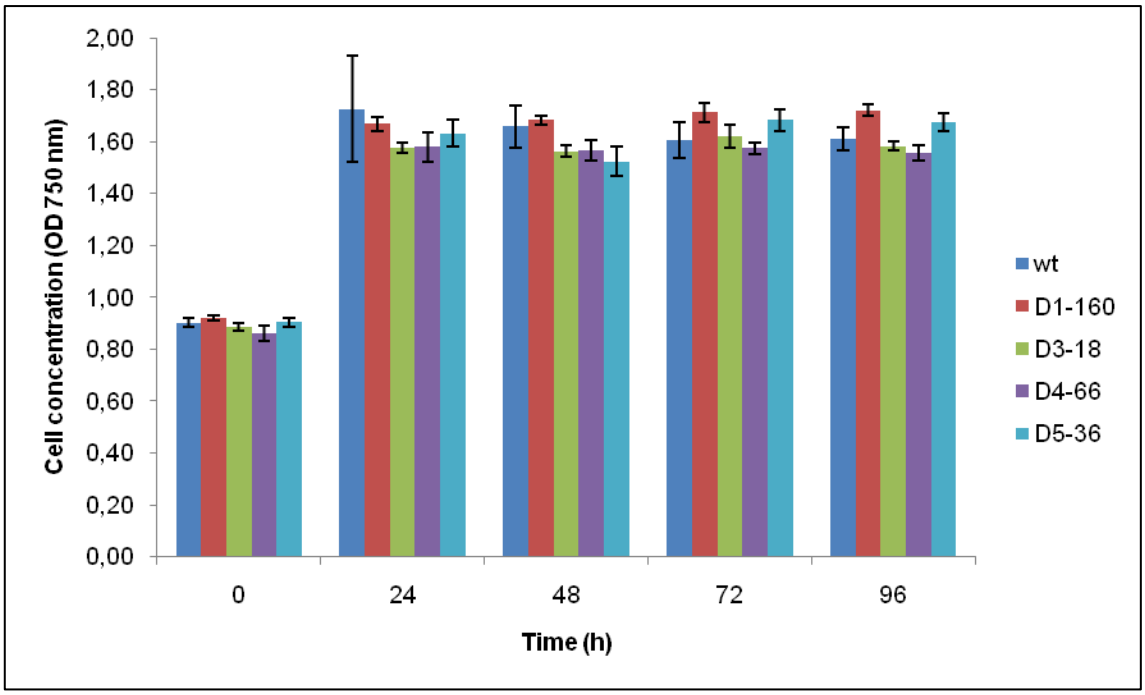


Figure 3.26. Cell concentration, expressed as optical density (OD_{750}), of cultures grown for 96 hours in nitrogen depletion. Data were the mean values and standard deviations of three replicates.

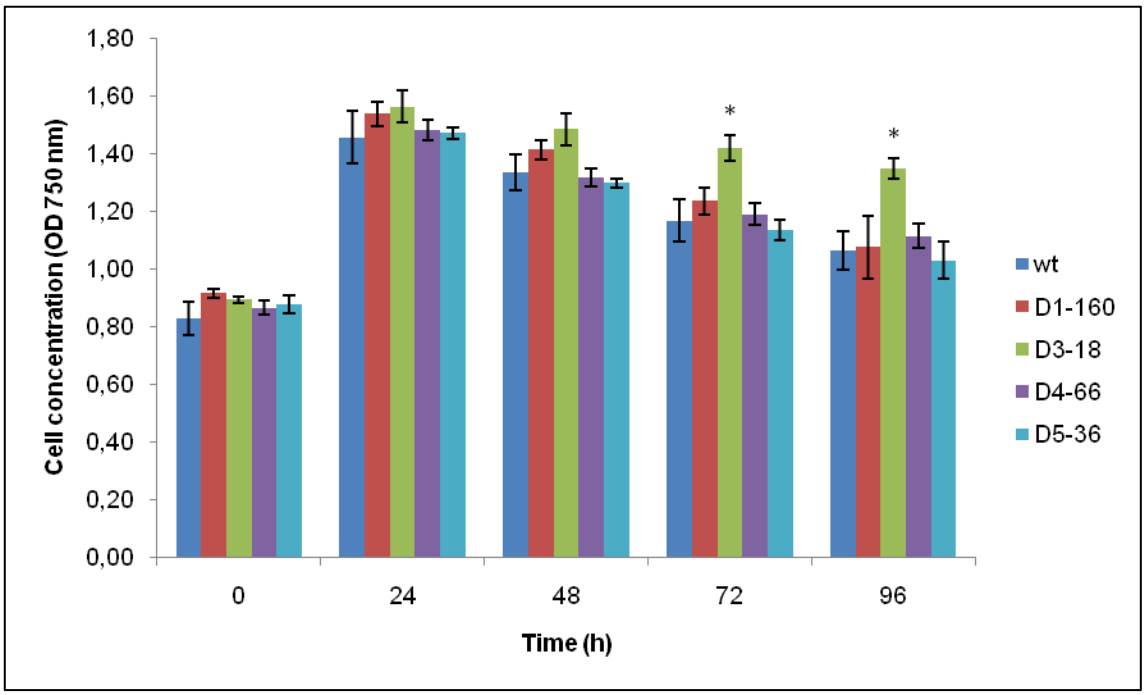


Figure 3.27. Cell concentration, expressed as optical density (OD_{750}), of cultures grown for 96 hours in sulphur depletion. Data were the mean values and standard deviations of three replicates. Stars indicate mean values significantly different from wild type ($p<0.05$).

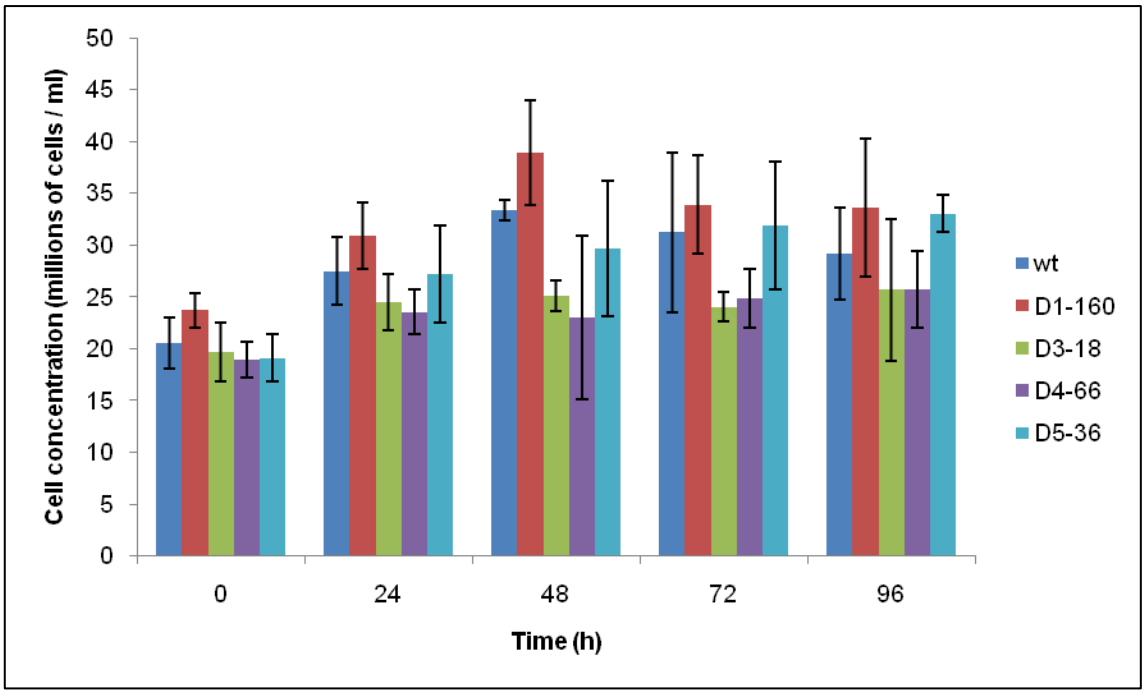


Figure 3.28. Cell concentration, expressed as millions of cells per ml^{-1}, of cultures grown for 96 hours during nitrogen depletion. Data were the mean values and standard deviations of three replicates.

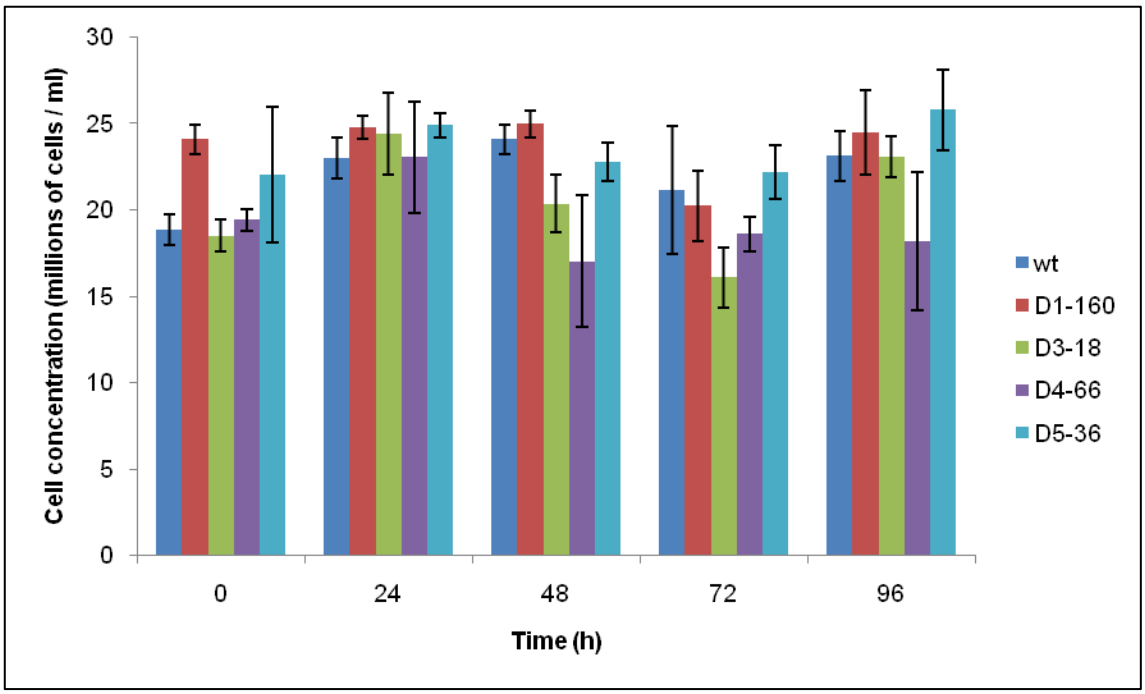


Figure 3.29. Cell concentration, expressed as millions of cells per ml^{-1}, of cultures grown during sulphur depletion for 96 hours. Data were the mean values and standard deviations of three replicates.

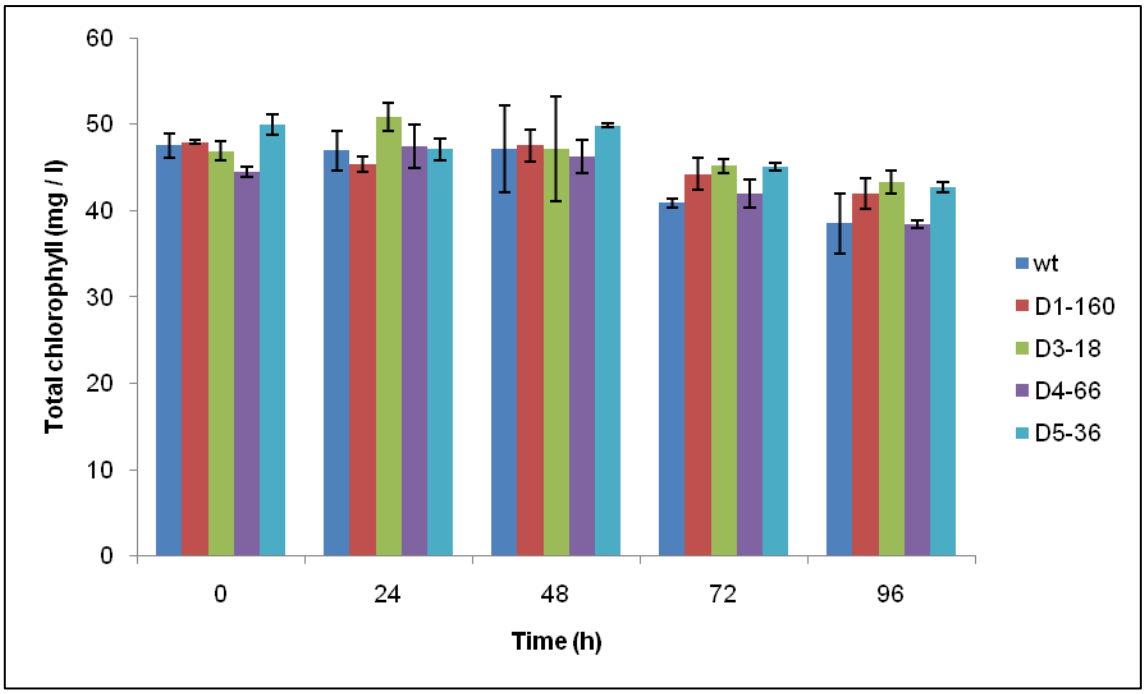


Figure 3.30. Total amount of chlorophyll, expressed as mg per l^{-1}, of cultures grown for 96 hours in nitrogen depletion. Data were the mean values and standard deviations of three replicates.

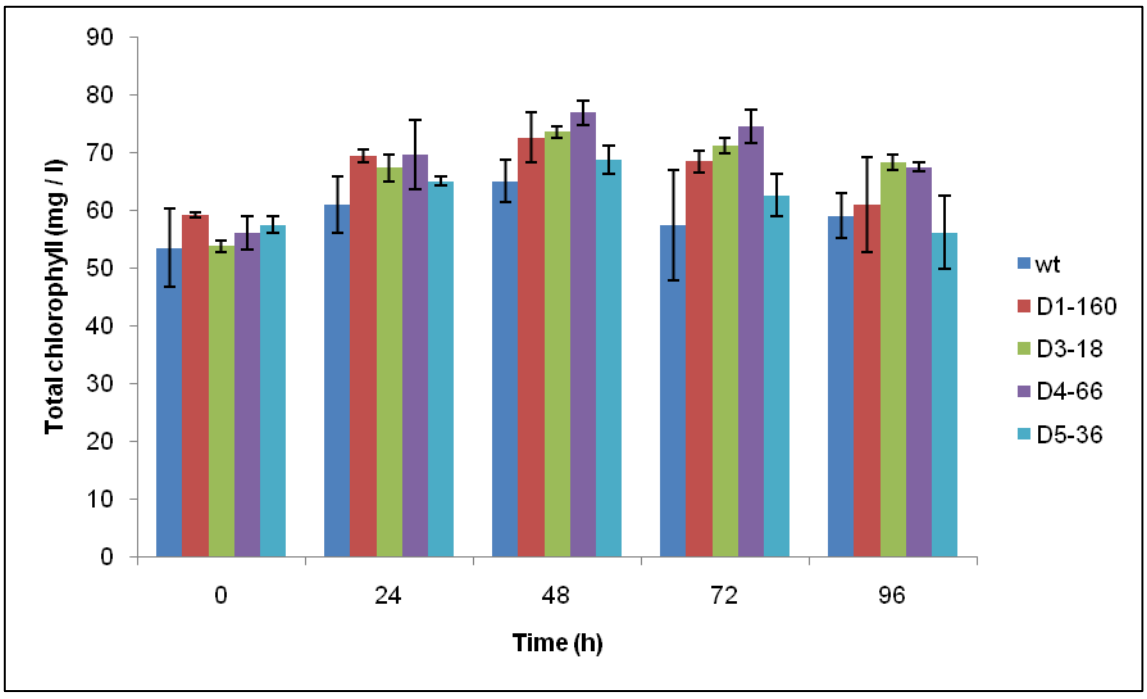


Figure 3.31. Total amount of chlorophyll, expressed as mg per l^{-1}, of cultures grown for 96 hours in sulphur depletion. Data were the mean values and standard deviations of three replicates.

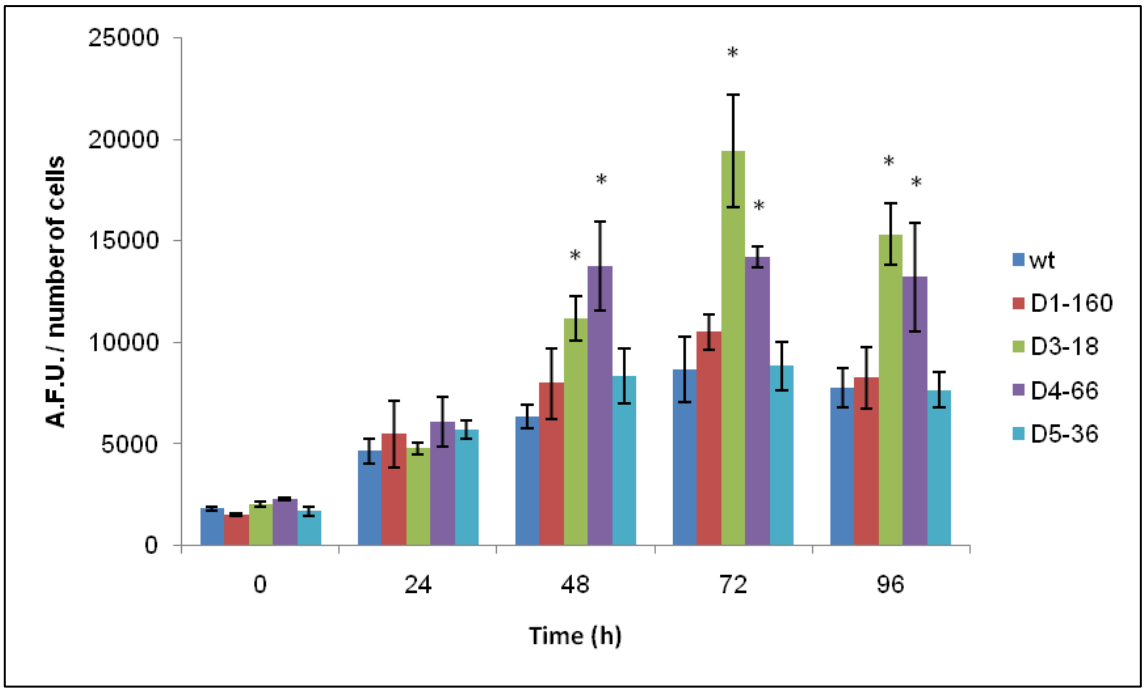


Figure 3.32. Fluorescence emission of Nile Red-stained cells grown for 96 hours in sulphur depletion. Data were the mean values and standard deviations of three replicates. Stars indicate mean values significantly different from wild type ($p<0.05$).

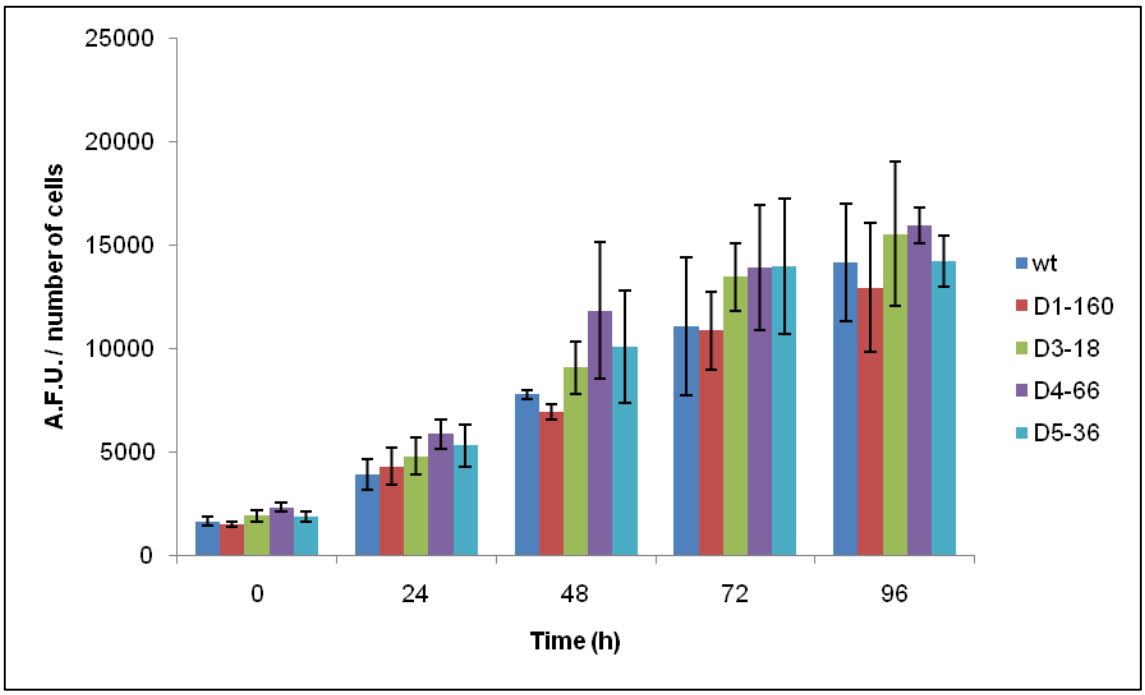


Figure 3.33. Fluorescence emission of Nile Red-stained cells grown for 96 hours in nitrogen depletion. Data were the mean values and standard deviations of three replicates.

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8. APPENDIX

Foreign experience: at the Algae Biotechnology & Bioenergy research group of the University of Bielefeld (Germany) since September 2008 to September 2009.

Publications:

- **La Russa M,** Palomba F, De Biasi MG, Pinto G, Pollio A, Filippone E. Microalghe con attività fenol-ossidasica extracellulare utili per il fitorisanamento di acque reflue. Giornate Scientifiche – Napoli, 22-23 settembre 2007

- **La Russa M,** Palomba F, De Biasi MG, Chiaiese P, Pinto G, Pollio A, Filippone E. Wastewaters phytoremediation: genomic analysis and screening of green microalgae species for extracellular laccase activity. 51° Congresso Annuale della Società Italiana di Genetica Agraria – Riva del Garda, 23-26 settembre 2007 – ISBN **978-88-900622-7-8**

- De Biasi MG, Palomba F, **La Russa M**, Chiaiese P, Pinto G, Pollio A, Filippone E. Extracellular laccase activity in several green microalgae species. IX Italian Federation Life Science Annual Congress – Riva del Garda, 26-30 settembre 2007

- Chiaiese P, **La Russa M,** De Biasi MG, Pollio A, Pinto G, Filippone E. Wastewater phycoremediation: screening of green microalgae aspecies for extracellular phenoloxidase activity. COST action 859 – Verona, 5-6 giugno 2008

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MICROALGHE CON ATTIVITA' FENOL-OSSIDASICA EXTRACELLULARE UTILI PER IL FITORISANAMENTO DI ACQUE REFLUE

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Il fitorisanamento consiste nell'impiego d'organismi verdi per la riduzione della presenza di sostanze inquinanti nell'ambiente. Recentemente, alcuni lavori hanno dimostrato come le microalghe possano essere impiegate per degradare sostanze inquinanti d'origine organica. D'altra parte, sono ancora pochi i lavori pubblicati che chiariscono quali siano gli enzimi coinvolti in quest'attività di degradazione. E' comunque importante rilevare che le Chlorophyceae sono rappresentate da ben 2.500 famiglie appartenenti a diversi gruppi tassonomici, che hanno colonizzato vari ambienti anche in condizioni estreme: quindi, c'è una notevole biodiversità che deve ancora essere del tutto studiata e che potrebbe essere utilmente impiegata per il fitorisanamento d'acque reflue. Presso il Dipartimento di Scienze Biologiche dell'Università di Napoli è disponibile un'ampia collezione di specie microalgali; abbiamo quindi dato inizio ad una ricerca volta a (a) saggiarne l'attività fenolossidasica extracellulare; (b) identificare enzimi extracellulari algali capaci di degradare xenobiotici quali coloranti sintetici ed IPA; (c) clonare e sovra-esprimere geni codificanti fenolossidati in sistemi omologhi ed eterologhi, principalmente per il fitorisanamento di acque di vegetazione provenienti da frantoi. Il nostro interesse è caduto sulle laccasi, un gruppo di fenolossidoriduttasi che sono capaci di catalizzare l'ossidazione di numerosi composti aromatici con la concomitante riduzione dell'ossigeno in acqua. Le specie algali selezionate sono state allevate in condizioni controllate in coltura liquida, partendo con un inoculo corrispondente a 0,1 OD a 600 nm. Dopo dieci giorni di coltura, per ogni specie ne è stata determinata la crescita attraverso lettura dell'OD. Il mezzo di coltura, privato delle cellule algali, è stato saggiato per attività laccasica attraverso saggio con ABTS e seguente lettura a 420 nm. Solo le specie algali mostranti la più alta attività laccasica sono state sottoposte ad altri saggi. L'attività laccasica è stata misurata in comparazione con l'attività della laccasi isolata dal fungo *Trametes versicolor*; quindi, l'attività laccasica è stata saggiata su substrati organici quali il colorante fenolico industriale di sintesi Remazol Brilliant Blue R (RBBR) ed un fenolo naturale (siringaldazina) con un'analisi della cinetica di degradazione. I risultati preliminari hanno mostrato che c'è differenza tra le specie algali nell'attività fenolossidasica sia nei confronti dell'ABTS sia dei composti organici saggiati. Infine, per clonare i geni delle fenolossidasi da queste specie algali, è stata eseguita un'analisi bioinformatica, allineando le sequenze di geni codificanti laccasi vegetali presenti in banca dati e costruendo coppie di primer in base alle sequenze maggiormente conservate ed a più alta identità. Le coppie di primer così disegnate sono state utilizzate per amplificare il DNA genomico delle specie algali risultate positive all'attività laccasica.

Proceedings of the 51st Italian Society of Agricultural Genetics Annual Congress
Riva del Garda, Italy – 23/26 September, 2007
ISBN **978-88-900622-7-8**

Poster Abstract – C.19

WASTEWATER PHYTOREMEDIATION: GENOMIC ANALYSIS AND SCREENING OF GREEN MICROALGAE SPECIES FOR EXTRACELLULAR LACCASE ACTIVITY

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microalgae, laccase, ABTS, phytoremediation

Phytoremediation deals with the use of plants, or other green photosynthetic organisms, to reduce organic or inorganic pollutant in the environment, mainly waters and soils (Pivetz. B.E. 2001. EPA
/540/S-01/500). Our research group is involved in studies regarding the use of plants for soil phytoremediation (Galante *et al.*, 2005. Proc. SIGA Congress, L04) and, more recently, of unicellular green algae species (green microalgae) for wastewater treatments. Some microalgae species have been recently tested to degrade an array of pollutants such as phenols, polyphenolic aromatic compounds (PAH) and even hormones (Pollio *et.al.*, 1994. Phytochemistry, *37*:1269-1272;. Pinto *et.al.*, 2003. Biotechnol Lett., *25*:1657-1659). It is worthy to note that about 2,500 species belong from Chlorophyceae, seldom living in contrasting habitat under severe environmental conditions. So far, a little has been done to exploit this genetic biodiversity bonanza; thus, few reports have been published on enzymes implicated in their degradative action (Semple *et.al.*, 1996. Appl. Envr. Micr, *62*:1265-1273).

Since a wide collection of green microalgae species are available at the University of Naples, Department of Biological Science, recently we have started a research aimed to (a) find algae species with extracellular phenoloxidase enzymatic activity; (b) identify extracellular enzymes able to degrade xenobiotic like synthetic dyes and other PAHs; (c) clone and overexpress genes producing phenoloxidases in homologous and in heterologous systems, in order to use these enzymes primarily for phytoremediation of milling oil wastewaters. Among phenoloxidaes, we focused our interest on laccases (EC 1.10.3.2) that are phenol-oxidoreductases able to catalyze the oxidation of various aromatic compounds (particularly phenols) with the concomitant reduction of oxygen to water.

Selected algae strains were grown in liquid culture at 22°C under continuous light conditions, starting with an inoculum of 0.1 OD. After ten days, the algal growth was measured as optical density at 600 nm. A screening was performed by detecting the laccase activity in the broth medium culture, deprived of algae cells, in the presence of 2,2-azino-bis 3-ethybenz-thiazoline-6-sulfonic acid (ABTS) at 420 nm. The laccase activity was referred to the polyphenol oxidase activity of *Trametes versicolor*; thus, each positive strain was assayed on industrial azo-dye Remazol Brilliant Blue R (RBBR) and on the natural phenol compound syringaldazine by kinetic analysis. Preliminary results, obtained comparing different species, showed a wide variation both within the same substrate and among the different microalgae.

Microalgae strains able to produce and secrete laccase enzymes were further chosen for more detailed genetic studies. To clone phenoloxidase genes from those species, we have started a bio-informatics approach, on the basis of highly conserved coding sequences of laccases already isolated and sequenced from several higher plants. Primers drawn on the alignment of those sequences have been used to amplify genomic DNA.

EXTRACELLULAR LACCASE ACTIVITY IN SEVERAL GREEN MICROALGAE SPECIES

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Photosynthetic organisms have been reported as useful tools for remediation of polluted environment. In the recent years, microalgae have been used to degrade an array of pollutants such as phenols and polyphenolic aromatic compounds (PAH); however, few reports have been published so far on enzymes implicated in this degradative action. In the other hand, Chlorophyceae have been classified in about 2.500 species belonging from different taxonomic groups, living in contrasting habitat, seldom under a severe environmental conditions: thus, they represent a source of biodiversity to be exploited for water phytoremediation.
Since a wide collection of algae species are available at the University of Naples, Department of Biology Science, recently we have started a research aimed to (a) find algae species with extracellular phenoloxidase enzymatic activity; (b) identify extracellular enzymes able to degrade xenobiotic like synthetic dyes and other PAHs; (c) clone and overexpress genes producing phenoloxidases in homologous and in heterologous systems, in order to use these enzymes primarily for phytoremediation of milling oil wastewaters. Among phenoloxidaes, we focused our interest on laccases (EC 1.10.3.2) that are phenol-oxidoreductases able to catalyze the oxidation of various aromatic compounds (particularly phenols) with the concomitant reduction of oxygen to water.
Selected algae strains were grown in liquid culture at 22°C under continuous light conditions, starting with an inoculum of 0.1 OD. After ten days, the algal growth was measured as optical density at 600 nm. A screening was performed by detecting the laccase activity in the broth medium culture, deprived of algae cells, in the presence of 2,2-azino-bis 3-ethybenz-thiazoline-6-sulfonic acid (ABTS) at 420 nm. Algae strains able to produce and secrete laccase enzymes were further chosen for more detailed studies. The laccase activity was referred to the polyphenol oxidase activity of *Trametes versicolor*; thus, each positive strain was assayed on industrial azo-dye Remazol Brilliant Blue R (RBBR) and on the natural phenol compound syringaldazine by kinetic analysis. Preliminary results, obtained comparing different species, showed a wide variation both within the same substrate and among the different microalgae.
To clone phenoloxidase genes from those algae species, we have started a bio-informatics approach, on the basis of highly conserved coding sequences of laccases already isolated and sequenced from several higher plants. Primers drawn on the alignment of those sequences have been used to amplify genomic DNA.

WASTEWATER PHYCOREMEDIATION: SCREENING OF GREEN MICROALGAE SPECIES FOR EXTRACELLULAR PHENOLOXIDASE ACTIVITY

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Objectives
Agro-industrial activities seldom release in the environment xenobiotic compounds that can be toxic for human and animal health and are not easy to degrade. In nature, organisms are able to degrade or completely remove pollutants: among them, microalgae have shown a certain degree of degradation of pollutants such as phenols, PAHs and even hormones. Thus, *phycoremediation* has been defined as the use of algae, mainly unicellular ones (microalgae), to reduce organic or inorganic pollutant content in waters. Since in Southern Italy are active several olive oil mills and tanning plants, producing a large amount of wastewaters rich of phenolic compounds, our research is aimed to identify microalgae species with strong extracellular phenoloxidase enzymatic activity and able to degrade xenobiotic like polyphenols, synthetic dyes and PAHs, in order to use them in wastewaters phycoremediation.

Materials and Methods
A collection of more than 300 different green microalgae species, available in our laboratory, has been submitted to a wide screening for extracellular phenoloxidase activity. So far, 100 microalgae strains culture broth has been tested for enzymatic activity in the presence of: ABTS; an industrial azo-dye (RBBR); syringaldazine, and 2,6-dimethoxyphenol (2,6-DMP). Microalgae were grown in liquid culture medium at 22°C under continuous light conditions, starting with an inoculum of 0.1 OD. After ten days, the algal growth was measured as optical density at 550 nm. A first screening round was performed on the broth medium culture, deprived of microalgae cells, in the presence of ABTS. Selected strains, showing the highest phenoloxidase activity measured by spectrophotometric assay at 420 nm, were further characterised for their degrading ability on the other three above-mentioned substances, always by means of spectrophotometric assays.

Results
Sixteen microalgae strains were selected after the first screening round. Among those strains, one was able to degrade RBBR (50 microM) to 50% in 72 hrs only, and another was capable to degrade both syringaldazine (10 microM) and 2,6-DMP (10 mM) after 15 minutes more efficiently than purified laccase from *Trametes versicolor* used as control at the concentration of 0,6 micro grams/ml. More analyses are in course to screen more microalgae strains from our collection and to identify extracellular enzymes involved in the phenol-oxidase degrading activity. Moreover, selected strains will be assayed on olive-mill wastewaters, in order to verify their potential in phycoremediation strategy.

extracellular phenoloxidase activity, microalgae, phycoremediation, screening

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ID 074

SCREENING OF GREEN MICROALGAE SPECIES FOR EXTRACELLULAR PHENOLOXIDASE ACTIVITY USEFUL FOR WASTEWATER PHYCOREMEDIATION

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ABSTRACT

In Mediterranean area there are several olive oil mills and tanning plants producing a large amount of wastewaters rich of phenolic compounds. Our research group is aimed to identify microalgae species with extracellular phenoloxidase activity to use them in wastewaters phycoremediation. In order to achieve our goal, we have started a screening of a microalgae collection available at the University of Naples (Department of Biological Science). So far, one hundred microalgae strains have been tested for enzymatic extracellular activity in the presence of: 2,2-azino-bis 3-ethybenz-thiazoline-6-sulfonic acid (ABTS); Remazol Brilliant Blue R (RBBR); syringaldazine; 2,6-dimethoxyphenol (2,6-DMP). Among them, two strains have shown promising results for phenols degradations.

1. INTRODUCTION

Industrial activities release in the environment a large quantity of xenobiotic compounds that can be toxic for human health and are not easy to degrade. However, in nature are present organisms capable to degrade or completely remove a lot of those pollutants. Among them, microalgae have been suggested as bioremediation tool for wastewater treatments, since they have shown to degrade varying pollutants such as phenol compounds and even hormones [3, 4]. Thus, phycoremediation is defined as the use of algae, mainly unicellular ones (microalgae), to reduce organic or inorganic pollutant content in waters. Despite the fact that *Chlorophyceae* are classified in 2,500 species, seldom living in contrasting habitat under severe environmental conditions, only few of those are used as remediation tool. Since a wide collection of green microalgae species is available at the University of Naples, Department of Biological Science, recently we have started a research aimed to find algae species with extracellular phenoloxidase activity and to identify extracellular enzymes able to degrade phenolic compounds and other xenobiotics like synthetic dyes.

2. MATERIALS AND METHODS

2.1 Algal cultures

Microalgae were grown in 50 ml of inorganic liquid culture (Bold Basal Medium) in 100 ml flask. Initial culture density was set up about 0.1 OD_{550}. Culture were left growth on a shaking apparatus which rocked them at a rate of 68 cycles per minutes as described by Shihira and Krauss [5] in a climate chamber at 23±2°C under continuous light conditions provided by fluorescent lamps (Philips TLD, 30W/55).

Algal growth was evaluated by growth index (G.I.) that was defined as: $(OD_{550}\ t_{10} - OD_{550}\ t_0) / OD_{550}\ t_0$, whereas $OD_{550}\ t_0$ was the culture absorbance at the inoculum time and $OD_{550}\ t_{10}$ was the culture absorbance 10 days after the inoculum.

2.2 Enzymatic assays

Enzymatic assays were carried out on supernatant obtained from 10 days-old microalgae cultures after centrifugation (4.000 rpm for 10 min at 4°C).

ABTS assay was carried out on 20 µl of supernatant in 200 µl of 1 mM ABTS and 100 mM Na-citrate buffer, pH 3.0. The reaction was followed measuring the absorbance at 420 nm using a Wallac Victor Multireader until 10 min at 25°C. Only algal culture supernatants showing phenol-oxidoreductase activity were chosen for more detailed studies.

2,6-DMP oxidation assay was performed on 1 ml solution containing 500 µl of the supernatant, 10 mM 2,6-DMP and 100 mM Na-tartrate buffer, pH 5.0 [6]. Oxidation of 2,6-DMP was followed by the absorbance increase at 469 nm.

Syringaldazine oxidation assay was performed on 1 ml solution containing 500 µl of the supernatant, 10 µM syringaldazine and 25 mM phosphate buffer, pH 6.5. Oxidation of syringaldazine was followed by the absorbance increase at 525 nm [1]. The latter two reactions were followed until 10 min at 25°C by using a Beckman spectrophotometer.

The RBBR decolourization assay was performed on 1 ml solution containing 750 µl of supernatant in 100 µM RBBR dissolved in 20 mM Na-acetate buffer, pH 4.5 [2]. The mixture reaction was incubate at 25°C and the decolourization was followed until 72 hours at 592 nm by using a Beckman spectrophotometer.

In order to compare the efficiency of different supernatants in presence of ABTS, 2,6-DMP and syringaldazine, we defined an efficiency index (E.I.) expressed as the ratio between the absorbance variation, detected at the end of reaction, and the G.I. For the RBBR assay we have also expressed the results as percentage of dye decolourization.

3. RESULTS AND DISCUSSION

After 10 days, the culture OD_{550} values were different among microalgae strains, as we expected. The growth index (G.I.) ranged continuously between 0.16 and 9.77.

Phenol- oxidase activity in presence of ABTS was find out in almost all microalgae supernatant, allowing us to group them in three classes: low activity, 84 strains, OD_{420} increase values of 0.1 ± 0.1; medium activity, 8 strains, OD_{420} 0.9 ± 0.3; high activity, 8 strains, OD_{420} 2.6 ± 0.7. On the basis of these results, we made a choice to perform the further experiments with microalgae supernatants belonging to the medium and high classes only. Those selected supernatants belonged to microalgae genera *Chlamydomonas*, *Chlorella* and *Scenedesmus*. Regarding the efficiency, two out sixteen samples (#78, #42) showed a significantly higher E.I., about 3.5 times than others (Figure 1).

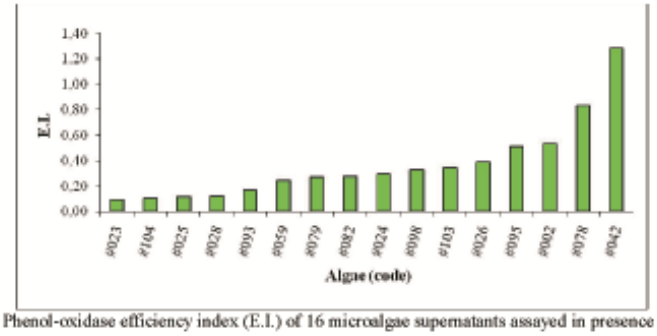

Figure 1. Phenol-oxidase efficiency index (E.I.) of 16 microalgae supernatants assayed in presence of ABTS.

In presence of 2,6-DMP the microalgae supernatants #42 and #79 showed the higher E.I. values, about 6 times more than others (Figure 2A). Instead, for the syringaldazine assay the best results were obtained with the microalgae supernatants #78 and #79; they showed average values about 4 times higher than the others (Figure 2B). Finally, the microalga supernatant #95 ranked the best RBBR-E.I. absolute value, about 3.5 times higher than the others (Figure 2C). This sample was able to reduce 50% of the initial concentration of RBBR in 72 hours (Figure 3). Twelve tested microalgae supernatants ranked the same in both decolourization results and E.I. values, whilst samples #02, #26, #59 and #98 showed a different pattern. The latter result confirmed that E.I. parameter described the capability of microalgae supernatants to degrade phenolic compounds better than the absorbance variation alone.

Comparing all data assays (Figure 2) no one microalgae supernatants ranked to the same position. However, in average, supernatants #95, produced by a strain of *Chlorella*, performed better than others for all compounds tested, while supernatants #79, produced by a strain of *Chlamydomonas*, ranked first for 2,6-DMP and syringaldazine but ineffective in RBBR decolourization.

Other analyses are now in course in order to screen more microalgae strains from our collection and to identify extracellular enzymes and their genes involved in phenols degradation. Moreover, selected strains will be assayed on olive-mill wastewaters, in order to verify their potential in phycoremediation strategy.

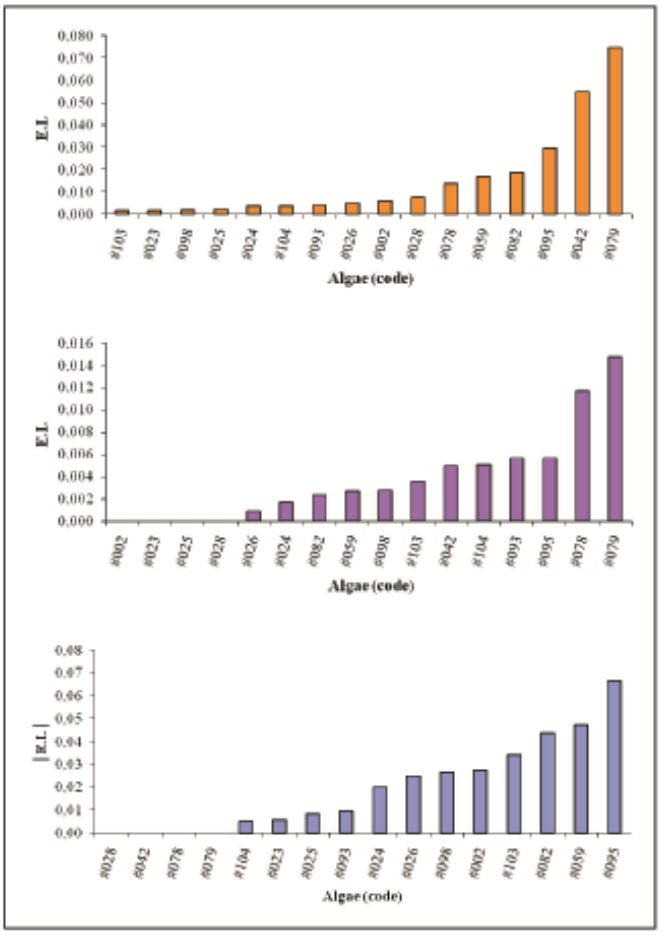

Figure 2. Phenol-oxidase efficiency index (E.I.) of 16 microalgae supernatants assayed in presence of 2,6-DMP (A), syringaldazine (B) and RBBR (C).

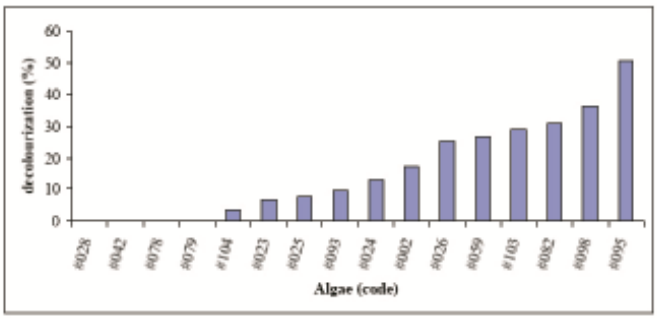

Figure 3. Decolourization of RBBR (%) by the sixteen selected microalgae supernatants

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