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INVESTIGATION OF FRESHWATER MICROALGAE FOR THEIR BIOACTIVE COMPOUNDS

By
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REQUIREMENTS FOR THE DEGREE OF DOCTOR
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APPROVAL

This dissertation satisfies all requirements for the degree of Doctor of Philosophy

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DECLARATION

I declare that this dissertation is written in accordance with the standards of the Karadeniz Technical University, Graduate School of Health Sciences thesis writing guide. I declare that this dissertation is the result of my own and that all sources or materials used for this dissertation have been duly acknowledged. I confidently declare that this dissertation has not submitted to this or any other university for the award of any academic degree, diploma or certificate.

02/05/2018

Sana TABBOUCHE

DEDICATION

To the friend, the brother and to the leader: Dr. Khaled Kamaleddine

To my special friend and sister: Neslihan Yılmaz Uyanık.

Mom & Dad

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ANOVA	Analysis of variances
3-oxo-HSL	N-(3-oxohexanoyl)-HSL
AHL	N-Acyl homoserine lactone
AIP	Auto-inducing peptide
AmB	Amphotericin B
AMP	Ampicillin
ATCC	American Type Culture Collection
B1	Thiamin
B12	Cobaltamine vitamin
B7	Biotine vitamin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CFU	Colony forming units
CH4	Methane
CLSI	Clinical & Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CFM	Comité Française de Microbiologie
CN	Gentamicin
CO_2	Carbone dioxide
CTAB	Hexadecyl Trimethyl ammonium bromide
ddH2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid
FAME	Methyl ester fatty acid
EtBr	Ethidium bromide
f.a.u	Fluorescence arbitrary unit
FL	Optical filter light detector
FSC	Forward scatter signal detector
g	Gram
GC	Gas chromatography
GHG	Greenhouse gases

Gt	Giga tons
HPLC	High-pressure liquid chromatography
HSD	Honestly Significant Difference
HSL	Homoserine lactone
L	Liter
LB	Luria Bertani
M	Molar
MeOH	Methanol
mg	Milligram
$MgCl_2$	Magnesium dichloride
MH	Mueller Hinton
MIC	Minimal inhibitory concentration
ml	Milliliter
mM	Millimolar
mm	Millimeter
m	Meter
MS	Mass spectrometry
nm	Nanometer
MW	Molecular weigh
NaCl	Sodium Chloride
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
O_2	Oxygen
OD	Optical density
PAR	Photosynthetic active radiations
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
PTFE	Polytetrafluoroethylene
PUFA	Poly unsaturated fatty acid
QS	Quorum sensing
rDNA	Ribosomal DNA
rpm	Round per minute
SDS	Sodium dodecyl sulfate
sp.	Species
SSC	Side scatter signal detector

TAE	Tris-EDTA-acetic acid
TAGs	Triacylglycerols
TLC	Thin-layer chromatography
TNF-α	Tumor necrosis factor-alpha
Tris-HCl	Tris-hydrochloride
U	Unit
V	Volt
β	Beta
%	Percent
°C	Degree centigrade
µl	Microliter
µm	Micrometer
µM	Micromolar
h	Hour
min	Minute
s	Second

1. ABSTRACT

Investigation of Freshwater Microalgae for Their Bioactive Compounds

Microalgal researches have increased recently due to their bioactive compounds like pharmaceuticals and nutraceuticals, which contributed to the microalgal industrial production. Today microalgae are known to harbor variety of bioactive molecules such as fat and fatty acids, proteins, carbohydrates, pigments, minerals, vitamins, sterols, and antioxidants. The aims of this study were to isolate and identify freshwater microalgae from different regions of Turkey and screen their metabolites for antimicrobial and anti-quorum sensing activities. In this study, 64 fresh water microalgal strains were isolated and characterized using standard methods. Three strains were identified as Cyanophyta, two strains belong to Bacillariophyta, one strain belong to Ochrophyta and the rest were members of phylum Chlorophyta. Fourteen strains were identified by partial sequence of the 18S rDNA using universal primers. Organic extraction of 34 strains were carried out using methanol and n-hexane, and the extracts were tested for their anti-microbial and anti-quorum sensing activities. The organic extracts from six isolates showed antimicrobial activity against *Acinetobacter haemolyticus,* nine extracts were active against *Bacillus subtilis*, and *Candida albicans*, three extracts were active against *Enterococcus faecalis* and one extract inhibited the growth of *Klebsiella pneumonia* with minimal inhibitory concentrations (MIC) between 3.9 and 1,250 µg/ml. The crude extracts of two strains at 78.13 µg/ml concentrations inhibited the pigment production of *Chromobacterium violaceum* strain as indication of anti-quorum sensing activity. Ten strains showed moderate to high content of lipid contents by Nile red staining and flow cytometry. In conclusion, the newly isolated and identified microalgae may be a potential source for new antimicrobials and anti-quorum sensing inhibitors that may be formulated into novel agents to combat infections.

Key Words: Antimicrobial Activity, Anti-Quorum Sensing Activity, Lipid Content, Microalgae

2. ÖZET

Tatlı Sulardan İzole Edilen Mikroalglerin Biyoaktif Madde Yönünden Araştırılması

Mikroalgal araştırmalar, ilaç ve nutrasötikler gibi biyoaktif bileşikleri taşımaları ve sanayi üretimine katkıları nedeniyle ile son yıllarda artmıştır. Bugün mikroalglerin, yağ ve yağ asitleri, proteinler, karbonhidratlar, pigmentler, mineraller, vitaminler, steroller ve antioksidanlar gibi çeşitli biyoaktif molekülleri ürettiği bilinmektedir. Bu çalışmanın amacı, Türkiye'nin farklı bölgelerinden tatlı su mikroalglerini izole ederek tanımlamak ve ürettikleri metabolitleri antimikrobiyal ve anti-quorum sensing aktiviteleri yönünden taramaktı. Bu çalışmada, 64 mikroalg suşu izole edilmiş ve standart yöntemler kullanılarak karakterize edilmiştir. Üç suş Cyanophyta, iki suş Bacillariophyta, bir suş Ochrophyta ve geri kalanları Chlorophyta filumlari olarak tanımlandı On dört suş, 18S rDNA'nın kısmi dizisi ile tanımlandı. Metanol ve n-heksan kullanılarak 34 suştan elde edilen ekstreler antimikrobiyal ve anti-quorum sensing aktiviteleri için test edildi. Altı izolattan elde edilen organik ekstraktlar *Acinetobacter haemolyticus*'a, dokuz ekstrakt *Bacillus subtilis* ve *Candida albicans'a,* üç ekstrakt *Enterococcus faecalis'*e ve bir ekstrakt da 3.9 ile 1250 ug / ml minimal inhibitör konsantrasyonlarla (MIC) *Klebsiella pnömoni'*ye karşı inhibitör etki gösterdi. İki suşun ham ekstrelerinin 78.13 µg/ml konsantrasyonlarda anti-quorum sensing aktivite göstergesi olarak *Chromobacterium violaceum* suşunun pigment üretimini inhibe ettiği saptandı. On suşun, Nil kırmızı boyaması ve akış sitometrisi ile orta ila yüksek oranda lipid içerdiği gösterildi. Sonuç olarak, yeni izole edilen ve tanımlanan mikroalgler enfeksiyonlarla mücadelede yeni antimikrobiyal ve anti-quorum inhibitörlerin formüle edilebilebileceği potansiyel bir kaynak olabilir.

Anahtar Kelimeler: Antimikrobiyal Aktivite, Anti-Quorum Sensing Aktivitesi, Lipit İçeriği, Mikroalg

3. INTRODUCTION AND AIMS

After the 1950s, with the medicinal and the industrial development, the world population rapidly increased and it was important to find alternative to the nutrient resources to supply the associated increased human needs. Algal species like *Nostoc* sp., *Arthrospira* sp. *(Spirulina* sp.) and *Aphanizomenon* sp. have been used in the early history as food and medicine (1). Then in the last decades, researches showed big potentials of microalgae as mean of bioactive compounds and industrial capacities. These findings attracted the attention of scientists toward microalgal biomass cultivation and industrial uses (1, 2). Today, human and animal foods, pharmaceuticals, cosmetics, biofuel, bioplastic and other compounds are produced from microalgae at commercial levels (3-5). Moreover, the progress of biotechnology, molecular biology and genetics permitted a better understanding of the algal physiology and the optimization of some species cultivation to ameliorate their benefits. Furthermore, many studies today are centered on developing a recombinant expression system in microalgae in the aim of producing recombinant proteins (2, 4).

On the other hand, the industrial progress and the modern lifestyle we are living today is going through maximizing our energy consumption, which is mostly of non-renewable resources like petrol, coal and natural gas. These resources are depleted day after day and they seem to be insufficient to supply the global needs in the near future (6).

The depletion of the petroleum energy is not the only reason propelling to the research of alternative energy resources. Fuel energy is an essential cause of the atmospheric pollution mainly by the greenhouse gases (GHG) emission and the accumulation of CO_2 and other toxic materials in the atmosphere, which contributed lately to alarming consequences such as the global warming (6, 7). For these reasons, investigations for alternative green fuel are gaining importance and some industries have already started using crops and seeds like sunflower, soybean and sugarcanes to produce biodiesel and bioethanol (8). Most of these resources are part of the human nutrition, thus because of the aforementioned population growth problem, a food non-competitive natural biofuel is in need (8). Today, microalgae are showing a big potential as a biofuel resource with zero toxicity and no GHG production (9).

Lately, algae have been excessively investigated for bioactive compounds and were found to involve lipids, proteins, polysaccharides and carbohydrates (9), pigments (10), minerals, vitamins (11), antioxidants (12) and polyphenolic metabolites (13). Moreover, some microalgal species are shown to have antioxidant and antitumor (14), anti-coagulant, anti-inflammatory, anti-microbial, anti-hypertensive, anti-diabetic (15) and algicidal activities as well as other pharmaceutical and nutraceutical abilities which make them great candidates for the next level of medicinal and pharmaceutical industry (16-18).

The aim of this study was to isolate different freshwater microalgae in different regions of Turkey and screen their compounds for antimicrobial and anti-quorum sensing activities. In addition, their neutral lipids contents were investigated using flow cytometry after staining with Nile red.

4. LITTERATURE REVIEWS

4.1. Microalgae

Algae are polyphyletic photosynthetic organisms that differ from plants by lacking roots, leaves, stems, and vascular networks. Macroscopic algae are similar to marine plant whereas microalgae are mostly unicellular and they are swimming or sliding microscopic organisms that constitute the phytoplankton of the marine ecosystem (19).

Algae comprise more than 30,000 species documented (http://www.algaebase.org) (20) and more than 43,000 species waiting for characterization (19). This large variability is the result of a heterogeneous assemblage of organisms evolving since the beginning of the life on our planet. Although the topic is still under discussion (21), it is believed that the non-oxygenic cyanobacterial ancestors were in charge of the formation of oxygen on the earth by their photosynthesis mechanism, using the atmospheric CO_2 as a primary product and producing O_2 as photosynthetic waste (22). The first eukaryotic microalgal cell is hypothesized to be generated by the symbiotic combination of a heterotrophic plankton or parasite with a photosynthetic cyanobacteria where this latter evolved thereafter to constitute the plastid (22).

4.2. Classification of Microalgae

The variability and the polyphyletic nature of microalgae make their classification less coherent with the traditional taxonomic sorting methods. In fact, the algal variability appears in every trait during the cell life cycle: in their morphology and structure, in their physiology, pigments and photosynthesis, in their reserves and motility. Microalgal cells may have a diameter of picometers like the case of unicellular phytoplankton or 60.m of length like the giant pelagic seaweeds (19). This parameter may also be extremely variable inside the same species especially during the different growth phases of the cell. Every one of the previously mentioned traits may be characteristic for the identification and the classification of algae and they are still used today as an essential step for the identification of genera and, in some cases, species. However, they are insufficient for the segregation at the subspecies grade and other molecular methods should be applied, particularly sequencing of the 18S ribosomal

DNA genes (23, 24). Despite all, we still do not have a universal classification method that integrates all the algal taxonomic groups.

Taking in consideration the different microalgal classes and identification methods, a microalgal species may belong to one of the four kingdoms: Bacteria, Plantae, Chromista and Protozoa (25) (Figure 1). Cyanobacteria are the only prokaryotic microalgae and, except for the photosynthetic characters, they are physiologically similar to the classical marine bacteria. As for the eukaryotic species, four algal phyla belong to the kingdom of Plantae, four belong to the Chromista and two phyla are part of the Protozoa. The different algal classes comprising the 11 phyla are shown in the Table 1, and their related pictures are listed in the Figure 2.

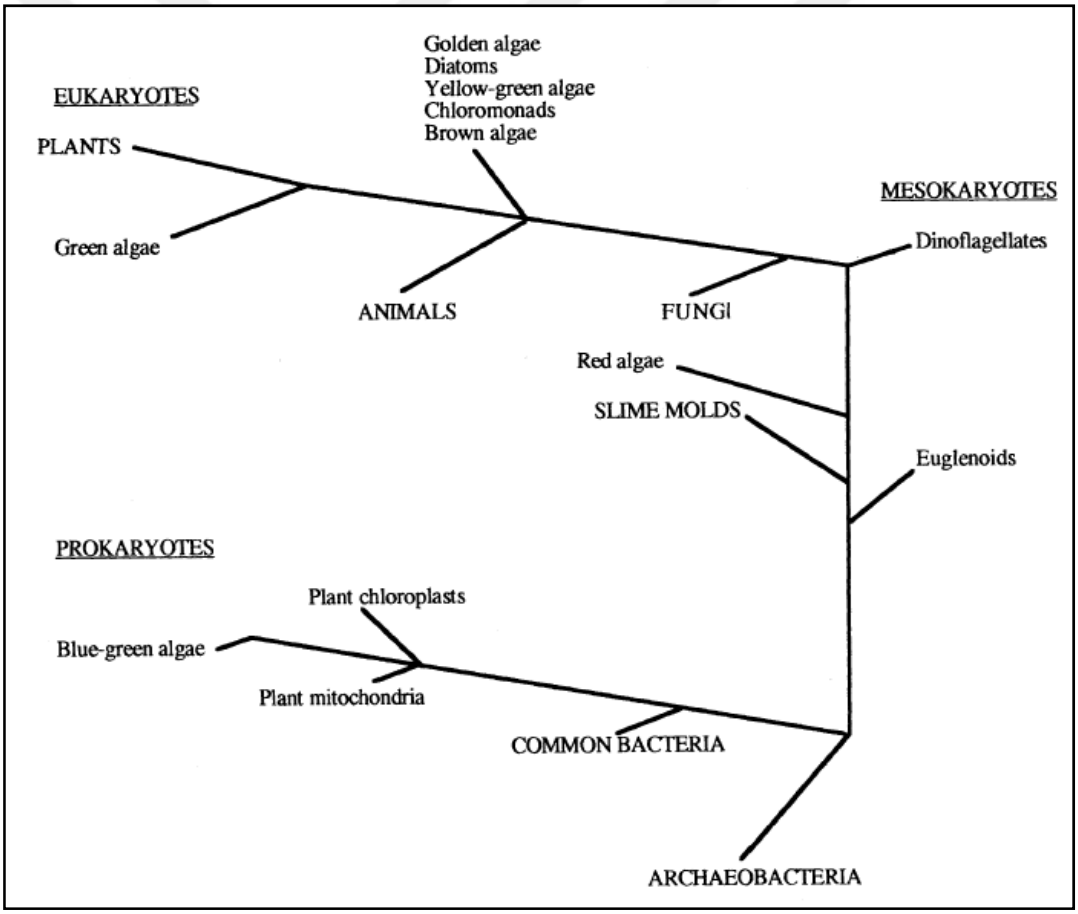


Figure 1. Phylogenetic tree based on the 18S ribosomal DNA sequences showing the variability of the microalgal taxonomic relations with plants, animals, bacteria and fungi (25).

Table 1. Classification of the different algal groups (19).

	Kingdom	Phylum	Class	Representative	Image*
Prokaryota	Bacteria	Cyanobacteria	Cyanophyceae	*Arthrospira*	a
Eukaryota	Plantae	Glaucophyta	Glaucophyceae	*Cyanophora*	b
		Rhodophyta	Bangiophyceae	*Porphyra*	c
			Compsopogonophyceae	*Erythrocladia*	d
			Cyanidiophyceae	*Cyanodioschyzon*	e
			Florideophyceae	*Phyllophora*	f
			Porphyridiophyceae	*Porphyridium*	g
			Rhodellophyceae	*Glaucosphaera*	h
			Stylonematophyceae	*Stylonema*	i
		Chlorophyta	Prasinophytes	*Pyramimonas*	l
			Mamiellophyceae	*Crustomastix*	m
			Nephroselmidophyceae	*Nephroselmis*	n
			Pedinophyceae	*Pedinomonas*	o
			Chlorodendrophyceae	*Tetraselmis*	p
			Chlorophyceae	*Scenedesmus*	q
			Ulvophyceae	*Ulva*	r
			Trebouxiophyceae	*Chlorella*	s
			Dasycladophyceae	*Acetabularia*	t
			Palmophyllales	*Palmophyllum*	u
		Charophyta	Mesostigmatophyceae	*Mesostigma*	v
			Chlorokybophyceae	*Chlorokybus*	z
			Klebsormidiophyceae	*Klebsormidium*	aa
			Charophyceae	*Nitella*	ab
			Coleochaetophyceae	*Coleochaete*	ac
			Zygnematophyceae	*Cosmarium*	ad
	Chromista	Haptophyta	Coccolithophyceae (Prymnesiophyceae)	*Umbellosphaera*	ae
			Haptophyta incerta sedis	*Coronocyclus*	af
			Pavlovophyceae	*Pavlova*	ag
		Cryptophyta	Cryptophyceae	*Rhodomonas*	ah
		Ochrophyta	Chryseophyceae	*Ochromonas*	ai
			Xanthophyceae	*Vaucheria*	al
			Eustigmatophyceae	*Nannochloropsis*	am
			Bacillariophyceae	*Cylindrotheca*	an
			Raphidophyceae	*Heterosigma*	ao
			Dictyochophyceae	*Distephanus*	ap
			Phaeophyceae	*Ascophyllium*	aq
			Pelagophyceae	*Chrysophaeum*	ar
			Bolidophyceae	*Tetraparma*	as
			Schizocladiophyceae	*Schizocladia*	at
			Chrysomerophyceae	*Gyraudiopsis*	au
			Picophagophyceae	*Picophagus*	av
			Pinguiophyceae	*Pinguiococcus*	az
			Placidiophyceae	*Placidia*	ba
			Phaeothamniophyceae	*Phaeothamnion*	bb
			Synchromophyceae	*Synchroma*	bc
			Synurophyceae	*Synura*	bd
			Aurearenophyceae	*Aurearena*	be
		Cercozoa	Chlorarachniophyceae	*Gymnochlora*	bf
	Protozoa	Myzozoa	Dinophyceae	*Porocentrum*	bg
				Lepidodinium	bh
		Euglenozoa	Euglenophyceae	*Euglena*	bi
				Phacus	bl
				Trachelomonas	bm
				Peranema	bn

* Related images are shown in Figure 2.

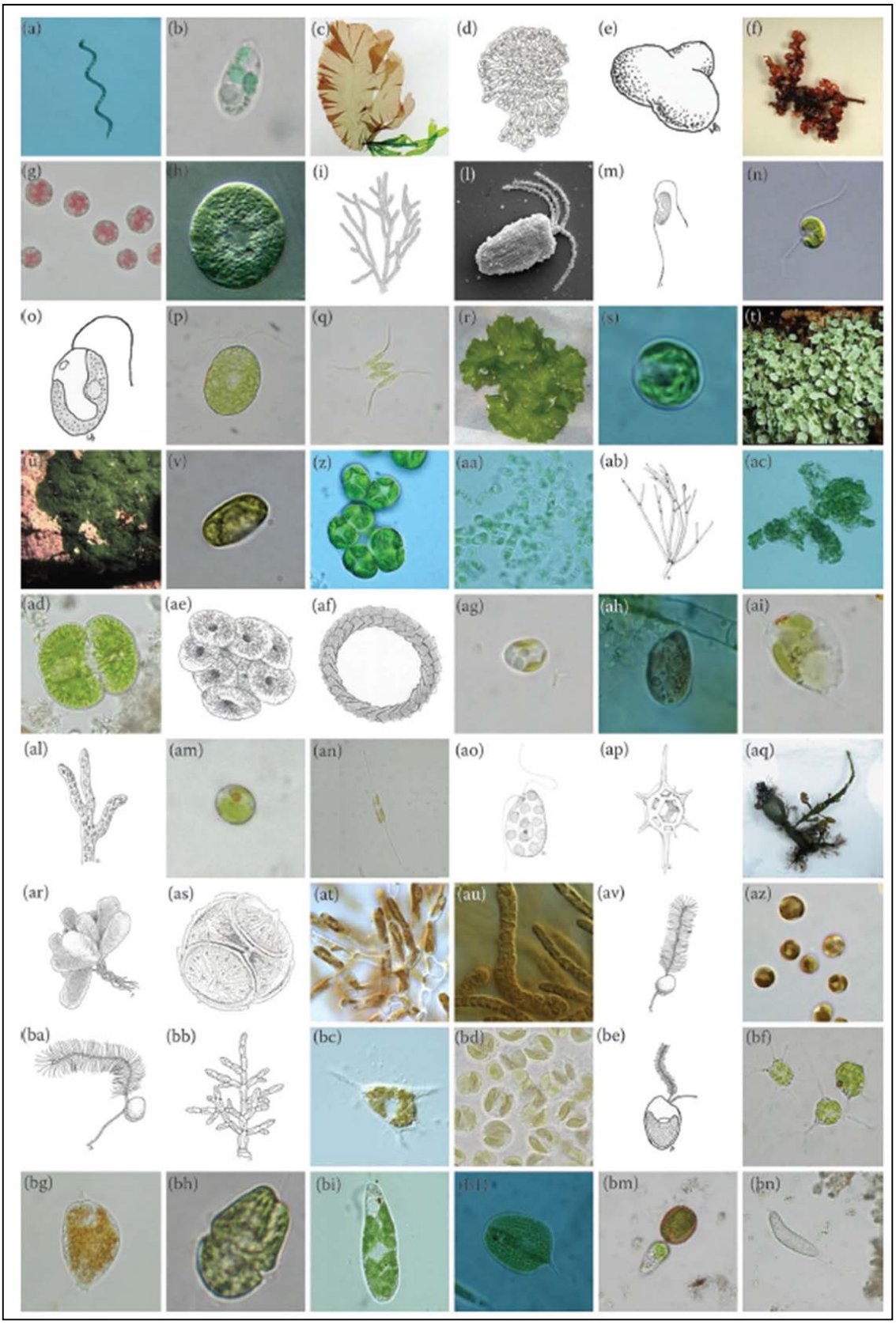


Figure 2. Images of the different algal classes (19). For legend, see Table 1.

Table 2. The different divisions of algae with their habitats (19)

Phylum	Common name	Marine	Fresh water	Terrestrial	Symbiotic
Cyanobacteria	Blue-green algae	Yes	Yes	Yes	Yes
Glaucophyta	n.a.	n.d.	Yes	Yes	Yes
Rhodophyta	Red algae	Yes	Yes	Yes	Yes
Chlorophyta	Green algae	Yes	Yes	Yes	Yes
Charophyta	n.a.	Yes	Yes	Yes	n.d.
Haptophyta	Coccolithophorids	Yes	Yes	Yes	Yes
Cryptophyta	Cryptomonads	Yes	Yes	n.d.	Yes
Ochrophyta	Golden algae Yellow-green algae Diatoms Brown algae	Yes	Yes	Yes	Yes
Cercozoa (Chlorarachniophycae)	n.a	Yes	n.d.	n.d.	Yes
Myzozoa (Dynophyceae)	Dynoflagellates	Yes	Yes	n.d.	Yes
Euglenozoa (Euglenophyceae)	Euglenoids	Yes	Yes	Yes	Yes

n.d., not detected; n.a., not available

4.3. Ecological Role of Microalgae

The diversity of algae is also reflected by the variability of their living conditions and growth environments. Aquatic algae may be found in marine environment and in fresh waters. They may be found in the oceans, lakes, rivers and almost everywhere even in hot or ice-cold regions (26, 27). Algae may also be subaerial and may be found in soils, on stones, over sands and deserts or (28) or in caves (29), on the trees and plants, on the urban building's walls (30) and even in the atmospheric spaces at 2000.m heights (31). They have a high tolerance to grow under variable conditions such as pH, temperature, turbidity, O_2 and CO_2 concentrations. Moreover, some heterotrophic or

mixotrophic algal strains are able to live in darkness using carbon or other elements as sole nutrient source (Table 2) (19).

Algae play an important ecological role since they constitute basic elements of the marine food chain and efficiently participate in the marine life cycles (32). They are main player in the formation and the conservation of the atmospheric oxygen balance where more than 45% of the planet photosynthetic oxygenic production is of algal origin. In addition, toxic algal species may grow in the polluted environments and form algal bloom, which are an essential reason of the death of birds, fishes, other photosynthetic organisms and may be toxic to human (33).

Microalgae are also professional mutualistic living creatures and they may grow in symbiotic state either with fungi (lichens) or with bacteria providing oxygen and complex nutrients to their partners and receiving in return physical protection or primitive nutrients (34). This mutualism provides for the symbiotic complex the endurance to survive the hard-living conditions and the ability to resist the dangerous situations from the external world (34).

4.4. Photosynthesis in Microalgae

One of the important characteristics of microalgae is their photoautotrophic capabilities, which provide them the energy required to synthesize their own nutrients. The microalgal cell contains pigments that absorb the photosynthetic active radiations (PAR) of the solar light and transform them into chemical energy for their own sake. PAR are the part of the visible light and their wavelengths range between 400.nm and 700.nm (35).

Pigments are chromophores that are excited by a determined wavelength of the visible light. This excitation will affect the atomic electrons movement, and will change their location from a lower to a higher energy orbital, and then when electrons come back to their original orbit they will emit another light energy with longer wavelength. Every chromophore has its specific excitation and emission wavelength ranges and this is what determines their color (35). In higher plants and algae, chlorophylls constitute the principal pigment and are responsible for the photosynthesis of light energy (35).

Beside chlorophylls, carotenoids also help in the photosynthetic light absorption mechanism. They constitute with the chlorophylls some protein complexes that harvest

the light and transform it into chemical energy (36). Carotenoids are also chromophores and have their own excitation and emission wavelengths (36).

Algae participate to the half of the global photosynthesis process using PAR to produce biological energy by a process of redox reaction (37):

$$CO_2 + H_2O + \text{nutrients} + \text{light energy} \rightarrow \text{Biomass} + O_2.$$

4.5. Bioactive Compounds from Microalgae

As microorganisms, microalgae are almost present everywhere on the planet and are part of the different ecosystems in the world; they have lived and evolved in different environments facing highly competitive conditions and they have been exposed to different grazers and microorganisms, thus they were obligated to find their ways to resist and survive. Allelopathy is a process where microalgae produce molecules called allelochemicals that kill or affect the other organisms. After been tested in the laboratories, some allelochemicals produced by algae showed to have pharmaceutical and biological effects that may be beneficial for human (38). Microalgae were found to involve important bioactive compounds like pigments, enzymes, polysaccharides, lipid and fatty acids, sterols, and vitamins that may be used as pharmaceuticals, cosmetics, nutraceuticals, functional foods, bioenergy resources and other industrial materials (38).

4.5.1. Microalgae as Functional Foods

Microalgal biomass is rich in pigments like chlorophylls and carotenoids that may be used for multiple purposes in human health (39). Some pigments for example constitute essential vitamins for human like the β-carotene, which is a vitamin A precursor, tocopherol E and ascorbic acid (40). Algae may also contain riboflavin, niacin, biotin, nicotinic acid, pantothenic acid, pyridoxine and folic acid thus they may constitute a valuable multi-vitamin food supplement (40). Pigments may also be consumed for the prevention of some degenerative and chronic diseases like cardiovascular diseases and cancer (39).

Pigments may also be used as natural dyes for food. In food industry, the carotenoids are widely used for their coloring capacity (40). The most famous are β-carotene and astaxanthin that are used in food industry for their coloration functions and

astaxanthin is particularly used in salmon and trout aquacultures. Lutein and β-carotene are also used in poultry for their yellow-orange coloration, which improves the coloring of the egg-yolk (40).

4.5.2. Pharmaceutic Metabolites in Microalgae

Microalgae include many bioactive metabolites with anti-cancer, anti-obesity, anti-inflammatory and anti-hypertensive activities with many other pharmaceutical effects (40, 41).

As for the anti-cancer metabolites, plenty of studies were done giving promising results (42). Microalgae are rich in phenolic compounds, which are known for their high anti-oxidant activity and they may provide some prevention for cancer suspected patients (43). Moreover, most of the microalgal pigments are anti-oxidant and help to fight and to prevent the development of cancerous cells (39). Chlorophyllin for example, which is a chlorophyll derivative, was reported to have anti-colon cancer activity when tested on human HCT116 cancer cells (44). On the other hand, some microalgal species like *Fucus vesiculosus* and *Adenocystis utricularis* were shown to contain sulfated polysaccharides that are characterized by their anti-cancer effects (14, 45). Furthermore, carrageenan, fucoidan, ulvan and other marine polysaccharides were isolated from red, brown and green algae (41) and were also demonstrated to have antioxidant, anti-allergic, anti-microbial, anti-inflammatory, anti-coagulant, anti-lipidemic and anti-cancer activities (9, 46). The fatty alcohol ester nonyl 8- acetoxy-6-methyl octanoate (NAMO) was isolated from *Phaeodactylum tricornutum*, and showed positive anti-cancer activity when tested on human leukemia (HL-60), lung carcinoma (A549) and on mouse melanoma (B16F10) cells (47). Adenosine isolated from the same diatom strain was shown to have anti-arrhythmic activity thus may be used to treat tachycardia (48).

The red microalgae *Phorphyridium* sp. contain polysaccharides with anti-inflammation activity that inhibits the activation of the nuclear factor-*κ*B (NF-κB) in the pro-inflammatory cytokine Tumor Necrosis factor-alpha (TNF-α) tested on human coronary artery endothelial cells (49). Monogalactosyl diacyl glycerols and digalactosyl diacylglycerols isolated from the marine green microalga *Isochrysis galbana* showed

positive anti-inflammatory activity by inhibiting the production of the TNF-α in the lipopolysaccharide-stimulated human (LPS) THP-1 macrophages (50).

Similarly, violaxanthin isolated from *Chlorella ellipsoidea* has anti-inflammatory activity by inhibiting the NF-*κ*B pathways by inhibiting the LPS-mediated NF-*κ*B.p65 subunit translocation into the nucleus. Moreover, violaxanthin showed inhibitory effect on the production of nitric oxide (NO) and of prostaglandin E2 (PGE2) (51). Carotenoids are also good stimulators of the immune system, which may help in the prevention of some chronic diseases like coronary heart disease and cancer (52). Moreover, lutein and zeaxanthin were found to have positive cognitive activities particularly when tested with the elderly (53), and epidemiological trials showed their incrimination in the prevention of some age-related degenerative diseases (53).

Fucoxanthin is a brown pigment that is responsible for the coloration of some algae like diatoms. It was found to have many medicinal bioactive functions such as antioxidant, anti-inflammatory, anti-cancer, anti-obesity, anti-diabetic, anti-angiogenic and anti-malarial activities (54).

4.5.3. Bioactive Compounds for Cosmetic Usages

Microalgae are rich in bioactive compounds like pigments, vitamins, lipopolysaccharides, phytohormones (auxin, abscisic acid, cytokinin, ethylene, gibberellins) and amino-acids and lot of anti-oxidant compounds that may be applied in cosmetic usages like sun protection screen, anti-aging factors, anti-oxidant, stress protection, and immune system booster (55). Particularly anti-oxidant compounds are principal reagents for anti-aging cosmetic materials; therefore, all the previously mentioned anti-oxidant compounds may also be used as supplement to prevent aging and age-related diseases (52).

Moreover, chlorophyll a may be used as ingredient in the body hygienic materials like deodorants and bad breath removal (56, 57).

Microalgae are also known for their good effect on skin and for their preventive activity against the sunlight ultraviolet radiations (57). Microalgal pigments may also be used as natural dye for the commercial production of make-up products (55).

4.5.4. Anti-Microbial Compounds

Microorganisms compete with each other to grow. They produce certain chemicals to compete with other organisms, which constitute potential source to find new antimicrobial compounds (38). In fact, the marine environments are highly rich in anti-microbial molecules that are produced as allelochemicals, like phlorotannins, fatty acids, polysaccharides, peptides, and terpenes (58).

Naviculan, for example is a sulfated polysaccharide isolated from the diatom *Navicula directa,* it is found to include anti-viral compounds against herpes simplex viruses (HSV) type 1 and 2, influenza A virus and against the cell entry of the human immunodeficiency virus (HIV) (59). Another study showed that ethanol and hexane extract of *Haematococcus pluvialis* and *Dunaliella salina* reduced the infectivity of HSV-1 tested on Vero cells (60). Organic extracts of some microalgal species from the Thamirabarani river, in the south India showed to have antimicrobial activity against *Staphylococcus aureus (S. aureus)*, *Escherichia coli (E. coli)*, *Salmonella typhi (S. typhi)*, *Proteus vulgaris (P. vulgaris)* and *Proteus mirabilis (P. mirabilis)* (61). Another work showed that methanol extracts of two freshwater microalgae (*Dunaliella salina* and *Pseudokirchneriella subcapitata*) had inhibitor effect on *P. aeruginosa*, *E. coli*, *Klebsiella* sp., and *S. aureus* including methicillin resistant strains isolated from otitis infection (62). Protein hydrolysates of *Scenedesmus obliquus* obtained by pepsin digestion inhibited also the viral activity of the Coxsackie B3 virus (CVB3) on Vero cell lines at 100.µg/ml (63).

The studies of the antimicrobial activity of microalgal isolates remain uncountable and many positive results are obtained, even though we still do not have any practical solution for the bacterial resistance and for the multi-drug resistant bacteria that are rapidly developing and threatening the humans and animals

4.5.5. Anti-Quorum Sensing Metabolites

4.5.5.1. Quorum Sensing

Our world is dominated by a vast variety of microorganisms most of them live in multiple species environments (oral cavity, intestine, soil, water, etc.). As previously mentioned, allelopathy is the communication mechanism used by microalgae to control its behavior towards other microorganisms (38). As for bacteria, they communicate with

each other using a cell-to-cell signalization mechanism mediated by diffusible chemical molecules called autoinducers with their cognate receptors. This system is called quorum sensing (QS) and it provides an intra- and extra- species communication between the microorganisms of the same species or even with other prokaryotic or eukaryotic organisms (64). QS signalization is mainly dependent on the bacterial growth, and it is activated by the increasing of the bacterial cells number that should exceed a threshold limit. In normal conditions, the bacterial QS signal molecules are produced in small quantities. The bacterial growth will be perceived by specific receptors on the bacterial cell membrane (64). The receptor been activated, it will induce a signaling pathway finishing by activating some translation factors promoting the bacterial growth with the production of some related factors and proteins, much of them are considered as pathogenicity or virulence factors for humans or other living organisms (65). Thus, QS system allows the bacterial cells to monitor their environment and to take reaction to provide themselves the necessary competitive tools to grow and survive (64).

4.5.5.2. Quorum Sensing Mechanisms

Although the QS mechanism steps are similar, the molecular schemes vary between the different bacterial groups, but still at the same time interconnected. The main players in each system are the autoinducer signals and their cognate receptors either on the same or on the other microorganisms (64). In gram-negative bacteria, the most common used autoinducers are the N-Acyl homoserine lactones (AHLs), which are constituted of a homoserine lactone (HSL) molecule bound to an acyl chain. HSL molecule is stable while the acyl chain may change between the bacteria by the number of carbon's atom that can go from 4 to 18, by saturation of the bonds of the chain or by the oxidation state of its third position (65) (Figure 3).

AHL autoinducer system was first discovered in the luminescent marine bacteria, *Vibrio fischeri,* which have become the prototype of this type of signalization with its LuxI/R autoinducer /receptor system. LuxI/R type is used to control the *luxI* gene and its repressor *luxR*, which are responsible for the control of the bacterial bioluminescence (66). LuxI enzyme constitutively synthesizes the autoinducer *N*-(3-oxohexanoyl)-HSL (3-oxo-HSL) in small quantities and the molecules continuously diffuse in and out of

the cell membrane. With the increasing of the bacterial cells density, the autoinducer concentration relatively increases until reaching a threshold level where the autoinducer starts binding to the LuxR receptor and activates its DNA binding domain. The activated LuxR binds to the promoter region of the *luxCDABE* operon to upregulate the transcription of the luciferase genes and the production of light (Figure 4). Simultaneously, LuxR signal activates *luxI* in a form of a positive feedback and will increase its own expression; this also means an increasing in the light production. The balancing of this phenomenon is achieved by a negative feedback regulation of the *luxR* gene by the level of the activated luxR protein. This, in turn, leads to a decrease in the production of LuxR and LuxI, thus decreases the expression of luciferase genes (67).

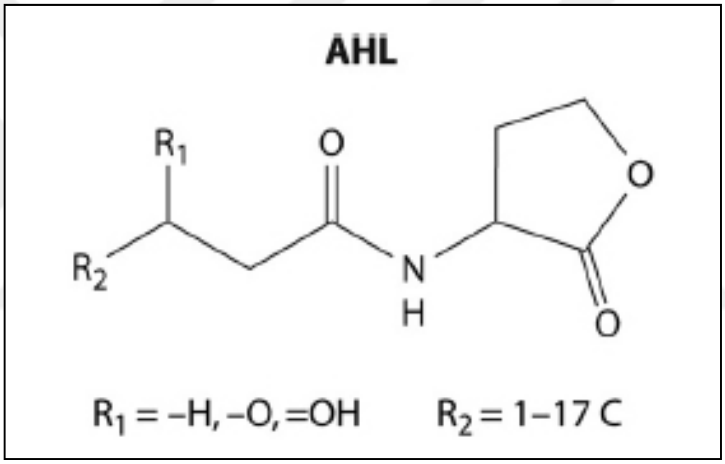


Figure 3. General structure of the AHL autoinducers (65).

The LuxI type QS is shared in common by most of the gram-negative bacteria using autoinducers and receptors homologous to luxI/R system, it is called autoinducer I system (AI-1) and it is species specific.

One of the famous AHL type QS model is used by *Chromobacterium violaceum* (*C. violaceum*) where the AHL C6HSL is synthesized by the *CviI* synthase gene with its cognate receptor gene *CviR* that regulates the production of a violet pigment called violacein (67). This system is very well studied and widely used in-vitro especially for the research of QS or anti-QS effectors (68, 69).

In gram-positive bacteria, QS signal molecules are oligopeptides ranging from 5 to 17 amino acids and are called auto-inducing peptide (AIP) (65). These oligopeptides

are coded as precursors and, like any normal protein; they are exposed to post-translational modifications (70). Since they cannot across the cellular membrane, they need active transporters to drive them through (64). The cognate receptors of the AIP may also be localized on the cellular membrane as a part of a histidine-kinase two-component system (70). When the receptor is activated by the AIP, it contributes to a phosphorylation pathway ending by the activation of the cognate operon which, in the most of the cases, encodes for the pro-AIP, the transporter, the histidine kinase receptor and for the response regulator factors that are responsible for the positive feedback activation (64, 71) (Figure 4A and 4B).

Quorum sensing is not a simple talkative mechanism and is not limited to the bacterial communication. The QS system is similar to a nervous system model in bacteria where it perceives the external conditions, communicates the information between the different bacterial cells, regulates the bacterial genetic expression, and adjusts it to adapt the new conditions and to surpass the inadaptable ones. In fact, QS is responsible for the regulation of most of the bacterial cell functions: its growth, pigmentation, sporulation, biofilm, cell division, genetic expression, motility, mutations and developing selective resistance.

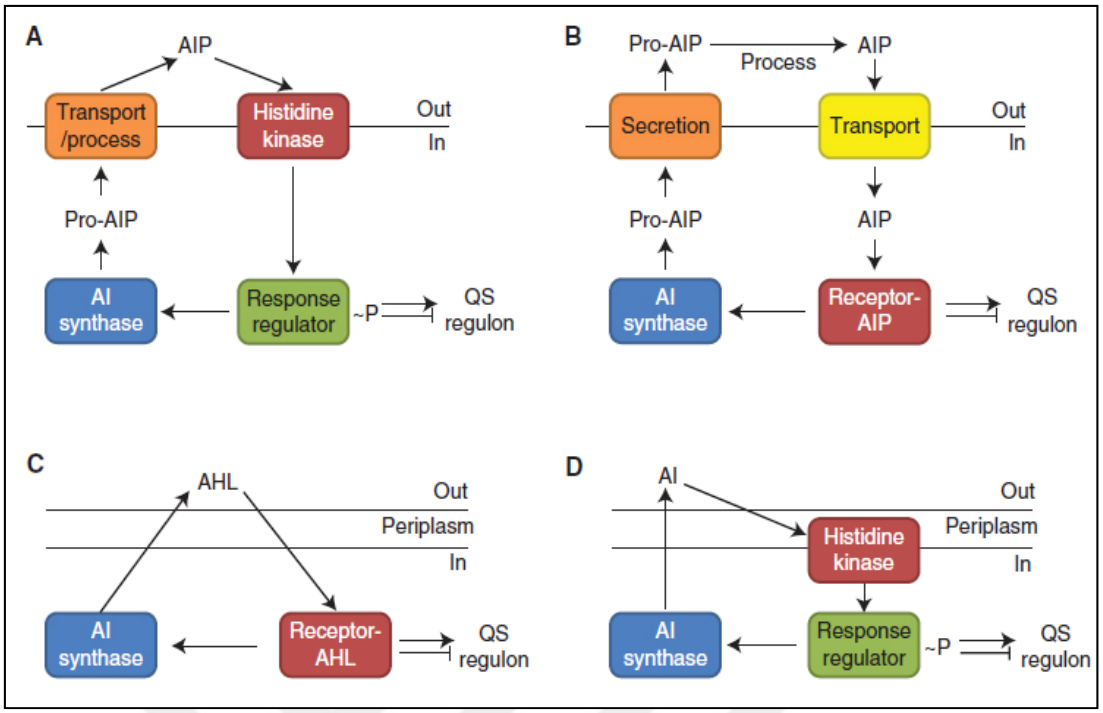


Figure 4. Representation of the different mechanisms of the QS systems in bacteria. A, two-component signaling auto-inducing oligopeptide type signalization; B, an AIP-binding transcription factor; C, small molecules QS in gram-negative bacteria by a LuxI/LuxR type system; D, two-component signaling QS system (71).

4.5.5.3. Interkingdom Quorum Sensing

Human body is inhabited by the microbiota whose cells number exceeds the number of the human body cells. Most of these are bacteria and are essential for the body's physiological functions, like the oral, intestine, and the vaginal microbiota where bacteria cooperate with the human cells through signalization system and help controlling and regulating their living environment, thus, the homeostasis of the region they belong to (72). Epidermal growth factors, steroids, amines and many other hormones are perceived as QS signals by the bacteria, and they affect its growth and its genetic expression (72). The situation is the same for the environmental bacteria, which cooperate with the other organisms to control their surrounding environment (73). Actually, phytoplanktons are the principal microorganisms marine bacteria interact with, where everyone benefit from the other without any competition (73). As for the pathogenic bacteria, QS encoded genes mostly constitute a part of the virulence and the pathogenicity factors and allow the bacteria to proliferate and to execute their infection in the host's body (65).

On the other hand, these signal molecules are essential for the bacterial symbiotic relationships with other microorganisms like the case of biofilms that are essentially controlled and derived by QS interaction between the different cells incriminated in the biofilm complex (74).

The observation of microalgal cells by electronic microscope proved the presence of bacterial cells attached to microalgal cell wall or present in the microenvironment surrounding the cells called phycosphere (75). This linkage is hardly separable and it indicates an obvious physical relationship, thus a benefit-based interaction between microalgal cell and bacteria (75).

4.6. Biofuels

In our era of technology, we become highly dependent on different forms of energy in every small act of our life. The big problem is that non-renewable fossil energy resources are quickly consumed and will not be able to cover the human needs in the near future. At the same time, the negative effects of these fuels are dreadfully increasing including the greenhouse gases (GHG) mainly carbon dioxide (CO_2) and methane (CH_4) that contributed lately to an alarming atmospheric pollution and the climate changing with the global warming we are living today are witnesses (7). Since the 1960's, researchers were looking for alternative fuel as solution to these problems and many country were managing policies to decrease their coal and fuel energy consumption and many solutions were proposed such as biofuel, solar energy and others (76).

4.6.1. Types of Biofuels

The Biofuel word in practical meaning refers to hydrocarbon-generated fuel, which means the production of energy from organic biomass by biological carbon fixation in a short period in comparison with the fossil fuels, which need millions of years to develop. Whereas the other types of energy like electricity, solar energy or nuclear fission are not based on hydrocarbons.

Biofuel concept has developed over time through 3 generations: First, primary biofuels consisted of unprocessed combustible material from nature such as firewood, wood chips, animal wastes, and forest and crop residues (77). Thereafter, biofuel concept has progressed through different generations in the aim to ameliorate the yield

of the process: the first generation of biofuel was obtained by fermentation of starches (wheat, barley, corn, potato) or sugars (sugarcane) to produce bioethanol and biobutanol, whereas biodiesel was produced by transesterification of oil crops (rapeseed, soybeans, sunflower, palm, coconut, used cooking oil, animal fats, etc.) (77, 78). Since the first generation compete with the human feeding sources, the second generation of biofuel consisted of using cellulose and lingo-cellulosic material from natural sources to produce bioethanol, biobutanol and syndiesel (77). The third and the newest generation of biofuels consisted of using microalgae and other microorganisms to produce bioethanol, biobutanol, biohydrogen and diesel (78) because of the ease of use of microorganisms in comparison to the higher plants, and since they grow faster and with least production costs.

Today, clean energy like biogas, bioethanol, hydrogen and biodiesel may be produced from microalgae. *Chlorella vulgaris* for example was found to comprise high ratio of starches that may be hydrolyzed to produce bioethanol (78). Meanwhile microalgal lipids may be an important resource for biodiesel production (79), especially for some microalgal species like chlorella that may contain a fat ratio up to 50 or 60% of the cells dry weight and the fat ratio may even reach 70% for some species under stress conditions (for example nitrogen deprivation) (79). Microalgal biomass may also be anaerobically digested to produce biogas or methane (78).

4.6.2. Biodiesel Production

Biodiesel is obtained by a transesterification (alcoholysis) reaction between a fatty acid and an alcohol to produce glycerol and methyl ester fatty acid; this latter constitutes the diesel (Figure 5) (80). Most of the cases, the fatty acid is a triglycerides and the alcohol used is methanol. The reaction is an equilibrium that requisites a large excess of methanol to ensure that the pathway is driven in the direction of production of methyl ester fatty acids (80). The process goes stepwise where triglycerides are first converted to diglycerides, then to monoglycerides and finally to glycerol. Catalysts are also needed to move the transesterification reaction. They may be acids, alkalis (81) or enzymes like lipases. Alkali catalysts like sodium and potassium hydroxide are considered the best since they manage the reaction about 4.000 times faster than acids

(82). The whole reaction applied at 60°C under atmospheric pressure and takes about 90.min to complete (82).

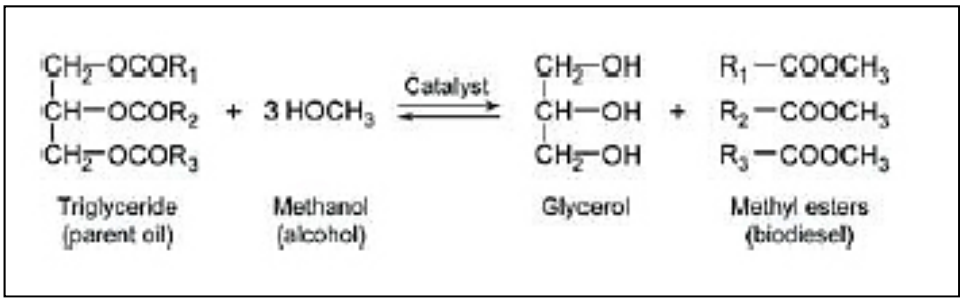


Figure 5. Transesterification of oil to biodiesel. R1–3 are hydrocarbon groups

4.6.3. Microalgal Lipids Contents

Poly unsaturated fatty acids (PUFAs) are mainly synthesized by microalgae (for example, omega-3 acid) and they are comsumed by human as sea food or food supplements. Microalgal PUFAs constitute today an essential source of nutritional and pharmaceutical interest. Microalgal omega-3-long chain PUFAs synthesis may reach over 20% of their total lipid (83). These PUFAs esterification by the cellular alcohol mainly glycerol produce triacylglycerols (TAGs) or polar lipids (i.e. phospholipids, glycolipids, carotenoids) that are involved in the regulation of the cellular membranes fluidity and functions.

4.6.4. Microalgae and Plants for Biodiesel Production

Today solar energy, crops and seeds and other green resources are slowly replacing the fuel energy particularly in the developing countries, but there is still no definitive solution for the problem (7). Solar energy needs a lot of global surface to be able to generate sufficient yields for big populations, and is always dependent on the weather conditions whereas crops and seeds are already unable to cover the food supply for the continuously increasing humans needs, thus they will not be able to encounter another competition area for fuel generation. Lingo-cellulosic feedstocks are also used for biofuel production since they are not edible and are considered as organic wastes (77). Yet they are still not cost effective and are unable to cover all the human needs, otherwise crops cultivation should be increased which means a need of arable lands, pesticide consumption and more water supply (77). Meanwhile microalgae grow faster than plants, they do not need arable lands and their culture may be better optimizable

than plants. Microalgal photosynthesis is more effective than plants as a mean of CO_2 consumption, since plants and other crops use approximately 77 giga tons (Gt) of CO_2 to produce 100.Gt of lignocellulose biomass whereas microalgae use 183.Gt to produce the same biomass, which means a better solution for the GHG problem (84).

4.6.5. Microalgal Lipids Investigation

Fatty acids in microalgae were widely investigated all around the world. Beginning with the gold standard gravimetric method of Bligh and Dyer (85), which consists of extracting the oils by a chloroform/methanol/water mixture then analyzing the extracted oil with chromatographic or spectrophotometric methods. Supercritical fluid extraction was also used with good extraction yields, and the fatty acids may also be purified and determined by chromatographic methods like HPLC or GC-MS. Fatty acids may also be transesterified using alcohol (methanol, ethanol), then a saponification mechanism is used to determine their initial quantity. Sulpho-phospho-vanillin method is also a spectrophotometric method that may be used to determine the content of fatty acids after extracting them by concentrated sulfuric acid (86).

Fluorescent dyes could be used for an indirect measurement of lipids as an easier and cheaper method that requires a much smaller amount of equipment and that saves much more time in comparison to the classical gravimetric and spectrophotometric methods (87). Nile red and BODIPY.505/515 lipid dyes are used today for a fast in-situ screening of potential oleaginous microalgae in order to identify promising sources for commercial biofuel production (87).

4.6.6. Flow Cytometry

Flow cytometry is a technique for counting and sorting cells according to their physical properties. It had been frequently used lately for the investigation of phytoplankton (35). Briefly, a cell suspension is driven into a fluidic stream with determined core dimension where the cell particles flow through. Then a light beam is opened to attack the flowing cells which diffract the light according to their size and consistency (35) (Figure 6).

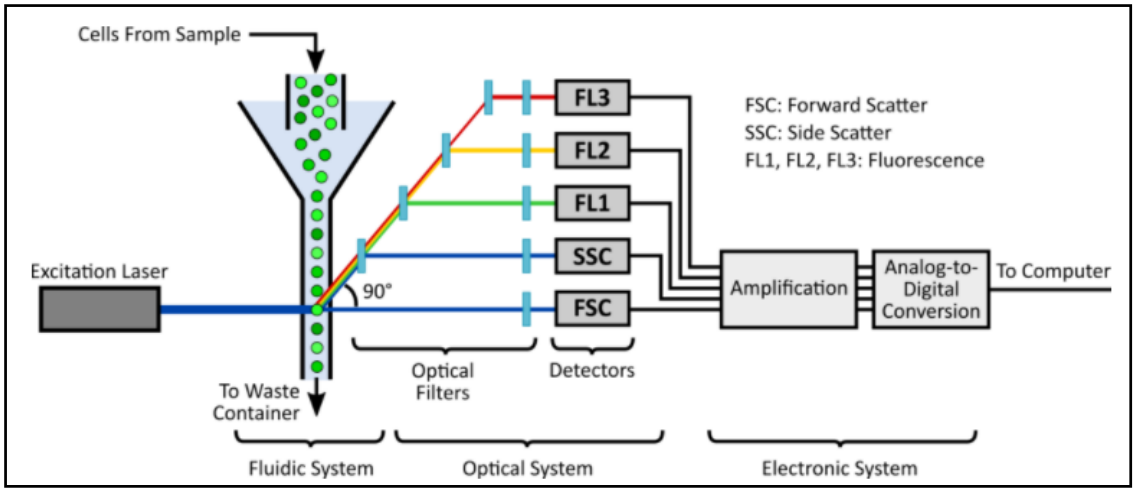


Figure 6. Classical flow cytometry system. 1, the fluidic system that drive the cells inside the core; 2, the optic system that contains the scatter detectors and the fluorescence mirrors; 3, the electronic system that transform the photo-information into electronic signals that could be analyzed in by software system (from https://benchling.com/pub/tabor-flowcal)

Most of the actual flow cytometric instruments works on laser light that produces a blue laser with excitation beam of 488.nm and another red laser with excitation beam of 640.nm. The scattered light is received by two photodiode detectors: the first located in the same axis forward to the incited light beam and is called Forward Scatter signal detector (FSC) (35). This detector receives the diffracted light depending on the cells size and shape and will consequently give an idea about these two characteristics. The second receptor is located at 90°C in the side of the light beam and detects the light scattered by the intracellular inclusions of the cells, it is called Side Scatter signal detector (SSC) (35). The photodiode is a photomultiplier that converts the detected photons into electrical signal that could be analyzed on a computer's software (Figure 6**)**. In addition to the FSC and the SSC detectors, mirrors with determined filters are appropriately located in the instrument to detect emissions with particular fluorescent absorbances (35).

When the light's spectrum encounters the cells absorption spectrum these latter will be excited and will emit another fluorescent light that will be detected by its suitable mirror (35). This setting helped with the molecular staining techniques to change the flow cytometry from been a cell counter and sorter method to be used in all type of molecular analysis.

5. MATERIALS and METHODS

5.1. Culture Media

Luria Bertani (LB) broth and agar used for the cultivation of bacterial strains and for anti-QS activity tests and Mueller Hinton (MH) broth and agar used for the anti-microbial activity tests were purchased from Lab M (Lancashire, UK). BOLD medium used for the microalgal strains culture was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The agar used for the different culture media was purchased from BioShop (Burlington, Canada).

Hepes buffer 1 M was purchased from Biological industries (Beit Haemek, Israel) and was used as buffer to prepare the vitamin solutions: 4.mg of vitamin B12 (MW.=.1355.38.g/mol), 9.mg of vitamin B1 (MW=.300.81.g/mol) and 7.3.mg of vitamin B7 (MW.=.244.31 g/mol) were separately dissolved in 1.5.mL of Hepes buffer (1.M) then every solution was completed to 30 ml by sterile water. Their pH was adjusted to.7.8 and they were filter sterilized by 0.22.µm pore diameter polytetrafluoroethylene (PTFE) filters. BOLD medium was prepared according to the manufacturer's recommendations, then autoclaved at 121^{o}C for 20 min. After cooling at 50^{o}C, 100 µl of thiamin (vitamin B_1, 1.mM), 100.µl of biotin (vitamin B_7, 0.1.mM) and 1.ml of cobalamin (vitamin B_{12}, 0.1.mM) were added for every 1.L of medium that was then poured in the petri dishes.

5.2. Chemicals and Reagents

Hexadecyl Trimethyl ammonium bromide (CTAB) was purchased from abcr GmbH (Karlsruhe, Germany); 2-mercaptoethanol, chloroform and Tris were purchased from AMRESCO (Solon, Ohio, USA). Methanol, n-hexane, acetic acid, isoamyl alcohol, Tris-HCl and EDTA were purchased from Merck (Kenilworth, New Jersey, USA). Hydrochloric acid (HCl) and sodium chloride (NaCl) were purchased from (Riedel de Haen, Germany). Phenol, chloroform, ethanol, Nile Red, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS) and sephadex.6-50 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Proteinase K and lyticase were purchased from New England Biolabs (Ipswich, Massachusetts, USA). Agarose was purchased from Accumedia (Neogen, Lansing, Michigan, USA). PCR amplification kit and Taq DNA polymerases were purchased from Promega Corporation (Madison, USA). Big Dye

Terminator v3.1 Cycle Sequencing Kit with the 5x sequencing buffer were purchased from Applied Biosystem (Foster City, California, USA). DNA molecular weight standards were purchased from BIORON GmbH (Ludwigshafen, Germany).

5.3. Microalgal Samples

Samples collection was performed from different ecosystems in different regions of Turkey including fountains, lakes, streams, cave soils and waters with a focus on the Black Sea region**.**

a. Soil samples were taken from the following caves: Akçakale, Kırklar, Köprübaşı (Altıntaş) caves are located in the province of Gümüşane; Ayvayini from Mustafa kemalpaşa, Doğanalan Köyü, Bursa and Toprakini from Güneysınır, Konya. These caves were non-urbanized and they have not been invested for any commercial usages before.
b. Water samples were collected from streams, lakes in the regions of Maçka, Akçaabat (Sera Lake and Çamlıca village), Zafanos, Çaykara, Araklı and Vakfıkebir in Trabzon province, Limni lake in Gümüşhane province, Aladağ, Bolu, Cehennem valley of Artvin, and Ekaterina spring water from Georgia.
c. Four samples were taken from a commercial drinking (Damla®) water left in the sunlight.

5.4. Microalgal Strains Isolation

Samples were incubated in BOLD liquid medium under specific lighting condition (16.h light / 8.h obscurity) for 2-6.weeks. The algal growth was controlled by macroscopic and microscopic observation:

a. Macroscopic observation: Cultures were observed for producing green color on the wall or in the bottom of the culture flask.
b. Microscopic observation: After mixing the culture, 15.ml were removed and centrifuged at 14,000.rpm for 5.min. Then a drop of the pellet was observed under the optical microscope in the search of algal cells.

In case of detection of algal culture, a drop of the centrifuged pellet was taken and cultivated by the quadrant method on BOLD agar plate enriched with vitamin B12, B1 and B7.

5.5.Microalgal Culture and Harvesting

Algal colonies grown on BOLD agar plates were isolated and re-cultivated until obtaining a pure culture. Single colonies were re-cultivated in 1.ml of BOLD medium for harvesting. Culture growth was checked under a stereo microscope and the cells were counted using a Thoma cell-counting chamber. When the number of cells reached to 10^6.-.10^7.cell/ml, fresh BOLD medium was added to the cultures to obtain larger volumes. For about 3.weeks of cultivation, 600.ml were taken and centrifuged at 8,000.rpm for 5.min to pellet the cells. The cells were washed 3.times with sterile water and dried at 50^{o}C, weighted and stored at 4^{o}C for further tests. Culture medium was added to the rest of the culture to continue the harvesting until the dry weight of the algal cells reached to minimum 500.mg each.

5.6. Organic Extractions of Bioactive Compounds

Microalgal powder was extracted in methanol and hexane separately and incubated for 24.h with shaking at 225.rpm. The extracts were centrifuged for 5.min at 8,000.rpm and the supernatant were transferred to a pre-weighted new tube and dried at 50°C. Extracts are weighted and dissolved as 100.mg/ml in dimethyl sulfoxide (DMSO) and stored at 4°C to be further tested for antimicrobial and anti- QS activities.

5.7. Anti-Microbial Activity Test

Antimicrobial activity was tested using first agar well diffusion method to screen the positive results, and then microdilution method was used to verify the results and to determine the concentrations of the extracts showing positive results.

5.7.1. Agar Well Diffusion Test

Antimicrobial activity was tested by agar well diffusion method in accordance to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (88).

Antimicrobial activity was tested against *E. coli* ATCC.25922, *S. aureus* ATCC.25923, *P. aeruginosa* ATCC.27853, *Bacillus subtilis (B. subtilis)* ATCC.6633, *Enterococcus faecalis* (*E. faecalis)* ATCC.29212, *Acinetobacter haemolyticus (A. haemolyticus*) ATCC.19002, *K. pneumoniae* ATCC.13883, and *Candida albicans (C. albicans)* ATCC.10231. LB broth was used to prepare an overnight culture of each bacterium and the bacterial suspensions were adjusted to 0.5.McFarland by controlling their density at OD_{600}.nm. Bacterial suspension was cultivated on MH agar by covering

all the petri's surface using sterile cotton swabs that had been submerged in the bacterial suspension. Wells were opened with a diameter of 7.mm using a sterile Pasteur pipette and 50.µl of 10.mg/ml of each extract was disposed in every well. DMSO was used as negative control and a proper antibiotic for each bacterium was used as positive control. The cultures were incubated at 37^{o}C for 18.-.24.h before been read and evaluated in accordance to the guidelines of the CLSI (88).

Anti-microbial activity was evaluated after the incubation period by measuring the inhibition zone around the well of the active extract for every bacterium. Since the negative control does not show any positive inhibitive activity against the tested bacteria, any inhibition zone observed was accepted to be a positive activity of the tested extract.

5.7.2. Minimal Inhibitory Concentrations Determination

Extracts showing positive antimicrobial results were tested to determine their minimum inhibitory concentration (MIC). For this purpose, microdilution method was applied on 96 micro-well plate in accordance to the guidelines of the CLSI (88).

Briefly, 100.µl of LB broth was transferred in every well in the microplate, then 100.µl of each extract was added to the first well of every row series. With the aid of micropipette channel and after well mixing, a 100.µl was transported from the first well to the second well of every row, then after mixing, a 100.µl was transported from the second well to the third and so on to continue the serial 1/2 dilution until the end of the row. The last well of every row series was left without extract to be used as positive culture control. Finally, an overnight culture of each bacterium is adjusted to the value of 0.5.McFarland (which is considered equal to 10^{8}.CFU/ml). The adjustment of the bacterial suspension was used referring to previous optimization of the bacterial count/OD_{600} for each bacterium.

The 0.5.McFarland bacterial suspension was diluted to the 1/100 factor, and a 5.µl is distributed to the test plates. The 11th well of every row in the plate was left without bacterial culture and considered as a negative control. For every bacterium, a suitable antibiotic was used as positive control and DMSO was tested as solvent control.

The MIC value was evaluated as the least concentration that inhibits the growth of the tested bacterium. Evaluation was done by observation of the culture turbulence and

the presence of bacterial pellet at the bottom of every well, and then the verification was done by taking 20.µl of the suspected concentrations after well mixing the culture and by cultivating them on MH agar plate. Finally, after incubation at 37°C for 18.-.24.h, only MIC values with zero colony cultivation were accepted.

5.8. Anti-Quorum Sensing Activity Tests

Anti-QS activity was tested against the bacterium *C. violaceum* ATCC.12472 using the microdilution method on a 96.micro-well plate as previously indicated *(see section 4.7.2)*. Quorum sensing activity indicator here is the pigment production by *C. violaceum*. In case of QS inhibition, the pigment production should be decreased, without affecting the bacterial growth. This latter was evaluated as mean of bacterial count. As for the pigment production evaluation, after been extracted by DMSO, its intensity was measured by spectrophotometer at OD_{585} (89).

Briefly, after the 24 hours of incubation, the MIC concentration was determined for every extract. Then the next smaller concentration's well (SubMIC) and ½ SubMIC were tested for anti-QS activity. For this purpose, a 10.µl was taken from each well and mixed in 90.µl of NaCl 0.9% to make a dilution of 1/10. Then a serial of 1/10 dilution was continued the same way until reaching the 10^{-8} dilution factor. After a brief mixing, 10.µl of the 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} diluted solutions were taken and dropped on MH agar then incubated at 37°C. Finally, the bacterial colonies are counted for every cultivated drop and the counted number was multiplied by the dilution factor and by 10 to obtain the final bacterial count as colony forming unit (CFU) in the 100.µl culture that was in the well plate. On the other side, the 96.well plate was dried at 50°C; a 200.µl of DMSO was added to every well and left for 1.-.2.h. at room temperature at obscurity to extract the pigments. After the pigments are completely extracted, DMSO solutions are transferred to a new well plate and their absorbance was measured at 585.nm.

Every test was repeated twice for every extract concentration and at least seven.times for the positive control. Vanilla was used as positive control for QS inhibitory activity.

5.9. Anti-Quorum Sensing Results Evaluation

Bacterial count and the pigment absorbance of the tested extracts concentrations were compared to the positive control's results (bacterial growth with 0 extract) and the differences were statistically evaluated using SPSS® Statistics software (IBM® Corporation, USA). The evaluation method used was the One-Way Analysis of variances (One-Way ANOVA). The homogeneity of variances was tested by Levene test. Then homogenously variable data were analyzed using the post-hoc Tukey HSD (Honestly

5.10. Neutral Lipids Investigation

Fatty acids were investigated by staining the microalgal cells with Nile red and analyzing their fluorescence using the BD Accuri™ C6 Flow Cytometer Instrument (Becton Dickinson and Company, USA). The protocol was taken from Satpati and colleagues with slight modifications (90). Strains were cultivated in 3.ml culture without adding any new medium for 14.-.17 days. Then 100.µl was taken and diluted in 900.µl of sterile distilled water then vortexed. Samples were centrifuged at 14,000.rpm for 10.min then washed again. Finally, 900.µl of sterile distilled water was added and after a vigorous vortexing, they were divided to 2 x 500.µl into 2 microcentrifuge tubes. One tube was left unstained for negative control measurement and the second tube wad stained with 5.µl of Nile red solution (100.mg/ml in DMSO) with 295.µl of 40% DMSO. The mixture was vortexed and left at obscurity under room temperature for 15.min before been measured by the flow cytometer.

Flow cytometry measurements were applied by excitation with a blue laser light at 488.nm. The cells flow rate was 35.µl/min in a core size of 16.µm. Emitted fluorescence was read at FL2 (585 ±40) and FL3 (670 LP) filters.

Stained and unstained strains were observed under fluorescence microscope (Euroimmun, Lübeck, Germany) with a blue LED light (460.-.490.nm).

5.11. Flow Cytometry Results Analysis

Nile red is a stain that binds to the neutral lipid molecules and has maximum emission fluorescence at 570.-.580 nm when dissolved in 25% DMSO (90). Unstained cells were used to remove the microalgal auto-fluorescence signals that are detected on

this channel by gating, and then stained cells fluorescence was measured and their intensity was calculated for each event (event = excited single cell) by the software's system and the data were expressed as fluorescence arbitrary unit per 100.event (90).

5.12. Molecular Identification of Microalgal Strains

5.12.1. Genomic DNA Isolation

For DNA extraction, 1.ml of dense microalgal culture was centrifuged at 14,000.rpm and the supernatant was discarded. Pellet was suspended in 300.µl of spheroblast buffer (Tris-HCl (10.mM) pH. 7.5; EDTA (1.mM); β-mercaptoethanol 0.2%; 0.1% lyticase). Glass beads of 1.mm diameter were added to the suspension (until covering the liquid volume) and the mixture was rigorously vortexed for 5.to.10.min before been incubated for half an hour at 37^{o}C. After the incubation, a 50 µl of proteinase K (2.5.mg/ml) and 50.µl of (SDS) (10%) were added and the mixture was vortexed rigorously again and left at 37^{o}C for half an hour. In the next step, a 400.µl of 5.M NaCl and 200.µl of CTAB were added and the tubes then vortexed and incubated at 65^{o}C for 30.min. After vortexing, for each tube the same volume of chloroform:isoamyl alcohol (24:1) was added (ca. 600.µl) and the tubes were mixed by inverting followed by centrifugation at 14,000.rpm for 10 min. The upper phase was taken to a new microcentrifuge tubes and remixed with 700.µl of phenol:chloroform:isoamyl alcohol (25:24:1) by inverting the mixtures then centrifugation at 14,000.rpm for 10.min. The upper phase was transferred to a microcentrifuge tube. An equal volume of chilled 96% ethanol was added to each tube and they were incubated for 10.min at -20^{o}C to permit the precipitation of the DNA. The DNA was precipitated by centrifugation at 12,000 rpm for 10 min and the supernatant was discarded. A final washing step was done by 70% ethanol and the pellet was dried at 50^{o}C. Finally, DNA pellets were suspended in 30 µl of elution buffer, electrophoresed on 1% (w/v) Tris-EDTA-acetic acid (TAE) agarose gel stained with ethidium bromide (EtBr) and visualized under a UV illuminator.

5.12.2. PCR Amplification

PCR amplification of the 18S rDNA sequences was applied using the universal primers: Alg1F (5′-CCA GCA (G/C)C(C/T) GCG GTA ATT CC-3′) and Alg1R (5′-ACT TTC GTT CTT GAT (C/T)(A/G)A-3′) that amplify a region of about 500 bp of the

domain V4 (91), and Alg2F (5'-GTC AGA GGT GAA ATT CTT GGA TTT A-3') and Alg2R (5'-AGG GCA GGG ACG TAA TCA ACG-3') that provide the multiplication of a region of 700.bp (92). PCR reaction was carried out using Promega GoTaq® Flexi DNA polymerase. A 20.µl of total reaction included 2.5.U/µl Taq DNA polymerase, 5X Taq reaction Buffer, 2.mM $MgCl_2$, 0.2.mM of each dNTP, and 0.4.µM of each primer. The parameters of the thermal cycling reaction for Alg1F and Alg1R primers were 60.sec for initial denaturation at 94°C, 35.cycles of denaturation at 94°C for 30. sec, annealing at 48°C for 1.min and extension at 72°C for 30. sec, and 10.min of final extension at 72°C. As for the Alg 2F and Alg2R primers, the PCR initial denaturation was done at 94.^{o}C for 130 sec then 35.cycles of denaturation at 94^{o}C for 30 sec, annealing at 56.^{o}C for 45.sec, and extension at 72^{o}C for 1.min, finally last extension was done at 72.^{o}C for 6.min. The PCR-products were visualized under a UV illuminator following agarose gels electrophoresis.

5.12.3. PCR Products Purification

PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH, Düren, Germany) according to the manufacturer's recommendations.

5.12.4. DNA Sequencing

DNA sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit. The reaction cycling included 2.min of initial denaturation at 96^{o}C, then a.25.cycle of denaturation at 96^{o}C for 15 sec, annealing at 50^{o}C for 15 sec and 2.min of extension at 60^{o}C, then a last extension was carried out at 60^{o}C for 3.min. The amplicons were purified by sephadex column and they were Sanger sequenced using the automatic DNA Sequence Analyzer (ABI PRISM 3130XL Genetic Analyzer, USA).

5.12.5. DNA Sequence Analysis

The sequencing results were read using Chromas 2.6.5 software (Technelysium Pty. Ltd., Australia) and were analyzed using the Basic Local Alignment Search Tool (BLAST) (NCBI, National Center for Biotechnology Information, Bethesda MD, USA). The distant phylogenetic trees were generated by the Maximum Likelihood Matrix

method based on the Jukes-Cantor model using the Molecular Evolutionary Genetics Analysis (MEGA7, Pennsylvania State University, USA) alignment analysis software.

5.12.6 Phenotypic Identification

The strains where identified by microscopic observation at 100X using an optical light microscope (Olympus Optical CH-30, Tokyo, Japan). Strains were identified referring to the following databases: Algabase (20) (http://www.algaebase.org), UTEX culture collection of algae (https://utex.org),PhycoKey collection, University of New Hampshire (http://cfb.unh.edu/phycokey/phycokey.htm), The database collection of the phycological research group in the Charles University, Prague (Faculty of Science, Department of Botany) (93). (https://botany.natur.cuni.cz/algo/database/content/strains).

Morphological identification was done taking in consideration cells shape, size, pigments color, presence of flagella, type of inclusion bodies, nucleus location, pyrenoid presence and shape, accumulation of chlorophyll in the cell, asexual reproduction phases, cells assemblage and their size and shape variations during the different life stages.

6. RESULTS

6.1. Isolation and Culturing of Microalgae

Microalgal isolation was first applied by cultivation on BOLD agar plates. Colonies appeared on the plates as early as two.weeks (for some samples a couple of months) were picked and cultivated in 1- 3.ml cultures in microplates (Figure 7B). Culture growth was monitored by microscopic observation and by cell counting as described before. The culture volume was doubled upon cell count reached to 10^8.-.10^9 cells/ml until obtaining 1.L.-.5.L of cultures. Figure 7 shows the different stages of microalgae isolation and cultivation.

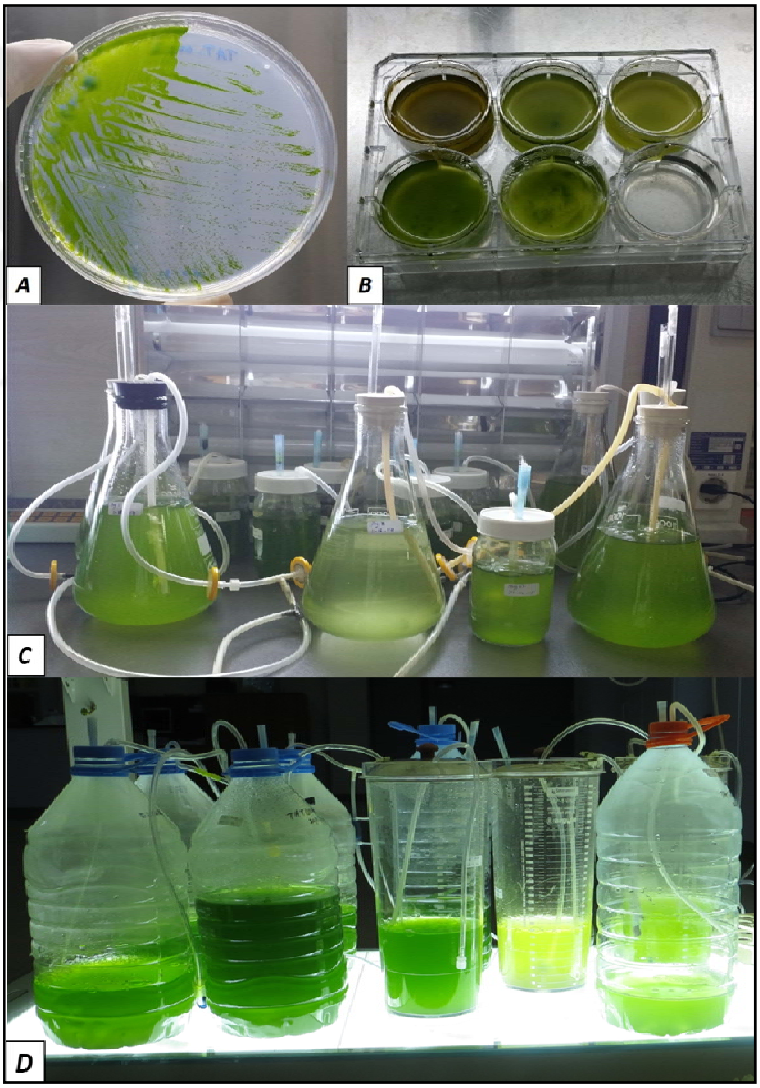


Figure 7. Microalgal cultivation system. A, microalgal isolation on BOLD agar; B, cultivation in 3 mL liquid culture; C, cultivation in 1 L volume; D, cultivation in 5 L and 10 L volumes.

6.2. Isolated Microalgal Strains

Among 64 microalgal strains that were isolated in this study, 17 were from cave soils and the rest were from different fresh water sources. Four strains were isolated from commercial drinking waters. The isolated strains and their sample locations are indicated in the Table.3. The morphological features from microscopic observations of the different strains are shown in the Figure.8.

Table 3. Isolated microalgal strains, their sampling locations and their phenotypic identification and 18S rDNA sequencing identification

Sample source (Location)	Strains	Genus ID (phenotypic)	Genus (18S rDNA)	Class	Family
Akçakale cave (Gümüşhane)	TALG 1	*Chlorella lobophora*	*Chlorella vulgaris*	Trebouxiophyceae	Chlorellaceae
	TALG 2	*Chlorella* sp.	*Elliptochloris subsphaerica*	Trebouxiophyceae	incertae sedis
	TALG 15	*Bracteacoccus* sp.	*Bracteacoccus* sp.	Chlorophyceae	Bracteacoccaceae
Kırklar cave (Gümüşhane)	TALG 3	*Chlorella* sp.	*Choricystis* sp	Trebouxiophyceae	Coccomyxaceae
	TALG 4	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
	TALG 6	*Chlorell*a sp.	*Desmodesmus subspicatus*	Chlorophyceae	Scenedesmaceae
	TALG 7	*Coccomyxa* sp.	ND	Trebouxiophyceae	Coccomyxaceae
	TALG 8	*Chlorella lobophora*	ND	Trebouxiophyceae	Chlorellaceae
	TALG 10	*Chlorella* sp.	*Elliptochloris subsphaerica*	Trebouxiophyceae	incertae sedis
	TALG 11	*Monoraphidium* sp.	*Monoraphidium* sp.	Chlorophyceae	Selenastraceae
	TALG 12	*Stichococcus* sp.	*Pseudostichococcus* sp	Chlorophyta classis*	Chlorophyta familia*
Köprübaşı cave (Gümüşhane)	TALG 13	*Ourococcus* sp.	*Chlorella vulgaris*	Trebouxiophyceae	Coccomyxaceae
Ayvayini cave (Bursa)	TALG 9	*Desmodesmus serratus*	ND	Chlorophyceae	Scenedesmaceae
	TALG 14	*Scenedesmus* sp.	*Asterarcys quadricellulare*	Chlorophyceae	Scenedesmaceae
	TALG 16	*Cyclotella meneghiniana*	*Pinnularia viridiformis*	Bacillariophyceae	Pinnulariaceae
	TALG 17	*Lobosphaera incisa*	*Kalenjinia* sp.	Trebouxiophyceae	Trebouxiaceae
Sumela (Maçka, Trabzon)	TALG 18	*Chlorococcum* sp.	ND	Chlorophyceae	Chlorococcaceae
	TALG 19	*Ankystrodesmus* sp.	ND	Chlorophyceae	Selenastraceae
Fountain (Zafanos, Trabzon)	TALG 20	*Oscillatoria* sp.	ND	Cyanophyceae	Oscillatoriaceae
	TALG 21	*Scenedesmus* sp.	*Scenedesmus vacuolatus*	Chlorophyceae	Scenedesmaceae
Fountain (Akçaköy, Akçaabat)	TALG 22	*Coccomyxa* sp.	ND	Trebouxiophyceae	Coccomyxaceae
	TALG 23	*Chlorella* sp.	*Chlorella vulgaris*	Trebouxiophyceae	Chlorellaceae

Table 3. Continued

Location	Strains	Genus ID (phenotypic)	Genus (18S rDNA)	Class	Family
Fountain (Vakfıkebir)	TALG 26	*Ankistrodesmus* sp.	ND	Chlorophyceae	Selenastraceae
	TALG 27	*Ankistrodesmus* sp.	ND	Chlorophyceae	Selenastraceae
	TALG 28	*Chlorella sorokiniana*	ND	Trebouxiophyceae	Chlorellaceae
Kazankıran (Çaykara)	TALG 30	*Scenedesmus* sp.	ND	Chlorophyceae	Scenedesmaceae
Çamlıca (Akçaabat)	TALG 31	*Chlorella sp.*	ND	Trebouxiophyceae	Chlorellaceae
Sera Lake (Trabzon)	TALG 24	Chlorella sorokiniana	ND	Trebouxiophyceae	Chlorellaceae
	TALG 34	*Chlorococcum* sp.	ND	Chlorophyceae	Chlorococcaceae
	TALG 35	*Chlorella vulgaris*	ND	Trebouxiophyceae	Chlorellaceae
	TALG 36	*Scenedesmus rubescens*	ND	Chlorophyceae	Scenedesmaceae
	TALG 37	*Coccomyxa* sp.	ND	Trebouxiophyceae	Coccomyxaceae
	TALG 38	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
	TALG 39	*Coccomyxa* sp.	ND	Trebouxiophyceae	Coccomyxaceae
	TALG 40	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
	TALG 41	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
	TALG 42	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
	TALG 43	*Nannochloropsis* sp.	ND	Eustigmatophyceae	Monodopsidaceae
	TALG 44	*Chloroidium* sp.	ND	Trebouxiophyceae	Trebouxiophyceae*
Santa Harebeleri (Trabzon)	TALG 45	*Trebouxia* sp.	ND	Trebouxiophyceae	Trebouxiaceae
	TALG 46	*Chlamydomonas* sp.	ND	Chlorophyceae	Chlamydomonadaceae
	TALG 47	*Scenedesmus* sp.	ND	Chlorophyceae	Scenedesmaceae
	TALG 48	*Chloroidium ellipsoidum*	ND	Trebouxiophyceae	Trebouxiophyceae*
	TALG 49	*Chloroidium ellipsoidum*	ND	Trebouxiophyceae	Trebouxiophyceae*

Table 3. Continued

Location	Strains	Genus ID (phenotypic)	Genus (18S rDNA)	Class	Family
Torakini cave (Konya)	TALG 5	*Chlorella lobophora*	ND	Trebouxiophyceae	Chlorellaceae
Limni Lake (Gümüşhane)	TALG 29	*Chlorococcum* sp.	ND	Chlorophyceae	Chlorococcaceae
Fountain (Vakfıkebir)	TALG 25	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
	TALG 50	*Scenedesmus* sp.	ND	Chlorophyceae	Scenedesmaceae
	TALG 51	*Scenedesmus* sp.	ND	Chlorophyceae	Scenedesmaceae
	TALG 52	*Coccomyxa* sp.	ND	Trebouxiophyceae	Coccomyxaceae
Karaca-Aladağ (Bolu)	TALG 53	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
	TALG 54	*Chloroidium ellipsoidum*	ND	Trebouxiophyceae	Trebouxiophyceae*
	TALG 55	*Cyanobacteria*	ND	Cyanophyceae	
Pleurotus ostreatur greenhouse	TALG 56	*Coccomyxa* sp.	ND	Trebouxiophyceae	Coccomyxaceae
Cehennem stream (Artvin)	TALG 57	*Chlorella* sp.	ND	Trebouxiophyceae	Chlorellaceae
Drinking water (SümelaSu®)	TALG 60	*Scenedesmus* sp.	ND	Chlorophyceae	Scenedesmaceae
Spring water (Georgia)	TALG 61	*Radiosphaera* sp.	ND	Chlorophyceae	Chlorococcaceae
Fountain (Araklı)	TALG 58	*Choricystis* sp.	ND	Trebouxiophyceae	Coccomyxaceae
	TALG 59	*Ourococcus* sp.	ND	Trebouxiophyceae	Coccomyxaceae
	TALG 62	*Pseudanabaena* sp.	ND	Cyanophyceae	Pseudanabaenaceae
	TALG 63	Diatom	ND	Bacillariophyceae	
Drinking water (Damla®)	TALG 32	*Chlorella vulgaris*	ND	Trebouxiophyceae	Chlorellaceae
	TALG 33	*Chlorella vulgaris*	ND	Trebouxiophyceae	Chlorellaceae
	TALG 64	*Chorysistis* sp.	ND	Trebouxiophyceae	Coccomyxaceae

*class or familia; ND, not determined.

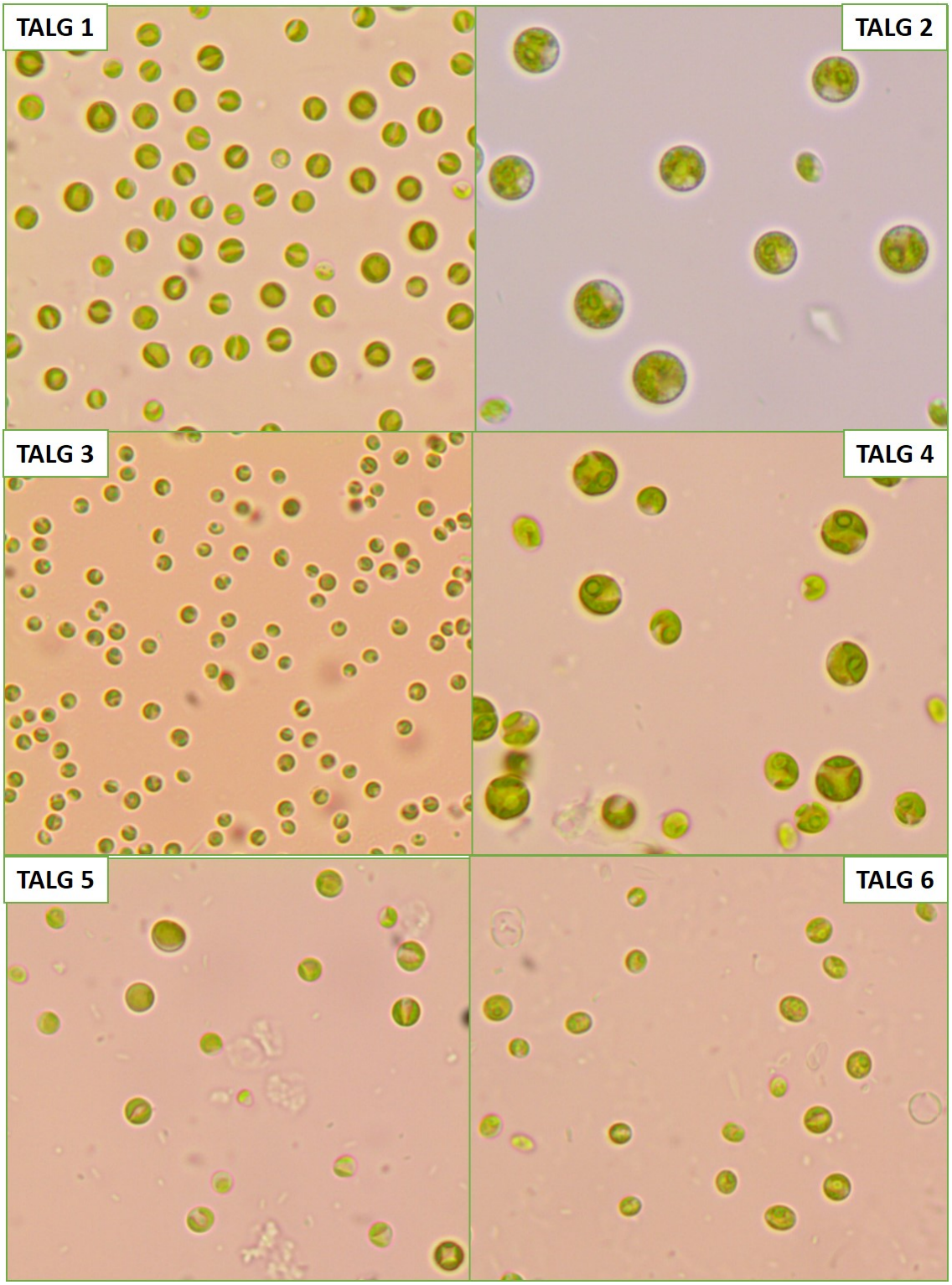
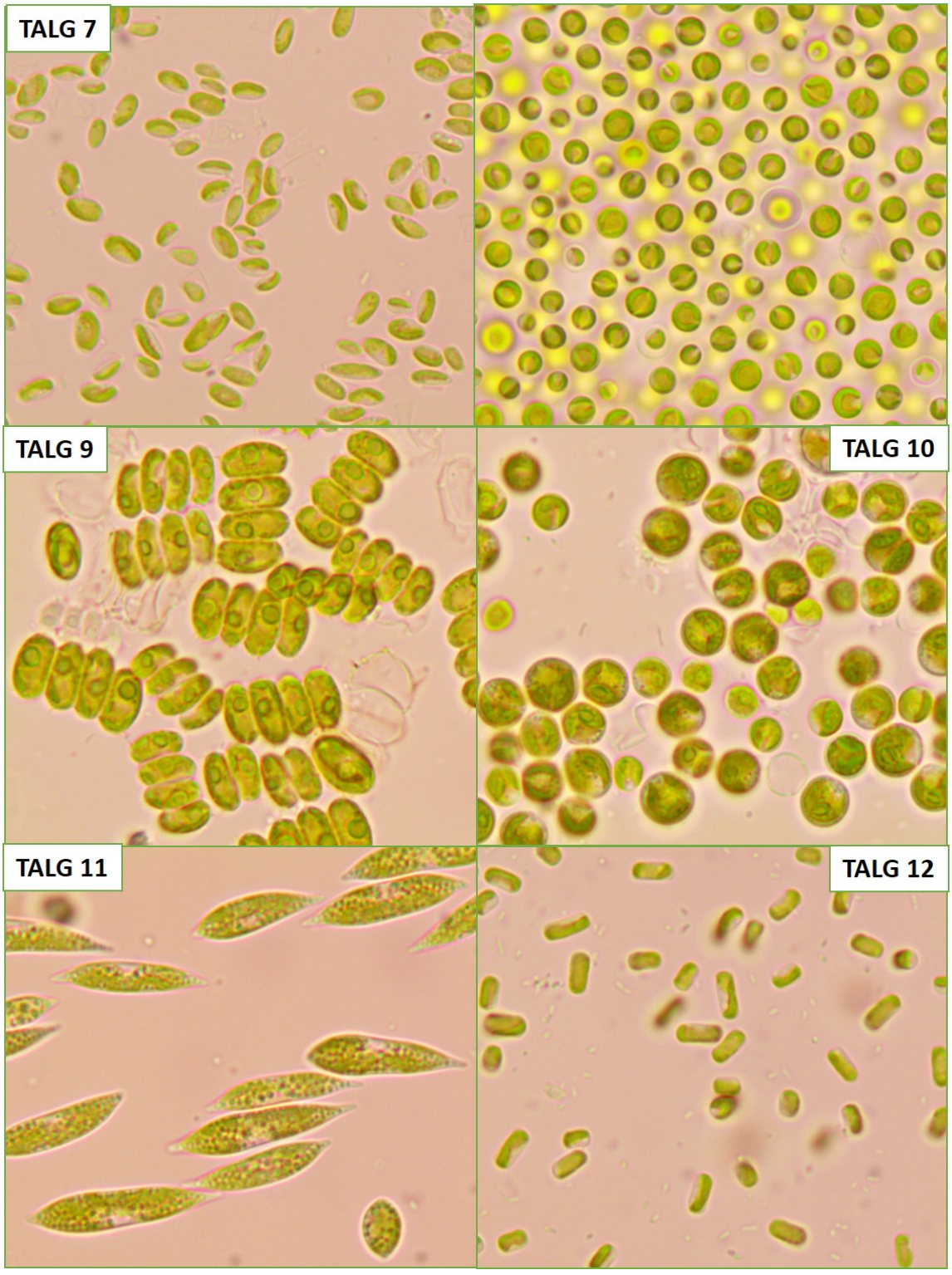


Figure 8. Cell morphologies of isolated microalgal strains observed under a light microscope (100X).



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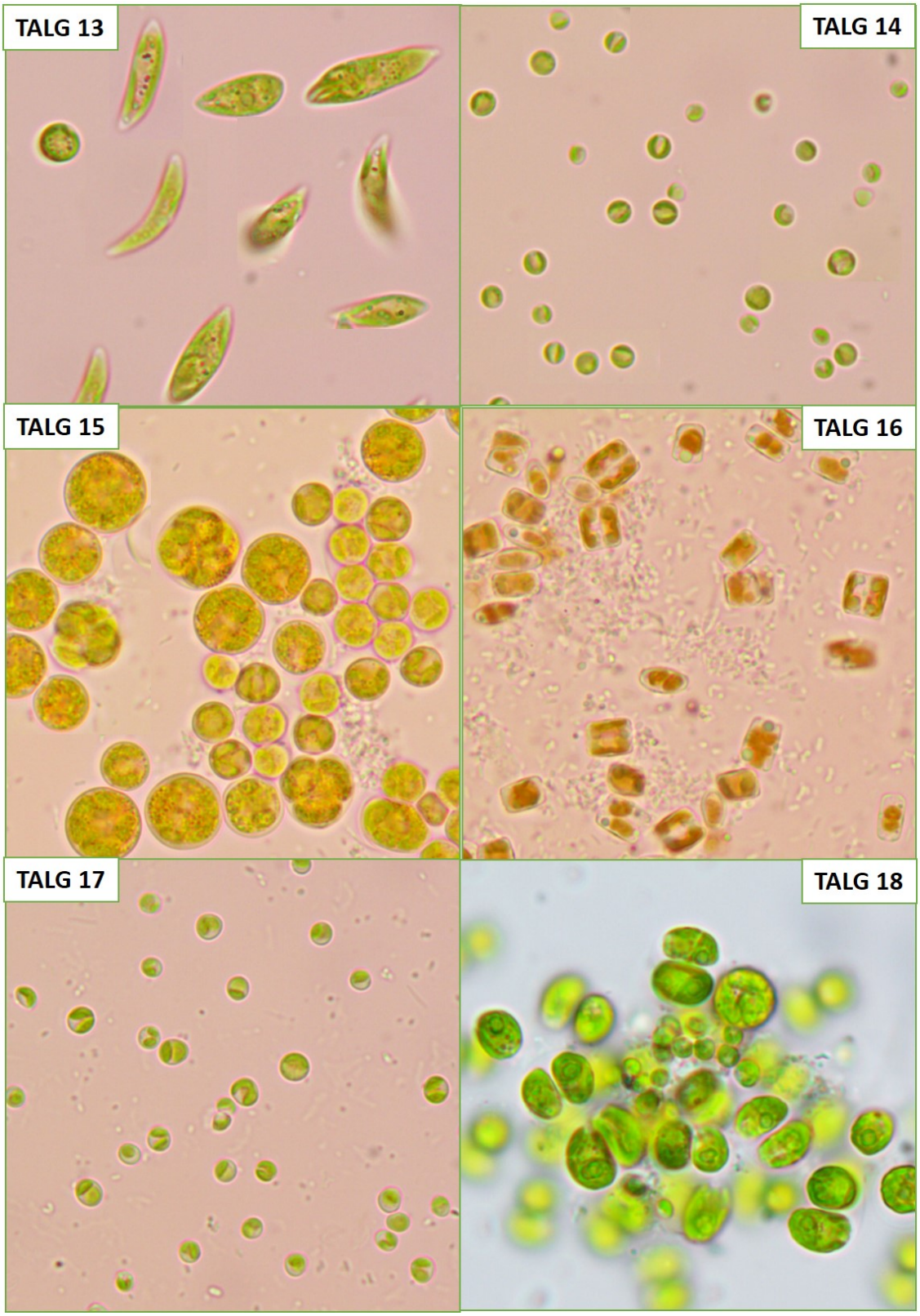

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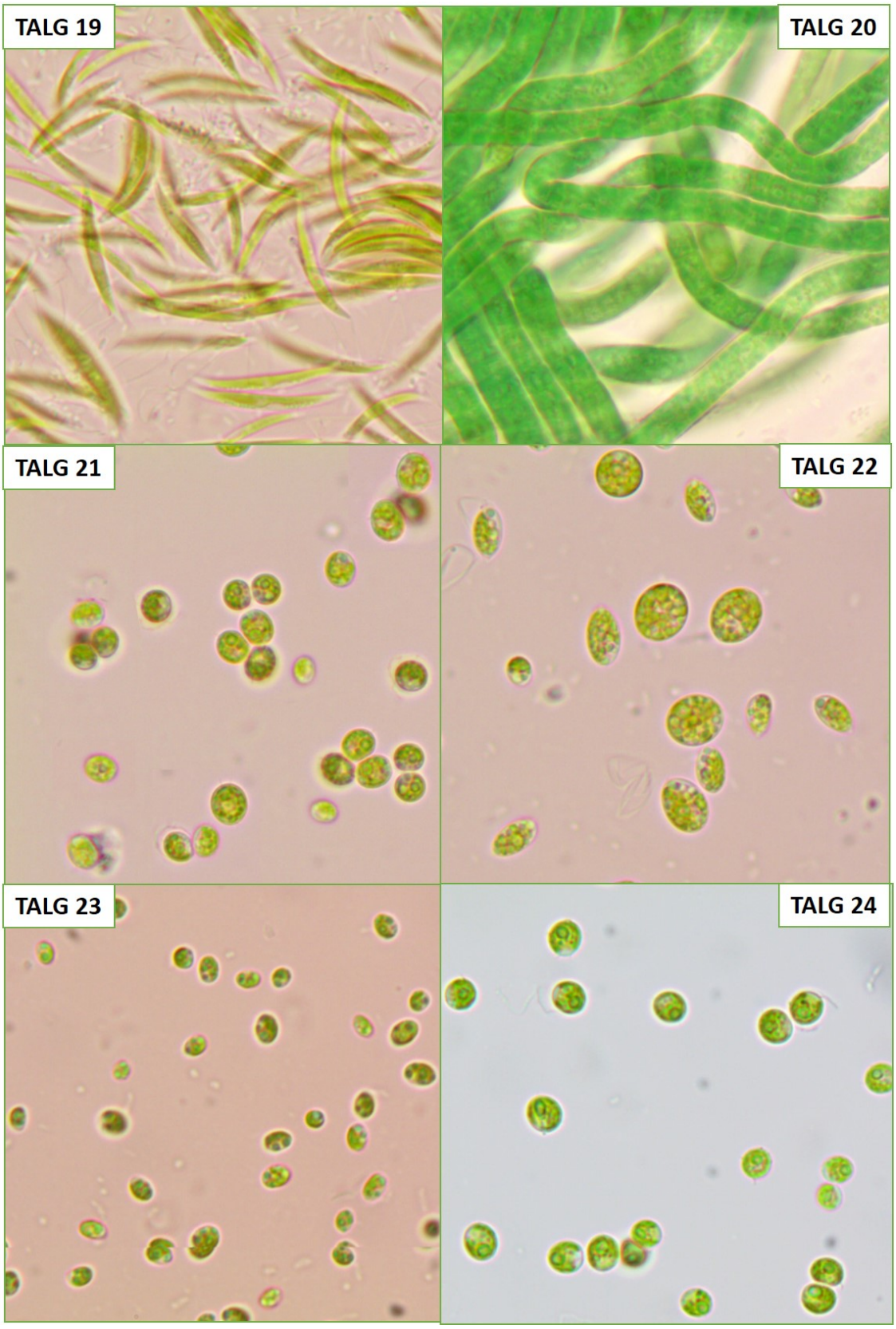

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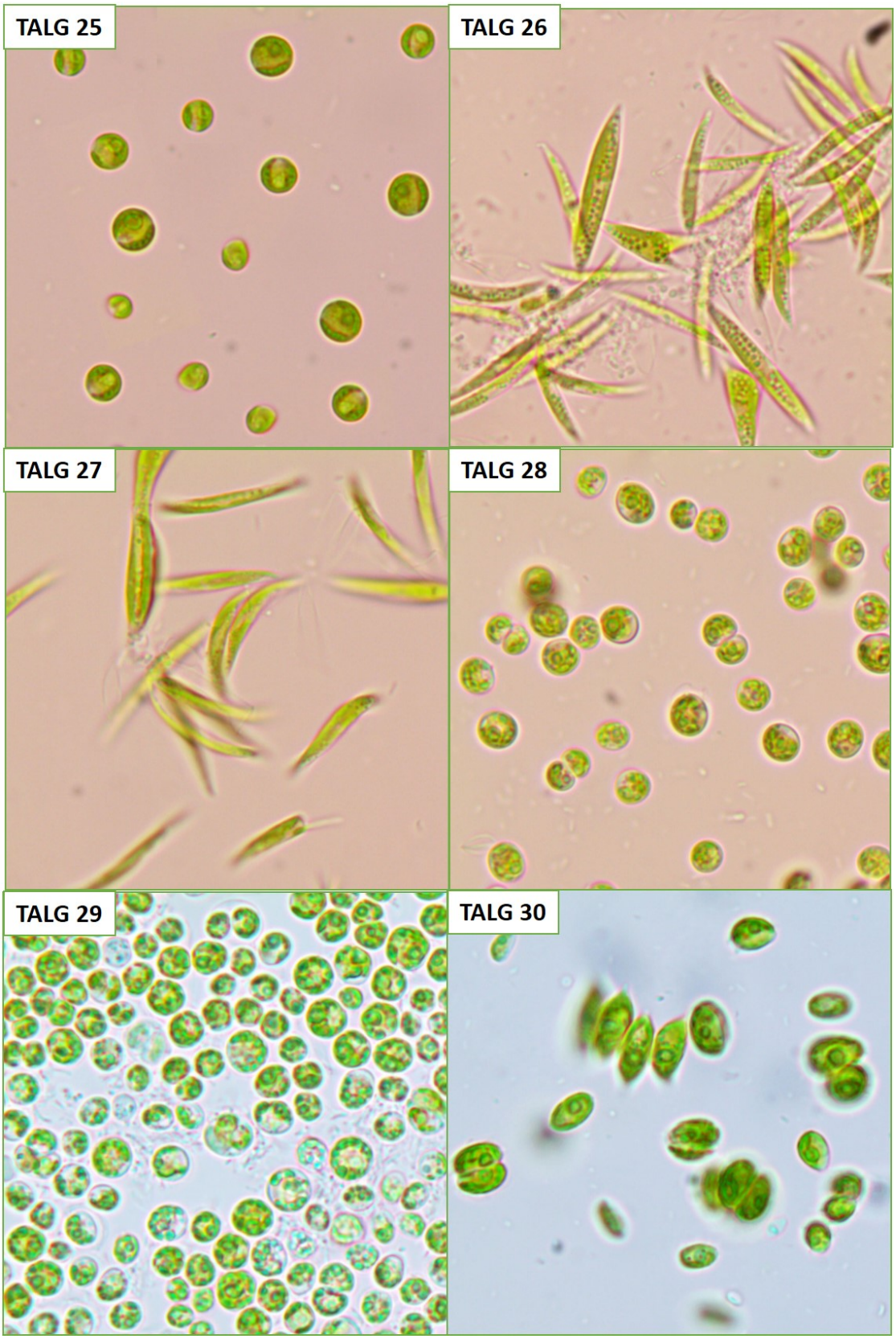

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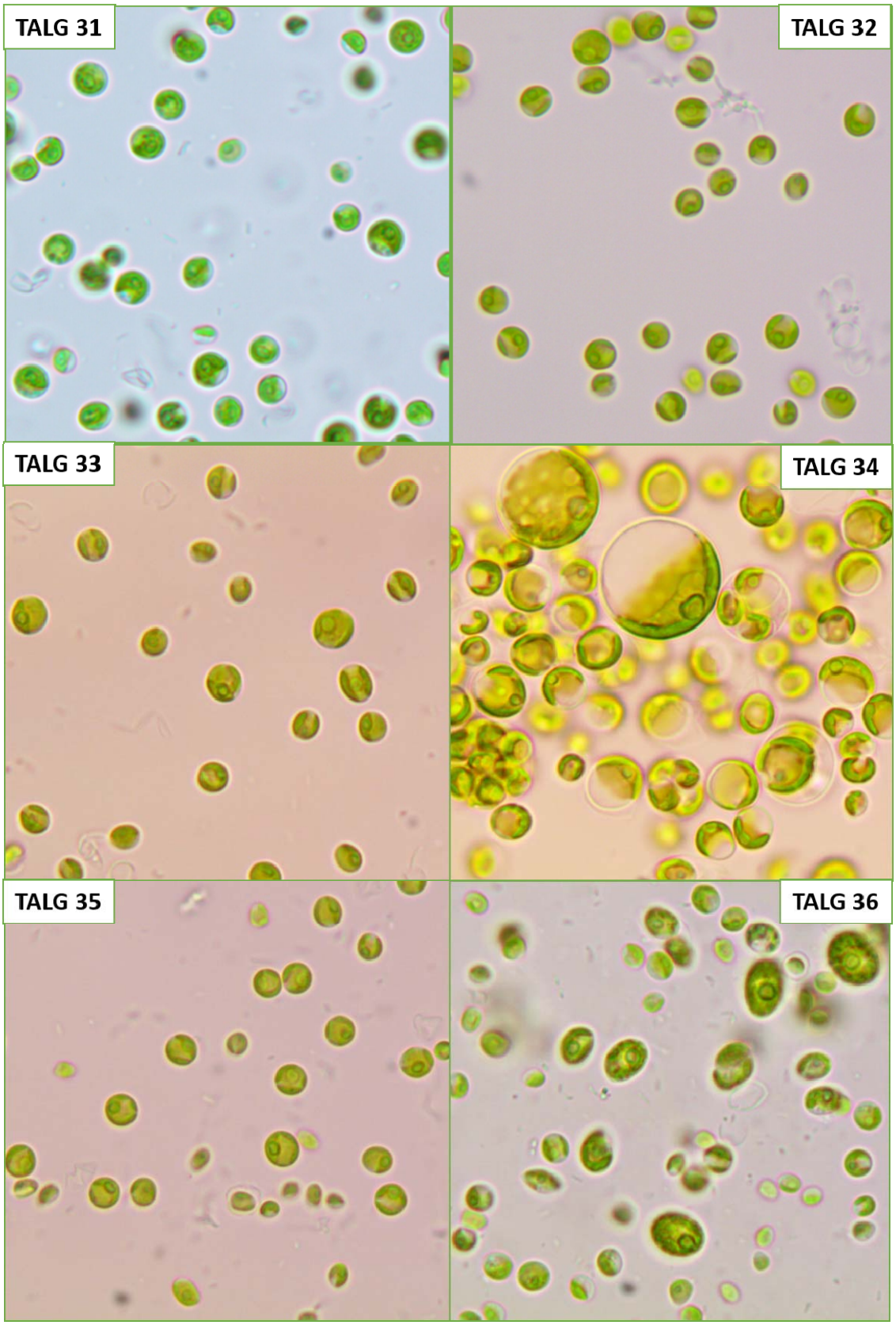

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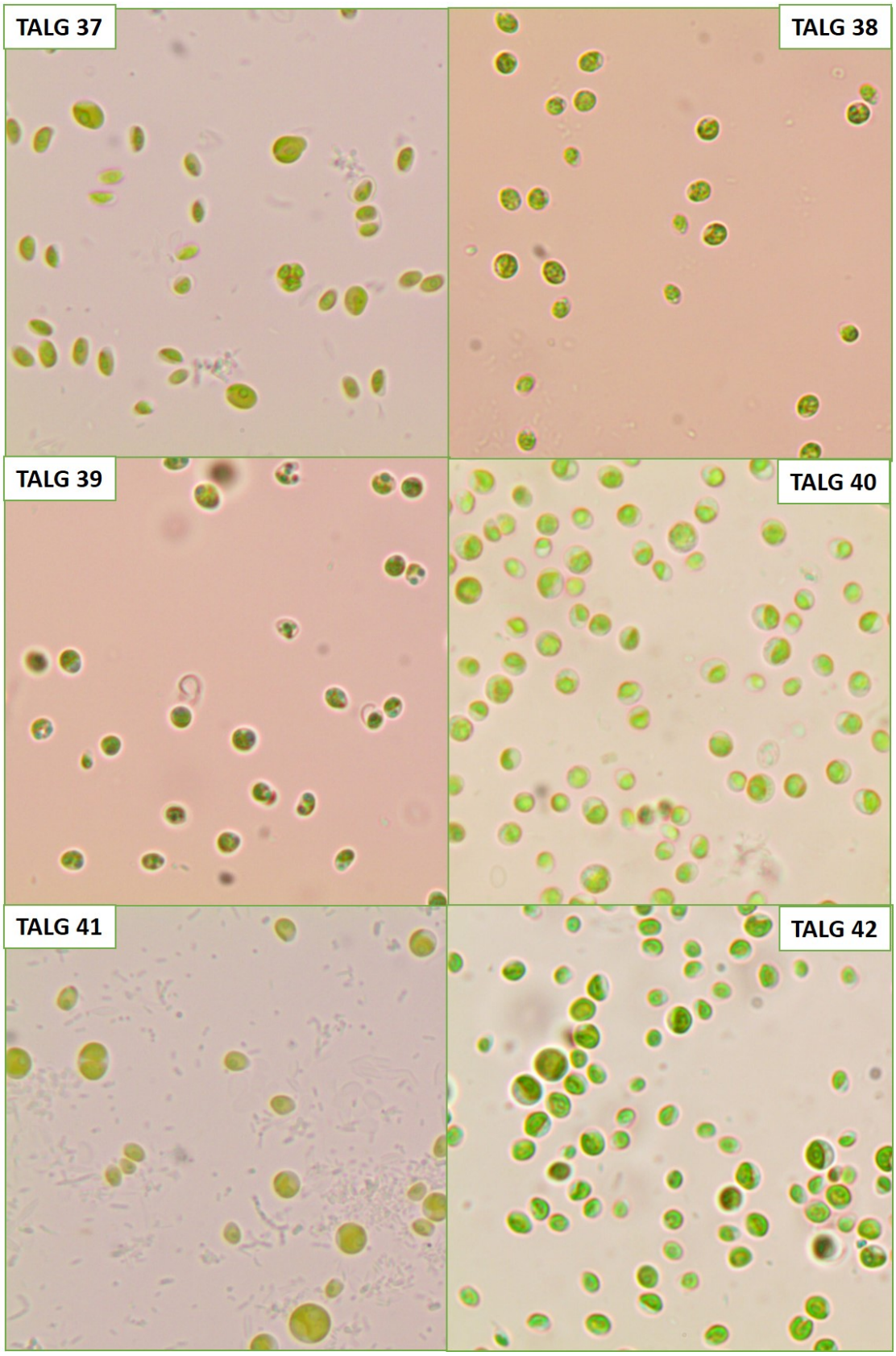

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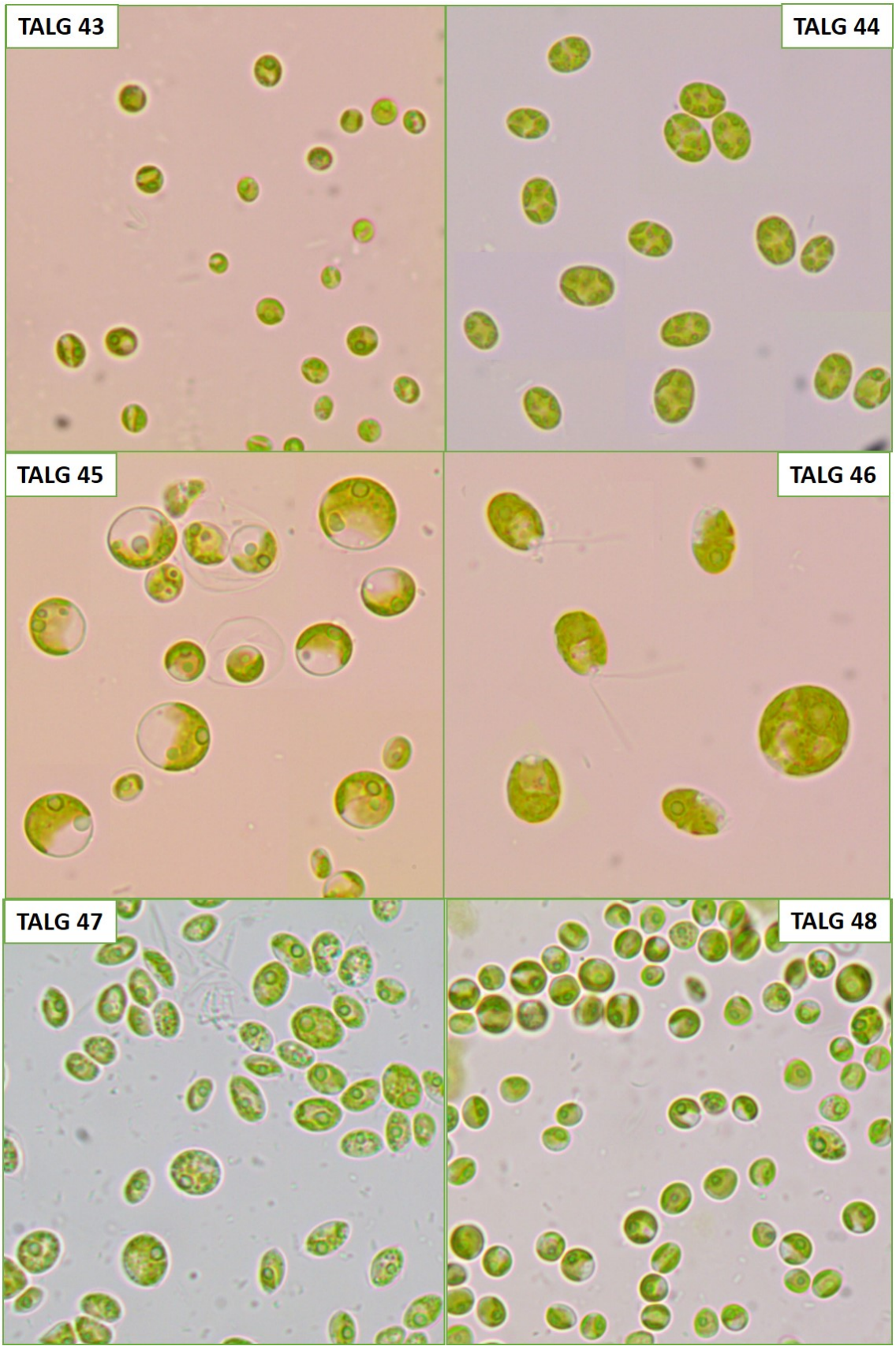

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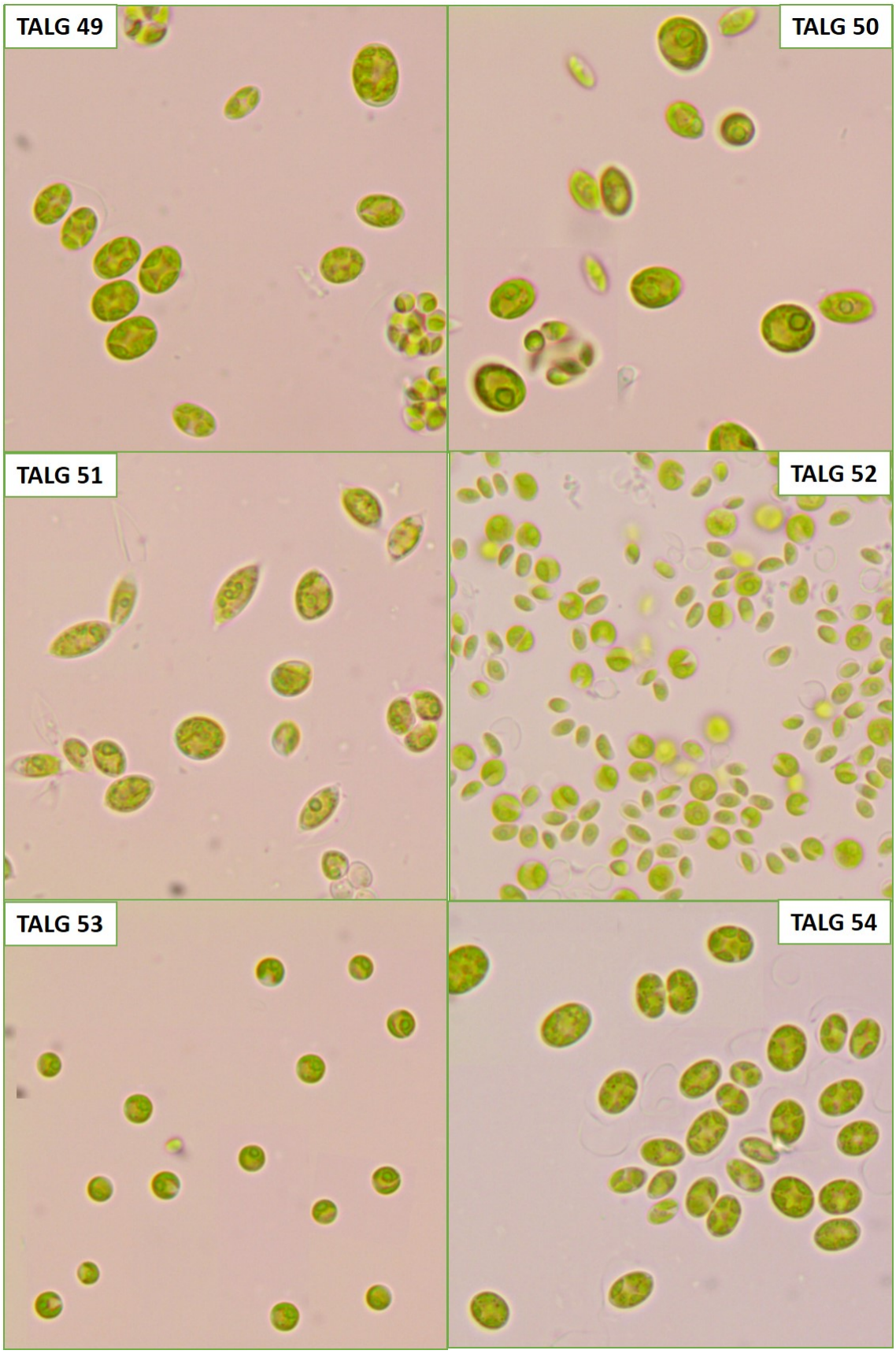

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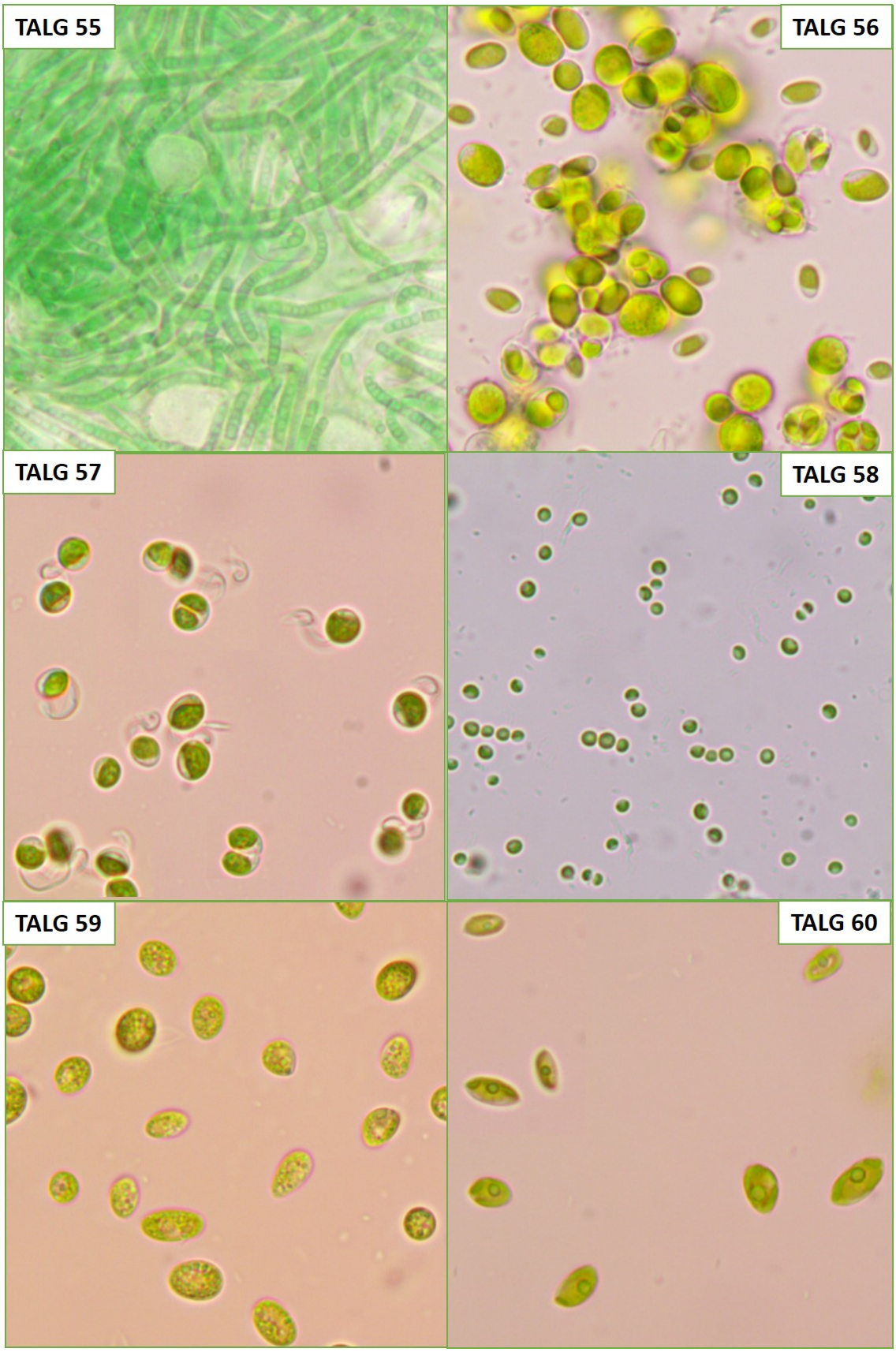

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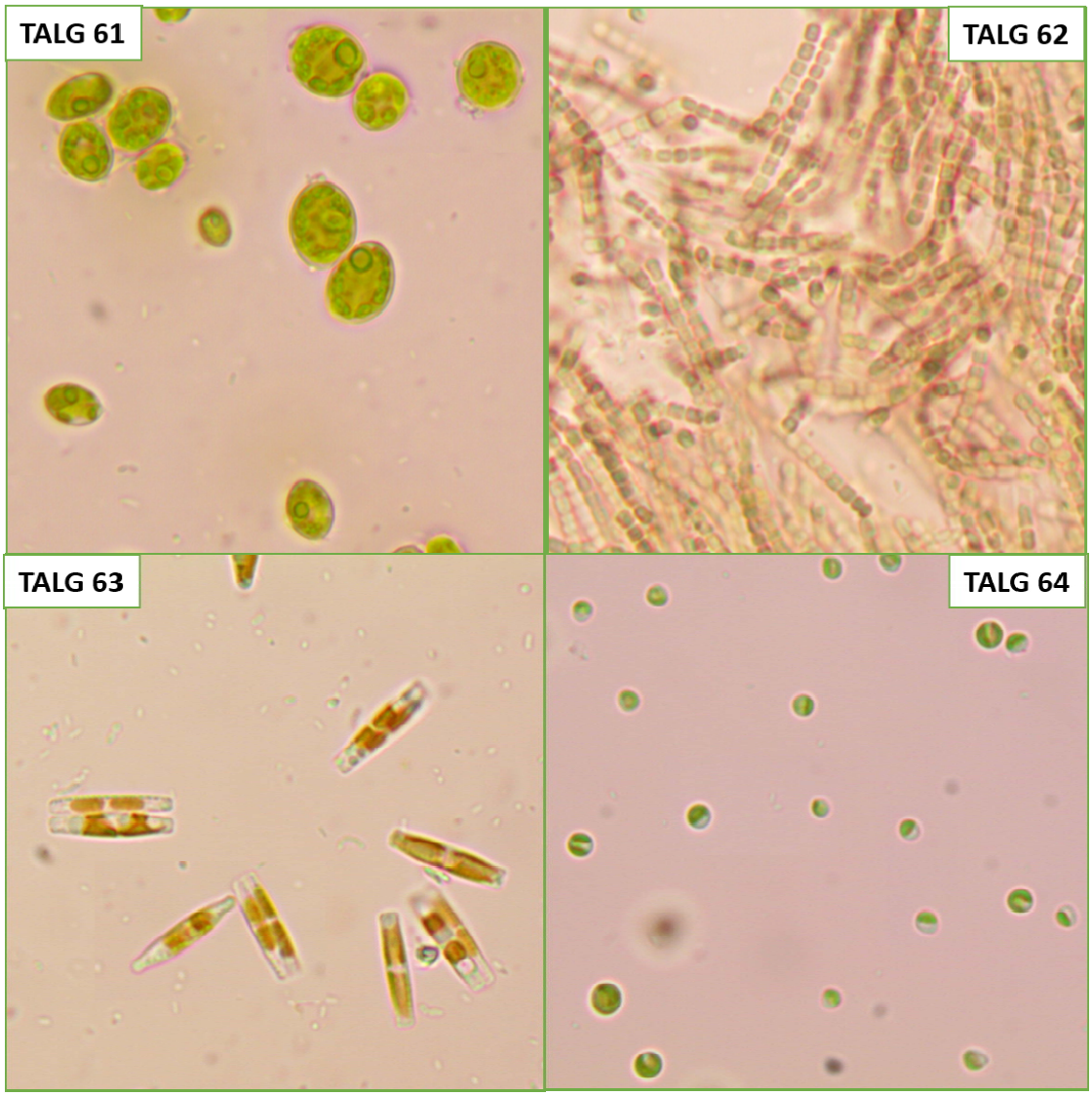

Figure 8. Continued

6.3. Organic Extraction of Algal Cells

About 2 to 30 mg of dry biomass was obtained from 100 ml of liquid culture after centrifugation and drying of the algal cultures. Of the total 64 microalgae isolated in this study, sufficient cell biomass was harvested from 34 strains and used for extraction and testing. These strains include; TALG 1, TALG 2, TALG 3, TALG 4, TALG 5, TALG 6, TALG 9, TALG 10, TALG 11, TALG 12, TALG 13, TALG 14, TALG 21, TALG 22, TALG 23, TALG 24, TALG 26, TALG 27, TALG 28, TALG 29, TALG 31, TALG 31, TALG 32, TALG 33, TALG 34, TALG 38, TALG 43, TALG 50, TALG 52, TALG 53, TALG 54, TALG 59, TALG 61, and TALG 62.

Organic extraction was applied as 10.mg : 1.ml (cell biomass : solvent). Methanol extracts yielded about 100.mg of extract from 100.mg of algal biomass. As for hexane, yields were between 1 and 10.mg for 100.mg of extracted algal biomass. All extracts were dissolved in DMSO at 100.mg/ml as stock solution then were 10-fold diluted with distilled sterile water and used as 10.mg/ml working solution. Low yielded extracts were dissolved as 10.mg/ml, 5.mg/ml or 1.mg/ml in DMSO and were directly tested. The working concentrations of the extracts are mentioned in the tests results (Table 4).

6.4. Antimicrobial Activity Test Results

Agar well diffusion test was applied for the 34 methanol and hexane extracts, 16 microalgal strains showed antimicrobial activity as shown in Table 4, and Figures 9 and 10.

Table 4. Antimicrobial activity tests results

Extract Source	Extract concentration used (mg/ml)	Solvent	Sensitive Microorganisms	Inhibition Diameter (mm)	MIC (µg/ml)
TALG 1	10	Hex.	*A. haemolyticus* ATCC 19002	9	1250
TALG 6	10	Hex.		12	1250
TALG 11	10	Hex.		10	1250
TALG 13	10	Hex.		10	625
TALG 27	10	MeOH		15	625
TALG 29	5	MeOH		13	0
Pos	0.2	CN		23	6.3
TALG 22	5	MeOH	*B. subtilis* ATCC 6633	12	1250
TALG 24	1	MeOH		12	4
TALG 27	10	MeOH		10	78
TALG 29	5	MeOH		10	2500
TALG 30	10	MeOH		10	39
TALG 31	10	MeOH		11	78
TALG 33	5	MeOH		11	312
TALG 38	10	MeOH		11	78
TALG 54	5	MeOH		12	19.6
Pos	0.2	AMP		20	1.6
TALG 10	10	Hex.	*C. albicans* ATCC 10231	15	625
TALG 12	10	Hex.		13	625
TALG 21	10	Hex.		12	625
TALG 24	1	MeOH		12	62.5
TALG 24	10	Hex.		14	1250
TALG 27	10	Hex.		12	1250
TALG 29	10	Hex.		10	0
TALG 31	10	Hex.		12	625
TALG 38	10	MeOH		12	1250
Pos	0.2	AmB		22	0.6
TALG 30	10	MeOH	*E. faecalis* ATCC 29212	9	156
TALG 31	10	MeOH		9	39
TALG 11	10	MeOH		11	2500
Pos	0.2	AMP		22	3
TALG 30	10	Hex.	*K. pneumonia* ATCC 13883	11	2500
Pos	0.2	CN		21	1.6

Hex, Hexane extract; MeOH, Methanol extract; MIC, Minimal inhibitory concentration; Pos, positive control; CN, Gentamicin; AMP; Ampicillin; AmB, Amphotericin B.

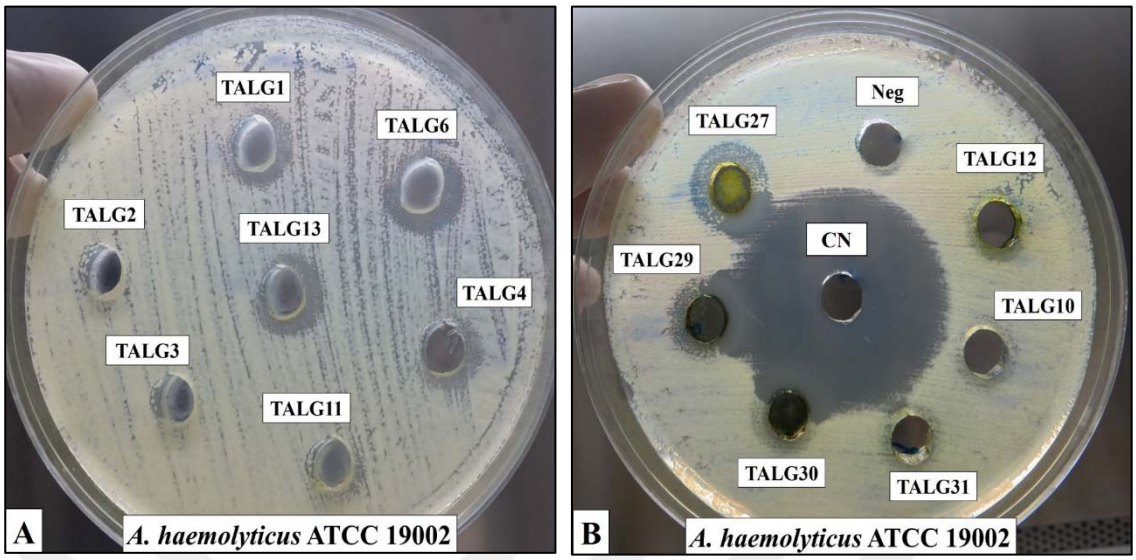

Figure 9. Agar well diffusion test results against *A. haemolyticus* ATCC 19002. A, hexane extracts; B, methanol extracts; CN, gentamicin (10.µg); Neg, negative control (DMSO).

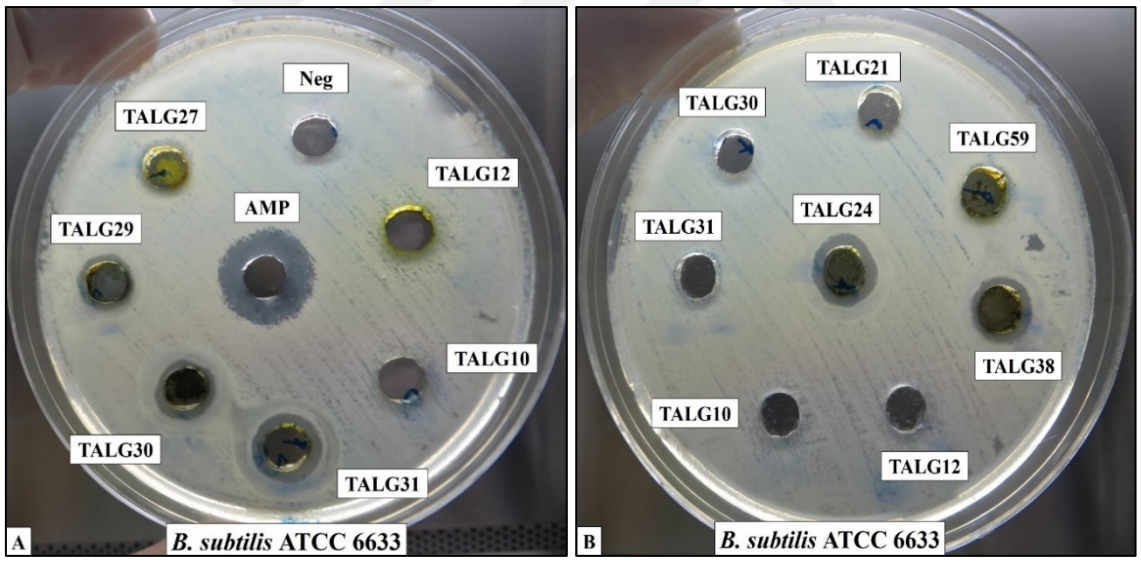

Figure 10. Agar well diffusion test results against *B. subtilis* ATCC.6633. A, methanol extracts. B, TALG 30, TALG 31, TALG 10 and TALG 12 are hexane extracts, TALG 38, TALG 59, TALG 21 and TALG 24 are methanol extracts; AMP, ampicillin (10.µg); Neg, negative control (DMSO).

6.5. Anti-Quorum Sensing Activity Test Results

The algal extracts tested for antimicrobial activities were also tested for their anti-QS activity against purple pigment production of the indicator strain of *C. violaceum*. A positive anti-QS effect will not affect the bacterial growth but will

decrease or eliminate its pigment production. While two of the methanol extracts (TALG 23 and TALG 34) had positive results for significant QS inhibition without cell growth inhibition, none of the hexane extracts exhibits any QS inhibitory activity.

Anti-QS activity of vanilla against *C. violaceum* at 50 µg/ml and 100 µg/ml concentrations shown in Figure 11 as control. Vanilla decreased the pigment production of the bacteria decreased to OD_{595} = 0.1 and 0.3 while bacterial growth were unaffected (around 10^8 and 10^9 CFU/ml) in comparison to the control.

The chart in the Figure 12 shows the QS inhibitory activity of the methanol extract of TALG 23 with the concentrations 78 and 39 µg/ ml. These concentrations inhibit the bacterial pigment production without significantly affecting the bacterial growth indicated by the bacterial count in comparison with the control. The similar results were obtained for TALG 34 (Figure 13) with the anti-QS activity exerted at 78 and 156 µg/ml.

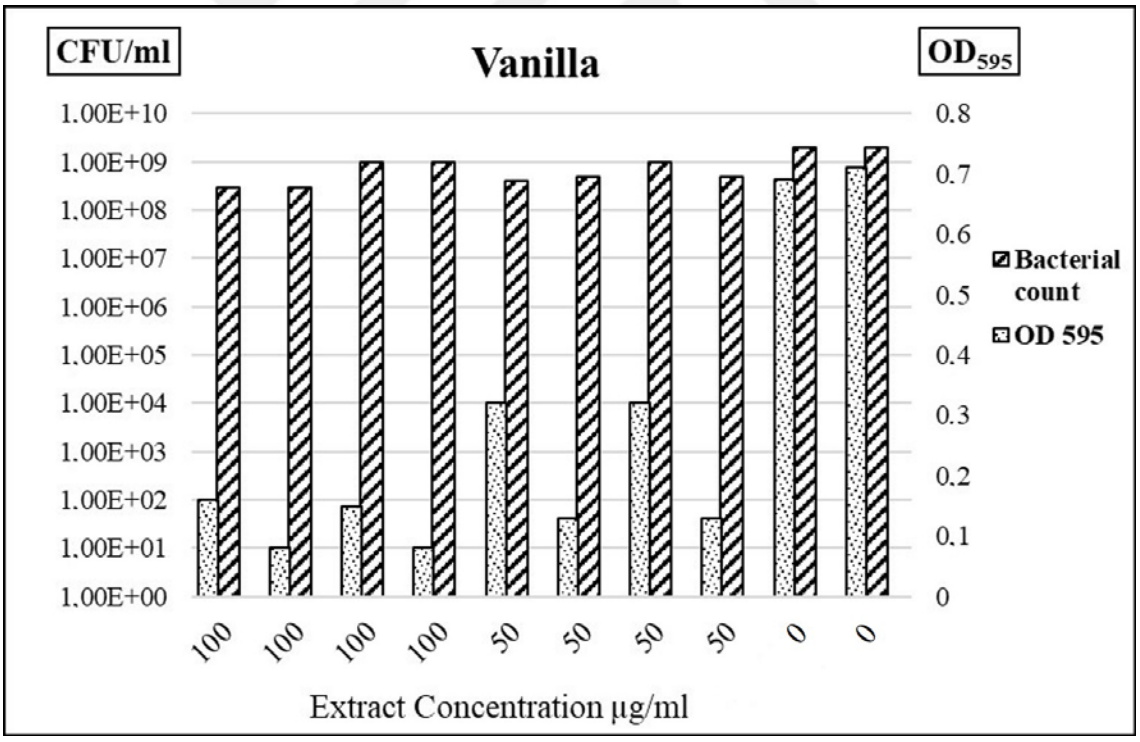

Figure 11. Anti-quorum sensing activity chart of the positive control vanilla

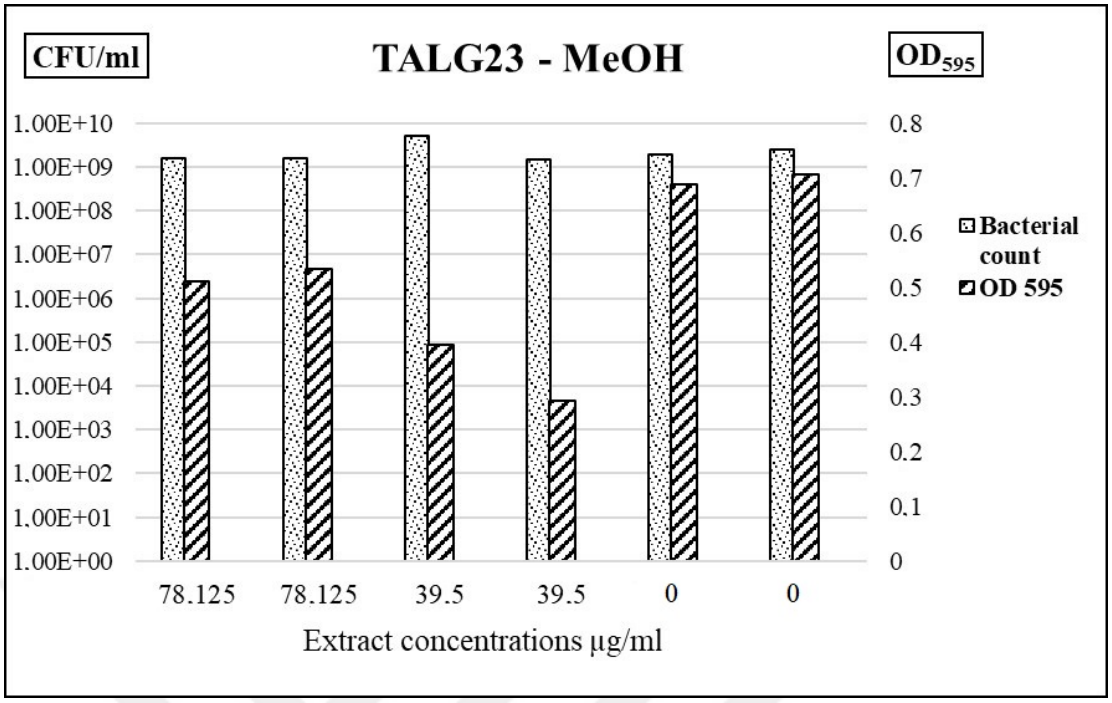

Figure 12. Anti-quorum sensing activity chart of TALG 23

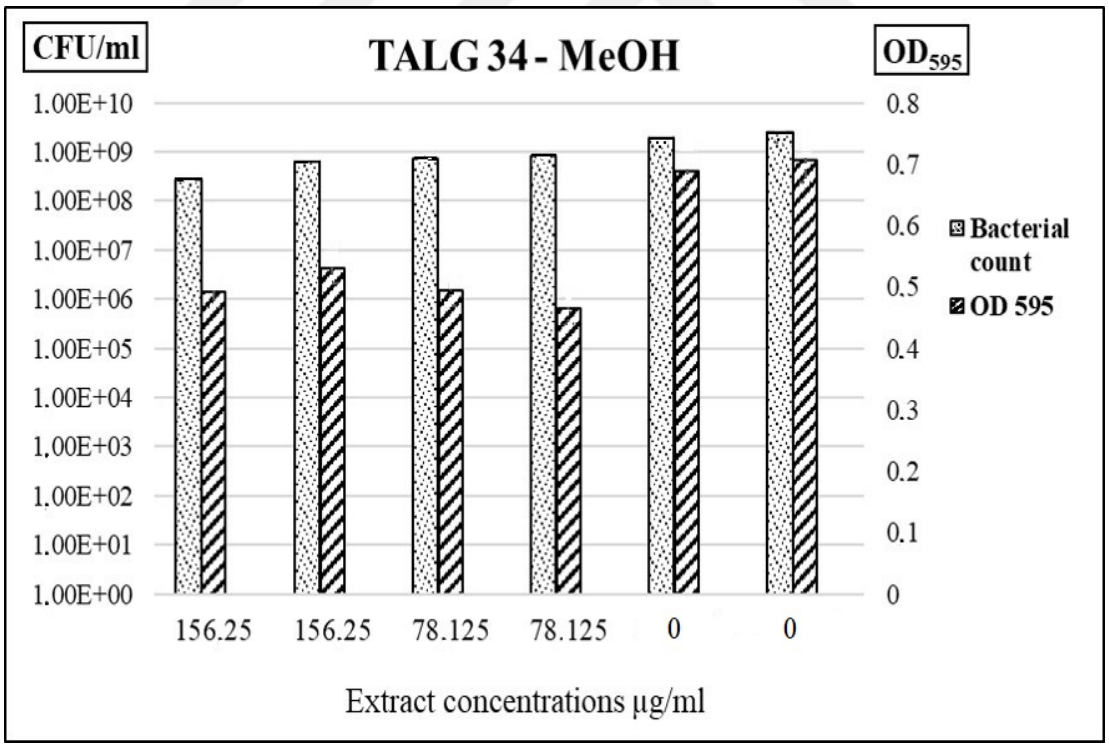

Figure 13. Anti-quorum sensing activity chart of TALG 34

6.6. Flow Cytometry Analysis of Intracellular Lipids

Nile red stained and unstained cells were visualized by fluorescence microscope with blue laser excitation. Lipids droplets appeared yellow inside the cells that emitted red fluorescence due to their chlorophyll contents. Flow cytometry detection was applied by blue laser excitation at 488.nm, and the emitted fluorescence was read at FL2 channel (585/40.nm). The unstained cells were considered as negative control to remove the erroneous auto-fluorescence signals by gating, then the stained cells were measured and the results were expressed as fluorescence arbitrary units per 100 events (Table.5 and Figures 14-34).

Among 32 algal strains tested, TALG 13, TALG 15, and TALG 26 had the highest intracellular lipid content as indicated by fluorescence arbitrary units (f.a.u.) as 1,322.4, 1,385.6, and 1,110.8, respectively.

Table 5. Lipid contents of selected algal strains

Sample	Unstained		Stained	
	Count	f.a.u./100 event	Count	f.a.u./100 event
TALG 1	49,950	0.18	63,864	10.3
TALG 2	8,051	4.28	19,697	175.1
TALG 3	50,748	0.7	98,092	15.3
TALG 4	31,971	1.5	23,048	558.1
TALG 5	46,692	0.9	8,323	106.8
TALG 6	120,877	0.2	67,256	25.5
TALG 7	13,852	1.8	6,922	428
TALG 8	22,608	2.9	33,760	65.5
TALG 9	13,333	18.2	7,436	481.3
TALG 10	5,060	19.7	6,962	418
TALG 11	55,922	2.6	40,490	0.2
TALG 12	20,452	0.9	19,861	100.8
TALG 13	6,924	99.4	14,953	1 322.4
TALG 14	150,490	0.3	73,708	3.2
TALG 15	12,158	1	31,619	1 385.6
TALG 21	4,215	16.8	4,807	298.1
TALG 22	16,220	1.8	9,461	292.5
TALG 23	10,750	0.4	16,259	20.1
TALG 26	12,386	10	14,027	1,110.9
TALG 27	27,823	4.8	12,272	491.6
TALG 28	165,447	0.7	55,030	9.6
TALG 30	73,434	0.6	27,423	150.7
TALG 32	76,587	0.4	66,003	21.2
TALG 33	41,189	0.6	72,125	15
TALG 34	65,688	1.5	20,810	71.4
TALG 38	122,460	0.3	60,809	52.6
TALG 43	99,519	0.33	47,602	13.7
TALG 50	68,916	1.33	23,005	34.8
TALG 52	47,789	0.53	123,239	5.7
TALG 53	56,231	0.43	165,399	3.6
TALG 54	100,541	0.4	45,185	219.7
TALG 61	62,274	1.1	25,918	27.27

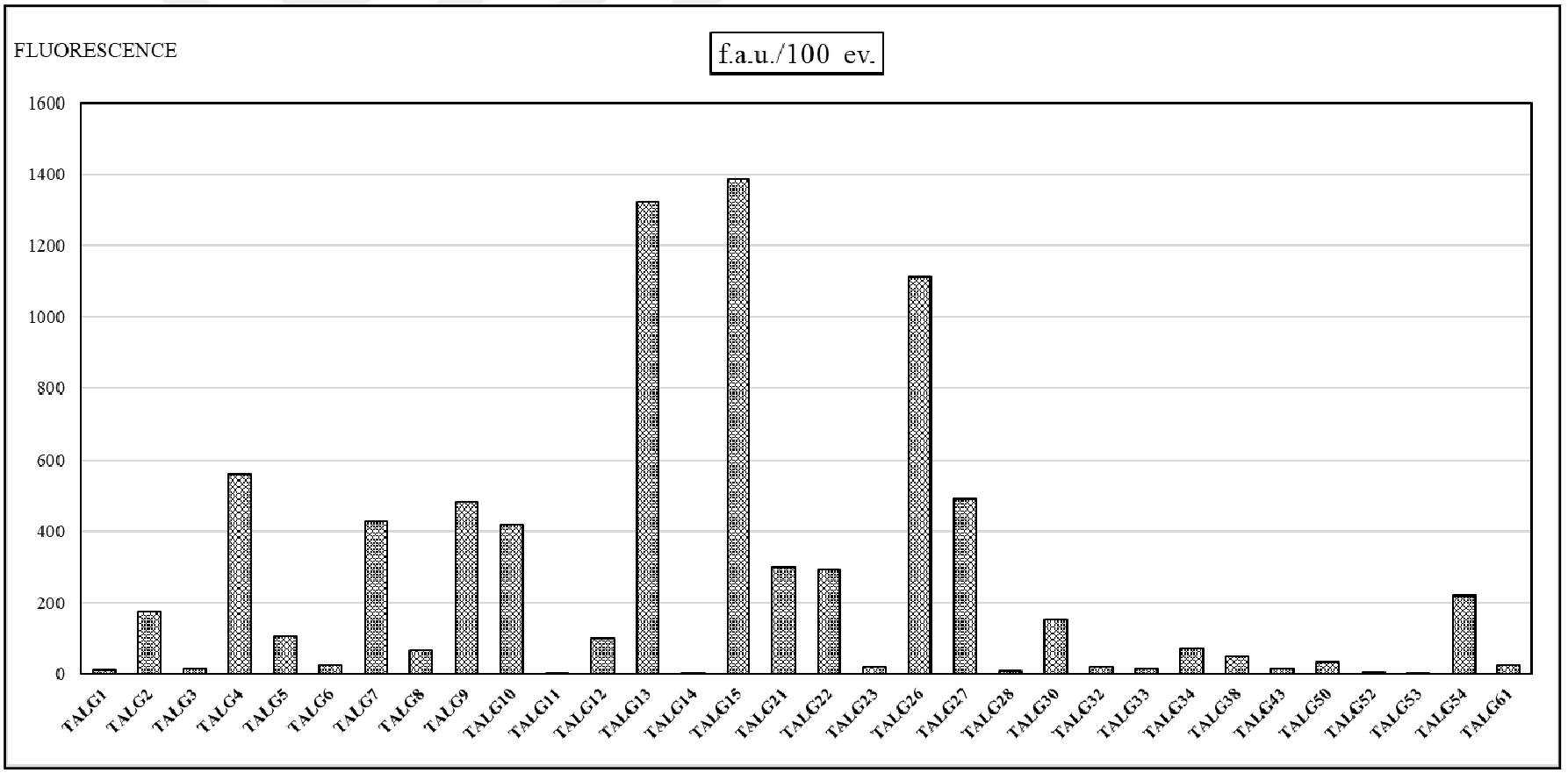

Figure 14. Neutral lipid contents in fluorescence arbitrary units (f.a.u.) per 100 events for selected microalgal cells.

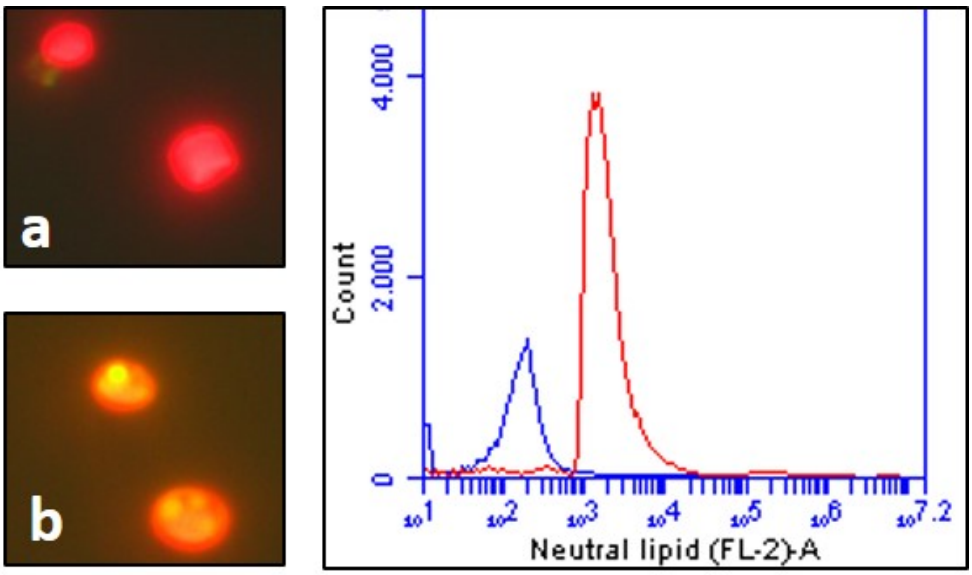

Figure 15. Lipid content observation of TALG 1 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 100-fold in the fluorescence arbitrary unit in the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

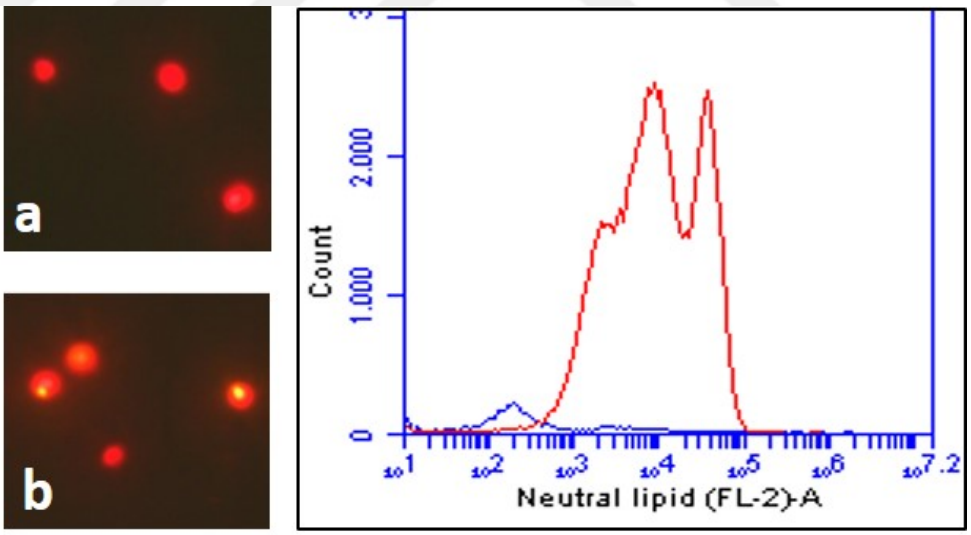

Figure 16. Lipid content observation of TALG 3 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 100 to 1000-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

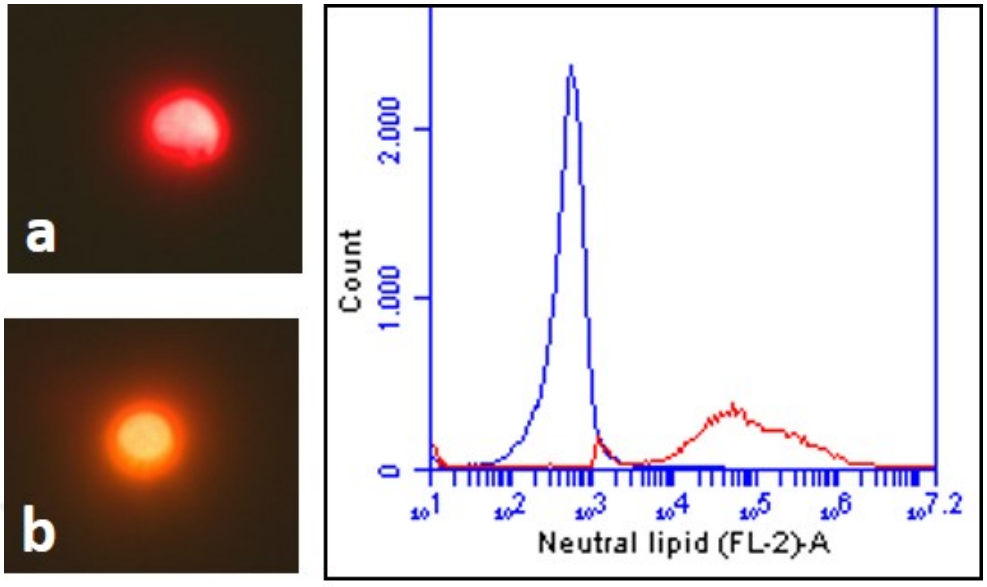

Figure 17. Lipid content observation of TALG 4 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 100 to 1000-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

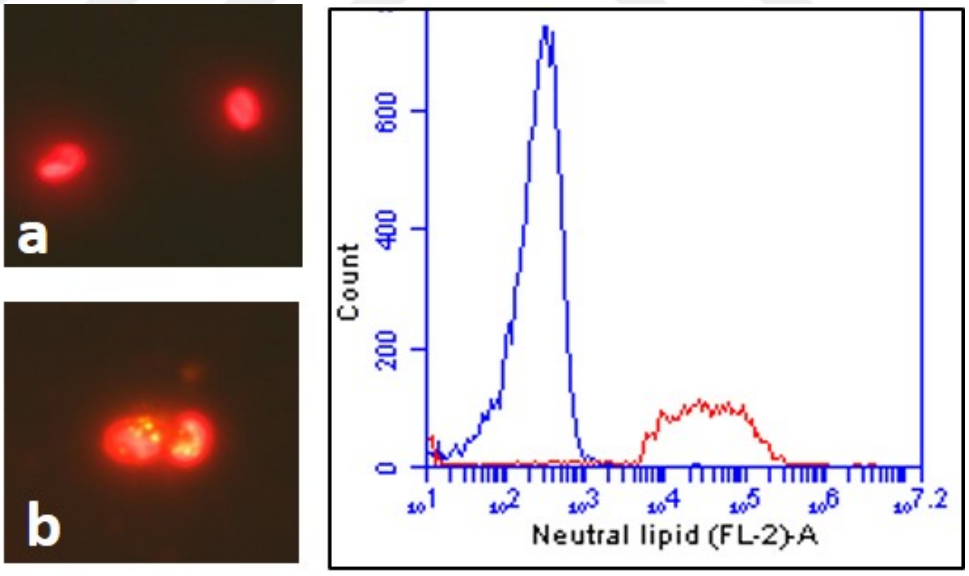

Figure 18. Lipid content observation of TALG 6 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 100-fold in the fluorescence arbitrary unit in the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

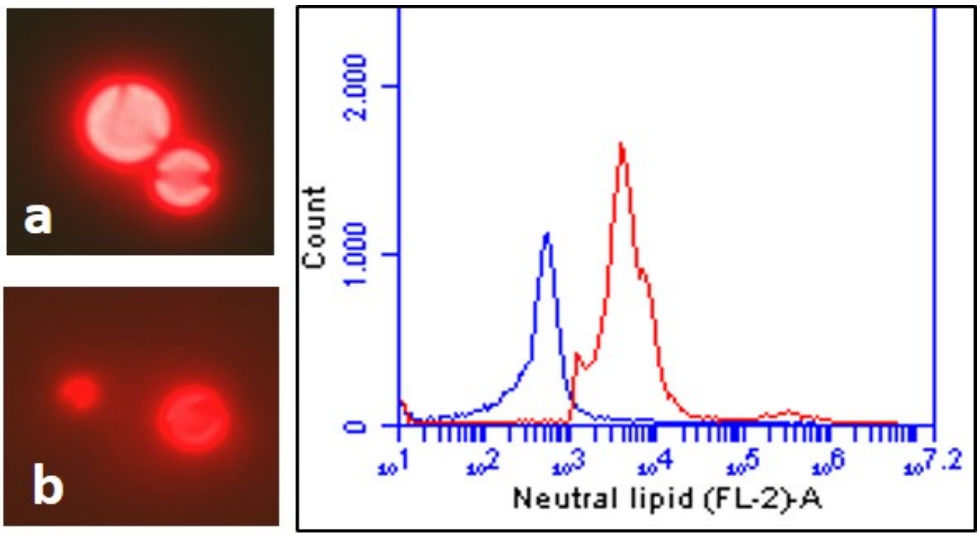

Figure 19. Lipid content observation of TALG 8 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 10-fold in the fluorescence arbitrary unit detected by FL2 channel after staining (red) in comparison with the non-stained cells (blue).

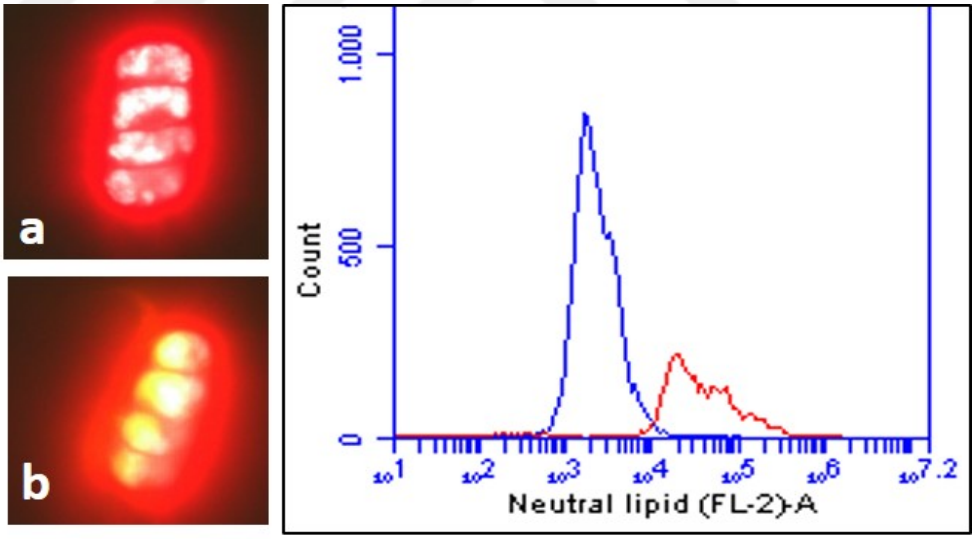

Figure 20. Lipid content observation of TALG 9 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 1000-fold in the fluorescence arbitrary unit detected by FL2 channel after staining (red) in comparison with the non-stained cells (blue).

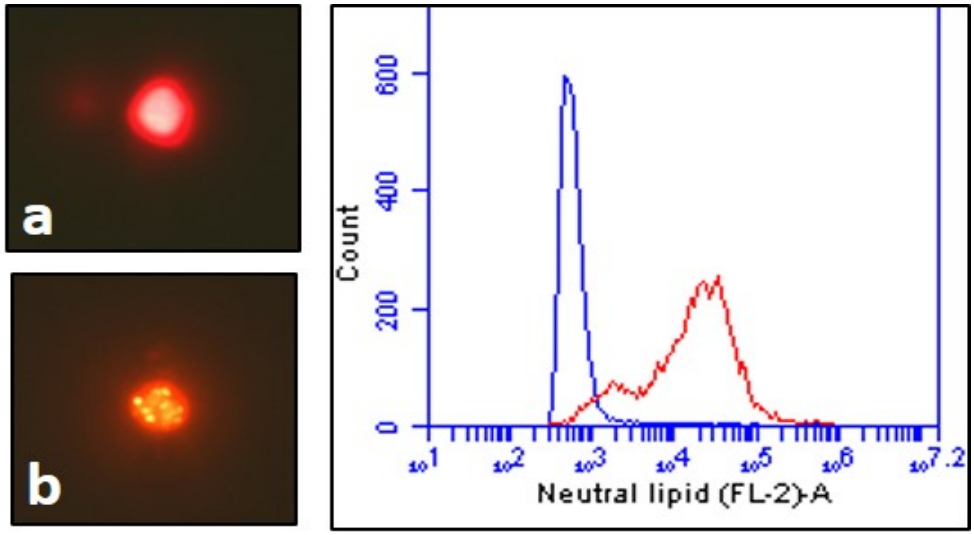

Figure 21. Lipid content observation of TALG 10 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 100-fold in the fluorescence arbitrary unit in the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

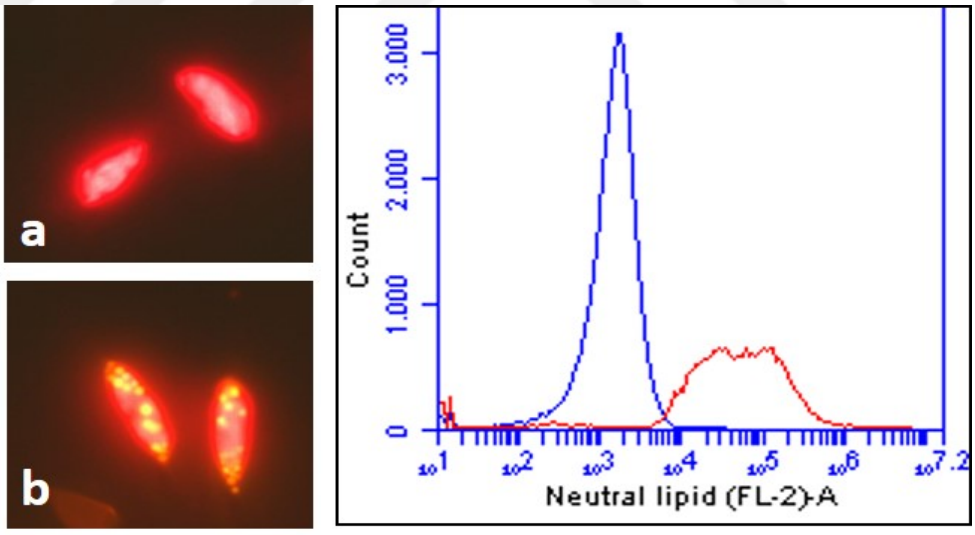

Figure 22. Lipid content observation of TALG 11 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 1000-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

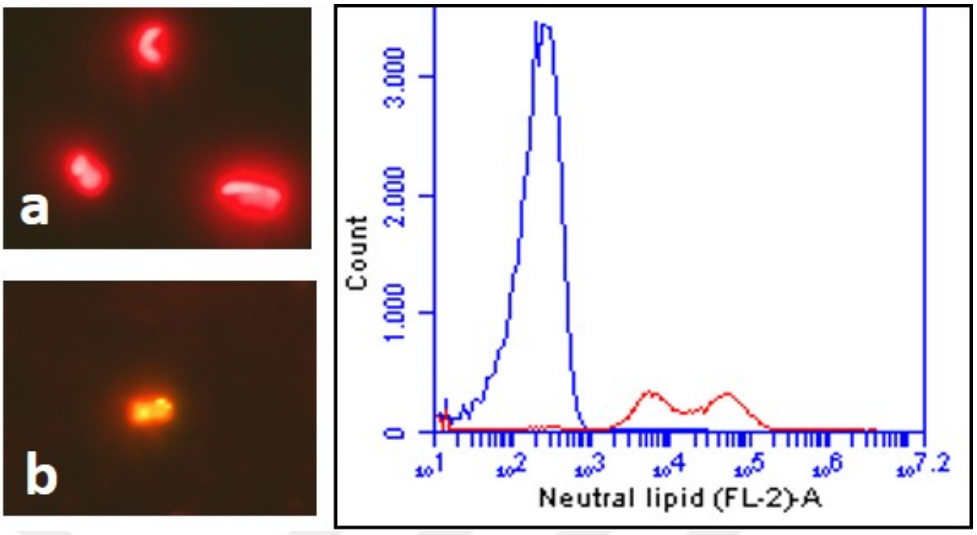

Figure 23. Lipid content observation of TALG 12 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 1000-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

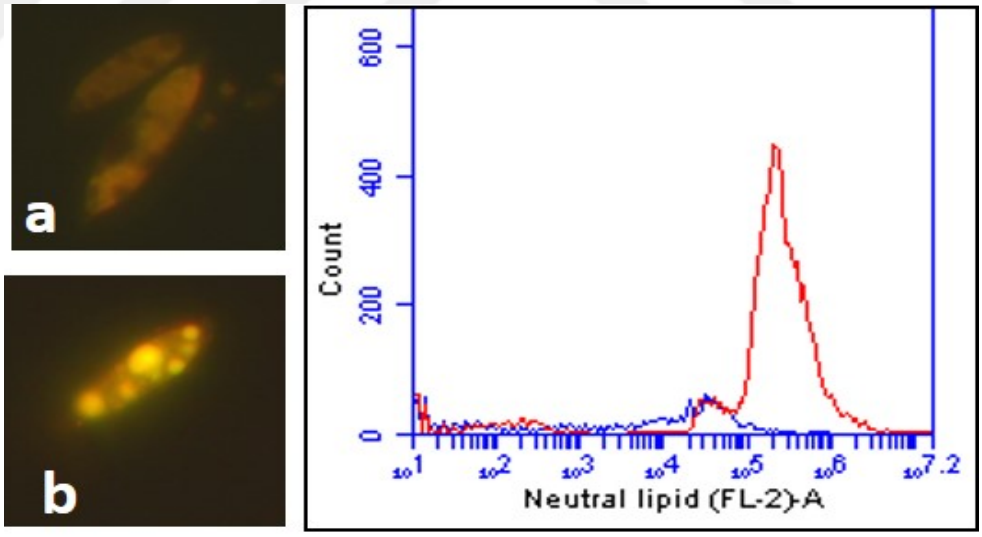

Figure 24. Lipid content observation of TALG 13 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 10-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

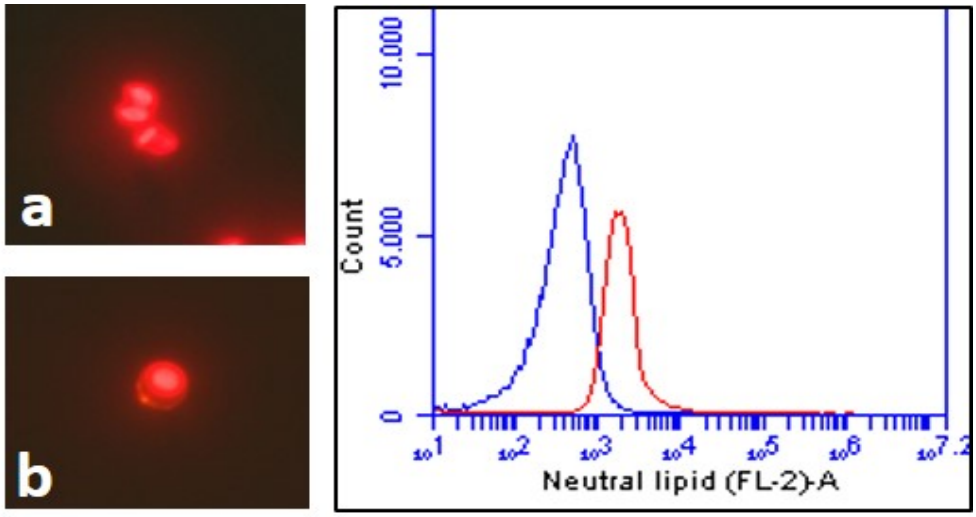

Figure 25. Lipid content observation of TALG 14 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing less than 10-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

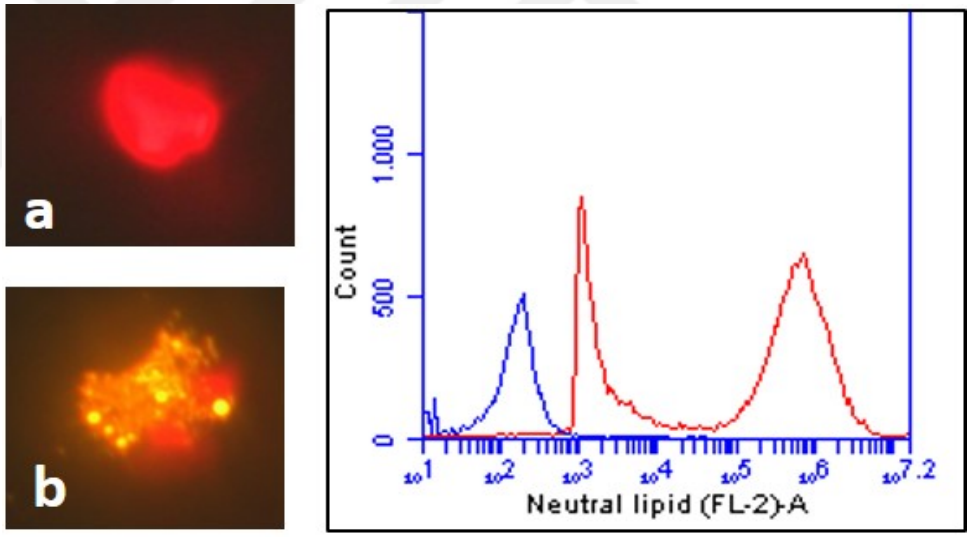

Figure 26. Lipid content observation of TALG 15 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about a 100,000-fold in the fluorescence arbitrary unit in the FL2 channel after staining (red) in comparison with the non-stained cells (blue). Although, 2 populations of neutral lipid stained cells are shown at 10^3 and $10^5 – 10^7$.

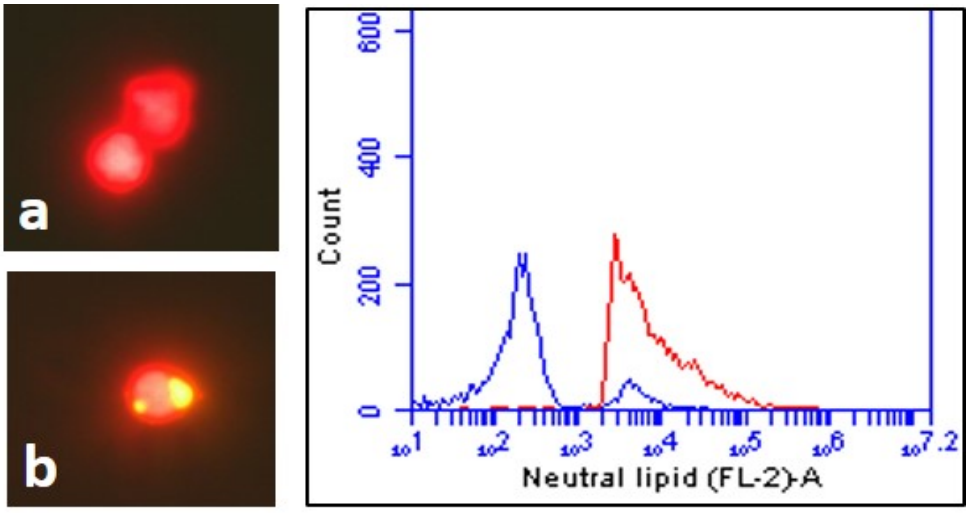

Figure 27. Lipid content observation of TALG 21 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing less than 10-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

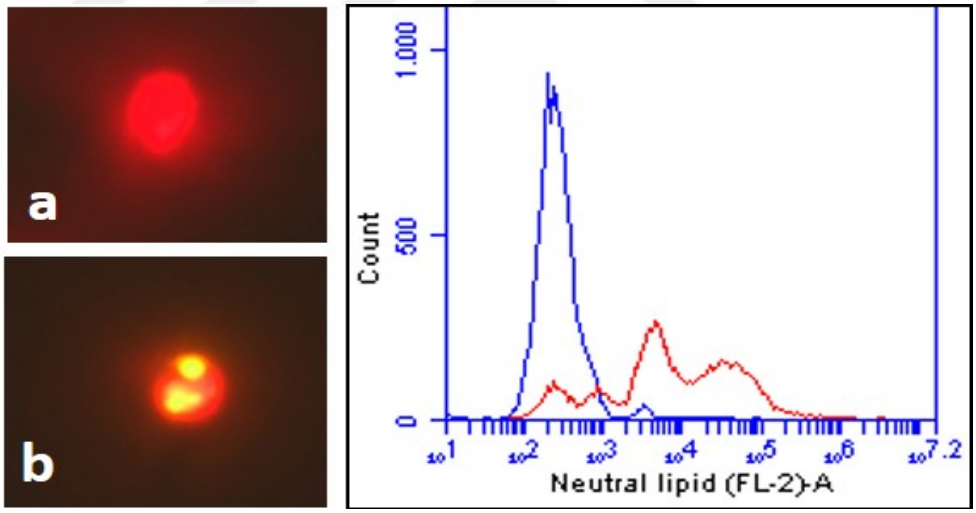

Figure 28. Lipid content observation of TALG 22 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing of about 100 to 10000-fold of the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

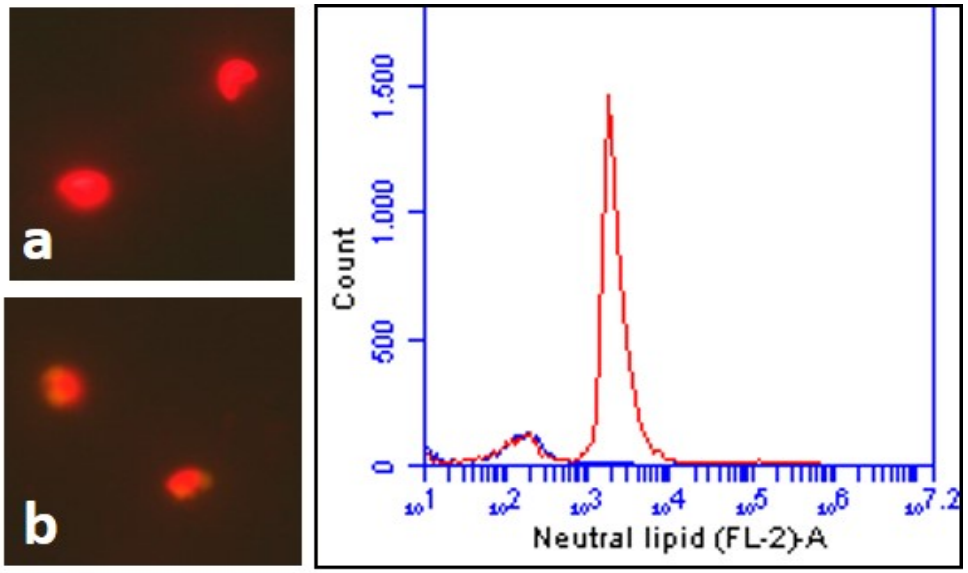

Figure 29. Lipid content observation of TALG 23 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing of about 100 to 10,000-fold of the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

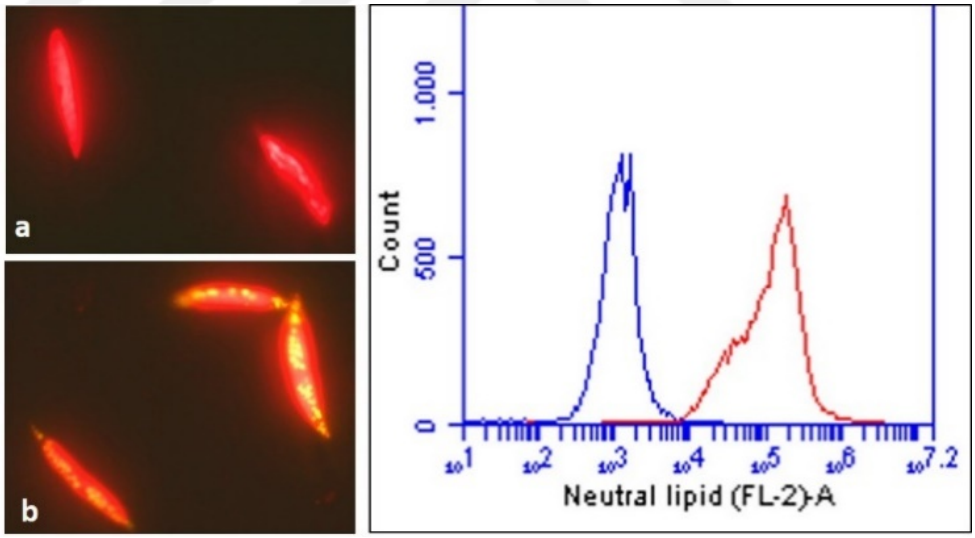

Figure 30. Lipid content observation of TALG 26 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing of about 100-fold of the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

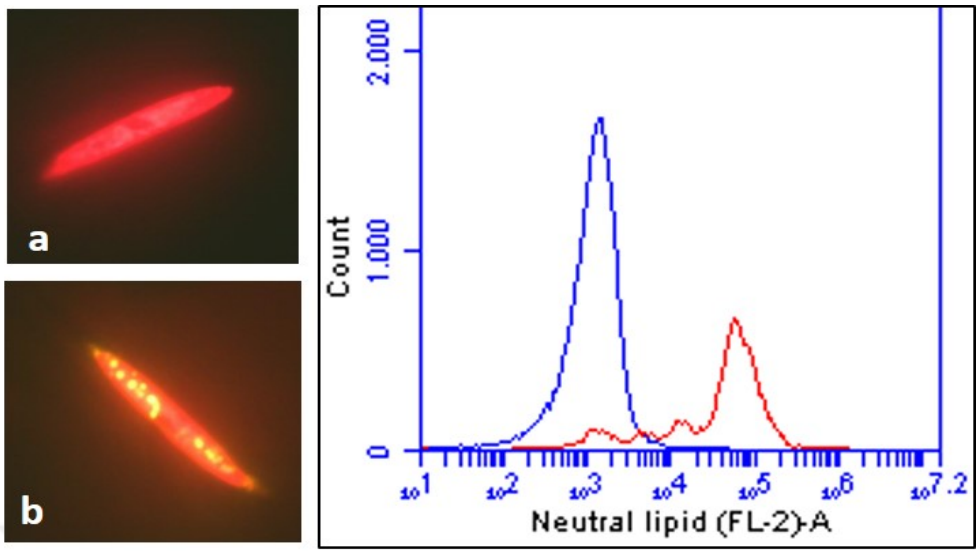

Figure 31. Lipid content observation of TALG 27 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing less than 10-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

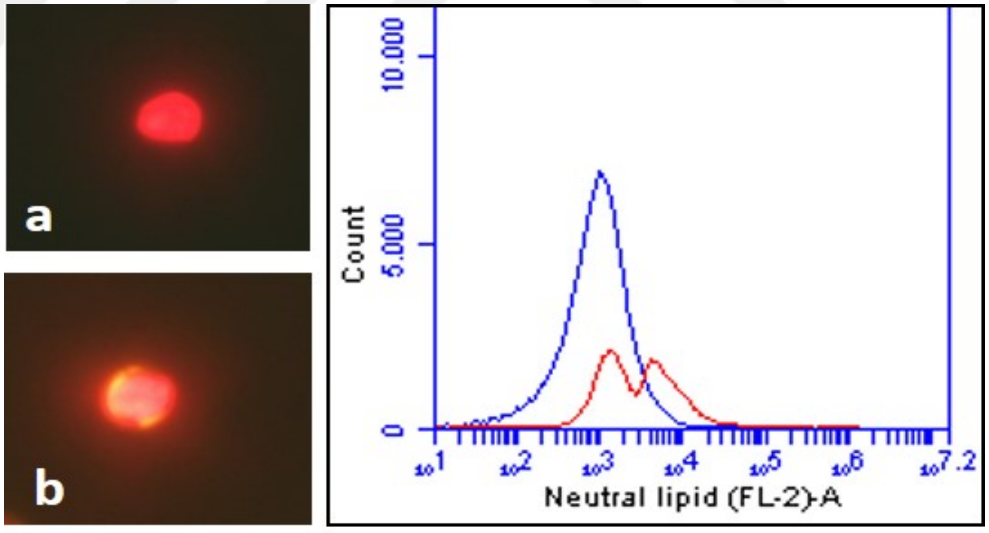

Figure 32. Lipid content observation of TALG 28 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing less than 10-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

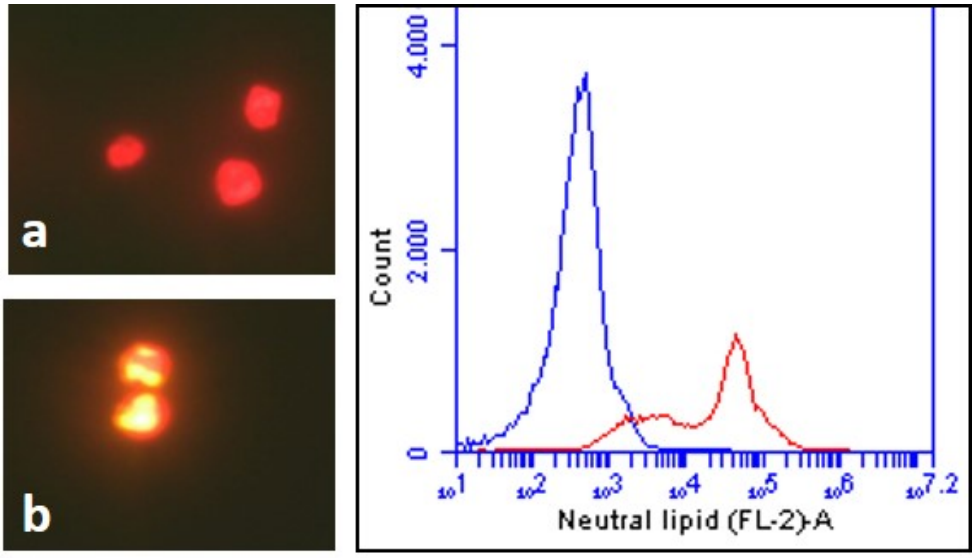

Figure 33. Lipid content observation of TALG 30 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing of about 100 to 10,000-fold of the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue).

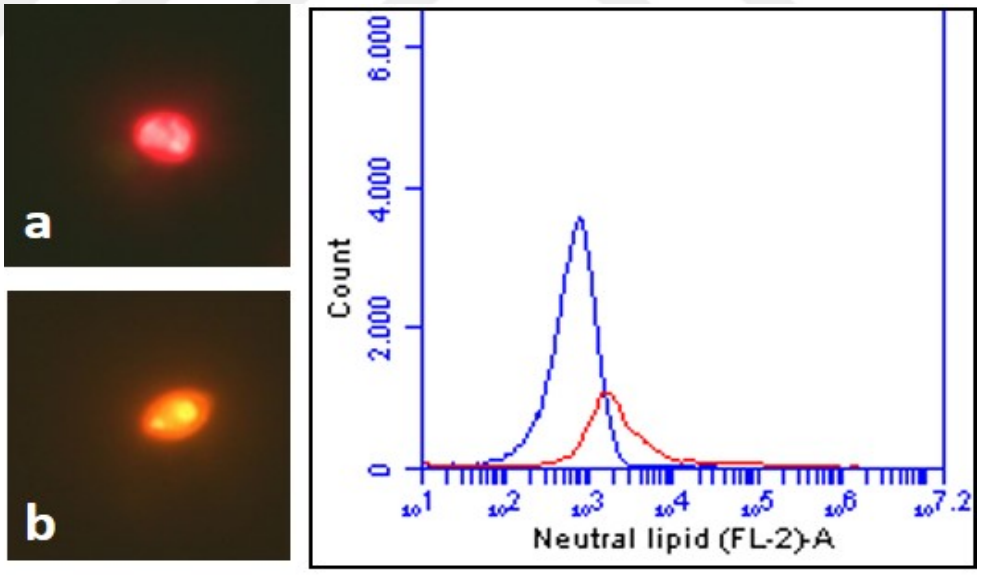

Figure 34. Lipid content observation of TALG 61 by fluorescence microscope. a, without staining; b, Nile red staining. The chart shows the increasing about 10-fold in the fluorescence arbitrary unit detected by the FL2 channel after staining (red) in comparison with the non-stained cells (blue)**.**

6.7. Strains Identification

6.7.1. Genomic DNA Isolation

Genomic DNA was isolated from 16.strains. Figure 35 shows the DNA bands visualized on 1% agarose gel after staining with EtBr.

6.7.2. Polymerase Chain Reaction (PCR)

PCR was applied using universal primers. TALG 1, TALG 10,.TALG 11,.TALG 12,.TALG 6,.TALG 13,.TALG 21,.TALG 23, TALG 27,.TALG 30, TALG 38, and TALG 54 were amplified by Alg1F and Alg1R primers that produce a partial segment of 500 bp of the 18S rDNA, whereas TALG 24,.TALG 30,.TALG 31 and TALG 33 were polymerized with Alg2F and Alg2R that amplify another segment with product size of 700 bp. Amplified PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit. Figure 36 shows the purified PCR products visualized on 1.2% agarose gel after staining with EtBr.

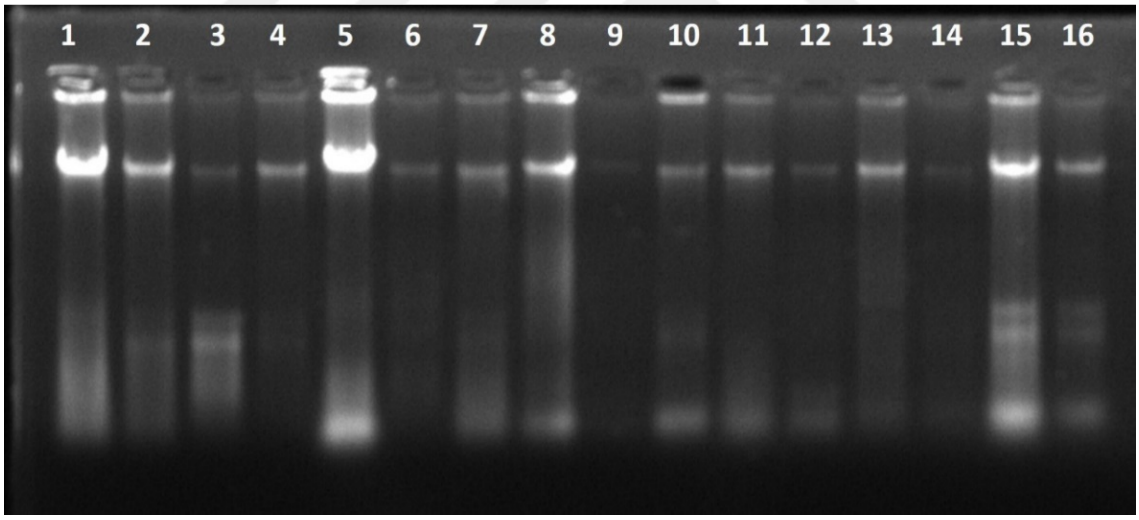

Figure 35. Agarose gel electrophoresis analysis of undigested algal genomic DNA. 1,.TALG 1; 2,.TALG 10; 3,.TALG 11; 4,.TALG 12; 5,.TALG 6; 6,.TALG 13; 7,.TALG 21; 8, TALG 23; 9,.TALG 24; 10,.TALG 27; 11,.TALG 30; 12,.TALG 31; 13,.TALG 33; 14,.TALG 34; 15,.TALG 38 and 16,.TALG 54.

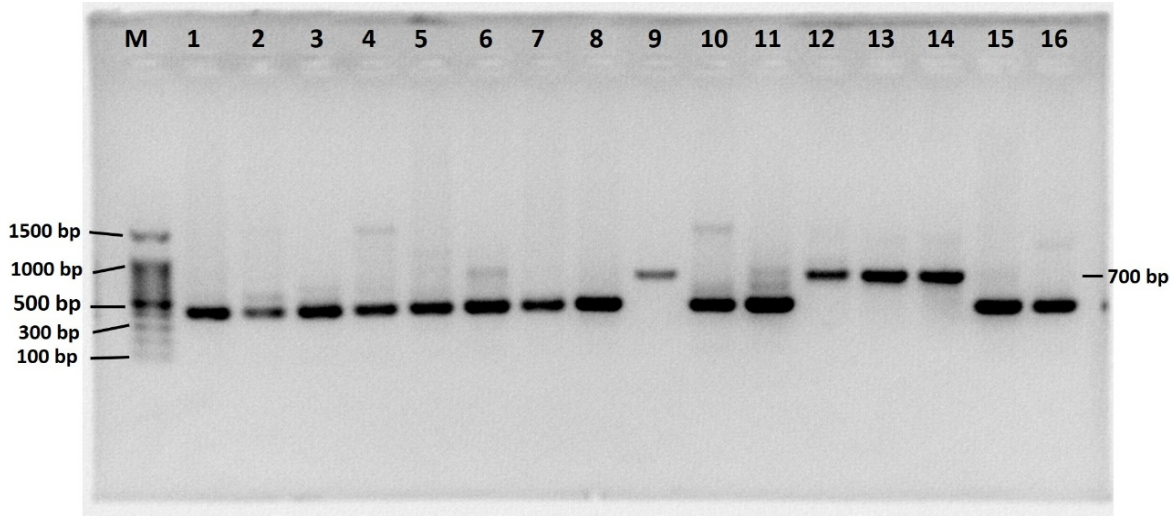

Figure 36. Agarose gel electrophoresis of purified PCR products from 18S rDNA genes of selected algae strains. 1,.TALG 1; 2,.TALG 10; 3,.TALG 11; 4, TALG 12; 5,.TALG 6; 6,.TALG 13; 7,.TALG 21; 8,.TALG 23; 10, TALG 27; 11,.TALG 30; 15, TALG 38 and 16,.TALG 54 (amplicon size of 500.pb). 9, TALG 24; 11, TALG 30; 12, TALG 31 and 13, TALG 33 (amplicon size of 700 bp).

6.7.3. DNA Sequencing Results

The results of the sequenced 18S rDNA genes are summarized in Table 6, and a phylogenetic tree was established based on the sequence data of the different algal strains (Figure 37). Evolutionary history was based on the Maximum-likelihood method using the Jukes-Cantor model. Evolutionary analyses were conducted in MEGA7 software.

Table 6. Algal strain identification based on 18S ribosomal DNA sequencing

Sample ID	Morphological identification	The most probable species (Accession number)	Score (Query cover)	Identity %
TALG 1	*Chlorella lobophora*	*Chlorella vulgaris* (MG022722.1)	763 (98%)	99%
TALG 2	*Chlorella* sp.	*Elliptochloris subsphaerica* (KM020033.1)	736 (99%)	98%
TALG 3	*Chlorella* sp.	*Choricystis* sp. (AH012393.2)	715 (100%)	98%
TALG 6	*Chlorella* sp.	*Chlorella vulgaris* (MG022722.1)	760 (96%)	99%
TALG 10	*Chlorella* sp.	*Elliptochloris subsphaerica* (KM020033.1)	773 (98%)	100%
TALG 11	*Monoraphidium* sp.	*Monoraphidium* sp (KF805717.1)	758 (97%)	99%
TALG 12	*Stichococcus* sp.	*Pseudostichococcus* sp (KX094813.1)	773 (98%)	100%
TALG 13	*Ourococcus* sp.	*Chlorella vulgaris* (MG022722.1)	754 (100%)	99%
TALG 14	*Scenedesmus* sp.	*Scenedesmus* sp. (KX818834.1)	689 (99%)	96%
TALG 15	*Bracteacoccus* sp.	*Bracteacoccus glacialis* (JQ259940.1)	667 (97%)	99%
TALG 16	*Cyclotella meneghiniana /Chaetoceros sp/ Sellaphora*	*Pinnularia viridiformis* (AM743108.1)	329 (100%)	86%
TALG 17	*lobosphaera incisa*	*Kalenjinia* sp (KU361146.1)	595 (93%)	93%
TALG 21	*Scenedesmus* sp.	*Desmodesmus* sp. (KX818837.1)	750 (79%)	99%
TALG 23	*Chlorella* sp.	*Chlorella vulgaris* (MG022722.1)	760 (100%)	99%

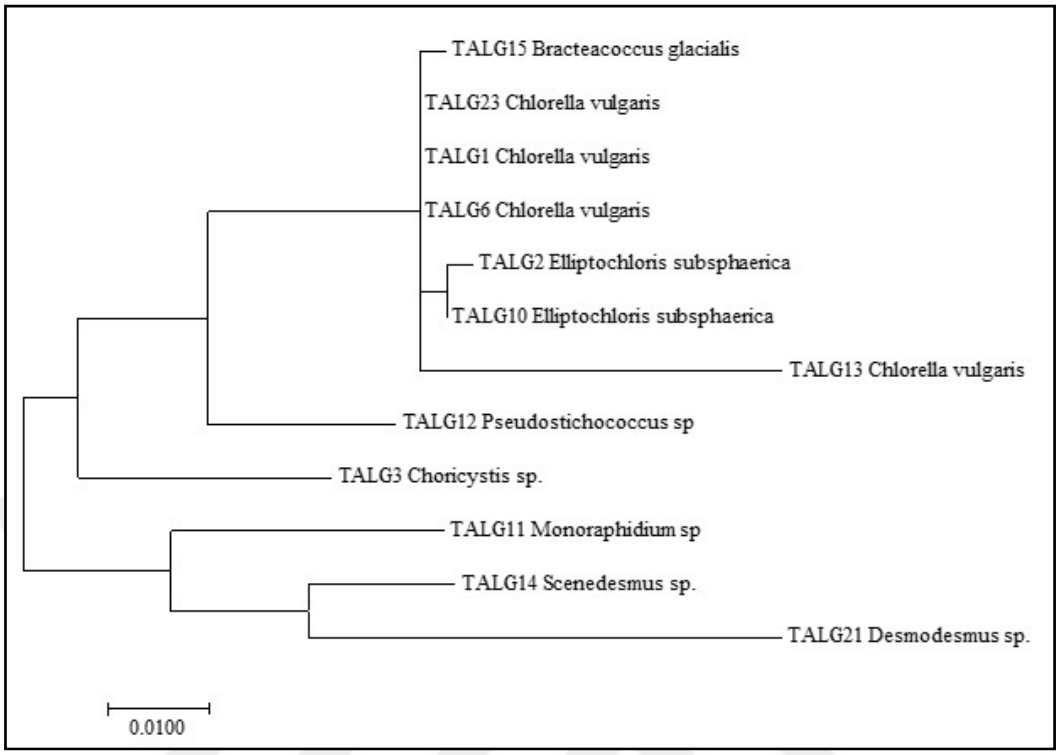

Figure 37. Phylogenetic tree based on the V4 regions of 18S rDNA genes of selected algal strains.

7. DISCUSSION

In this project, 64 microalgal strains were isolated from different regions of Turkey. Most of the strains isolated belong to Chlorophyta, the rest included 3 Cyanophyta, 2 Bacillariophyta and 1 Ochrophyta. Samlpes were taken from different sources including non-urbanized cave soils closer to the entrance, streams, lakes, fountains, spring waters, in addition to some commercial drinking water samples (Table 3).

Several studies were previously done and most of them aimed to identify freshwater microalgae in Turkey. Demircan and his collaborators studied the variation of the phytoplankton species and their growth from the coast of the Black Sea in Sinop province of Turkey (94). Another team identified 244 marine algal taxa (including macro and microalgae) in the Bosphorus coasts of Istanbul (95). A computer database system was established by Şen et al. (96) including 627 strain of freshwater microalgae with their taxa, images, taxonomic and ecological information. The database included species that belonged to Bacillariophyta, Charophyta, Chlorophyta, Chrysophyta, Cryptophyta, Cyanophyta, Dinophyta, Euglenophyta, Prasinophyta, Rhodophyta, and Xanthophyta. Sevindik et al. (97) recorded 24 freshwater microalgal taxa from the Çaygören reservoir in Balıkesir province. The microalgal divisions found were Cyanobacteria, Chlorophyta, Charophyta, Euglenophyta, Heterokontophyta, and Dinophyta. Microalgal flora from the Germencik-Alangüllü thermal water in Aydın province generated 27 taxa from Cyanobacteria, Bacillariophyceae and Conjugatophyceae (98). Varol and colleagues collected 84 phytoplankton samples from the Tigris River in the Anatolia region of Turkey, phenotypically identified them and registered 4 new taxa that were added to the recorded microalgal flora in Turkey (99). These taxa are: 1 (*Oscillatoria nitida*) to Cyanoprokaryota, 2 (*Micractinium bornhemiense* and *Lagerheimia wratis laviensis*) to Chlorophyta and 1 (*Audouinella chalybaea*) to Rhodophyta (99). An investigation of the phytoplanktonic flora was done in Eğidir Lake between 2012 and 2013 where 104 taxa were found including Bacillariophyta, Chlorophyta, Charophyta, Cyanobacteria, Dinophyta, Euglenozoa and Ochrophyta (100).

As for our results, no new strain was recorded as shown in Table 3. The limited variety and number may be explained by using one cultivation medium to isolate all the samples and by using the same incubation conditions, which may have promoted the growth of similar strains and delimited the growth of those that needs particular conditions to grow. It is also recommended to collect the samples during the different seasons of the year, which was not the case in our study, since microalgal growth may be affected by the seasonal conditions.

Among the isolated strains, 17 isolates were isolated from cave soil samples, 12 of them were from caves in Gümüşhane province (Akçakale, Kırklar and Köprübaşı), one from Toprakini cave in Konya province, and 4 isolates from Ayvayini cave in Bursa province. These caves have not been open to public visit and not been investigated for microalgal flora. There have been reports in the literature in which microalgae and cyanobacteria were isolated (101, 102). Microalgae are generally considered as detrimental and unwelcomed contaminant to the caves via indirect transport by humans, animals, wind or water flows (29). Meanwhile from a microbiological perspective, caves constitute a harsh ecological environment for microalgae in terms of light, humidity, temperature or even microbiota. Therefore cave grown microalgae had to surpass the surrounding extreme conditions to survive, which means they may contain important traits that are not found in the ordinary marine and freshwater microalgal species. However, caves scientists are barely attracted by such type of investigations since their interests are more targeted toward algicidal materials and methodologies, especially in the aim to eliminate the microalgal biofilms formed inside of the caves (103).

Caves in Turkey have never been investigated for microalgae before, which means the strains isolated in this project are the first data obtained in Turkey and are worth taking in consideration for further investigation.

To investigate the antimicrobial compounds, algal extracts were prepared using organic solvents. As shown in Table 4, positive antimicrobial activity was obtained by a number of extracts against *A. haemolyticus, B. subtilis, C. albicans,* and *K. pneumoniae*, most of them had their MIC below 1250.µg/ml, whereas no positive activity was observed against *S. aureus*, *E. coli* or *P. aeruginosa*. These results are

promising since the testing was done with crude extracts. Except for TALG 11, cave strains showing positive results were all of hexane extracts, and most of them were inhibitor of *A. haemolyticus*. Most of these extracts showed heteroresistance profile on agar well diffusion test where some colonies grew inside the inhibition zone (TALG 6 and TALG 13) (Figure 9-A). Although MIC test showed low active concentrations for these extracts (625.µg/ml and 1250.µg/ml), they cannot be considered positive since the bacterial strain is apt to develop a resistance any time against the active compound.

Methanol extracts showed no clear activity against *A. haemolyticus*, even though, a heteroresistance profile is observed with TALG 27 and TALG 29 extracts (Figure 9-B). However, we can clearly notice the inversion of these resistances by a positive synergy between the extracts and gentamicin, which was used as positive control. The same synergy also inverted the resistance of the bacteria against TALG 30. This synergetic profile obviously advocates the conclusion that methanolic compounds extracted from these 3 strains are functionally different from the methanolic compounds extracted from the other algal strains (Figure 9-B), even if the strains seem similar under the microscope.

Only methanol extracts showed positive antimicrobial activity against *B. subtilis* and *E. faecalis*; this may indicate a similarity of the active ingredients in these extracts. MIC results showed very low active concentrations; for *B. subtilis*: 3.9.µg/ml from TALG 24, 78.1.µg/ml from TALG27, 39.1.µg/ml. from TALG 30, 78.1.µg/ml from TALG 31, 312.µg/ml from TALG 33, 78.1 µg/ml from TALG 38, and 19.6.µg/ml from TALG 54. For *E faecalis*: 156.3 µg/ml from TALG 30, 39.1 µg/ml from TALG 31, and 2500.µg/ml from TALG 11. These results may promote powerful antibacterial compounds that are worth to be purified and further characterized.

An incoherence between the agar well diffusion and the MIC test results is observed with some strains like TALG 30, TALG 31 and TALG 38 the inhibition diameter in agar well diffusion tests are similar (around 10-12.mm) but the MIC values are highly variable (from 39.1 to 2500.µg/ml). This incoherence may be explained as following: Agar well diffusion and micro-dilution tests standards defined by the CLSI or by other antimicrobial or microbiological committees (European Committee on Antimicrobial Susceptibility Testing (EUCAST), Comité Française de Microbiologie

(CFM), which are designed with the purpose to test pure material like antibiotic, whereas working with unknown crude extracts may impose other conditions depending on the techniques used to test the antimicrobial activity (104), on the tested microorganisms (105, 106), or to the nature of the tested material (104). It should be mentioned that the tested materials are crude extracts where the yield of the active ingredient may vary between the samples due to the difference of the extraction's efficacy between the strains, or to the difference of the ingredient's original quantity for every strain. Since the nature of the active compound is unknown then the mechanism is also unknown and needs to be elucidated, because the mechanism of action may be variable between the testes. In fact, in the micro-dilution method the bacteria is directly exposed to the extract whereas the agar well diffusion method is dependent on the extract diffusion through agar.

The extracts from two strains, TALG 30 (*Scenedesmus* sp.) and TALG 31 (*Chlorella* sp), showed similar positive activity against *B. subtilis* and *E. faecalis* with different MICs.

As for the antifungal activity tests, 9 extracts showed inhibition of *C. albicans* growth, only 2 of them were methanolic extracts whereas the rest were hexane extracts. The best activity was obtained with the methanol extract of TALG 24 with a MIC of 62.5.µg/ml, meanwhile the hexane extract of the same strain showed antimicrobial activity with a MIC of 2500.µg/ml. The same strain's methanol extract showed activity against *B. subtilis* with MIC of 3.9 µg/ml.

Other studies have also investigated antimicrobial activity in microalgae. For example, ethanol and hexane extracts of *Haematococcus pluvialis* obtained by pressurized liquid extraction at 50^{o}C, 100^{o}C, 150^{o}C and 200^{o}C were in-vitro bactericidal against *E. coli, S. aureus*, *C. albicans* and *Aspergillus niger* with minimal bactericidal concentrations higher than 10.mg/ml for the hexane extracts (107). Although pressurized liquid extraction may be more efficient than the classical method used here, temperatures higher than 45^{o}C may be detrimental for some chemical compounds.

Another study used a protocol similar to ours to test the antimicrobial activity of methanol, acetone, petroleum ether and water extracts from 2 cyanobacteria (*Anabaena oryzae* and *Nodularia spumigena*) isolated from Telangana State in India. Their results

showed effective antimicrobial activities of acetone, methanol, petroleum ether and chloroform extracts of *A. oryzae* against *S. aureus, Bacillus cereus, Micrococcus luteus, Salmonella typhi and Klebsiella aerogenes* (108).

In another investigation, 32 microalgal strains were isolated from Arabian Sea and their water and acetone extracts were tested against *E. coli*, *P. aeruginosa*, *S. aureus*, *E..faecalis* and *Streptococcus agalactiae.* Two extracts of *Skeletonema marinoi* strains inhibited *S. aureus* by 96% and 97% (109).

In a study carried out in Spain marine microalgae (*Skeletonema marinoi*, *Thalassiosira* sp*., Chaetoceros* sp., *P. tricornutum)* and microalgae isolated from urban wastewater (*Coelastrum* sp*., Scenedesmus quadricauda and Selenastrum* sp*.)* extracts demonstrated antibacterial activity against *Pseudomonas fluorescens, Serratia marcescens*, *E. coli*, *M. luteus, B. subtilis* and *S. epidermidis.* The algal strains were extracted by methanol, ethanol, hexane, and by a mixture of water: methanol: hexane. The different extracts showed positive antibacterial activity against *S. epidermidis S. marcescens and P. fluorescens* whereas no inhibitory effect was observed on the other strains (110).

As for the investigations carried out in Turkey, few studies were working on microalgae whereas the majority of the studies focused on macroalgae such as seaweeds extracts (111-113).

In 2011, 5 microalgal species (*Oscillatoria limosa*, *Oscillatoria limnetica*, *Phormidium tenue*, *Chlorella vulgaris*, *Spirulina major*) were investigated for their antimicrobial activities by Demiriz and collaborators (114). They extracted 500.µg of microalgae with different solvents (methanol, ethanol, acetone, *n*-butanol, hexane and *tris*-HCl (0.5.M, pH = 8.00). Positive antimicrobial activity was obtained against *E. coli*, *S. aureus* and *Salmonella enteritidis* and it was noticed that hexane extract of *C. vulgaris* had inhibitory activity against *E. coli* and *S. aureus,* which is contradictory to our findings (TALG 1, TALG 6, TALG 13, TALG 14, TALG 17 and TALG 23). In fact, the two studies cannot be objectively comparable since they have used larger quantity of algal powder in the extraction without mentioning the volume of the solvent used. Therefore, the contradiction of the results may be due to a difference of the extraction yields (114).

Another study carried out in 2014 isolated the marine microalgae *Dunaliella salina* from the lake Tuz in Turkey and investigated it for its bioactive compounds (115). *D. salina* was extracted by n-hexane, dichloromethane, ethanol, and methanol. Antimicrobial activity was tested by agar disc diffusion and 500.µg of extract were impregnated in each disc. Most of the tested microorganisms were inhibited by most of the tested extracts including *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis*, and *C. albicans* with MIC around 1.25 and 2.5.mg/ml for methanol extracts, and for 10.mg/ml for the hexane extracts (115).

Desmodesmus protuberans from Eğirdir Lake in Isparta (Turkey) was isolated and its its methanol extract was tested against *Bacillus cereus (*ATCC 7064), *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 6538P), *Staphylococcus epidermidis* (ATCC 12228), *P. aeruginosa* (ATCC 27853), *Enterobacter cloacae* (ATCC 13047), *E. coli* (ATCC 29908), *Salmonella typhimurium* (CCM 5445[1]), *E. faecalis* (ATCC 29212) and *C. albicans* (ATCC 10239) (116). The inhibitory activity was observed against *B. subtilis, B. cereus*, *C. albicans* and *E. faecalis* and these results are in coherence with the results we obtained with the methanolic extracts of our *Scenedesmus* like species (TALG 30) (Table.4).

Ang Li and his colleagues (117) worked on freshwater microalgal strains including microalgal genus similar to ours (*Chlorella sorokiniana*, *Scenedesmus* sp. *Monoraphidium dybowskii*, *Micractinium reisseri*, *Scenedesmus obliquus*, *Chlorella zofingiensis* and *Scenedesmus ecornis*. The strains were extracted by acetate and 95% ethanol, and antimicrobial activity was obtained against *S. aureus* and *E. coli.*

Freshwater cyanobacteria *Anabaena variabilis and 3* chlorophytes *(Chlorella vulgaris, Scenedesmus quadricauda* and *Selenastrum capricornatum*) were extracted with methanol, ethanol and water, and dichloromethane with isopropanol and their antimicrobial activity was tested against *B. subtilis*, *Bacillus pumilus*, *E. coli* and 2 subspecies of *Fusarium* (118). Their extracts showed good inhibitory activity against Nostoc and against Fusarium strains. Methanolic extracts showed no antibacterial

*The collection number written in the referenced paper was CCM 544. Since such strain does not exist, we assume this is *Salmonella typhimurium* CCM 5445.

activity even though they were active against *Fusarium* sp. and other algal species (algicidal activity) (118).

In conclusion, investigations on antimicrobial activity of microalgae in Turkey are limited especially for the freshwater microalgal samples. Thus, this study may contribute some data to the subject regarding the bioactive metabolites of microalgae from the fresh water resources in Turkey. In addition, the results showed potential application of extracts since their effective mean minimal inhibitory concentration (MIC) being less than 1.mg and inhibiting *B. subtilis*, *C. albicans* and *E. faecalis*. Taking in consideration that two algal strains showed inhibition of *E. faecalis* with MIC around of 156.3.µg/ml for TALG 30 and 39.1.µg/ml for TALG 31. As previously indicated, the heteroresistance profile obtained in the results with *A. haemolyticus* prove the difference of the active ingredients of these two extracts. Therefore these two antimicrobial compounds with strong activity even as crude extracts deserve further characterization. On the other hand, other organic solvent may be used like DCM:iso (Dichloromethane:isopropanol) or ethyl acetate since they seem to capture powerful antibacterial, antifungal and algicidal active compounds (117, 118).

To our knowledge, this is the first report regarding the antimicrobial compounds been investigated in karstic cave microalgal species in Turkey.

In terms of anti-QS activity of algal extracts, methanol extracts of two strains, TALG 23 (*C. vulgaris*) and TALG 34 (*Chlorococcum* sp.) presented anti-QS activity with 78.µg/ml. In a study reported from Turkey demonstrated anti-QS activity from *C. vulgaris* extracts against pigment production of *C. violaceum* and against the swarming function of *P. aeruginosa* PAO-1 (119). These results support our findings found from TALG 23 strain extract. Detection of anti-QS activity from *Chlorococcum* sp. extract in our study may represent the first record about this strain. Similarly, Jha et al. (120) investigated the anti-QS activity in seaweed species against *C. violaceum* pigment production and positive activity was obtained with *Asparagopsis taxiformis* strain.

Another study conducted by Natrah and coworkers (121) reported positive anti-QS activity from *Chlorella saccharophila* and *C. vulgaris* strains, which were also tested against *C. violaceum* pigment production. The extraction was applied on the supernatant of the algal culture using ethyl acetate acetonitrile as solvent.

Microalgae are pigmented organisms living in a world crowded with other microorganisms where they established strong mutualism. In addition, they are under the true threat comes from the grazers of phytoplankton, zooplanktons and fish. Although they play a significant role in food chain in the aquatic environment, algal anti-quorum sensing compounds were rarely investigated in microalgae.

In this project, we highlighted the importance of the QS mechanism by trying to detect the presence of quorum quenching active compounds from the microalgae isolated from water and soil samples collected from different regions of Turkey. Among the screened algal strains, anti-QS activity was detected in two strains extract which prevented pigment production of *C. violaceum*. Further studies are needed to test the quorum quenching activities of these strains with particularly other human pathogenic strains like *E. coli*, *S. aureus P. aeruginosa, E. faecalis* and others in planktonic as well as biofilm form. Quorum quenching mechanism of the extracts should also be investigated at the molecular level as recommended elsewhere (122).

The lipid contents of the selected microalgal strains were screened by flow cytometry. The results showed that three strains contained high neutral lipid contents. These high lipid containing strains are; TALG 13 with 1322.4 f.a.u./100 events, TALG 15 with 1385.6 f.a.u./100 events, and TALG 26: 1110.9 f.a.u./100 events (Table 5). Seven strains showed moderate amount of fatty acid contents with their f.a.u per 100 events between 200 and 600. These strains include TALG 4, TALG 7, TALG 9, TALG 10, TALG 21, TALG 22, and TALG 27. These results are verified by the microscopic visualization before and after the Nile red staining, where TALG 13; TALG 15 and TALG 26 showed high fluorescence emission comparison to the non-stained samples (Figures 15-34).

To note that most of the strains showing high lipid content were isolated from cave samples (TALG 4, TALG 7, TALG 9 TALG 10, TALG 13 and TALG 15). This may be due to the lack of light and aeration in the cave habitat, which may constitute a harsh environment for microalgae. These results indicate that the cave microalgal strains may present a better potential for biofuel production. These strains should be further investigated and their lipids content should be characterized using chromatographic methods.

Flow cytometry is a simple and easy to handle screening method that can be used after a quick staining with a fluorescent dye (90). Moreover, the new cytometer models included a sorting system permitting the isolation of simple cells from the positive substrates for further uses (35). Nile red or BODIPY 505/515 may be used as non-polar dyes to stain neutral fatty acids, then a flow cytometer may be used to quantity of fluorescence emitted by the cells. Today, fluorescent dyes are proved to detect neutral lipids and to give accurate results when compared to other techniques (87). Nile red staining have been used for longtime for the screening of the fatty acids, and the ratio of polar/neutral lipids, was used to determine the relative fatty acid content and was accepted as an accurate indicator of the relative PUFA contents (87). The problem is still to eliminate the interference generated by the autofluorescence of the microalgal pigments particularly chlorophyll and carotenoids. These pigments can absorb the laser light at 488.nm and they emit a green fluorescence between 505 nm and 530 nm for chlorophyll and a red fluorescence for carotenoids whose emitted fluorescence is detected by filters for more than 650 nm (123). For this reason, a critical step of compensation is required to eliminate the erroneous fluorescent signals.

Usually, a positive control of known strain with known lipid content is used for compensation, but in this project, it was quite hard to provide positive control for all the strains since I was working on unknown isolates and molecular identification could not be applied for the 64 strains together. Therefore, I adjusted the system using the gating function to remove the autofluorescence captured by the mirrors FL3 and FL4 while measuring the negative control, then I measured the fluorescence of the stained cells on FL2 (90), and I accepted it as the pure fluorescence emitted by the neutral lipids. The results were always relative to the number of the cells passing through the core and to the coloration yield of Nile red.

Another drawback of the flow cytometry method is to not have an absolute measure of the tested criteria; therefore, we were not able to have an absolute quantification of the neutral lipids in every strain and the results will always be relative. Despite all, our aim was to screen strains that are highly rich in fatty acids and this was easily done by flow cytometry with verification via fluorescent microscopic observation. Flow cytometry is still a semi quantitative, easily manipulated, quick and a

simple method that permitted to detect 8 strains rich in neutral lipids, 5 of these strains were from cave samples. These strains should be investigated by other quantitative techniques, fatty acid may be extracted and their structure and quantities of these strains should be accurately determined by chromatographic or spectrometric methods like HPLC or GC-MS (124, 125).

Freshwater ecosystems may be too small to have a global impact. However, this study proves that microalgae are part of soil, cave, stream, lake and any freshwater resource as much as ocean and marine ecosystems. Considering that water constitute more than 70% of our planet that is draining in pollution, GHG and other problems driving toward critical problems like the climate change and the thermal expansion.

Photosynthesis is free, and its efficacy is much better in microalgae than higher plants considering that microalgal biomass cultures are considered harmless and reparative for the nature and their cost are very effectives in comparison to the other biomass culture candidates.

Turkey is one of the favorable places for potential algal cultivation and farming with its high thermal climate and wealth in land and water. Particularly because microalgal cultivation does not require large spaces and in case of restriction of freshwater cultivation may be applied using wastewater.

This study proved that Turkish microalgal strains comprise big potentials for bioactive compounds and must encourage the researcher for intensive investigation of this subject.

8. CONCLUSIONS and RECOMMENDATIONS

- In this study 64 fresh water microalgal strains were isolated from different regions of Turkey.
- Among the isolated strains**,** three strains belong to Cyanophyta, two strains belong to Bacillariophyta, one strain belong to Ochrophyta and the rest were members of Chlorophyta.
- Organic extraction of 34 strains were carried out using methanol and n-hexane.
- Agar well diffusion test was applied for the 34 methanol and hexane extracts, 16 microalgal strains showed antimicrobial activities.
- Six extracts were active against *A. haemolyticus*, 9 methanol extracts were active against *B. subtilis*, 9 extract inhibited *C. albicans*, and 3 methanol extract inhibited the growth of *E. faecalis*. One extract inhibited the growth of *K. pneumoniae*.
- Anti-QS activity was tested by microdilution against *C. violaceum*. The extracts from two strains showed positive anti-QS activity with MIC of 78.13.µg/ml.
- Strains were investigated for their neutral lipid contents by Nile red staining and measuring the fatty acids emitted fluorescence by flow cytometry. The lipid contents of the algal strains were also visualized under fluorescent microscope**.**
- All 64 strains were morphologically identified referring to microalgal databases, and 14 strains were identified by partial sequence of the 18S rDNA using universal primers.
- The molecular identification of the remaining 50 isolated strains should be completed by sequencing the entire18S rDNA, and a phylogenetic tree of algal strains which is showing the strains/species diversity of Turkey should be constructed.
- The extracts with antimicrobial activity should be purified and characterized, and their activity should be tested on additional multi-drug resistant pathogenic strains.

- The extracts with anti-QS sensing activity should be characterized and tested for their effects on other QS indicator strains as well as on the reduction of microbial biofilms.
- Antimicrobial and anti QS activity mechanisms of the extracts should be further characterized.
- The microalgal strains highly rich in PUFAs should be tested by quantitative methods and the lipid content should be determined using chromatographic methods by saponification.
- Natural resources and different ecological resources of Turkey should be investigated for microalgal isolation, identification and bioactive molecule screening.

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10. CURRICULUM VITAE

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Education

Degree/Field	Institution	Graduation Year
MSc / Medical Microbiology	Lebanese University, Doctoral school of Sciences and Biotechnology	2010
BSc / Medical Laboratory	Lebanese University, Institute of Health Sciences,	2008
Secondary School	Andre Nahhas High School	2003

Certificate

Turkish Language Proficiency, Ankara University TÖMER, Turkey (Attended from October 2012 to June 2013)

Work experience

Profession/duties	Institution	Duration
Bacterial strains stock culture collection	Lebanese University, Doctoral School of Sciences and Biotechnology	2010-2012

Publications

1. **Tabbouche Sana**, Khudary Rami, Beyrouthy Racha, Dabboussi Fouad, Achkar Marcel, Mallat Hassan, Hlais Sani, Hamze Monzer (2011). Detection of Genes TEM, OXA, SHV and CTX-M in 73 Clinical Isolates of *Escherichia coli* Producers of Extended Spectrum Beta-lactamases and Determination of Their Susceptibility to Antibiotics. The Intern Arabic J of Antimicrob Agents; 1:1-5. DOI: 10:3823/704.
2. Yılmaz Ayşenur, Sibel Yıldız, **Sana Tabbouche**, Ali O. Kiliç, Zehra Can (2016). Total Phenolic Content, Antioxidant and Antimicrobial Properties of *Pleurotus ostreatus* grown on lime (*Tilia Tomentosa*) Leaves. Hacettepe J Biol Chem, 44 (2), 119–124. DOI: 10.15671/HJBC.20184417585.
3. **Tabbouche Sana**, Ayşenur Gürgen, Sibel Yildiz, Ali O. Kiliç, Münevver Sökmen (2017) Antimicrobial and Anti-Quorum Sensing Activity of Some Wild Mushrooms Collected from Turkey. MSU J of Sci, 5(2): 453-457. DOI:10.18586/msufbd.347692.
4. Sibel Yıldız, Ayşenur Yılmaz, Zehra Can, **Sana Tabbouche**, Ali O. Kiliç, Ertuğrul Sesli (2017). Some Bioactive Properties of Wild and Commercial Mushroom Species. J Food Health Sci, 3(4):161-169. DOI: 10.3153/JFHS17019.

Posters

1. **Tabbouche Sana**, Enis Fuat Tüfekci, Jaleel Samanje, Mujib Abdurrahman, Ahu Reis, Ali O. Kılıç. *Callosobruchus maculatus* (Fasülye Böceği) Bağirsak Florasindan İzole Edilen Bakterilerin Morfolojik ve Genetik Karakterizasyonu ve Antimikrobiyal Duyarliliklarinin Saptanmasi", 36. Türk Mikrobiyoloji Kongresi, Antalya, Türkiye, 12-16 Kasım 2014, ss.243-244

2. Khorshidtalab Mona, **Sana Tabbouche**, Enis Fuat Tüfekci, Ayla Çamurşen, Tuğçin Palabıyık, Ali O. Kılıç, "Trabzon İli Ve Çevresi Tatlı Su Kaynaklarından İzole Edilen Mikroalg Ekstraktlarının Antimikrobiyal Özelliklerinin Araştırılması", 36. Türk Mikrobiyoloji Kongresi, Antalya, Türkiye, 12-16 Kasım 2014, ss.243-243
3. **Tabbouche Sana**, Tuğçin Palabıyık, Enis Fuat Tüfekci, Mona Khorshidtalab, Ali O. Kılıç "Trabzon İli Çevresindeki Tatlı ve Tuzlu Sulardan İzole Edilecek Mikroalglerin Antimikrobiyal Madde Üretimi Yönünden Araştırılması", Ekoloji 2015 Sempozyumu, Sinop, Türkiye, 6-9 Mayıs 2015, ss 445-445.
4. Yıldız Sibel, Ayşenur Yılmaz, **Sana Tabbouche**, Ali O. Kılıç, Sevgi Kolaylı. Kahramanmaraş ve Erzurum'den toplanan Agaricus campestris Mantarının Toplam Polifenol, Antioksidan ve Antimikrobiyal Özelliklerinin Belirlenmesi, Ekoloji 2015 VI Ulusal Ekoloji Sempozyumu, Sinop, Türkiye, 6-9 Mayıs 2015, ss 444.
5. Ayşenur Yılmaz, Sibel Yıldız, **Sana Tabbouche**, Ali O. Kılıç, Zehra Can. Ihlamur (*Tilia tomentosa*) Yapraklarında Yetiştirilen *Pleurotus ostreatus* Mantarının Toplam Polifenol, Antioksidan ve Antimikrobiyal Özelliklerinin Belirlenmesi, Ekoloji 2015 VI Ulusal Ekoloji Sempozyumu, Sinop, Türkiye, 6-9 Mayıs 2015, ss.142-142
6. **Tabbouche Sana**, Ayşenur Gürgen, Sibel Yildiz, Ali O. Kiliç and Münevver SÖKMEN; Antimicrobial and Anti-Quorum Sensing Activity of Some Wild Mushrooms Collected from Turkey. International Multidisciplinary Congress of Eurasian (IMCOF) Rome, Italy. 23-25 August 2017.
7. Gürgen Ayşenur, Sibel Yildiz, **Sana Tabbouche**, Ali O. Kiliç, Münevver Sökmen. Trabzon'dan Toplanan Bazı Mantarların Anti-Mikrobiyal Özellikleri ve Mikroorganizmalar Arası Çoğunluğu Algılama (Quorum Sensing) Mekanizmasını Engelleme Kapasitelerinin Belirlenmesi. IV. Ulusal Ormancilik Kongresi, Antalya, Türkiye, 15-16 Kasım 2017.