FIRST NATIONAL WORKSHOP ON MARINE BIOTECHNOLOGY AND GENOMICS

24-25 May 2012

Bodrum, Muğla – TURKEY

Edited by

Cemal TURAN

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PREFACE

We have exploited much resource on land, but there still remain some unexploited materials, possibly deep in rainforests. In seas, needless to say, there is still so much to learn and find as 70 % of our globe is covered by water. As Turkish Marine Research Foundation, we are proud of organizing the First National Workshop on Marine Biotechnology and Genomics in the beautiful town of Bodrum.

Issues related to marine biotechnology and genomics are growing rapidly in the world. 'Blue Biotechnology' is a new term referring to the biotechnological potential of organisms from the seas. Surprisingly, even in a small market in the countryside, we can find products originated in seas, such as *Spirulina* as food, sea water, several drugs, dietary supplements, cosmetics, and so on. Isolation of algae and bacteria has also become important due to the industrial utilization. Turkey is surrounded by four different seas but has not yet explored them in terms of Blue Biotechnology.

Our aim of this workshop is to exchange information and ideas between scientists, companies and investors in this field, so that there will be fruitful collaboration among these stakeholders in the future. Finally, I sincerely thank **Prof. Dr. Cemal TURAN** for his effort in organizing this workshop and editing this book, and **Mr. Ali UYANIK** for workshop logo illustration.

24 May 2012
Bodrum, TURKEY

Prof. Dr. Bayram ÖZTÜRK
Director, TÜDAV

Keynote Speech

Blue Biotechnology: Bridges Between Marine and Maritime Sectors

Laura Giuliano

CIESM, The Mediterranean Science Commission

INTRODUCTION

Oceans occupy 70% of the Earth's surface with practically untouched fauna and flora and host ~87% of life on earth. Marine environments, such as deep sea sediments, from the seashore to the depths of 10 000 meters are rich sources of microbes as soil samples. Several millions of undiscovered organisms, microorganisms for the most part, are speculated to exist in oceans. A detailed investigation of the marine biosphere was initiated only in the last 15–20 years, when advances in molecular methods made it possible to study in depth the huge variety of adapting mechanisms / processes that allow marine organisms to thrive in the oceans.

For millions of years, marine life has continuously interacted with its physical, chemical and biological environments. Planktonic organisms (including most marine microorganisms) need to behave opportunistically, as they are 'strolling around', mainly transported by currents. They often develop chemotactic movements or the capacity to duplicate faster and faster when placed in their optimal environmental conditions. Some of them float only for some part of their life span and then need to settle, displaying *ad-hoc* induced adhesive capacities. Such adaptive mechanisms rely on complex membrane structures, with trans membrane molecules playing pivotal roles while mediating communication between the outside environment and the regulation of some cell metabolic pathways. Sessile organisms, typically exposed to very high risks of aggression (*i.e.* infections/biofouling), need to develop sophisticated defensive mechanisms to survive. They may produce bioactive molecules (so called 'secondary metabolites') acting as signalling or antimicrobial agents. In addition, microbes interact with other living organisms, as studies have shown that these organisms communicate with each other. All marine organisms (with special emphasis on marine microbes) produce compounds (*i.e.* the so called 'secondary metabolites') that serve as the chemical interface between the producer organism and other living organisms or the environment.

Analyses of the diverse types of natural molecules produced by marine organisms, the clear implication of their role in nature, their specific building blocks, possible active sites have shown logical interlinks between the real biosynthetic mechanisms and the marine life processes, and have provided a better understanding of structure–activity relationships. In general, several facts complicate the analyses as bioactivities are displayed on a molecular level, using diverse interactions with various targets under variable conditions. Sometimes, these constraints may pave the way to important opportunities. For example, at different sub inhibitory concentrations, some metabolites may display different activities. While creating obvious difficulties related to experimental reproducibility, this 'flexible' pattern enlarges the range of potential applications for those compounds

Marine bioprospecting based pharmaceutical sector – focus on antibiotics

After the 'golden age' from the '60s the efficiency of antibiotic research decreased and productivity of classical screening methods failed. The declining trends in natural products research call for a refocusing of this research area. The increasing resistance of antibiotics was the first problem derived from the widespread and uncontrolled application of antibiotics. The largest advantage microbes have against antibiotics is their incredible adaptability to changes in their environment due to their flexible metabolic power. More than 90% of the studies performed by large pharmaceutical companies between 1980 and 2003 resulted in decreasing profits due to increasing research expenses, the small number of new leads and regulatory obstacles. As a result, Governments and almost all large pharmas moved away from shortterm antibiotics to the profitable 'lifestyle' drug market, and those used to treat chronic diseases. One reason for the declining trends in antibiotic research is the negative effect of several unrealized scientific expectations. For example, the chemical profile of metabolites produced in laboratories, especially by industrial strains, may be quite different due to numerous artificial mutations compared with strains growing in the natural environment. There is still no explanation as to why antibiotic production is restricted to several distinct species. In addition to the previously discussed factors, increasing research costs and frequent misconceptions in the usage and marketing of antibiotics, there are other purely technical reasons, namely licensing and regulatory hurdles, which have a role in the withdrawal of large pharmas from antibiotic research. The main patents of popular antibiotics have expired or will expire in the near future, and undesirable competition with generic drugs is a risk for large pharmas. The introduction of a new compound takes at least 10–12 years and costs

billions of euros (€600–1500 millions per drug), while the lifetime of these drugs on the market is only 8–10 years, providing additional fiscal challenges (Katz *et al.* 2006).

Natural products, including those with chemical and genetic modifications, are compounds that will be successful therapeutics in the future. The biggest advantage of natural or naturally derived compounds is the existence of a basic similarity between the enzyme and other systems of evolutionally developed producers and hosts. Thus, these compounds are preeminent drug leads. Renewing natural products research requires inexhaustible natural resources, as well as new genetic techniques and microbial sources, including endophytic microbes. Invertebrate marine animals, such as sponges, molluscs, ascidians, bryozoa, coelenterates and echinoderms, and/or their symbiotic assembled fungi and bacteria are projected to lead to tens of thousands of new interesting compounds. In particular, the biosynthetic capacity of marine microbes, especially actinobacteria, seems inexhaustible. In addition to the basic pathways (for example, polyketide synthase), the microbes are able to produce new types of compounds via extensive branching, and a series of alternative reactions by different enzymes, condensations, alkylations, oxidations, isomerizations and simple decoration. The activation of hidden/silent microbial genes, as well as new variations and skeletons of compounds are likely to be discovered in the future (Hong 2011). According to conservative estimations, at least 30,000 compounds, including many interesting new structures, have been isolated from marine organisms (Simmons *et al.* 2008; Hu *et al.* 2011; Liu *et al.* 2010; Imhoff *et al.* 2011). Discovery of new types of organisms, biosynthetic methods and clarified microbial biosynthetic machinery will lead to discovery of new bioactivities and types of structures. The mostly undiscovered marine world surely will provide us with a number of pleasant surprises.

At the forefront on Maritime industries – the potential of marine biotechnology

Bioactive molecular metabolites from the sea have potential importance for various industrial sectors other than the ones directly related to human health (*i.e.* the more classical pharmaceutical, nutraceutical, cosmeceutical and medical categories). Particularly, due to the features that justify their production and existence in the marine environments, these metabolites may express some kind of chemical or biological activities that shall logically benefit the maritime industrial sectors at different levels. I describe below a few examples of important applications of marine biotechnologies in the maritime sectors.

In the aquaculture sector, one of the most dramatic areas of improvement has been in the field of fish vaccines where the salmon

farming industry did pioneer the way. For example, until the early 1990s, two bacterial diseases, *vibriosis* and *furunculosis,* constantly caused problems in farmed salmon and in other species too. Though vaccines had been developed, they were not very effective until it was found that they should be injected into the body cavity of juvenile fish as an emulsion in vegetable oil. This discovery spurred rapid development by pharmaceutical companies, such as Alpharma and Novartis (Aquahealth), and vaccines against at least six fish diseases are in use today. Vaccines are presently under development against common parasitic infestations and viral diseases in several species of fish in different countries (Xu *et al.* 2009). They will provide future offshore aquaculturists with powerful new health management tools. Oral vaccines, in particular, if they can be developed, would greatly simplify and extend how vaccines are used in aquaculture. Besides, progress in the understanding of pathogen recognition molecules and elements of signaling pathways involved in marine organisms immune responses and inflammatory processes will improve the animals' immune response by less invasive therapies (de Lorgeril *et al.* 2011; Venier *et al.* 2011).

New marine bioactive metabolites such as inducible promoters and specific cell signaling factors can also help to adjust cultures (including breeding conditions) optimally. Some marine metabolic processes are already in use to perform better diagnostic tools (*i.e.* especially for viruses).

Renewable energy is another pillar of marine biotechnology. The abundant pigment protein membrane complex PSI photosystems are at the heart of the Earth's energy cycle. They are the central molecules converting sunlight into the chemical energy of life. Algae and cyanobacteria contain different pigments that increase their flexibility to use the available light at various depths along the water column. Among those pigments, the very well known green fluorescent protein (GFP) (Chiragwandi *et al.* 2006) absorbs at UV wave length while the new pigment 'chlorophyll f', recently obtained from extracts from ground up stromatolites captures light in the infrared wavelength (Chen *et al.* 2010). Commandeering this intricately organized photosynthetic nanocircuitries and rewiring them to produce electricity carries the promise of very efficient and environmentally friendly solar cells. To date, none of the current solar cells can absorb IR light, which accounts for over half of the sun's rays. Enlarging the usable wavelength can help to create devices yielding energy / electrical power density orders of magnitude higher than any photosystembased biophotovoltaic to date. At a different scale, minuscule size / low power output marine bio inspired fuel cells may be used for medical nanobots that could one day patrol our bloodstreams and treat our illnesses from within.

More recently, marine organisms have become inspiring cutting edge research in the design of new materials at nanoscale. Scientists expect that by learning the fundamental mechanisms used in nature, those mechanisms could be translated into practical and low cost manufacturing methods. Such "biomimetic" approaches will eventually be used in industry. Among various examples figures the use of a filamentous catalysing protein from Spongae to control the synthesis of silica needles at low temperature (Kisailus 2005). Silicon chips are fundamental components of computers and telecommunications devices. In combination with oxygen, silicon forms fiber optics and drives other hightech applications. Biosynthesis is remarkable because this nanoscale precision cannot be duplicated by man. Besides this remarkable precision, nature manages to produce silica at a low temperature, in an environmentally friendly way without the use of caustic chemicals, whereas man must use very high temperatures, high vacuums, and dangerous chemicals requiring costly remediation.

The marine world also provides unique examples of inherently fouling resistant surfaces based on physical properties. For instance, shark's skin (Ball 1999) and whale skin (Baum *et al.* 2001) are two examples where natural microtextures contribute to the reduction in fouling settlement. Gorgonian corals (Figuerido *et al.* 1997), crab carapaces and brittle stars (Bers and Wahl 2004) as well as dogfish egg cases (Davenport 1999) may also have potential physical fouling resistant properties. By far the best researched models of natural fouling resistant surfaces are the mussels *Mytilus galloprovincialis* (Scardino and de Nys 2004) *and Mytilus edulis* (Baum *et al.* 2001).

Marine microorganisms have been launched as optimized machineries for carrying out bioremediation activities in the oceans and coastal areas. Specialised single cell organisms, found in seawater all over the planet, aid in keeping the ocean healthy by eating naturally seeping oil. Without their presence, the world's oceans would be covered by a thick film of oil. The organisms surge in response to hydrocarbons, molecules made up of bonded hydrogen and carbon, which are naturally found in oil. Forefront research in this sector has already produced thousands of patents (Yakimov *et al*. 2007).

Marine genetic resources economics and legal framework

Since the later part of the twentieth century, the foreseen impact of marine genetic resources (MGRs) bioprospecting contracts and patenting on social welfare, fuels a brand new outlook on biodiversity, with living organisms potentially having a high economic value (Bouchet, 2006). In the context of marine genetic resources the term "bioprospecting" is more accurately defined as including the entire research and development

process from sample extraction by publicly funded scientific and academic research institutions, through to full scale commercialization and marketing by commercial interests such as biotechnology companies.

Since researchers and entrepreneurs have realized the untapped potential of MGRs, we are experiencing the beginning of an era of marine exploration, extraction, experimentation and commercialization. The main categories for MGRs related patents concern pharmaceuticals, nutraceuticals, cosmeceuticals and medical research sectors. There is a steady increase in the number of MGRs patents dealing with 'cutting edge' research, with emphasis on nanobiotechnological products inspired by marine organisms.

Nevertheless, the real MGRs related exploited products are still low in number. While academic researchers dealing with marine bioresources have ultimately boosted research and development, patenting and marketing of the wonder products derived from the MGRs, the major biotechnology companies are still reluctant to invest efforts into marine bioprospecting activities. This may be attributed in part to the fact that MGRs exploitation has a much shorter history than their terrestrial counterpart (with emphasis on terrestrial plants) (Blunt *et al.* 2010). Also, many countries continue to struggle with a number of unresolved issues over the strengthening of their patent regimes, including reconciling indigenous knowledge and patenting, benefit sharing and balancing public needs with property rights (Quach *et al.* 2006).

The Convention on Biological Diversity (CBD) is the source of new attitudes and new regulations, and is changing the way academic and non academic communities inventory, document, safeguard and use species of fauna and flora. The Convention on Biological Diversity has highlighted the imbalance between the distribution of biodiversity and the distribution of knowledge on that biodiversity. Most known and unknown biodiversity is in tropical countries, most of them developing or emerging countries of the South, whereas most of the knowledge and resources on that biodiversity is in the developed countries of the North. Although interesting and valuable to the cause of conservation, there is a growing sentiment that the 'price' being paid under these arrangements is too low. Among various reasons, it has been argued that the real MGRs value is obscured by the fact that those resources are largely open access (Nijkam *et al.* 2008). Some analyses have shown that, under conditions of appropriate quality control, the use of standard contracts against misappropriation and an initial investment in the creation of social networks, managing use and exchange of global genetic resources as a commons can be a desirable and effective solution to provide essential knowledge assets with major benefits both in developing and industrialized countries (Dedeurwaerdere 2010).

CONCLUSION

Different types of marine bioproducts may possibly have some kind of inherent feature that justifies their production and existence in Nature. These compounds may express some type of undiscovered chemical or biological activity. Numerous data reflect the very complicated, diverse and sometimes unknown biosynthetic machinery, especially in the case of unculturable species. The value of marine biodiversity as source of patentable compound / processes with potential application in various industrial sectors is getting better and better documented. The real task is to access marine organisms, building blocks and biosynthetic machineries. When this enormous task is completed, we will have access to an almost endless number of new, useful compounds for the benefit of humankind.

Currently, very few discoveries related to the use of MGRs are being developed into marketable products. Difficulties related to the governance of MGRs access and benefit sharing related issues may be one of the reasons. Among the disadvantages of MGRs related products figures also the need of well trained teams of scientists from different disciplines and of specific equipment. In addition, the scale up of natural products is problematic. In particular, natural product extracts are always complicated mixtures, making the isolation of compounds challenging. Real success can only be achieved using biotechnological and semisynthetic approaches or their logical combination.

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Oil Hydrocarbon Degradation Effects of Some Bacteria Isolated from Various Environments in Turkey

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ABSTRACT

Since bacteria have an essential role in the providence of organic pollutants in natural environments, biodegradation of petroleum hydrocarbons by aquatic microorganisms has become an issue of great interest. In this study, with the aim of obtaining oil degrading bacterial strains from marine regions, in total, 288 units of sea water samples were collected from different marine locations in Turkey: the western Black Sea, the Istanbul strait, the eastern part of the Sea of Marmara, the northern part of the Sea of Marmara, the Oludeniz Lagoon, the Aegean Sea at different periods of time from February, 2002 to June, 2007. A total of 103 bacterial strains were isolated from the sea water samples and the soil surrounding petroleum refineries in the city of Batman, Turkey.

The isolates firstly were screened with the ″Minimum Inhibition Concentration″ (MIC) test against Batman crude oil and against different oil samples which were imported from Lebanon (Light ESSIDER), Iran (Heavy), Russia (Export Blend Middle, REB), and Siberia (Light SBLT) to Turkey in 2006-2007.

According to multi resistance findings of the isolates in various petroleum oil concentrations, five units of isolates and their mixed consortiums were selected for further analyses.

Different parameters were tested: growth profiles of the isolates in the media with 25% and without crude oil, hemolytic activities, pH values, the emulsification index and the oil layers' thickness during the 30 days of incubation. With the aim of improving their oil degradation capacities, which were detected by GC-MS measurements, the isolates were adapted into the media with 35% and 50% Batman crude oil.

The study findings indicated that Gr (+) bacilli BR02, Gr (-) rod MD03, Gr (+) coccus GA01, Gr (-) shape MDK04, BR03, and their mixed consortiums were efficient in using crude oil as the sole source of carbon and energy. The standard oil degrading bacterial strains *Alcanivorax borkumensis* (CIP 105606, France) and *Vibrio cyclitrophicus* (CIP 106644, France) were used for positive

controls in the tests. Adapted bacteria cultures and their mixed consortiums exhibited better degradation activities than the standard strains. This study contributed to our knowledge about the possibility of the use of natural bacterial isolates *in situ* bioremediation of crude oil spills.

Keywords: Bacteria, Oil Hydrocarbon, Emulsification, MIC, biodegradation, hemolytic activity

INTRODUCTION

Many bacteria are capable of degrading organic contaminants to obtain food and energy, typically degrading them into simple organic compounds, carbon dioxide, water, salts, and other harmless substances. The degradation of oil hydrocarbons by microorganisms has been studied with respect to their biochemical and genetic aspects (Wolfe 1977; Anon., 1991). The determination, isolation and characterization of those microorganisms which degrade oil hydrocarbons are of great importance with respect to rehabilitation of the environment in a shorter period of time.

Biodegradation by marine bacteria can play an important role in the fate of various types of complex molecules. The biodegradation of many components of petroleum hydrocarbons by bacteria has been reported in a variety of terrestrial and marine ecosystems (Byrom and Beastall 1971 Farrington 1980; Lee 1980; Fusey and Oudot 1984; Oudot 1984, Okoh and Hernandez 2006).

Although oil hydrocarbon degrading bacteria are indigenous to marine environments, there are many different environmental factors that restrict bacterial activities in marine environments. One of the limiting factors in this process is the bioavailability of various fractions of oil. Since the biosurfactants can improve the bioavailability of hydrocarbons into the bacterial cells, many bacteria can produce biosurfactants (Gerson 1993). As a result, biodegradation rates change according to geographical conditions and bioremediation strategies must be modified by considering the environmental stress of the specific location. This situation is important for developing petroleum-hydrocarbon remediation strategies to assess the potential for in situ microbial degradation of contaminants (Stevenson and Cole 1999; Ferguson *et al.* 2007).

Products composed of bacteria are used throughout the world in the improvement of areas polluted with oil hydrocarbons. However, efficiency in the bioremediation of environments polluted by petroleum hydrocarbons still remains to be a challenge. It is well known that ecological factors are influential on the development of and efficiency in the degradation ability of bacteria. Isolates of local bacteria were used with successful results in Alaska in 1989.

Turkey is under the risk of oil pollution due to its heavy marine traffic. In the case of an accident in the marine environment or in the

petroleum pipelines, local organic products will be useful to remediate the environment. The purpose of this study is to find possible candidates of local bacteria isolates which in turn will lead to the production of those products which may be necessary to remediate environmental pollution issues resulting from accidents involving petroleum. With the aim of identifying the most efficient oil-degrading bacteria and acquiring suitable bacterial candidates, 103 unit strains which were isolated from various marine areas of Turkey were screened.

MATERIALS AND METHODS

Sampling

The sea water samples were collected from various marine regions in Turkey: the western Black Sea (1), the Istanbul strait and the northern part of the Sea of Marmara (2), the eastern part of the Sea of Marmara (3), the northern Aegean Sea (4), the Oludeniz Lagoon, the Aegean Sea (5), at different periods of time from February, 2002 to June, 2007 (Figure 1).

The soil samples from the petroleum contaminated sites nearby Batman Petroleum Refinery in southeastern Turkey were collected into pre-sterilized glass bottles and immediately transported to the laboratory in Istanbul University for analyses.

Bacteriological analyses in the sea water samples

The sea water samples were collected in sterilized glass bottles, serial dilutions were prepared to 10^{-5} in 9 ml amounts of sterile seawater (artificial seawater, Sigma) and were inoculated (0.2 ml) in duplicate on marine agar (Difco) and the plates were incubated for five days at 22±0.1°C (Bianchi *et al*. 1992).

After incubation, different colonies were picked and restreaked several times to obtain pure cultures. The pure isolates were identified using GN, GP and BCL cards in the automated biochemical identification system VITEK 2 Compact 30 (Biomereux, France).

Bacteriological analyses in the soil samples surrounding the petroleum refinery

The soil samples taken from the surrounding regions of Batman Petroleum Refinery were incubated for 48h at 25±0.1°C in pre-enrichment media, Nutrient Broth (NB) and Mueller Hinton Broth (MHB). After the enrichment process, the samples were incubated for 24 or 48h on Nutrient Agar. Following the incubation, the pure cultures were obtained by picking from different colonies. The pure isolates were identified using the automated biochemical identification system VITEK 2 Compact 30 (Biomereux, France).

Minimum Inhibition Concentration (MIC) Values of the isolates
Bacterial suspension (50 µl: McFarland No: 1 standard solution) + 50 µl MSM (Mineral Salt Medium) was added into 96 well micro-titer plates. Crude oil was added to 12 unit micro-wells in decreasing amounts ranging from 100µg/ml to 0.09µg/ml. After incubation at 37 ºC for 18h, the petroleum concentration of the well without turbidity was accepted as the minimal inhibitory concentration (MIC) in oil (Anon 1997).

Besides Batman crude oil, Lebanon Light oil (ESSIDER), Russian Export Blend- Middle oil (REB) and Siberian Light oil (SBLT) which were imported from the other countries to Turkey in 2006-2007 were used in the MIC analyses.

Biodegradation potential of the bacterial strains
25 ml crude oil, MSM (Mineral Salt Medium) and 25 ml bacterial suspension (McFarland No: 1 standard solution: $3x10^8$) were incubated for 30 days at 25ºC on a shaker (150 rpm) (Rahman *et al*. 2002). The growth profiles of bacterial strains were determined by measuring the CFU of the isolates by means of the spread plate technique in media +/- crude oil (APHA, 1999). The hemolytic activity of bacterial cells was tested using a Blood Agar medium according to Bicca *et al*. (1999). The colonies with a transparent zone around them were evaluated as positive in terms of hemolytic activity. The Emulsification Index (E_{24}) of the culture samples was determined as the ratio of the height of the emulsified layer (mm) to the total height of the liquid column (mm) according to Tabatabaee *et al*. (2005) and Sarubbo (2006). The chosen bacterial isolates were screened against Batman crude oil, Lebanon light oil (ESSIDER), Iran heavy oil, Russian blend middle oil (REB) and Siberia light oil (SBLT). Batman crude oil was used for further biodegradation assays. The tested bacterial individual strains were adapted into 35% and 50% Batman crude oil media using incubation method. All the tests mentioned above were also applied to the adapted cultures.

Figure 1. Sampling Area.

Figure 2. Batman crude oil in laboratory scale microcosm experiment.

RESULTS AND DISCUSSION

The bacterial strains of *Klebsiella pneumoniae, Serratia marcescens*, *Enterobacter cloacae and Escherichia coli* that showed MIC values equal to or more than 50 µl against different oil samples were imported from other countries to Turkey in 2006-2007; these were shown in Table 1.

The various bacterial strains that showed MIC values equal to or more than 50 µl against Batman crude oil were shown in Table 2.

The percentage of the emulsification index (E24) of the bacterial isolates that were able to use crude oil as a sole carbon source at a late exponential phase was shown in Table 3.

The growth profile (CFU) of the chosen isolates in 25% crude oil and without crude oil media was shown in Figure 3.

The growth profile (CFU) of the adapted isolates in 35% and 50% crude oil and without crude oil media were shown in Figure 4.

The changes in pH and oil layers' thicknesses were recorded every 72 hours during the incubation period of 30 days. An example of the oil layers' thicknesses (cm) and pH values which were obtained from *S. haemolyticus* GA 01 and reference strains (*A. borkumensis* and *V. cyclitrophicus*) were shown in Figure 5.

Table 1. The bacterial strains that showed MIC values equal to or more than 50 µl against different oil samples were imported from other countries to Turkey in 2006-2007.

	Micro-wells	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	Crude Oil	**100**	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.56**	**0.78**	**0.39**	**0.19**	**0.09**	**0**
	MSM (µl*)	**-**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**
OIL TYPE	**Bacterial suspension (µl*)**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**
ESSIDER	*K. pneumoniae* 01	+	+	+	+	+	+	+	+	+	+	+	+
	S. marcescens OZEY0706	+	+	+	+	+	+	+	+	+	+	+	+
	E. cloacae 03	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli MDK03	+	+	+	+	+	+	+	+	+	+	+	+
	The mixed culture	-	-	+	+	+	+	+	+	+	+	+	+
IRAN HEAVY	*K. pneumoniae* 01	-	+	+	+	+	+	+	+	+	+	+	+
	S. marcescens OZEY0706	+	+	+	+	+	+	+	+	+	+	+	+
	E. cloacae 03	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli MDK03	+	+	+	+	+	+	+	+	+	+	+	+
	The mixed culture	-	-	-	+	+	+	+	+	+	+	+	+
REB	*K. pneumoniae* 01	+	+	+	+	+	+	+	+	+	+	+	+
	S. marcescens OZEY0706	+	+	+	+	+	+	+	+	+	+	+	+
	E. cloacae 03	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli MDK03	-	+	+	+	+	+	+	+	+	+	+	+
	The mixed culture	+	+	+	+	+	+	+	+	+	+	+	+

Table 1 Continued.

SBLT	*K. pneumoniae* 01	+	+	+	+	+	+	+	+	+	+	+	+
	S. marcescens OZEY0706	+	+	+	+	+	+	+	+	+	+	+	+
	E. cloacae 03	-	+	+	+	+	+	+	+	+	+	+	+
	E. coli MDK03	+	+	+	+	+	+	+	+	+	+	+	+
	The mixed culture	-	-	+	+	+	+	+	+	+	+	+	+

(+): Bacterial growth was observed (-): Bacterial growth was not observed. **ESSIDER:** Lebanon Light**, REB**: Russian Export Blend- Middle, **SBLT:** Siberia Light

The first micro-well (without MSM and containing only crude oil and bacteria) was used to detect bacteria that could survive in media containing crude oil as the only carbon source. The last micro-well without crude oil (bacterial suspension and MSM) was used as a positive growth control.

Table 2. The bacterial strains that showed MIC values equal to or more than 50 µl against Batman crude oil.

Screening Isolates	**Amount of crude oil (µl) in the micro wells**											
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.19	0.09	0
Enterococcus faecalis WBS01	-	-	+	+	+	+	+	+	+	+	+	+
E. coli WBS02	-	+	+	+	+	+	+	+	+	+	+	+
The mixed culture*	-	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus epidermitidis IB1	-	-	+	+	+	+	+	+	+	+	+	+
S. epidermitidis IB2	-	-	+	+	+	+	+	+	+	+	+	+
Pseudomonas oryzihabitans IBM24008	-	+	+	+	+	+	+	+	+	+	+	+
Proteus mirabilis IBM1M06İ	-	+	+	+	+	+	+	+	+	+	+	+
Enterobacter sakazaki IBA23KT06	-	+	+	+	+	+	+	+	+	+	+	+
Bacillus subtilis IB03	-	+	+	+	+	+	+	+	+	+	+	+
The mixed culture*	-	+	+	+	+	+	+	+	+	+	+	+
S. aureus EMS01	-	+	+	+	+	+	+	+	+	+	+	+
S. epidermitidis EMS02	-	+	+	+	+	+	+	+	+	+	+	+
Aeromonas hydrophila EMS03	-	-	+	+	+	+	+	+	+	+	+	+
Citrobacter freundii EMS04	-	-	+	+	+	+	+	+	+	+	+	+
Providencia spp. EMS05	-	-	+	+	+	+	+	+	+	+	+	+
The mixed culture*	-	-	+	+	+	+	+	+	+	+	+	+
S. marcencens MD01	-	+	+	+	+	+	+	+	+	+	+	+
K. pneumoniae MD02	-	+	+	+	+	+	+	+	+	+	+	+
V. fluvialis MD03	+	+	+	+	+	+	+	+	+	+	+	+
E. coli MDK04	+	+	+	+	+	+	+	+	+	+	+	+

Table 2 Continued.

K. oxytoca SAEK0805	-	+	+	+	+	+	+	+	+	+	+	+
S. haemolyticus GA01	+	+	+	+	+	+	+	+	+	+	+	+
The mixed culture*	+	+	+	+	+	+	+	+	+	+	+	+
Eikenella corrodens OZEY0705	-	-	+	+	+	+	+	+	+	+	+	+
S. marcescens OZEY0706	-	-	+	+	+	+	+	+	+	+	+	+
E. coli OZEY0707	-	+	+	+	+	+	+	+	+	+	+	+
E. faecalis OZEY0708	-	-	+	+	+	+	+	+	+	+	+	+
Burkholderia cepacea OZEY0709	-	-	+	+	+	+	+	+	+	+	+	+
The mixed culture*	-	-	+	+	+	+	+	+	+	+	+	+
E. coli BR01	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis BR02	+	+	+	+	+	+	+	+	+	+	+	+
P. aeruginosa BR03	+	+	+	+	+	+	+	+	+	+	+	+
The mixed culture*	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis BR02 *P. aeruginosa* BR03 *S. haemolyticus* GA01 *V. fluvialis* MD03 *E. coli* MDK04 = A mixed bacterial consortium of the strains selected for degradation analyses	+	+	+	+	+	+	+		+	+	+	+
CIP 105606** *A. borkumensis*	-	+	+	+	+	+	+	+	+	+	+	+
CIP 106644** *V. cyclitrophicus*	-	+	+	+	+	+	+	+	+	+	+	+

(+): Bacterial growth was observed, (-): Bacterial growth was not observed *A mixed culture prepared using the strains isolated from the above mentioned regions

**Standard Strains: Centre de Ressources Biologiques de l'Institut Pasteur (France)

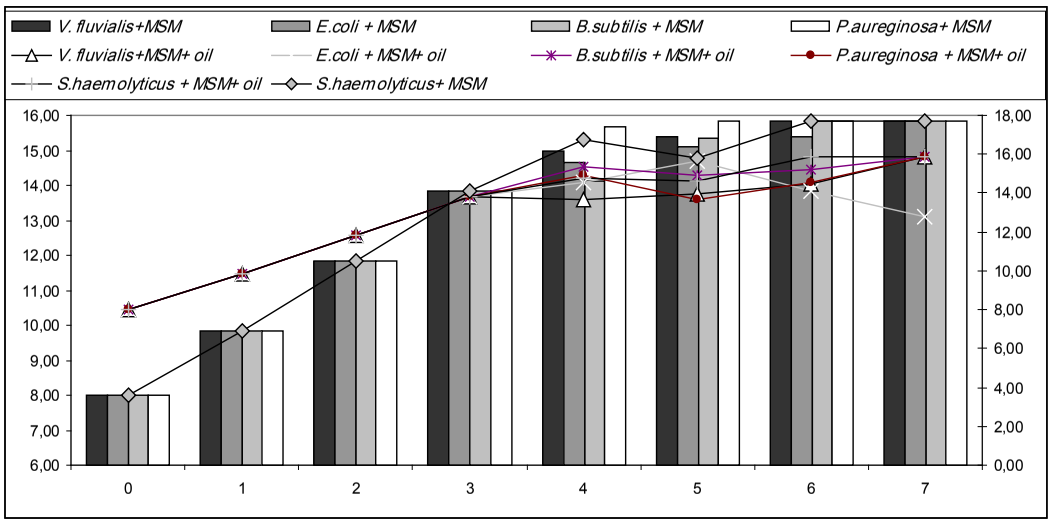

Figure 3. Growth profile (CFU: Colony Formed Unit) of the isolates tested in 25% crude oil and without crude oil media.

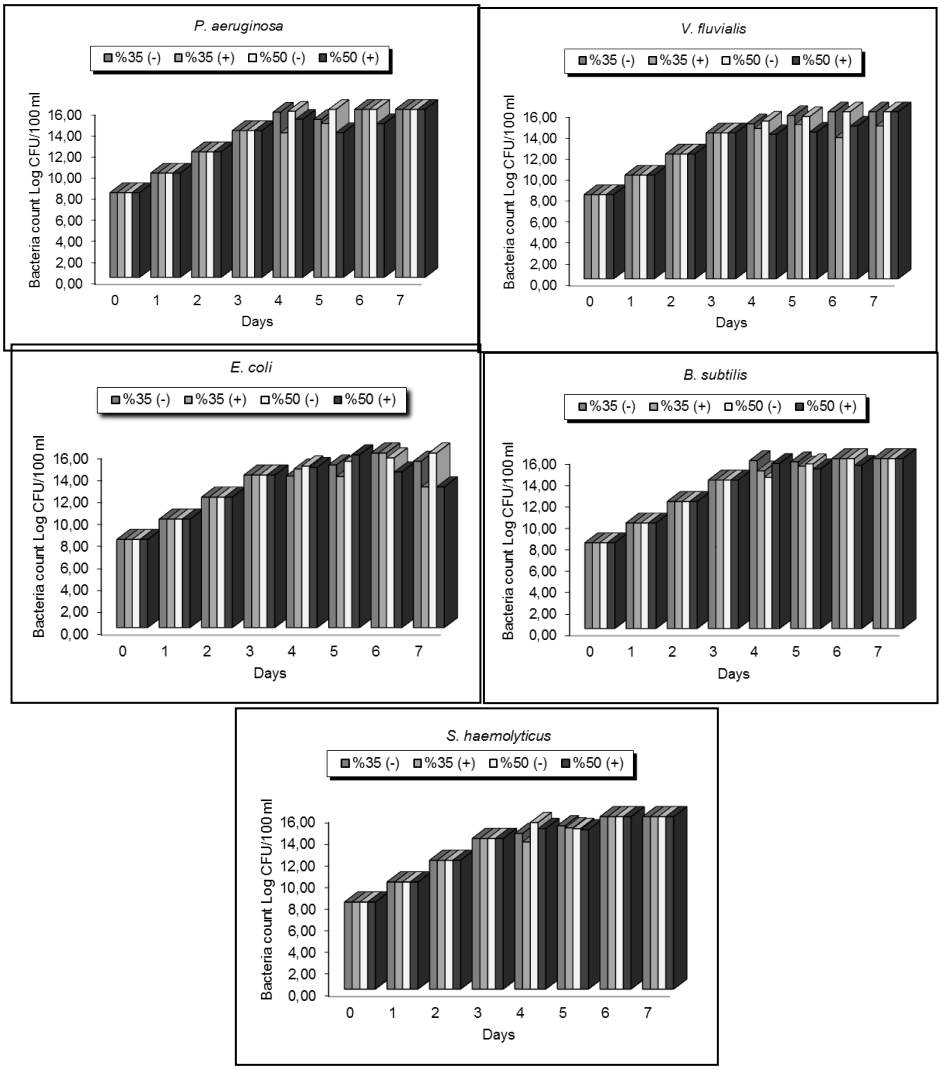

Figure 4. The growth profile (CFU) of the isolates adapted into crude oil media (35% and 50%) and without crude oil media.

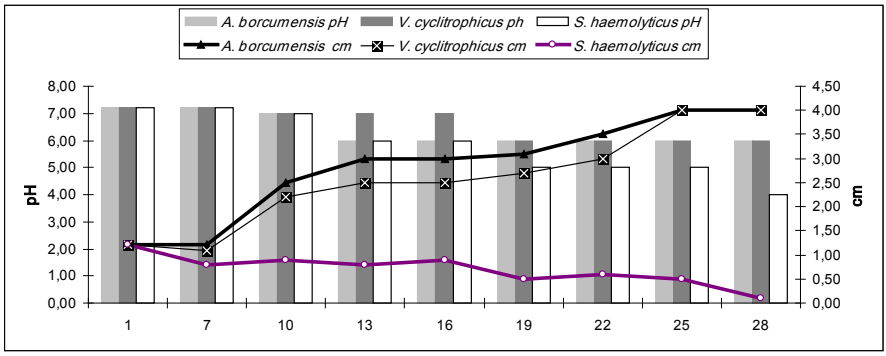

Figure 5. An example of a comparison of oil layers' thicknesses (cm) and pH values obtained from an isolate of *S. haemolyticus* GA 01and reference strains of *A. borcumensis* and *V. cyclitrophicus*.

The thicknesses of the oil layers were calculated and found to be lower in the medium of *S. haemolyticus* GA01 as compared to the reference strains. At the beginning of the incubation, pH values were recorded to be about 7. Depending on the incubation time, slight decreases were recorded.

Table 3. The percentage of the emulsification index (E24) of the bacterial isolates that were able to use crude oil as a sole carbon source at a late exponential phase.

Bacteria	**E_{24} %**
P. aeruginosa BR03	95
E. coli MDK04	90
S. haemolyticus GA01	85
B. subtilis BR02	85
V. fluvialis MD03	45
Negative Control	0

Leahy and Colwell (1990) reported that bacterial strains with better hydrocarbon degradation capacities are dominant in regions contaminated with hydrocarbons and furthermore, the population size of hydrocarbon degrading bacteria shows alterations according to the type and level of pollutants. In this study, the areas which were bacteria isolated have different trophic character. Depending on the pollution rate of the areas, dominant bacteria species rates were found to be different. The dominant species were selected and screened for their potential oil hydrocarbon degradation capacities. The strains which were isolated from eutrophic areas exhibited higher MIC values than the other isolates. For example, the bacterial strains which were isolated from the Istanbul Strait and the northern part of the Sea of Marmara showed higher MIC values as

compared to relatively less polluted marine areas such as the northern part of the Aegean Sea and the eastern part of the Sea of Marmara. The largest bacterial population sizes of the tested bacterial strains were also observed in the samples from the soil from the oil refinery region.

One main factor that controls the level of petroleum in bacterial degradation processes is the bioavailability of the petroleum hydrocarbons to the bacteria. Emulsification properties of bacteria induce solubility first and then induce the bioavailability of oil hydrocarbons via forming micelles. Consequently, the occurrence of surfactants may increase the microbial degradation of pollutants. Hemolytic peculiarities have been used as a rapid method in screening bacteria for potential biosurfactant production (Lin, 1996; Tabatabae *et al.* 2005). It was reported that (Prieto *et al*. 2008) biosurfactants have a strong emulsion capacity and a stable activity under neutral or alkaline environments. Observed pH values for all the isolates used in this study were in the permissible range for emulsion activity. Supporting this conclusion, all of the selected isolates showed a good level of hemolytic activity and emulsification indices. The highest E_{24} value was found (as 95%) in the isolate of *P. aeruginosa* BR03.

The measured total amount of petroleum hydrocarbons showed slight fluctuations in the media containing individual bacterial strains and the mixed bacterial consortium during the 72h interval during the 30 days. The fluctuations in petroleum hydrocarbon levels were correlated with changes in bacterial growth profile in the flasks. The measurements of the n-alkane order bacteria belonging to the aliphatic chains of the bacterial strains using Gas Chromatography–Mass Spectrometry (GC-MS) (*data not shown*) show that crude oil was used at a progressively increasing rate by all the isolates until the 42nd day of incubation.

Biodegradation of some oil hydrocarbons by microorganisms has been biochemically and genetically investigated. Gene locations for some of these pathways are frequently found in extra - chromosomal elements. In this study, the sequence analyses of a plasmid from *Bacillus* BR02 yielded some genes such as AcylCoA oxidase and superoxide dismutase that are likely to be critical for the oil degradation capacity. However, those findings that were obtained in the framework of this study, findings which were related to plasmid curing, proteomic profile and sequence analyses were not shown in this article.

Adapted bacteria culture is used for improved degradation rates of pollutants by bacteria in oil hydrocarbon contaminated areas. Using adapted bacteria cultures has become widespread during recent years with the latest developments in biotechnology. The capacity of bacterial strains to thrive in a pure crude oil medium is the most important criterion for determining the biodegradation efficiency of oil hydrocarbons. In this

study, the isolates which were adapted into 35% and 50% Batman crude oil exhibited similar growth profiles to the isolates in media containing 25% crude oil. Experimental results show that the tested adapted bacteria cultures and a mixed bacterial consortium of *P. aeruginosa* BR03, *E. coli* MDK04, *S. haemolyticus* GA01, *B. subtilis* BR02, and *V. fluvialis* MD03 may be used as potential candidates for biodegradation of oil hydrocarbons.

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Characterization of Some EPS-producing Biofilm Bacteria on the Panels Coated by Different Antifouling Paints in the Marinas

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ABSTRACT

The goal of the research was characterization of marine bacteria which could produce extracellular polymeric substances (EPSs) on static panels and to determine the minimum inhibitory concentrations (MICs) of the antifouling agents preventing the growth of biofilm bacteria. To create surfaces suitable for settlement, steel panels were manufactured by the contact method, more widely known as the hand lay-up method. Zinc oxide, copper oxide, fluorine and triazine diamine based additives were used as the antifouling agents in the paints. The biofilm bacteria were scraped from the surface of the panels and were inoculated on ZoBell agar medium and R2A agar medium by the spread plate method. Bacterial growth was detected on all test panels and twenty-six strains were cultured. Phylogenetic analysis using 16S rDNA sequences indicated that all of the strains belonged to the γ-Proteobacteria. However, six strains were investigated in respect of the MICs of paints and ability of EPSs producing. The EPS producing ability, sugar and protein contents of the EPSs were determined. Strain C 91 showed the highest EPS productivity, 0.80 (dried EPS weight/dried cell weight). Cell growth and EPS productivity were found to be negatively correlated ($r=-0.90$, $p=0.05$). The paint, which contains triazine diamine and copper oxide, showed strong antibacterial activity against each individual strain. The lowest MICs of the paints against the bacterial isolates were between 1/4 and 1/16 times dilutions. Individual biofilm bacteria, like our bacterial isolates, are very sensitive to antifouling paints in vitro. It was thought that bacteria had more resistance in environmental systems because they were isolated after the development of bacterial biofilm on test panels.

Keywords: Marine bacteria; Biofilm; Extracellular polymeric substances; Antifouling paints

INTRODUCTION

Extracellular polymeric substances (EPSs) sequester nutrients from the environment as part of a general microbial strategy for survival under oligotrophic conditions (Wolfaardt *et al*. 1998; Decho 2000). EPSs also

have the potential to physically prevent penetration by certain antimicrobial agents by acting as ion exchangers, thereby restricting diffusion of compounds into the biofilm (Gilbert *et al.* 1997). Therefore, biofilm formation on heat exchangers, pipelines, ship surfaces and other industrial devices (fouling) causes serious problems and consumes large amounts of time and money for its removal or mitigation (Costerton *et al*. 1987). There are literature reports on the marine microorganisms that cause microfouling, such as *Zoogloea* sp., *Pseudomonas* sp., *Vibrio fischeri*, *Cyanothece* sp., and *Alteromonas macleodii* (Ko *et al*. 2000). Most frequently, members of the *Pseudoalteromonas* genus have been isolated from sources such as marine eukaryotes, seawater, sea ice, etc. (Nichols *et al.* 2004; Nam *et al.* 2007). Studies have focused on the isolation of *Pseudoalteromonas* from diverse habitats and analysis of its biopolymer (including EPS) production and biofilm formation (Nichols *et al*. 2004; Nandakumar *et al*. 2004).

Recently, Nichols *et al*. (2004) isolated bacteria belonging to the *Pseudoalteromonas* genus from Antarctic waters and studied its EPS production in batch cultures. Bacteria with EPS-producing ability may have an advantage in successfully colonizing surfaces as primary colonizers (Saravanan *et al*. 2008). In addition, control of biofilm formation has also been an important topic of interest (Lindberg *et al*. 2001). The use of antifouling paints is very common in order to prevent fouling problems on surfaces immersed in water. The purpose of antifouling paints is primarily to prevent attachment of macrobial organisms, e.g. barnacles. Existence of microorganisms on a surface can accelerate the attachment of shellfish larvae to a submerged surface (Tang and Cooney 1998). Both organotin and copper based antifouling paints effectively prevent fouling problems by macroorganisms, although organotin-based paints are effective for longer periods than copper-based paints (Champ and Lowenstein 1987). However, both organotins and copper can be toxic to non-target marine species, such as dog-whelks (Gibbs and Bryan 1986) and oysters (Axiak *et al*. 1995). Tributyltin (TBT), in particular, causes imposex, a condition where females grow male sex organs, in dog-whelks and other shellfish.

In this study, marine bacterial fouling communities growing on artificial substrates that were coated by antifouling paints in two marinas (Izmir Marina and Cesme Marina) in Izmir, Turkey were investigated by i) the isolation of bacteria from the initial stage of biofilm that formed on the test panels that had been exposed to natural seawater, ii) identification of bacterial strains, iii) determination and characterization of the EPS produced by the strains.

MATERIALS AND METHODS

Study area

Izmir Bay (Western Turkey) is one of the great natural bays of the Mediterranean basin. This region used to be exposed to pollution due to industrial wastewater discharges (Kucuksezgin *et al*. 2006). Cesme, which has oligotrophic seawater, is about 80 km away from Izmir. Surrounded on three sides by the Aegean Sea, is the western most town in Turkey and about 80 km from Izmir. Settlement and recruitment of biofilm bacteria were studied at two marinas located in Izmir Bay and Cesme.

Preparation of static panels and coating systems

To create surfaces suitable for settlement, 30-cm x 30-cm square steel panels were manufactured by the contact method, more widely known as the hand lay-up method (Bernetsson and Jonsson 2003; Valkirs *et al*. 2003). Three types of marine paints and one type of rustproof paint, which were provided from a local paint Manufacturing and Trading Co. Inc., were used in this study. After cutting, trimming tolerances and maintaining a sufficient gap between panels, each type of paint was applied to the surfaces by brush, yielding 1.5 mm of total thickness. Static panels were then submerged to a depth of 1.5 m from the surface in the two marinas.

The antifouling paints were as follows: Supplier formulation number 279.2710: (basic component; triazine diamine and copper oxide 47 %, total solid matter; 76-77 %, solvent; xylene 23-24 %). Supplier formulation number 088.1154: (basic component; zinc oxide and copper oxide 35 %, total solid matter; 70-71 %, solvent; toluene 29-30 %). Supplier formulation number 088.1155: (basic component; fluorine resin 30%, total solid matter; 67-70 %, solvent; ethyl acetate 30-33%). Rustproof: synthetic resin based with alkyd and anticorrosive pigments.

Isolation and characterization of bacterial isolates

The biofilm bacteria were scraped from the surface of the panels and suspended in 20 ml of sterilized aged seawater. A dispersed and diluted sample (10^{-1}-10^{-8}) was inoculated on ZoBell agar medium and R2A agar medium by the spread plate method. The agar plates were incubated at 25°C for 5 days. Bacterial colonies showing different morphological characteristics were purified on ZoBell agar plates (Kwon *et al*. 2002; Lee *et al*. 2003). All isolates were characterized in terms of morphology, gram-staining and some biochemical tests.

Analysis of 16S rRNA gene sequences

Genomic DNA was extracted from logarithmic phase cells using a Fungal/Bacterial DNA Kit (Zymo Research, USA) after incubation in ZoBell broth for 1 day at 26 °C. The universal bacterial primers 27F and 1522R were used for polymerase chain reaction (PCR) amplification of 16S rDNA. FastStart Taq DNA Polymerase, the dNTPack Kit (Roche, Germany) and 200 mmol l^{-1} primers and 10 ng of template DNA were used for PCR (Kwon *et al*. 2002; Lee *et al*. 2003). The DNA sequence analyses of purified PCR products were performed using BigDye terminator technology and an automatic sequence analyzer system (ABI Prism 3100) (REFGEN Biyoteknoloji, Turkey).

Phylogenetic analysis of bacterial isolates

The single-stranded 16S rRNA gene sequences of the biofilm isolates were matched with those in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) by BLAST searching. A phylogenetic tree was constructed by the neighbor-joining method and was evaluated by bootstrap sampling (1,000 replicates) using the MEGA 4 program (Tamura *et al*. 2007).

Nucleotide accession number

The 16S rDNA sequence data of the isolates C 91, I 2, I 96, I 97, I 50 and C 73 reported in this article were submitted to GenBank and assigned the accession numbers FJ040185, FJ040186, FJ040187, FJ040188, FJ040189 and FJ040190, respectively.

Ability of EPS production

The bacterial strains were inoculated in YMG broth and pre-incubated at 25 °C for 24 h. A 1-ml volume of culture broth was transferred into 100 ml of YMG broth and incubated at 25 °C for 5 days at 120 rpm. Cultures were centrifuged at 10000 x g for 20 min, and the supernatant obtained was mixed with 3 volumes of propan-2-ol; after incubation at 4 °C for 4 h, the mixture was centrifuged at 10000 x g for 20 min at 4 °C. The weight of the precipitated EPS was measured after lyophilization (Kwon *et al*. 2002).

Characterization of EPS

Monosaccharide composition of the EPS was analyzed by TLC (Kwon *et al*. 2002) and samples were prepared by the method of Staneck and Roberts (1974). Polysaccharide samples (50 mg) were hydrolyzed with 4 ml of 2 N H2SO4 at 100oC for 2 h and were neutralized by adding saturated $Ba(OH)_2$. After centrifugation (17,000´g, 4^oC, 10 min), the supernatant was filtered through a 0.22 mm membrane filter, concentrated

by evaporating under reduced pressure, and placed on TLC plates. Saccharose, lactose, glucose, mannose, rhamnose, galactose, fructose, arabinose, and ribose were used as standards. Lyophilized extracellular polymeric substances were assayed for total sugar content using the phenol-sulphuric acid method, as described by Dubois *et al*. (1956), and for total protein content using the Bradford method (Bradford 1976). The Pearson correlation procedure was applied for cell growth and EPS productivity analyses (Sokal and Rohlf 1995).

2. 9 Determination of minimum inhibitory concentrations (MIC) of antifouling paints MIC Values of the antifouling paints used in this study were determined according to the method described by Zgoda and Porter (2001) with some modifications. A series of dilutions, ranging from 1/2 to 1/1024, of the three antifouling paints (coded as 279.2710, 088.1154, 088.1155) and the rustproof paint were prepared in test tubes and were then transferred to broth in 96-well microtiter plates. Before inoculation, the bacterial strains were adjusted to the turbidity of the 0.5 McFarland standards and diluted 1:100 in ZoBell marine broth. Plates were incubated at 26 °C for 24 h whereas the MIC values of the paints were defined as the lowest concentration that prevented bacterial growth, minimum bactericidal concentrations (MBCs) of the paints were determined by plating samples from clear wells onto ZoBell marine agar.

RESULTS

Characterization of biofilm-forming bacteria

All test panels coated by different antifouling paints were observed to be slimy during sampling period in both study areas. All isolates were gram-negative, non-spore forming and oxidase positive bacteria. Some morphological, biochemical and other features of the isolates are shown in Table 1. Phylogenetic analysis using 16S rDNA indicated that the six strains were γ-Proteobacteria (*Pseudoalteromonas agarivorans, P. haloplanktis, P. marina, Alteromonas genoviensis, A. alvinella* and *Klebsiella pneumonia*). Strain I 97 showed 100% homology to *Pseudoalteromonas agarivorans*, an agarolytic marine isolate. Strain I 2 was 99% identical to *P. haloplanktis*, C 91 showed 100% resemblance to *P. marina* and strain C 73 showed 98% homology to *A. alvinella*. Strain I 96 showed 100% homology to *A. genoviensis* and I 50 was 99% identical to *Klebsiella pneumonia* (Table 2). Based on the homology of the 16S rRNA gene of the bacteria, a phylogenetic tree was constructed containing the isolated bacteria and the related species of γ-Proteobacteria (Figure 1).

Table 1. Some phenotypic and biochemical characteristics of bacterial isolate.

Tests	Bacterial isolates					
	I 50	I 97	I 2	C 91	I 96	C 73
Gram's reac.	-	-	-	-	-	-
Colony color	white	cream	cream	cream	camel	cream
Pigmentation	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Nitrat reduction	+	-	-	-	-	-
Utilisation of-						
D-Glucose	+	+	-	-	-	-
D-Mannitol	+	+	-	-	-	-
Inositol	+	-	-	-	-	-
D-Sorbitol	+	-	-	-	-	-
L- Rhamnose	+	-	-	-	-	-
D-Saccharose	+	+	-	-	-	+
D-Melibiose	+	+	-	-	-	-
L-Arabinose	+	-	-	-	-	-
L-Arginine	-	+	-	-	-	-
L-Lysine	+	-	-	-	-	-
Gelatine	-	+	+	+	+	+
Production of						
H_2S	-	-	-	-	-	-
Indol	-	-	-	-	-	-

Note: + present, - absent, + can utilize, - cannot utilize

Table 2. List of bacterial species isolated from marine biofilms on coated panels.

Bacterial isolates	Paint no.	16S rDNA similarity %	Closest match
I 50	Rustproof	99	*Klebsiella pneumonia*
I 97	279.2710	100	*Pseudoalteromonas agorivorans*
C73	088.1154	98	*Alteromonas alvinella*
I 2	Rustproof	99	*Pseudoalteromonas haloplanktis*
C 91	088.1154	100	*Pseudoalteromonas marina*
I 96	088.1155	100	*Alteromonas genoviensis*

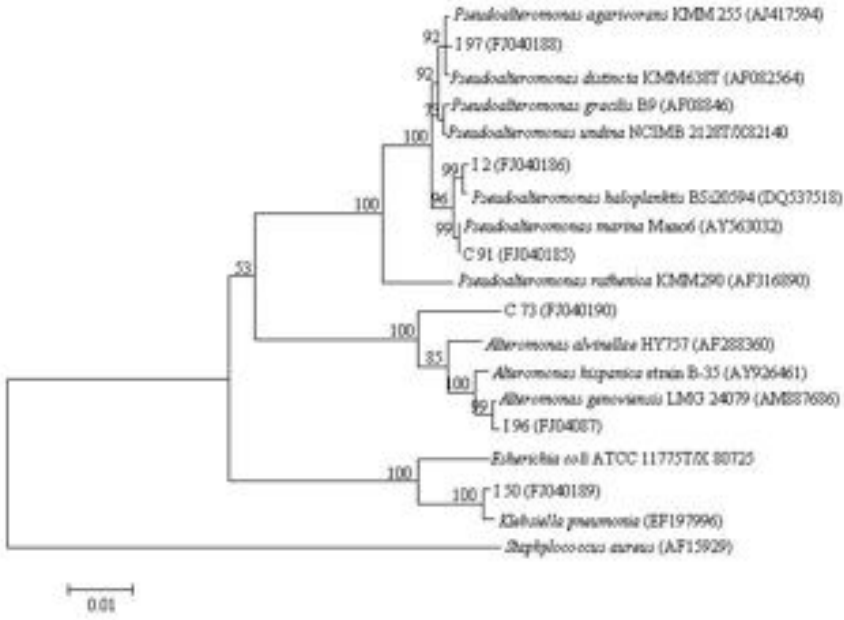

Figure 1. Phylogenetic trees based on partial 16S rDNA gene sequence data (approximately 1400bp) showing isolated bacteria from test panels.

EPS-producing ability and total sugar and protein analyses of the bacterial strains

The dry cell weight of the six strains ranged from 2.10 ± 0.24 (C91) to 4.00 ± 0.24 (C 73) g/l, and the dry weight of the EPS ranged from 1.40 ± 0.12 (C 73) to 2.20 ± 0.12 (I 50) g/l after 5 days of incubation in YMG broth. Strain C 91 showed the highest EPS productivity, 0.80 (dried EPS weight/dried cell weight). The amounts of EPS produced by the other 5 strains were less than the amount produced by strain C 91 (Figure 2). In this study, it was found that cell growth and EPS productivity were negatively correlated (r=-0.90, p=0.05). As indicated in Table 3, the EPS of all the isolates contained sugars and proteins. The maximum amounts of total sugar and protein were detected in EPS samples collected from strain I 50, 425.6 mg/l and 407.4 mg/l, respectively. Although strain C 91 showed the highest EPS productivity, it contained less sugars and proteins than I 50. Very low amounts of sugar and protein in the EPS of the isolates were found in our research. All strains contained glucose. EPS from strains I 2 and I 96 are composed of only two sugar monomers (glucose and galactose). The major sugar components of these biofilm bacteria were reported as glucose, galactose, fructose and rhamnose. The major sugar components of our strains were similar.

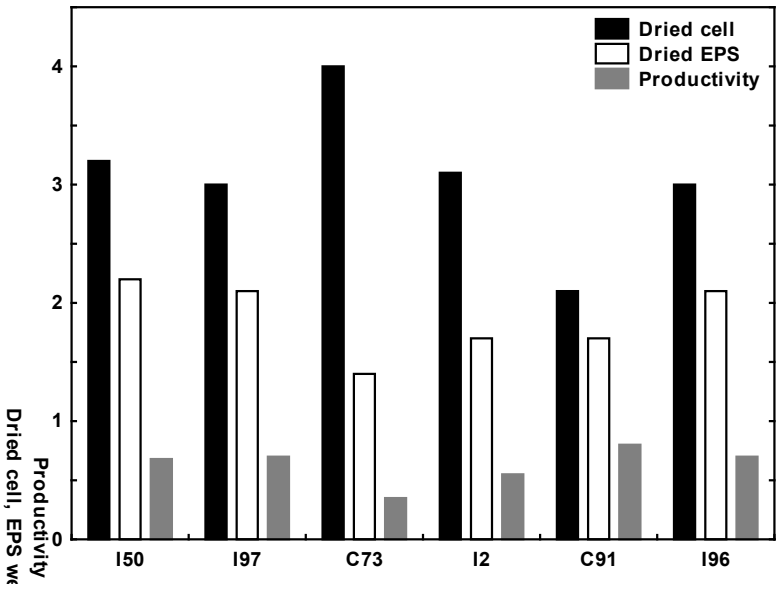

Figure 2. Comparison of dried cell weight, dried EPS weight, and productivity of EPS by biofilm-forming marine bacteria. ■ Dry cell weight (g l^{-1}); □ dry EPS weight (g l^{-1}); ■ productivity.

Table 3. Total sugar and total protein content of the isolated EPS.

Bacterial isolates	Total sugar (mg l^{-1} of EPS)	Total protein (mg l^{-1} of EPS)
I 50	425.6	407.4
I 97	410.4	153.8
C 73	183.2	22.4
I 2	349.7	51.2
C 91	180.6	93.9
I 96	134.9	28.4

Minimum inhibitory concentrations (MICs)

The microdilution method was used to determine the MICs and MBCs of the antifouling paints and rustproof paint used in this study. The paint numbered 279.2710, which contains triazine diamine and copper oxide, showed strong antibacterial activity against all individual strains. None of the strains were able to grow in any of the concentrations (1/2 to 1/1024). The lowest MICs and MBCs of 088.1154, which includes zinc oxide and copper oxide, and 088.1155, which contains fluorine resin, were the 1/16 dilutions against strains I 50 and I 97. Strains C 73, I 2, C 91 and I 96 did not grow in any of the concentrations. Rustproof paint showed weak antibacterial activity against all the bacteria tested. The lowest MICs and MBCs for the strains I 50, I 97, C 73, I 2, C 91 and I 96 were 1/4, 1/4, 1/8,

1/16, 1/16 and 1/16 dilutions, respectively. In this study, the paint (279.2710) with triazine diamine and copper oxide was found to be most effective against the bacteria. Strains I 2, C 91 and I 96 were found to be very sensitive to all the antifouling paints, except the rustproof paint (Table 4).

Table 4. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) (ml ml^{-1}), of antifouling paints against biofilm bacteria.

Bacterial isolates	Paint no. 088.1154	088.1155	279.2710	Rustproof
I 50	1/16	1/16	-	1/4
I 97	1/16	1/16	-	1/4
C 73	1/16	1/16	-	1/8
I 2	-	-	-	1/16
C 91	-	-	-	1/16
I 96	-	-	-	1/16

DISCUSSION

Biofilms represent biological systems with a high level of organization and where bacteria form structured coordinated functional communities (Saravanan *et al*. 2008). α-Proteobacteria, γ-Proteobacteria and CFB (*Cytophaga, Flavobacterium, Brevibacterium*) group bacteria are dominant bacterial groups in the marine environment (Kelly and Chistoserdov 2001) and have been reported in various marine biofilms. These groups of bacteria are dominant in dead coral surfaces (Frias-Lopez *et al*. 2002) and are associated with tubeworms living in deep-sea hydrothermal vents. Several species, such as *Cytophaga laterculai, Pseudoalteromonas agarivorans, P. piscicida* and *Vibrio pomeroyi*, have been reported to have been isolated from seawater and marine organisms (Romanenko *et al*. 2003; Thompson *et al*. 2003; Lee *et al*. 2003).

Bacteria with EPS-producing ability may have an advantage in successfully colonizing surfaces by becoming primary colonizers (Saravanan *et al*. 2008). In our study, cell growth and EPS productivity were negatively correlated. One notable phenomenon was that strains exhibiting superior cell growth showed lower EPS productivity (Kwon *et al*. 2002). According to other similar studies, in liquid culture modified with glucose, the *Pseudoalteromonas* genus produced EPS, which when chemically analyzed by colorimetric techniques, was shown to have similarly low amounts of protein and neutral sugars and uronic acids. Similar biochemical compositions were observed in previous studies of EPS from the *Alteromonas* genus isolated from hydrothermal vent communities (Raguenes *et al*. 2003; Nichols *et al*. 2004). Ko *et al*. (2000) stated that EPS production depends on microbial species, optimal C/N

ratio, cultivation temperature and time. Sugar composition could be changed by the culture medium composition (Lindberg *et al*. 2001).

Antifouling paints are commonly applied in order to prevent fouling problems on surfaces immersed in water (Tang and Cooney 1998). It is well known that any surface at static conditions, even when protected by biocides (e.g., copper, zinc and TBT-based compounds), becomes rapidly covered by a microbial biofilm (Yebra *et al*. 2006). Grasland *et al*. (2003) have found that early settlers on biocide-free surfaces are typically belonged to subdivisions α and γ-Proteobacteria, which impart specific physical properties to the surface, probably influencing subsequent attachment processes and the biofilm response to present compounds, such as the biocides. According to Tang and Cooney (1998), both copper and TBT inhibited development of a biofilm by *P. aeruginosa PAO-1* in its early stages, but the inhibition only occurred during early stages. Biofilm development on surfaces containing copper or TBT may involve growth of resistant cells. Bacterial exopolysaccharide product may protect cells from the toxicity of copper.

Consequently, individual biofilm bacteria, like our bacterial isolates, are very sensitive to antifouling paints in vitro. It was thought that bacteria had more resistance in environmental systems because they were isolated after the development of bacterial biofilm on test panels.

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Screening of Potential Anti-Bacterial Activity of Marine Sponge Extracts from Gökçeada Island, Aegean Sea, Turkey

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ABSTRACT

The antimicrobial activity of the methanolic extracts of the 11 unit marine sponge samples belonging to six different species: *Sarcotragus* sp., *Cacospongia scalaris* (Schmidt, 1862), *Axinella cannabina* (Esper, 1794), *Ircinia* sp., *Chondrosia reniformis* (Nardo, 1847), *Agelas oroides,* (Schmidt, 1864) were screened against Gram positive (*Staphylococcus aureus* SA1*, S. aureus* SA2), Gram negative (*Brucella melitensis* B37*, Vibrio vulnificus* GK23) and pathogenic standard strains (*Escherichia coli* O157:H7 ATCC 35150, *Salmonella enterica* subsp. *enterica* serovar *typhi* ATCC 167).

The diversity of the viable epibiotic bacterial communities of the marine sponge samples was also investigated. The marine sponge samples were collected via scuba diving at a depth of 8 to 15 meters around Gökceada Island, Aegean Sea, Turkey in December 2011. The samples were extracted using cold methanol extraction technique. The agar disc diffusion method was used on Marine Agar to determine the anti-bacterial activity of the sponge extracts. The antibacterial activity of the extracts was further characterized by determining the minimum inhibitory concentration (MIC) against pathogenic bacterial strains.

Among a total of 550 units bacterial strains, 11 aerobic heterotrophic bacterial species belonging to 7 different familia and three bacterial classes: Gamma Proteobacteria (54.55%), Alfa Proteobacteria (27.27%) and Flavobacteria (18.18%) were detected.

All of the 11 methanolic extractions of the marine sponge samples showed 100% antibacterial activity against *S. aureus* SA1- SA2*, B. melitensis* B37 and *V. vulnificus* GK23. While the antibacterial activities of the all marine sponge samples were recorded to be 72.73% against *S. enterica* subsp. *enterica* serovar *typhi*, it was recorded 36.36% against *E. coli* O157:H7. The highest antibacterial activity was found against all the tested pathogenic bacterial strains in the marine sponge samples of *A. cannabina, S. spinosulus, C. scalaris* and *Ircinia* sp. The samples of *C. reniformis* and *Agelas oroides* showed the second highest antibacterial activity against five pathogenic bacteria out of six. The samples 4 and 11 followed them showing antibacterial activity against four pathogenic bacteria.

Preliminary assays of the encouraging antibacterial activities, observed in this study, indicated that the samples exhibited antibacterial activity. This situation offers us an opportunity to obtain potential sources of antibacterial compounds for further studies of Turkish sponges surrounding Gökçeada Island.

Keywords: Marine sponge, antibacterial activity, pathogenic bacteria, methanolic extract, *Salmonella typhimurium, Brucella melitensis, Vibrio vulnificus, Staphylococcus aureus, Escherichia coli* O157:H7

INTRODUCTION

Marine sponges can host heterotrophic bacteria, archaea, cyanobacteria and unicellular algae (Hentschel *et al*. 2001). As a general rule, microorganisms, symbionts included, found in association with a sponge host are named ″associated microorganisms″. These microorganisms are of an important role in sponge nutrition, metabolic transportation (Wilkinson and Garrone, 1980) and a defense mechanism against biofouling formation and predators (Unson *et al*. 1994; Bewley *et al*. 1996).

Marine sponges are constantly exposed to water-borne bacteria, including pathogenic, opportunistic pathogenic forms and fouling microorganisms. However, marine sponges successfully suffer these threats due to their defense mechanisms mentioned above.

Among various sources for the development of new drugs, natural products are of particular significance (Proksch *et al*. 2003). Since marine sponges can produce a variety of unusual chemical compounds for their defense mechanisms, they are considered as possible resources to provide future drugs against diseases, such as cancer, malaria, and antibiotic-resistant infections. It has been well documented that 5000 different forms of compounds such as anti-bacterial, anti-cancer, anti-fungal, anti-inflammatory and anti malarial have been isolated from 500 species of sponges (Muller *et al*. 2004; Rafai *et al*. 2005; Sipkema *et al*. 2005; Azavedo *et al*. 2008). Among all the marine organisms screened, marine sponges produce the largest number of structurally diversified natural products (Zhang *et al*. 2003). These secondary metabolites are not crucial to the life of producing organisms and are produced by primary metabolites (Gudbjarnason 1999).

Recently, marine organisms have also been identified as a source of novel molecules of bioactive substances. This is because of the longer evolutionary history of marine organisms which may possess advanced molecular diversity compared to their terrestrial counterparts and plant biodiversity (Belarbi *et al*. 2003). Besides, sponges are assumed to be extremely rich sources of novel steroids, terpenoids, peptides, macrolides and alkaloids (Andersen *et al*. 1996).

Although culture–based techniques have been accepted to be insufficient for detecting bacterial diversity (Amann *et al*. 1995; Friedrich *et al*. 1999, 2001; Holben and Harris, 1995; Webster *et al*. 2001; Hentschel *et al.* 2002), microbial cultivation is essential to search for new bioactive compound producing strains. Furthermore, although culture

independent studies have served as common applications in detecting bacterial diversity, there are also a number of studies in which it has been shown that cultured strains of marine bacteria can represent significant fractions of the bacterial biomass in sea water (Rehnstam *et al.* 1993; Pinhassi and Hagström 1997).

Although it is widely known that marine sponges habitually have rich microbial consortia, there are no studies considering the composition of bacterial communities associated with marine sponges in Turkish waters. Only a few studies were reported on the bioactive compounds of Turkish marine sponges (Tasdemir *et al*. 2007; Wätjen *et al*. 2009).

In this preliminary study, for the first time, the sponge-associated viable heterotrophic aerobic bacteria compositions were investigated in the marine sponge samples which were collected from the surroundings of Gökçeada Island. The purpose of the investigation was to determine any possible anti-bacterial activity of the methanolic extracts of the marine sponge samples against the drug-resistant pathogenic bacteria strains.

MATERIALS AND METHODS

Sponge collections

The marine sponge samples were collected via scuba diving at a depth of 8 to 15 meters around Gökçeada Island, Aegean Sea, Turkey in December 2011. The sampling sites were identified via the global positioning system. The co-ordinates of the sampling region of Yelkenkaya, Gökçeada, Turkey were recorded to be *$40^0 14' 256'' N, 25^0 55' 271'' E$*. The collected materials were immediately frozen at -20 °C for one night prior to extraction.

Taxonomic designation of the sponge samples

Taxonomic designation of the sponge samples were carried out using histological sample preparation according to standard Rützler method (Boury-Esnault *et al*. 1992)

Sample extraction

The samples were extracted using the cold extraction technique. Ten grams of washed and dried samples were macerated with 100 ml of methanol (CH_3OH) for 24 hours. This was repeated three times to maximize the extraction. The methanol extract obtained was filtered and the solvent was evaporated to dryness instead of using evaporator (during the evaporation processes minor modifications were made) under a light airstream at room temperature. Then, the extract was re-dissolved in 10m1 of 4: l MeOH:H_20, thereby producing a solution containing the extract at the volumetric concentration of the original tissue (Izzati *et al*. 2011).

Bacterial Strains

The antimicrobial activity of the methanol extracts of the marine sponges was tested against:
Gram positive bacteria: *Staphylococcus aureus* SA1*, S. aureus*, SA2 (Clinical isolates)
Gram negative bacteria: *Brucella melitensis* B37*, Vibrio vulnificus* GK23, (marine isolates, Altuğ *et al*. 2010)
Standard human pathogen bacterial strains: *Escherichia coli* O157:H7 (ATCC 35150), *Salmonella enterica* subsp. *enterica* serovar *typhi*, (ATCC 167), **(**Centre de Ressources Biologiques de l'Institut Pasteur″, France).

The Agar Disc Diffusion Method
The agar disc diffusion method was used to determine the anti-bacterial activity of the sponge extracts. The overnight cultures of chosen strains in marine broth (Oxoid) at 37ºC were prepared. After incubation, the broth cultures were spread on 2216 Marine Agar (Oxoid) plates. 30 µl methanolic extract of sponge samples was applied onto the sterile paper disc (Whatman, 6mm diameter) and left to dry. This process was repeated for 3 times (a total of 90 µl of extracts for each disc). All the discs were put on inoculated agar plates and incubated at 37ºC for 24 hours. The antibacterial activities of the extracts were quantified by measuring the diameters (in mm) of the zones of inhibitions. The positive (standard antibiotic: Tetracycline of 5 µg/mL) and negative (methanol) controls were examined (Jorgensen and Turnidge, 2007).

Screening of Strains - MIC Values
The bacterial suspension cultures which were incubated overnight in marine broth medium were diluted to $3x10^8$ cfu/mL by using McFarland No: 1 standard solution. A volume of 50 µl of bacterial suspension and 50 µl of marine broth medium (except the control well) was then added into 96 well micro-titer plates. The extract of the sponge was added to 12 unit micro-wells in decreasing amounts ranging from 100 µL to 0.09 µL. After incubation at 37 ºC for 18 h, the extract concentration of the well without turbidity was accepted as the minimal inhibitory concentration (MIC) in extract of the sponge according to National Committee for Clinical Laboratory (2000).

Isolation of Sponge-Associated heterotrophic aerobic bacteria
10 g of the samples of marine sponge were incubated for 48 h at 25±0.1°C in pre-enrichment media Marine Broth. After the enrichment step, the samples were incubated for 24 or 48 h on Marine Agar. Following the incubation stage, different colonies were picked and restreaked several

times to obtain pure cultures. The pure isolates were identified using the automated biochemical identification system VITEK 2 Compact 30 (Biomereux, France). The pure isolates were Gram stained and then identified using GN (Gram-negative fermenting and non-fermenting bacilli), GP (Gram-positive cocci and non-spore-forming bacilli) and BCL (Gram-positive spore-forming bacilli) cards in the automated micro identification system VITEK 2 Compact 30 (Biomereux, France). The identification cards are based on established biochemical methods and newly developed substrates. There are biochemical tests (46 tests for BCL, 43 tests for GP, 47 tests for GN) measuring carbon source utilization, enzymatic activities, inhibition, and resistance. Calculations were performed on raw data and compared to thresholds to determine reactions for each test. On the VITEK 2 Compact, test reaction results appear as "+"," –", "(–)" or "(+)". Reactions that appear in parentheses were evaluated as an indicator of weak reactions that are too close to the test threshold (Pincus, 2005).

RESULTS AND DISCUSSION

The marine sponge species which were collected from Gökçeada Island used for antibacterial activity test were listed on Table 1.

Table 1. The marine sponge species screened for antibacterial activities.

Sample No	Marine Sponge
1	*Sarcotragus* sp.-1
2	*Cacospongia scalaris*-1 (Schmidt, 1862)
3	*C.scalaris* -1 (Schmidt, 1862)
4	*C. scalaris* -2 (Schmidt, 1862)
5	*Axinella cannabina* (Esper, 1794)
6	*Sarcotragus* sp -.2
7	*C. scalaris* -4(Schmidt, 1862)
8	*Ircinia* sp.-1
9	*Chondrosia reniformis* (Nardo, 1847)
10	*Ircinia* sp.-2
11	*Agelas oroides* (Schmidt, 1864)

11 unit marine sponge samples belonging to six different species were used for antibacterial activity tests. Epibiotic aerobic heterotrophic bacteria compositions of the marine sponge samples were summarized on Table 2.

Distribution of the isolated culturable heterotrophic bacteria species associated with the marine sponge samples were shown in Figure 1. The percentage of the isolated aerobic heterotrophic bacteria species associated with the marine sponge samples according to their classes were shown in Figure 2.

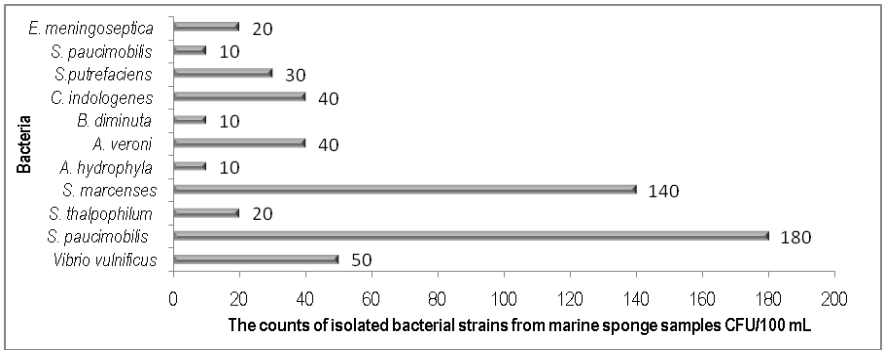

Figure 1. Distribution of the isolated culturable heterotrophic bacteria species associated with the marine sponge samples.

Frequency of isolation with respect to the count of the isolated bacteria, *S. paucimobilis* and *S. marcescens* was found to be higher than the other isolates.

Table 2. The distributions of the culturable heterotrophic aerobic bacteria species associated with the samples of marine sponge collected from Gökçeada Island, 2011.

Isolated Bacteria	**Marine Sponge Sample No**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Aeromonas hydrophila		+		+	+	+		+		+	+
Aeromonas veronii	+		+		+		+		+	+	+
Brevundimonas diminuta/vesicularis		+	+	+		+		+			
Chrysobacterium indologenes	+				+		+		+		+
Elizabetkingia meningoseptica			+	+		+		+		+	
Serratia marcescens	+	+	+		+	+	+		+	+	+
Shewanella paucimobillis	+		+	+			+				+
Shewanella putrefaciens		+		+	+	+			+		+
Sphingomonas paucimobilis	+	+	+	+			+	+	+	+	+
Sphingomonas thalpophilum	+		+		+	+					+
Vibrio vulnificus	+	+					+	+		+	

The frequencies of the similar culturable heterotrophic aerobic bacteria species associated with the different marine sponge samples were different.

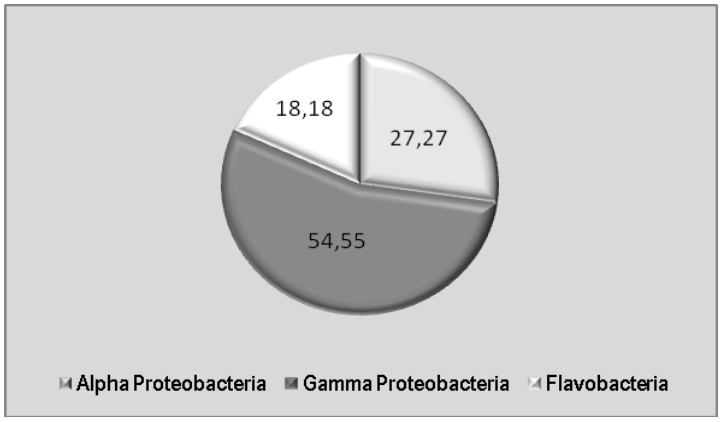

Figure 2. The classes of the isolated heterotrophic aerobic bacteria species associated with the marine sponge samples.

The minimal inhibitory concentration (MIC) values of methanolic extract of the sponge samples against the tested bacterial strains were shown in Figure 3.

The methanolic extract of *A. cannabina* (sample 5) showed the highest antibacterial activity to the *S. aureus* SA1 with a value of MIC 0.87 µg mL^{-1}. *C. scalaris* (Sample 2, 3, 4*)* and C. *reniformis* followed them with values of 3.18 MIC µg mL^{-1} to *S. aureus* SA1*,* and 3.5 MIC µg mL^{-1} against *S. aureus* SA2*,* respectively.

All of the 11 methanolic extractions of the marine sponge samples showed 100% antibacterial activity against *S. aureus* SA1- SA2*, B. melitensis* B37 and *V. vulnificus* GK23. While the antibacterial activities of the all marine sponge samples were recorded to be 72.73% against *S. enterica* subsp. *enterica* serovar *typhi*, it was recorded as 36.36% against *E. coli* O157:H7.

Proteobacteria which live as associated microorganisms in marine sponges have different effects on nitrogen fixation (Burnett and Mckenzie, 1997) and host reproduction (Stouthamer *et al*. 1999). Kalinovskaya *et al*. (2004) reported that Proteobacteria have significant potential to produce biologically active compounds with antimicrobial and surface-active properties. Proteobacteria were also found to produce enzymes at high levels; this is used for degrading protein and polysaccharides (Groudieva *et al*. 2004). The reported studies indicate a relationship between sponge bioactivity and occurrence of Proteobacteria in sponges (Li *et al*. 2006). In this study, 11 bacteria species belonging to 7 different familia and 3 classes were isolated from the marine sponge samples. Of the *bacteria*

isolated, 81.87% belonged to phylum *Proteobacteria* (Alpha and Gamma).

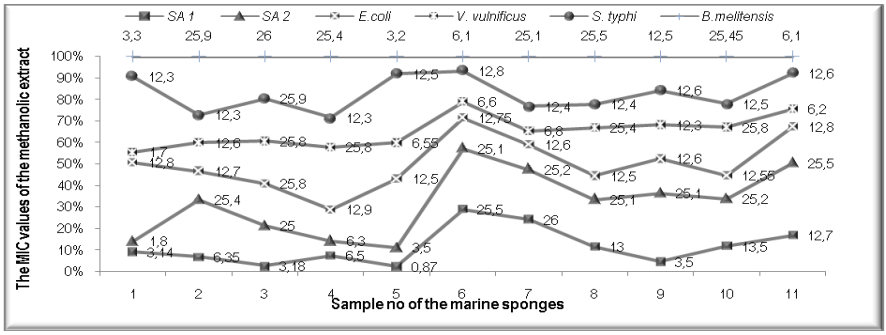

Figure 3. The minimal inhibitory concentration (MIC) values of methanolic extract of the sponge samples against the tested bacterial strains.

Table 3. Diameter (mm) of the zones of inhibition of the tested bacteria against methanolic extracts of the marine sponge samples.

Sample no of the marine sponges	**Diameter (mm) of the zones of inhibition**						**Mean**
	***S.aureus* SA1**	***S. aureus* SA2**	***V.vulnificus* GK23**	***B.melitensis* B37**	*S. enterica* subsp. *enterica* serovar *typhi*	*E. coli* O157:H7	
1	5.0	4.0	4.0	2.0	1.0	0	2.67
2	6.0	3.0	5.0	2.0	5.0	0	3.50
3	6.0	4.0	5.0	3.0	3.0	0	3.50
4	5.0	4.0	6.0	4.0	1.0	0	3.33
5	**11**	**10**	8.0	8.0	3.0	9.0	**8.17**
6	5.0	3.0	4.0	3.0	4.0	**10**	4.83
7	5.0	5.0	4.0	3.0	1.0	8.0	4.83
8	**10**	**11**	2.0	7.0	1.0	**11**	**7.0**
9	2.0	4.0	2.0	3.0	1.0	0	2.0
10	3.0	4.0	4.0	2.0	1.0	0	2.33
11	6.0	4.0	8.0	5.0	0	0	3.83
* Control	0	0	0	0	0	0	0
** Control	1.0	0	2.0	0	0	0	0.50

*Methanol was used for negative controls.

** 5 µg/mL of Tetracycline, as a standard antibiotic, was used for positive controls.

All of the 11 methanolic extractions of the marine sponge samples exhibited significant antibacterial activity against the tested pathogenic bacteria.

A total of 550 aerobic heterotrophic bacterial strains, belonging to 3 classes, 7 familia and 11 species were isolated from the marine sponge samples. Of the isolated bacterial strains, 98% were mucoid and pigmented (red, yellow, orange, and brownish). The mucoid colonies and the sponge species from which they were isolated most often exhibited the same or similar pigmentation. Wilkinson and Garrone (1980) reported bacteria isolated from marine sponges from the Mediterranean and the Great Barrier Reef also exhibited this characteristic. The data found in Wilkinson's research supports in several cases that symbioses between host sponges and their bacteria are mutualistic; it is beneficial not only for the bacteria (using the sponge as a habitat), but also for the sponge. Bacteria associated with marine sponges represent a rich source of bioactive metabolites. It has been hypothesized that numerous natural products from marine invertebrates may be of microbial origin (Faulkner, 2002; Santos *et al*. 2010), suggesting the possibility of using sponge associated bacteria instead of the sponge itself for the production of biologically active substances. In this study, though similar bacteria were found in different sponge samples, the sponge-associated dominant bacteria species were different. The findings suggest that the isolated strains may contribute to the search for new sources of antibacterial substances, a significant approach for developing alternative treatment regimes to infections caused by drug-resistant bacteria.

Thakur and Anil (2000) reported that the chemical nature and production of antibacterial compounds produced by sponges or their associated bacteria are managed by the environment. Furthermore, an inverse relationship was reported between the abundance of bacteria associated with marine sponges in marine environments and the antibacterial activity exhibited by the sponge extracts (Walls *et al*. 1993; Thakur and Anil 2000).

It has been documented that during the summer and spring seasons, in response to the higher number of bacteria in the surrounding water, sponges increase the production of antibacterial metabolites (Bakus *et al*. 1985). However, Thakur and Anil (2000) reported that in January, despite low bacterial density in the water column, the observed antibacterial activity of crude extract was greater. However in May, despite higher bacterial density in the surrounding water, antibacterial activity of the crude extract was minimal. In this study, the samples of marine sponges were collected in the winter season, and as shown in Table 3, methanolic extracts of the all of eleven sponge extracts screened for antibacterial activities exhibited significant activity against tested pathogenic bacteria.

The resistance of pathogenic bacteria to existing antibiotics is observed increasingly on a global scale and therefore has become a widespread public health problem. Attempts to overcome the issue are

underway. Research efforts are now addressing the matter of discovering novel and efficient antibacterial compounds (Devasahayam *et al*. 2010). It is widely known that a large number of linear polyprenylhydroquinones (antibacterial activity) have been isolated from marine sponges (De Rosa *et al*. 1994, Mihopoulos *et al*. 1999). However there are few studies reported that are related to bioactive compounds and there are no reports related to the diversity of sponge associated bacteria in the Turkish marine environments. Tasdemir *et al*. (2007) reported that the Turkish sponge *Agelas oroides* from Gökçeada Island has natural products that inhibit the enoyl reductases from *Plasmodium falciparum, Mycobacterium tuberculosis* and *E. coli*. Wätjen *et al.* (2009) reported that three cytotoxic and antioxidative derivates (hexaprenyl-1,4-hydroquinone, heptaprenyl-1,4-hydroquinone and nonaprenyl-1,4-hydroquinone) were isolated from the Turkish sponge (*S. muscarum* and *I. fasciculata*) from the Eastern Mediterranean Sea. In this study, while all the sponge species collected from Gökçeada Island exhibited strong antibacterial activity, *A. cannabina* (Esper 1794) was the most capable species. *Ircinia* spp., *C. scalaris* (Schmidt, 1862) and *C. reniformis* (Nordo, 1847) followed it.

This is the first preliminary study that reports Turkish sponge-associated bacterial strains with a high potential for producing antimicrobial substances against pathogenic bacteria. In conclusion, assays of encouraging antibacterial activities, observed in this study, indicated that the samples which exhibited antibacterial activity offer us an opportunity to obtain potential sources of antibacterial compounds for further studies in the Turkish sponges of Gökçeada Island. Our further studies may also contribute to clarify: the effectiveness of antibacterial activity related to environmental stress; inter-colonial or individual specimens of sponges; and bacterial abundance associated with marine sponges. The marine sponges from Gökçeada Island will be subjected to detailed research for the isolation of biologically active molecules along with the search for new antibacterial compounds. The present study provides baseline data for suggesting the possibility of finding potential antibacterial compounds.

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Biogenic Amine Production Ability of Lactic Acid Bacteria in European Barracuda, Common Pandora, Striped Mullet Infusion Broth

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ABSTRACT

Ammonia and biogenic amine production by eight lactic acid bacteria (LAB) strains (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactobacillus acidophilus*, *Pediococcus acidophilus* and *Lactobacillus delbrueckii* subsp. *lactis*) in three different seawater fish (European barracuda-*Sphyraena sphyraena*, common Pandora-*Pagellus erythrinus*, striped mullet-*Mullus barbatus*) infusion broth were investigated using HPLC methods. Significant differences in ammonia and biogenic amine production were found among the lactic acid bacteria strains and fish infusion broth ($P<0.05$). The all used bacteria produced more than 380 mg/L of ammonia. LAB strains produced eleven amine tested in fish infusion broth, although *Lb. acidophilus* did not accumulate tryptamine in European barracuda infusion broth. The most accumulated amines by lactic acid bacteria were dopamine, tyramine and serotonin. Histamine production by *Lc. cremoris* and *Lb. plantarum* in European barracuda infusion broth and *Lb. delb.* subs. *lactis* in striped mullet infusion broth were more than 60 mg/L, whilst lactic acid bacteria had lowest ability to produce histamine in common Pandora infusion broth.

INTRODUCTION

Biogenic amines are formed through the decarboxylases released from microbial populations associated with seafood (Rawles *et al*. 1996; Ndaw *et al*. 2007). Histamine and tyramine have been the most studied biogenic amines due to the toxicological effects derived from their vasoactive and psychoactive properties. Other biogenic amines such as the diamines, putrescine and cadaverine, may boost the toxicity of the above amines (Bovercid and Holzapfel 1999). Putrescine and agmatine also considered to be potential precursors for carcinogenic nitrosamines in the presence of nitrites (Hotchkiss *et al*. 1977; Arena and Manca de Nadra 2001).

The genes encoding the various decarboxylation pathways responsible for biogenic amines formation have been extensively studied in different bacterial groups and especially in lactic acid (Lucas *et al*.

2007; de las Rivas *et al*. 2008; Coton and Coton, 2009; Coton *et al*. 2010). Lactic acid bacteria frequently produce biogenic amines mostly histamine and tyramine in processed fish, cheese, fermented vegetables and beverages (Stratton *et al*. 1991; Leisner *et al*. 1994; Thapa *et al*. 2007). During fermentation and maturation of foods, biogenic amines are formed. The amount of histamine formed is strongly dependent on quality management during the production process (Gafner 2002). Maximum permissible limits of biogenic amines for fish and fish products are limited to 200 mg/kg of the food in Germany, whilst it is only 100 mg/kg in Canada, Finland and Switzerland (Paleologos *et al*. 2003; Brink *et al*. 1990). The United States Food and Drug Administration has indicated that defect levels of 50 ppm histamine in sea foods is acceptable (FDA 2011; Priyadarshani and Rakshit 2011)

Between LAB, strains belonging to the genera *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Pediococcus*, *Lactococcus* and *Leuconostoc* may be able to decarboxylate amino acids in several fermented foods (Maijala 1993; Bover-Cid and Holzapfel 1999; Moreno-Arribas *et al*. 2003 Russo *et al*. 2010). Some strains of LAB synthesize histamine, ability strictly related to the possession of the histidine decarboxylase gene (Landete *et al.* 2005; Lucas *et al.* 2005). Calles-Enríquez *et al*. (2010) found that two *Streptococcus thermophilus* strains CHCC1524 and CHCC6483 from a total of 69 *Streptococcus thermophilus* strains screened showed the capacity to produce histamine. *Oenococcus oeni*, *Lactobacillus hilgardii*, *Lactobacillus mali*, *Leuconostoc mesenteroides* and *Pediococcus parvulus* can contribute to the histamine synthesis in wine (Landete *et al*. 2007). The production of biogenic amines by LAB to be selected as starter cultures is not a desirable property (Buchenhuskes 1993). Before the use of LAB in the fish product, the quantitative analysis of biogenic amine is very essential to confirm production of biogenic amine. Thus, the aim of the study was to investigate biogenic amine production ability of LAB strains in some Mediterranean fish infusion broth.

MATERIALS AND METHODS

Lactic Acid Bacteria and Fish species

The used LAB species were *Lactococcus lactis* subsp. *cremoris* (MG 1363), *Lactococcus lactis* subsp. *lactis* (IL 1403), *Lactobacillus plantarum* (FI8595) and *Streptococcus thermophilus* (NCFB2392). They were obtained from Sutcu Imam University, Kahramanmaraş, Turkey in BGML stock culture. *Leuconostoc mesenteroides subsp. cremoris* (DSMZ 20346), *Lactobacillus acidophilus* (ATCC 11975), *Pediococcus acidophilus* (ATCC 25741) and *Lactobacillus delbrueckii subsp. lactis*

(ATCC 10697) were purchased from Institute of Refik Saydam Hifzisihha (Ankara, Turkey).

In the present study, fish decarboxylase infusion broth was prepared using three different fish species which were European barracuda (*Sphyraena sphyraena*), common pandora (*Pagellus erythrinus)* and striped mullet (*Mullus barbatus*).

Chemical reagents

All ammonia and biogenic amine standards were purchased from Sigma–Aldrich (Munich, Germany). The mobile phase consisted of acetonitrile and HPLC grade water for amine analyses.

Biogenic amine analysis Culture media and extraction

Fish infusion broth was prepared according to method of Okuzumi *et al*. (1982) with minor modifications. Two hundred fifty grams of fish flesh was homogenised with 2 volumes of water (w/v), steamed at 100^{o}C for 1 hour and filtered. The filtrate was enriched with 1% glucose and 0.5% NaCl. In order to decarboxylate amino acid by bacteria, 3 mg pyridoxal HCl addition was made in each infusion broth before autoclaving process.

MRS and M17 broth were used for propagation of LAB cultures. Lactic acid bacterial strains were incubated at 37^{o}C for 24 hour which after 0.5 mL of these bacterial cultures was removed and put into the fish IDB to decarboxylate amino acid.

To remove the bacterial strains present in the growth broth, 5 mL of the fish IDB containing LAB strains were removed to separate bottles and then 2 mL trichloroacetic acid was added. They were centrifuged at 3000xg for 10 min and then filtered through a Whatman filter paper (Whatman GmbH, Dassel, Germany). After that, 4 mL of bacterial supernatant was taken for derivatisation from each of LAB bacterial strains.

Preparation of standard amine solution

Histamine dihydrochloride (165.7 mg), tyramine hydrochloride (126.7 mg), tryptamine hydrochloride (122.8 mg), putrescine dihydrochloride (182.9 mg), 2-phenylethylamine (PHEN) hydrochloride (130.1 mg), cadaverine dihydrochloride (171.4 mg), spermidine trihydrochloride (175.3 mg), spermine tetrahydrochloride (172.0 mg), 5-hydroxytryptamine (Serotonin) (133.9 mg), 3-hydroxytyramine hydrochloride (Dopamine) (123.8 mg), agmatine sulphate (175.4 mg), trimethylamine (TMA) hydrochloride (161.7 mg) and ammonium chloride (296.9 mg) were dissolved in 10 mL HPLC grade water. The final concentration of free base for each amine was 10 mg mL^{-1} solution.

Derivatisation of bacterial extraction

A stock solution was prepared by dissolving 2% benzoyl chloride in acetonitrile to enhance the reaction with amines. For derivatisation of standard amine solutions, 100 µL was taken (4 mL for extracted bacterial cultures) from each free base standard solution (10 mg mL^{-1}). 1 mL of sodium hydroxide (2 M) was added, followed by 1 mL of 2% benzoyl chloride (dissolved in acetonitrile) and the solution mixed on a vortex mixer for 1 min. The reaction mixture was left at room temperature for 5 min and then centrifuged for 10 min. After that, the benzoylation was stopped by adding 2 mL of saturated sodium chloride solution and the solution extracted twice with 2 mL of diethyl ether. The upper organic layer was transferred into a clean tube after mixing. Afterwards, the organic layer was evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1 mL of acetonitrile and 10 µL aliquots were injected into the HPLC.

Analytical method

Biogenic amine analysis was done using the method of Özogul (2004) and measured in mg amines per litre broth. The confirmation of BAs production was accomplished using a rapid HPLC method with a reversed phase column by using a gradient elution program. Same analytic method was used for ammonia and TMA separation.

HPLC apparatus and column

A Shimadzu Prominence HPLC apparatus (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A diode array detector and two binary gradient pumps (Shimadzu LC-10AT), auto sampler (SIL 20AC), column oven (CTO-20AC), and a communication bus module (CBM-20A) with valve unit FCV-11AL was used. The column was a reverse-phase, ODS Hypersil 5µ, 250x4.6 mm (Phenomenex, Macclesfield, Cheshire, UK) for the BA analyses.

Statistical analysis

The mean value and standard deviation were calculated from the data obtained from the three samples for each treatment. One way ANOVA was used to determine the significance of differences at $P<0.05$. All statistics were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL. USA).

RESULTS AND DISCUSSION

The all LAB strains examined had an ability to produce ammonia and biogenic amine in fish infusion broth (Tables 1-3). Significant differences in ammonia and biogenic amine production were found among lactic acid bacteria strains and fish infusion broth ($P<0.05$). Ammonia production by LAB strains was in range of 167 mg/L by *Lc. lactis subs. cremoris* in European barracuda infusion broth and 918 mg/L by *Lc. lactis subs. cremoris* in Striped mullet infusion broth. In histidine decarboxylase broth, ammonia production by *Lc. lactis* subsp. *cremoris*, *Strep. thermophilus, Lb. plantarum, Lc. lactis* subsp. *lactis* was above 520 mg/L (Özogul 2011).The ammonia accumulation by LAB strains in tyrosine decarboxylase broth were relatively high, ranging from 453 to 677 mg/L (Kuley and Özogul 2011). Thapa *et al*. (2006) reported that none of the strains of LAB including *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Lb. plantarum*, *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* isolated from traditionally processed fish products produced biogenic amines. Bunkova *et al*. (2009) found that *Lc. lactis* subsp. *cremoris*, *Strep. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* produce tyramine although the other tested biogenic amines such as histamine, putrescine, cadaverine, agmatine, spermidine and spermine were not detected. LAB strains such as *Lb. plantarum, S. thermophilus, Lc. lactis. subs. lactis* except for *Lc. lactis* subsp. *cremoris* did not produce histamine but all of them produced putrescine, spermidine, phenylethylamine, dopamine, agmatine and ammonia in histidine decarboxylase broth (Özogul 2011). In the current study, LAB strains produced eleven amine tested in fish infusion broth, although *Lb. acidophilus* did not accumulate tryptamine in European barracuda infusion broth. Straub *et al*. (1995) reported that *Lactococcus* spp., *Pediococcus* spp., *Streptococcus* spp. and *Leuconostoc* spp. did not have amino acid decarboxylase activity. Serotonin and dopamine were the most accumulated amine by LAB strains in fish infusion broth followed by tyramine and spermidine. Putrescine production by *Leu. mes. subs. cremoris* was the highest (771 mg/ L) in striped mullet infusion broth, whereas the lowest putrescine production by bacteria was observed in common pandora infusion broth (<35 mg/L).

Table 1. Ammonia and biogenic amine production by lactic acid bacteria in European barracuda (*Sphyraena sphyraena*) infusion broth (mg/L).

	Lb.delb.*subs. *Lactis	***Leu.mes.* subs. *crem***	***Lb.acidophilus***	***Ped.acidophilus***	***Lc. lactis* subs. *cremoris***	***Lc. lactis* subs. *lactis***	***Lb.plantarum***	***S.thermophilus***
Ammonia	394.81±25.40^{a}	356.87±29.76^{a}	334.62±15.44^{a}	331.27±7.67^{a}	167.99±9.26^{b}	379.63±20.26^{a}	419.30±157.17^{a}	402.95±25.22a
Putrescine	44.61±4.03^{b}	26.62±2.14^{d}	5.31±0.13^{f}	23.17±1.93^{d}	3.56±0.08^{f}	35.60±3.18^{c}	94.34±3.15^{a}	11.39±1.05e
Cadaverine	13.52±1.25^{d}	21.67±1.92^{c}	32.13±1.39^{b}	16.15±1.42^{d}	6.13±0.33^{e}	19.70±1.62^{c}	38.69±1.78^{a}	5.16±0.37e
Spermidine	44.05±2.68^{d}	193.54±13.33^{a}	20.26±1.74^{e}	97.56±8.41^{b}	3.22±0.16^{f}	69.98±5.58^{c}	112.60±7.25^{b}	14.69±1.06ef
Tryptamine	3.44±0.21^{d}	2.65±0.07^{d}	0.00±0.00^{e}	8.59±0.28^{b}	0.17±0.01^{e}	6.19±0.19^{c}	20.51±1.13^{a}	0.27±0.01e
Phenylethylamine	10.61±0.38^{d}	26.90±1.42^{d}	5.68±0.27^{e}	37.21±2.34^{c}	43.72±3.60^{b}	10.28±0.82^{de}	57.23±3.11^{a}	7.18±0.54de
Spermine	10.25±0.18^{e}	44.91±2.52^{c}	14.56±1.13d^{e}	60.82±3.77^{b}	18.03±1.46^{de}	24.34±1.89^{d}	140.24±12.32^{a}	21.69±1.85^{de}
Histamine	22.31±1.53^{e}	41.95±2.25^{d}	21.96±1.15^{e}	46.46±3.28^{d}	89.01±5.28^{a}	57.17±4.36^{c}	68.88±4.36^{b}	24.79±0.88^{e}
Serotonin	74.90±5.97^{d}	510.24±32.48^{a}	69.35±4.29^{d}	169.85±7.81^{c}	479.64±19.57^{a}	143.29±2.79^{c}	141.29±12.34^{c}	417.00±33.31^{b}
Tyramine	14.88±1.22^{e}	130.49±10.36^{cd}	61.49±2.25^{de}	112.31±8.39^{cd}	206.77±16.69^{c}	905.10±65.48^{b}	1095.79±86.97^{a}	153.90±8.40^{cd}
TMA	0.62±0.02^{e}	2.27±0.26^{d}	2.64±0.16^{d}	13.12±0.72^{a}	5.97±0.45^{b}	0.93±0.06^{e}	2.32±0.18^{d}	3.51±0.17^{c}
Dopamine	98.34±7.28^{de}	215.54±13.35^{a}	119.27±8.21^{cd}	81.08±4.76^{e}	165.94±10.64^{b}	134.41±9.86^{c}	110.0±49.14^{d}	109.83±4.66^{d}
Agmatine	23.89±1.46^{e}	59.65±2.85^{b}	52.88±1.14^{c}	64.93±3.73^{a}	25.12±1.99^{e}	27.90±2.22^{de}	23.65±1.50^{e}	32.51±1.18^{d}

**Lactococcus lactis* subsp. *cremoris* MG 1363. *Lactococcus lactis* subsp. *lactis* IL 1403. *Lactobacillus plantarum* FI8595. *Leuconostoc mesenteroides subsp. cremoris DSMZ 20346. Lactobacillus acidophilus ATCC 11975. Pediococcus acidophilus ATCC 25741. Lactobacillus delbrueckii subsp. lactis ATCC 10697* and *Streptococcus thermophilus,* AMN, ammonia; PUT, putrescine; CAD, cadaverine; HIM, histamine; SPD, spermidine; TRPT, tryptamine; PHEN, 2-Phenyl-ethylamine; SPN, spermine; SER, serotonin; TYM, tyramine; TMA, trimethylamine; DOP; Dopamine, AGM, agmatine. ^{x} Mean value. ^{y} Standard deviation (n = 4). Different lowercase letters $^{(a–g)}$ in a row indicate significant differences ($P < 0.05$) among bacteria.

Table 2. Ammonia and biogenic amine production by lactic acid bacteria in common pandora (*Pagellus erythrinus*) infusion broth (mg/L).

	Lb.delb.*subs. *lactis*	***Leu.mes.* subs. *crem***	***Lb.acidophilus***	***Ped.acidophilus***	***Lc. lactis* subs. *cremoris***	***Lc. lactis* subs. *lactis***	***Lb.plantarum***	***S.thermophilus***
Ammonia	414.67±14.53^{c}	444.09±31.07^{c}	471.85±39.25^{bc}	614.73±33.42^{a}	486.13±39.61^{bc}	541.75±47.38^{ab}	486.77±33.77^{bc}	459.73±39.50^{bc}
Putrescine	7.05±0.63^{c}	4.74±0.11^{cd}	32.80±2.67^{a}	3.80±0.28^{cd}	2.98±0.24^{d}	18.16±1.16^{b}	19.40±1.65^{b}	20.63±1.92^{b}
Cadaverine	8.82±0.71^{e}	1.33±0.01^{f}	19.15±1.09^{b}	36.79±1.83^{a}	1.66±0.04^{f}	15.58±0.99^{c}	13.18±0.53^{d}	12.01±0.27^{d}
Spermidine	70.08±3.19^{d}	39.72±1.24^{e}	166.59±14.12^{a}	131.09±7.24^{b}	51.06±4.99^{e}	45.60±3.28^{e}	99.51±4.60^{c}	105.43±3.27^{c}
Tryptamine	4.72±0.38^{e}	2.23±0.45^{e}	14.52±1.41^{c}	9.73±0.92^{d}	9.55±0.73^{d}	37.17±2.42^{b}	42.85±3.31^{a}	15.87±0.92^{c}
Phenylethylamine	20.55±2.03^{f}	6.61±0.52^{g}	30.48±2.73^{de}	27.57±1.63^{e}	86.04±3.77^{a}	50.25±2.16^{b}	41.83±1.90^{c}	35.54±3.25^{d}
Spermine	46.13±3.83^{c}	26.30±2.19^{de}	23.36±1.21^{e}	33.63±3.02^{d}	58.26±4.32^{b}	72.60±3.86^{a}	67.59±2.64^{a}	49.65±4.73^{c}
Histamine	67.70±2.25^{a}	45.68±2.17^{d}	58.86±2.21^{b}	52.30±2.87^{c}	4.51±0.41^{g}	21.14±0.84^{e}	13.42±0.81^{f}	55.43±4.10^{bc}
Serotonin	258.91±12.06^{de}	208.78±16.90^{e}	263.74±7.98^{de}	631.12±39.93^{b}	327.8±412.62^{d}	526.96±40.42^{c}	830.21±47.39^{a}	696.09±34.04^{b}
Tyramine	152.73±9.55^{cd}	248.32±18.90^{a}	146.25±10.48^{cd}	193.08±9.28^{b}	169.74±.91^{bc}	136.27±5.73^{de}	139.73±10.66^{de}	115.53±8.37^{e}
TMA	1.74±0.10^{d}	7.77±0.37^{d}	3.12±0.24^{d}	0.33±0.02^{d}	255.70±19.12^{a}	16.77±1.18^{cd}	30.31±1.31^{bc}	42.08±2.29^{b}
Dopamine	135.17±8.22^{d}	155.46±5.35^{d}	223.02±9.80^{b}	263.53±10.78^{a}	92.10±4.84^{e}	268.04±12.81^{a}	205.28±13.39^{bc}	193.99±4.27^{c}
Agmatine	44.69±2.64^{ef}	54.63±2.44^{cd}	38.19±1.44^{f}	30.00±2.40^{g}	90.59±4.16^{a}	60.14±1.94^{c}	75.48±4.71^{b}	47.74±4.31^{de}

**Lactococcus lactis* subsp. *cremoris* MG 1363. *Lactococcus lactis* subsp. *lactis* IL 1403. *Lactobacillus plantarum* FI8595. *Leuconostoc mesenteroides subsp. cremoris DSMZ 20346. Lactobacillus acidophilus ATCC 11975. Pediococcus acidophilus ATCC 25741. Lactobacillus delbrueckii subsp. lactis ATCC 10697* and *Streptococcus thermophilus,* AMN, ammonia; PUT, putrescine; CAD, cadaverine; HIM, histamine; SPD, spermidine; TRPT, tryptamine; PHEN, 2-Phenyl-ethylamine; SPN, spermine; SER, serotonin; TYM, tyramine; TMA, trimethylamine; DOP; Dopamine, AGM, agmatine. ^{x} Mean value. ^{y} Standard deviation (n = 4). Different lowercase letters $^{(a–g)}$ in a row indicate significant differences ($P < 0.05$) among bacteria.

Table 3. Ammonia and biogenic amine production by lactic acid bacteria in striped mullet (*Mullus barbatus*) infusion broth (mg/L).

	***Lb.delb.*subs. *lactis* ***	***Leu.mes.* subs. *crem***	***Lb.acidophilus***	***Ped.acidophilus***	***Lc. lactis* subs. *cremoris***	***Lc. lactis* subs. *lactis***	***Lb.plantarum***	***S.thermophilus***
Ammonia	547.56±17.58^{d}	397.10±15.95^{e}	753.86±13.42^{c}	721.64±14.56^{c}	918.11±11.77^{a}	741.33±19.70^{c}	807.32±32.44^{b}	760.37±19.89^{c}
Putrescine	18.91±0.54^{bc}	771.50±13.96^{a}	10.47±0.77^{c}	25.79±1.55^{b}	14.19±0.72^{bc}	20.96±1.24^{bc}	10.06±0.80^{c}	12.94±0.22^{c}
Cadaverine	17.62±0.84^{d}	10.88±0.54^{e}	20.91±2.07^{d}	75.97±2.11^{a}	32.33±2.27^{b}	27.18±2.21^{c}	19.64±1.66^{d}	18.87±0.41^{d}
Spermidine	37.81±1.35^{e}	42.23±3.15^{e}	87.67±2.92^{c}	119.21±11.35^{b}	44.34±3.33^{e}	133.44±5.03^{a}	62.58±3.89^{d}	83.51±5.95^{c}
Tryptamine	7.44±0.67^{a}	2.59±0.16^{d}	3.53±0.04^{c}	6.59±0.2	0.21±0.01^{f}	7.19±0.34^{ab}	1.63±0.06^{e}	3.41±0.29^{c}
Phenylethylamine	16.15±0.98^{c}	4.10±0.15^{e}	7.58±0.36^{d}	20.21±1.44^{b}	197.37±2.28^{a}	18.64±0.42^{b}	9.64±0.67^{d}	8.16±0.32^{d}
Spermine	28.36±1.26^{f}	43.42±1.24^{cd}	48.77±2.63^{c}	57.68±3.34^{b}	82.57±4.73^{a}	82.77±1.90^{a}	36.96±0.98^{e}	42.24±1.97^{de}
Histamine	4.12±0.24^{e}	6.96±0.59^{d}	1.77±0.09^{f}	17.20±1.49^{b}	21.13±0.98^{a}	14.31±1.15^{c}	1.86±0.11^{f}	8.12±0.33^{d}
Serotonin	78.10±5.18^{c}	65.74±2.20^{d}	52.93±2.42^{e}	120.67±9.25^{b}	135.79±5.23^{a}	78.80±6.42^{c}	49.32±1.71^{e}	35.07±1.87^{f}
Tyramine	25.24±1.97^{de}	23.43±1.58^{e}	16.18±0.86^{e}	36.64±2.97^{d}	217.20±13.06^{a}	54.18±4.33^{c}	66.60±2.37^{b}	18.66±0.99^{e}
TMA	4.98±0.48^{b}	5.08±0.45^{b}	1.34±0.11^{de}	5.92±0.43^{a}	2.14±0.10^{c}	1.85±0.15^{cd}	2.41±0.11^{c}	0.99±0.05^{e}
Dopamine	113.30±10.15^{d}	222.99±13.81^{b}	111.66±8.56^{d}	157.59±3.06^{c}	750.20±22.29^{a}	56.17±4.55^{f}	68.57±1.58^{ef}	89.38±3.82^{de}
Agmatine	37.80±2.52^{b}	24.28±0.95^{d}	24.23±1.21^{d}	56.60±3.21^{a}	52.43±4.59^{a}	30.37±2.39^{c}	20.94±1.93^{d}	22.88±0.92^{d}

*****_Lactococcus lactis_ subsp. _cremoris_ MG 1363. _Lactococcus lactis_ subsp. _lactis_ IL 1403. _Lactobacillus plantarum_ FI8595. _Leuconostoc mesenteroides subsp. cremoris DSMZ 20346. Lactobacillus acidophilus ATCC 11975. Pediococcus acidophilus ATCC 25741. Lactobacillus delbrueckii subsp. lactis ATCC 10697_ and _Streptococcus thermophilus,_ AMN, ammonia; PUT, putrescine; CAD, cadaverine; HIM, histamine; SPD, spermidine; TRPT, tryptamine; PHEN, 2-Phenyl-ethylamine; SPN, spermine; SER, serotonin; TYM, tyramine; TMA, trimethylamine; DOP; Dopamine, AGM, agmatine. ^{x} Mean value. ^{y} Standard deviation (n = 4). Different lowercase letters $^{(a–g)}$ in a row indicate significant differences ($P < 0.05$) among bacteria.

There was not significant differences in cadaverine production among *Lb. delb.* subs. *lactis*, *Lb. acidophilus, Lb. plantarum* and *S. thermophilus* in striped mullet infusion broth. *Ped. acidophilus* had the highest activity to produce cadaverine in striped mullet and common pandora infusion broth (75 vs 36 mg/L). Cadaverine was one of the most abundant amines in fish sauce with maximum reported value of 1429 ppm (Zaman *et al*. 2010). Peteja *et al*. (2000) indicated that cadaverine production during storage of fermented rainbow trout were higher in the group without any LAB inoculation than other groups with three different LAB inoculations with staphylococci.

Spermidine was one of the most accumulated amines. *Ped. acidophilus* had remarkable ability to produce spermidine in all of the fish infusion broth with value above 95 mg/L spermidine. The highest spermidine production was observed for *Leu. mes.* subs. *cremoris* (193 mg/L) in European barracuda infusion broth. Spermidine and spermine contents in fish and fish sauce were generally low (Kalac and Krausova 2005; Tsai *et al*. 2006). Spermine production by bacteria was between 10 mg/L by *Lb. delb*. subs. *lactis* and 140 23 mg/L by *Lb. plantarum* in European barracuda infusion broth. *Lc. lactis subs. cremoris* and *Lc. lactis subs. lactis* produced statistically similar amount of spermine in striped mullet and European barracuda infusion broth.

LAB strains produced histamine more than 1.5 mg/L. *Lc. cremoris* accumulated the highest levels of histamine in European barracuda infusion broth (89 mg/L), although histamine produced the lowest level by *Lc. lactis subs. cremoris* in common pandora infusion broth (4.5 mg/L). *Lb. delb. subs. lactis* and *Lb. acidophilus* were the main bacteria produce high level of histamine with value of 68 and 59 mg/L. There was not significant differences in histamine production between *Lb. acidophilus* and *Lb. plantarum* ($p>0.05$), which accumulated about 1.8 mg/L histamine, respectively. *Strep. thermophilus* and *Lb. plantarum* produced low concentrations of HIS (~1.6 mg/L) (Kuley and Özogul 2011).

Histamine and tyramine production for LAB strains such as *Leuconostoc gelidum, Lc. piscium, Lb. fuchuensis* and *Carnobacterium alterfunditum* were below the detection threshold of 5 mg L-1 (Matamoros *et al*. 2009). Lactic acid bacteria strains isolated from vacuum-packaged and cold-smoked salmon were not able to produce histamine, but *Enterococcus faecium* ET05, *Lactobacillus curvatus* ET06 and *Lactobacillus curvatus* ET30 produced tyramine (Tome *et al*. 2008). In the present study, the LAB strains also had a high ability to produce tyramine in fish infusion broth. *Lb. plantarum* (1096 mg/L), *Lc. lactis*

subs. lactis (905 mg/L), *Lc. lactis subs. cremoris* (207 mg/L) in European barracuda infusion broth. Masson *et al*. (1996) found tyramine production by *Lactobacillus plantarum*. Da Silva *et al*. (2002) reported *Lc. lactis* subsp. *lactis* as strong tyramine producing LAB strains, with corresponding value of 646 mg/L. *Strep. thermophilus* and *Lb. plantarum* were the main producers of tyramine, while *Lc*. *lactis* subsp. *cremoris* produced the lowest tyramine in tyrosine decarboxylase broth (Kuley and Özogul 2011). Marino *et al*. (2008) reported that *Strep. thermophilus* was a tyramine producer strain. In common pandora infusion broth tyramine production ranged from 115 mg/L by *S. thermophilus* and 248 mg/L by *Leu. mes*. subs. *cremoris*, whereas tyramine production by LAB strains in striped mullet was generally lower than 67 mg/L except for *Lc. lactis* subs. *cremoris* which produced 217 mg/L of tyramine.

High phenylethylamine production was observed in striped mullet by *Lc. lactis subs. cremoris* (197 mg/L), although other LAB strains accumulated phenylethylamine in the range of 4 and 87 mg/L. Serotonin was one of the main amines formed by LAB strains. *Lb. plantarum* (830 mg/L), *S. thermophilus* (696 mg/L), *Ped. acidophilus* (631 mg/L) and *Lc. lactis* (527 mg/L) in common Pandora, and *Leu. mes*. subs. *cremoris* in European barracuda (510 mg/L) induced high level of serotonin accumulation. *Lb. acidophilus* did not produce tryptamine in European barracuda infusion broth. In common Pandora infusion broth the LAB strains tested accumulated higher tryptamine than that of other infusion.

Little amount of TMA, tryptamine and serotonin were produced by LAB strains in histidine decarboxylase broth (Özogul 2011). In the current study, TMA was generally produced below 17 mg/L by bacteria in fish infusion broth, although *Lc. lactis subs. cremoris*, *S. thermophilus* and *Lb. plantarum* accumulated TMA at level of 256, 42 and 30 mg/L, respectively.

Dopamine production by bacteria was above 50 mg/L for all infusion broth. The highest dopamine production was observed for *Lc. cremoris,* with value 750 mg/L. Agmatine production by *Lc. lactis cremoris* and *Ped. acidophilus* were the highest in common Pandora and European barracuda (90 vs 65 mg/L).

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Biologically Active Seaweed Resources of Turkey for Industrial Applications

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ABSTRACT

Seaweeds (or macroalgae) are rich sources of structurally new and biologically active metabolites for many industrial applications, including pharmaceuticals, nutraceuticals (*e.g.* marine lipids and antioxidants), cosmetics (*e.g.* antimicrobial preservatives, UV-absorbing compounds), agrochemicals (*e.g.* plant growth regulators, nutrients, biocides, herbicides, algicides, antifoulants, antibiofilm agents), biosensors (*e.g.* marine toxins) and other speciality chemicals (*e.g.* antibiofilm agents, antifoulants).

An EU Project, called *MAREX* (Exploring Marine Resources for Bioactive Compounds: From Discovery to Sustainable Production and Industrial Applications), supports isolation, characterization and sustainably exploitation new compounds from extracts prepared from marine organisms, including microalgae, macroalgae and cyanobacteria distributed along the coast of Turkey. In the first year of the study, a total 131 taxon (126 seaweeds, 3 seagrasses, 2 aquatic plants and 1 cyanobacteria) were collected from different locations, including Urla, Seferihisar, Bostanli, Inciraltı, Bodrum, Fethiye, Marmaris, Gokova, Side, Antalya, Olympus, Burhaniye, Akcay, Edremit, Gemlik, Mudanya and Asos. After the classification process, they were also deposited in a Herbarium.

The extracts prepared from 24 species of red seaweeds (*Rhodophyta*), 50 species of brown seaweeds (*Ochrophyta*), and 45 species of green seaweeds (*Chlorophyta*) are now being subjected to a highly representative and diverse panel of multidimensional bioactivity assays, and most promising extracts showing bioactivities will be subjected to chromatographic separation. Isolated marine compounds will be characterized thoroughly in terms of analytical purity and chemical structure.

Vegetative tissue and reproductive cell cultivation of seaweeds, such as *Gracilaria verrucosa* and *Ulva rigida* were started to establish in laboratuary and outdoor conditions. The cultivation of other strains, such as *Gigartina, Hypnea*, *Porphyra*, *Laurancia, Asparagopsis, Sargassum*, *Cystoseira*, *Dictyopteris*, *Ulva, Codium* and *Caulerpa* are being under invastigation. In the next study program, the most interesting compounds obtained from seaweeds will be produced via

biotechnological processes or optimized culture conditions in a sustainable medicinal chemistry programme with the aim of developing seaweed-based agents to the stage where they are available for various industrial applications.

Keywords: Seaweeds, seaweed cultivation, bioactive metabolites, biotechnology, industrial applications

INTRODUCTION

Marine macroalgae, or seaweeds, have been used as sea vegetables, medicines and fertilisers for centuries. Natural products extracted from seaweeds include the gelling polysaccharides agar and carrageenan (extracted from the *Rhodophyta*) and alginate (from members of the *Ochrophyta*); sometimes these are referred to as hydrocolloids or phycocolloids (Boisvert 1988; McHugh 2003).

The range of polysaccharides present in macroalgae is wide and complex and the biological activities they elicit are just as varied. The best known galactans (polysaccharides of animal or plant origin that yield galactose on hydrolysis), agar and carrageenan, have received much research attention focussed on it structural and functional properties that make them useful in diverse medical, pharmaceutical, cosmetic, food and industrial applications (De Roeck-Holtzhauer 1991; Donadiu and Basire, 1985).

These hydrocolloids, although immensely important, will not receive much attention unless they show specific biological activities. The polysaccharides that are of interest in medical and pharmaceutical fields usually have varying degrees of sulphation, and in the years since the world-wide spread of HIV/AIDS, three groups have received much attention in the medical literature for its possible use as antiviral substance, *viz*. carrageenan, xylomannan and galactan sulphate. These and a variety of lesser-known polysaccharides such as fucoidan and laminarin that exhibit other types of bioactivities (Indergaard and Ostgaard 1991).

Fucoidans are a group of highly heterogeneous highly branched polysaccharides consisting predominantly of sulphated a-L-fucose residues with varying amounts of galactose, mannose, glucose and xylose, and are distributed in the intercellular matrices of many members of the *Ochrophyta*. Laminarin, usually obtained from Brown seaweeds, is as receptor-ligand for reactions involving binding to β-glucan. Glucoronorhamnoxyloglycan, or simply ulvan, is a cell-wall matrix polysaccharide found in some members of the Ulvales. Porphyrans constitute a family of agaroids extracted from *Porphyra* spp. and is composed of alternating 3-linked -D-galactose units and 4-linked -L-

galactos eresidues or its derivatives; they contain sulphate groups only on the C6 of the –Lresidues (Ingaard and Ostgaard 1991).

A host of terpenoids are present in marine organisms, and biological activities for many of these have been reported for those extracted from invertebrates and seaweeds. Perhaps the best known terpenoid is the sesquiterpene, caulerpenyne, from the invasive green alga, *Caulerpa taxifolia*, and from other members of the Caulerpales Polyols (sugar-alcohols) such as altritol, dulcitol, xylitol, mannitol and sorbitol are produced as photosynthetic products in seaweeds. A group of macroalgal proteins that have found widespread application in biology research because of their spectral properties are the biliproteins (phycoerythrins, phycocyanins, allophycocyanins and phycoerythrocyanin) that are extracted from the red algae Polypeptides (kahalalides), for example kahalalide F, a cyclic depsipeptide, is produced by green seaweeds Lectins are present in all seaweeds. Those with haemagglutinating properties have been found in a variety of red, green and brown algae (Indergaard and Ostgaard 1991; Skjak and Martinsen 1991).

Many intertidal seaweeds are exposed to dangerous levels of high-energy radiation that could overload their photosystems or cause damage to proteins and DNA; humans (*Homo sapiens*) by nature also tend to congregate in intertidal areas or surface waters around beaches, and so face a similar problem. Seaweeds are constantly faced with microbes, spores and invertebrate larvae that attempt to settle on the thallus surface; similarly, submerged man-made structures such as ships' hulls also form attractive substrata for propagule settlement. The mechanisms algae employ to overcome these problems (fouling, photodamage) can also be adapted for human use, resulting in new technologies (natural antifoulants, novel UV sunscreens). It is clear that investigations of appropriate natural models can provide a more efficient way for the discovery and development of useful metabolites.

The aims of our study are:

- to collect and process of seaweeds from the Mediterranean Sea
- to prepare crude extracts and fractions from the collected seaweeds
- to promote taxonomy and cataloguing of collected seaweeds
- to develop suitable and sustainable cultivation and harvesting processes for selected seaweeds (scale dependently and including basic analysis for quality control purposes) of interest for therapeutic, cosmetic, nutraceutical, or agrochemical applications

- to develop sustainable biotechnological production protocols and long-term storage methods (culture collection) for promising seaweed cultures
- to use larger scale seaweed cultures to provide sufficient biomass for the isolation of pure compounds by bioactivity-guided fractionation for strains from which extracts showing promising bioactivity have been obtained; to optimize culture conditions to facilitate sustainable and economic production on an industrial scale.

MATERIALS AND METHODS

Collection and processing seaweeds

Between September 2010 and September 2011, the sample collection of seaweed species was perpormed and the collection area covered selected, mainly coastal parts of the Mediterranean Sea, including Aegean and Marmara Seas. During the sample collection, samples of cyanobacteria and microalgae/dinoflagellaes were also deposited addition to seaweeds. The sample types have been chosen to provide a balanced selection of organisms from different ecosystems and phyla. According to available literature and the expertise of the project partners these strains were among the most promising producers of bioactive compounds. Then, some of this collected seaweeds were used for cataloguing study, rest of the material was identified taxonomically in order to determine their voucher number and prepared crude extracts for biological screening study.

Preparation crude extracts from the collected seaweeds

All samples were identified, classified, washed with tap water and distilled water, weighed, frozen and stored at -20^0C. The lyophilised samples were extracted with 80% ethanol using Soxhlet apparatus. After 6 h of extraction the solvent were evaporated from crude extract by rotary evaporator. Each seaweed sample provided material for at least 9 separate crude extracts for each partner applies a wide variety of tools to detect bioactive molecules from seaweeds.

Developing suitable and sustainable cultivation methods for promising seaweeds

Cultivation studies of seaweeds are being established in controlled conditions in the EGE laboratories in accordance with protocols established by Kawai *et al*. (2005) and West (2005). Collected specimens were transported in plastic bags, bottles, or containers suitable to their

size, avoiding excess irradiation and temperature shocks relative to the prevailing habitat conditions. Most intertidal seaweeds are tolerant of stresses such as desiccation and rapid temperature changes, compared with subtidal seaweeds. Fertile specimens are being collected under desiccating conditions, they release reproductive cells (zooids, eggs, spores, *etc.*).

Either vegetative tissue or the cells released from reproductive structures were being used to establish cultures. In either case, cleaner plants with few epiphytes and epizoa, and fertile plants in the latter case, were selected in the field. Fertile portions were detected from the gross appearance; only those portions were cut and transported to the laboratory.

For unialgal cultures, glass and plastic petri dishes, beakers with glass covers, test tubes with screw caps, and clear thin polystyrene cups (so-called ice cream cups) were used. For small plants (less than several centimeters), plastic (polystyrene) petri dishes of 60 or 90 mm in diameter were used. For larger plants, beakers, ice cream cups, or various glass containers were used. Cultures were started to maintaine in climate-controlled culture chamber that regulates the temperature at 18 ^{0}C and were illuminated by daylight-type, white fluorescent tubes. Light intensities of 10–100 μmol · m^{-2} · s^{-1} were used for seaweed culture collection studies.

When the collected specimens do not immediately release reproductive cells, or as a preliminary step in isolation from vegetative tissues, the whole plant or a part of the plant was cultured (maintained) in the laboratory. These are often called crude cultures. To clean the surface of the plants, fine paintbrushes were helpful before starting the culture. Enriched seawater media with Guillar's F/2 medium was used, as for unialgal cultures, but to avoid overgrowth of epiphytes, plain sterilized seawater or reduced enrichment is being used. To suppress the growth of diatoms and cyanobacteria, germanium dioxide (GeO_2) and antibiotics were added to the media.

RESULTS AND DISCUSSION

Collection and processing seaweeds

As a result of the field studies for a 12-month period, 47 seaweed strains were collected from North Aegean and Marmara Sea (Gemlik, Erdek, Canakkale, Assos and etc.), 36 strains were collected from 22 different stations in the South Aegean Sea and Mediterranean Sea (Bodrum, Marmaris, Fethiye, Antalya and *etc.*) and 47 strains were collected from different stations near Izmir (Urla, Bostanli, Inciralti and etc.), so a total of

130 seaweed strains were added to the field data collection and were deposited in a Herbarium.

Preparation crude extracts from the collected seaweeds

A total of 409 crude extracts prepared from 24 species of red seaweeds (*Rhodophyta*), 50 species of brown seaweeds (*Ochrophyta*), and 45 species of green seaweeds (*Chlorophyta*) were sent to other partners and are now being subjected to a highly representative and diverse panel of multidimensional bioactivity assays, and most promising extracts showing bioactivities will be subjected to chromatographic separation. Isolated marine compounds will be characterized thoroughly in terms of analytical purity and chemical structure.

Developing suitable and sustainable cultivation methods for promising seaweeds

Vegetative tissue and reproductive cell cultivation of seaweeds, such as *Gracilaria verrucosa* and *Ulva rigida* were obtained in laboratuary and outdoor conditions Unialgal cultures of *Gigartina, Hypnea*, *Porphyra*, *Laurancia, Asparagopsis, Sargassum*, *Cystoseira*, *Dictyopteris*, *Ulva, Codium*, *Caulerpa* etc., are being also established by cutting off vegetative tissues (cells) and cleaning them in the course of their growth. However, in practice epiphytic algae or cyanobacteria, which may be very difficult to remove from the surface of the tissues, grow faster and more vigorously than the desired algae. Therefore, isolation from vegetative tissues is usually restricted to taxa with apical meristematic growth, siphonous green algae, and some taxa with rapid cell division. In other cases, unialgal cultures are being established from zooids such as zoospores and planogametes, or zygotes, carpospores, tetraspores, or aplanospores.

Since, the biomass and bioactive molecule yields of many seaweeds are controlled by environmental conditions, such as temperature, salinity, enrichment media and daylength conditions, the next study period will cover the areas of developing large or industrial scale seaweed cultivation to provide sufficient biomass for the isolation of pure compounds by bioactivity-guided fractionation for strains from which extracts showing promising bioactivity. The other sudy programs will be optimizing seaweed culture conditions to facilitate sustainable and economic production on an industrial scale, developing suitable harvesting processes, which may be scale dependent and should include basic analysis for quality control purposes and developing long-term storage (culture collection) methods for promising cultures.

CONCLUSION

Macroscopic marine algae (seaweeds or sea vegetables) form an amazing living resource of the near-shore environment. For millennia, people have collected seaweeds for food, fodder for animals, as well as fertilizers and soil enhancers. More recently, seaweeds have become important sources of various biochemicals, such as phycocolloids, marine lipids, antioxidants, antimicrobial preservatives, UV-absorbing compounds, plant growth regulators, nutrients, biocides, herbicides, algicides, antifoulants, antibiofilm agents, marine toxins, antibiofilm agents and antifoulants are important in medicine and biotechnology. The seaweed resources of Turkey are very rich, with approximately 1,000 species of seaweed within the classes of red seaweed (*Rhodophyta*, 60%), brown seaweed (*Ochrophyta*, 21%) and green seaweed (*Chlorophyta*, 19%). Of these, *Gracilaria*, *Gigartina, Hypnea*, *Porphyra*, *Laurancia, Asparagopsis, Sargassum*, *Cystoseira*, *Dictyopteris*, *Ulva, Codium* and *Caulerpa* have the highest potential for many industrial applications, including pharmaceuticals, nutraceuticals (*e.g.* marine lipids and antioxidants), cosmetics (*e.g.* antimicrobial preservatives, UV-absorbing compounds), agrochemicals (*e.g.* plant growth regulators, nutrients, biocides, herbicides, algicides, antifoulants, antibiofilm agents), biosensors (*e.g.* marine toxins) and other speciality chemicals (*e.g.* antibiofilm agents, antifoulants).

Turkey is still in its infancy and its potential in mariculture is far from being fully exploited. From the point of view of seaweed aquaculture, Turkey is just using its marine aquaculture potential at minor levels. However, it will undoubtedly expand in the years to come, by seaweed cultivation systems where with 15% dry weight content, 5 ton fresh weight seaweed/ha/year in sea-based and 110 ton fresh weight seaweed/ha/year in land-based systems can be cultivated (Turan 2009).

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An Eco-Friendly Bio-Stimulator for *Zea mays* from Invasive *Caulerpa racemosa* var. *cylindracea*

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ABSTRACT

Caulerpa racemosa var. *cylindracea* is an invasive green marine alga in the Mediterranean Sea, where 13 countries are currently under the thread of this species. *C. racemosa* var. *cylindracea* negatively affects sublittoral ecosystem of the Mediterranean Sea. Unfortunately, no validated eradication method has up to date existed in the scientific literature on this invasive seaweed. The extract of this species contains many trace elements, vitamins, amino acids and antioxidants which are necessary for plant growth and health. The present study proposes an alternative approach to the evaluation of the biomass of *C .racemosa* var. *cylindracea* as a seaweed fertilizer and a possible antioxidant supplement for plant growth. According to the results obtained, the extract of *C.racemosa* var.*cylindracea* can stimulate the growth and enzyme-based antioxidants of *Zea mays* seedlings. The best effect of *C. racemosa* var. *cylindracea* extract was observed in water soaked and extract treated *Zea mays* seedlings. The positive effects suggest that *C. racemosa* var. *cylindracea* extract might stimulate antioxidant activities and may lead to the productivity of the *Zea mays* with the presence of essential elements. Therefore, *C. racemosa* var. *cylindracea* extract can be used as an eco-friendly fertilizer in organic agriculture and might contribute to the healthy crop improvement.

Keywords: Biological invasion, *Caulerpa racemosa* var. *cylindracea*, eco-friendly biostimulator, marine seaweed, Mediterranean, organic agriculture.

INTRODUCTION

Caulerpa racemosa var. *cylindracea* is a well-known green invasive alga in Mediterranean Sea. Up to 1990, the spread of *Caulerpa racemosa* was not identified as an invasive species; therefore it was speculated as a Lessepsian migrant. After 1990, the invasive *Caulerpa racemosa* was observed in 13 Mediterranean countries including Albania, Algeria,

Croatia, Cyprus, France, Greece, Italy, Libya, Malta, Monaco, Spain, Tunisia and Turkey (Klein *et al.* 2008). According to Verlaque *et al*.'s DNA analysis, this species was identified as *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman et Boudouresque (Verlaque *et al.* 2003). The invasion of *C. racemosa* var. *cylindracea* threats the biodiversity of the Mediterranean Sea negatively (Piazzi and Balata 2008). The secondary metabolite of this species can be associated with its secondary metabolite caulerpenyne (CYN). Previous studies report the cytotoxic, antiproliferative, antiviral and apoptotic effects of CPN on some animals and cell lines (Nicoletti *et al.* 1999; Barbier *et al.* 2001; Cavas *et al.* 2006). Unfortunately, no published and valid eradication method exists in the scientific literature on the eradication of invasive *Caulerpa racemosa* var. *cylindracea* from the Mediterranean Sea. The lack of eradication method for this species motivated us to perform some alternative experimental approaches to the use of this over-produced biomass of *C.racemosa* var. *cylindracea* in Turkish coastlines (Caparkaya *et al*. 2009). In our previous scientific reports, *C.racemosa* var. *cylindracea* biomass was also proposed to be used for methylene blue adsorption (Cengiz and Cavas 2008), for malachite green adsorption (Bekçi *et al*. 2009) bovine serum albumine immobilization (Cengiz *et al.* 2008). In the present study, liquid seaweed fertilizer of *Caulerpa racemosa* var. *cylindracea* was aimed to be used in organic agriculture. Seaweeds are one of the important marine living resources which have been used as a source of food, feed and medicine for many years (Crouch and Staden 1992). In recent years, intense interest has been aroused around the effect of seaweed fertilizer on the plant growth in agriculture. There are many countries (Australia, France, Great Britain, India, Japan, New Zealand, Scotland, Spain and USA) where liquid seaweed-based fertilizers are used commercially (Zahid 1999). Here are the names of some well-known liquid fertilizers and the countries they are used: Maxicrop in United Kingdom, Kelpak 66 in South Africa, Algifert in Norway, Seagro in New Zealand and Seasol in Tasmania (Sivasankari *et al.* 2006). The role of seaweed fertilizer as a plant growth promoter has long been recognized, as it contains growth promoting hormones (IAA and IBA), cytokinins, trace elements (Fe, Cu, Zn, Co, Mo, Mn and Ni), vitamins and amino acids (Challen and Hemingway 1965; Sivasankari *et al.* 2006). In a previous study, the use of seaweed extracts was found to affect the crop quality positively, reduce the insect attack and also provide resistance to stress conditions (Berlyn and Russo 1990). Rising population, urbanization, industrialization and global warming have caused a decrase in agriculturel areas in the world. In addition to these, chemicals and synthetic fertilizers are highly used in the limited areas to raise the crop productivity. However, the damaging effects of these

fertilizers on human and animal health are not taken into consideration. As a result, the demand for organically produced products has become important day by day (Crouch and Staden 1993). In recent years, organic farming has benefits including water holding capacity, increasing food quality and lowered environmental stresses when compared to conventional farming (Cardellina 1986). The environmental stresses (biotic and abiotic) induce plants to produce reactive oxygen species (Posmyk 2005). Both biotic stresses (infection and/or competition by other organisms) and abiotic stresses (chilling, drought, UV, salt and heavy metal *etc.*) cause damage in plants. In plant metabolism, there are many sources of reactive oxygen species (ROS) such as photosynthesis and photorespiration, fatty acid oxidation and senescence (Kanazawa *et al.* 2000; Becana *et al*. 2000; Foyer and Noctor 2000; Vitória *et al.* 2001; Zhu *et al.* 2004). Inasmuch as the ROS such as superoxide radical anion, nitrite oxide and hydroxyl radical have very short half life, they are very reactive and directly attack the important components of aerobic cells such as cell membrane, DNA *etc.* (Halliwell and Gutteridge 2003). To get rid of the harmful effects of ROS, all aerobic living organisms have developed antioxidant systems. Among enzyme based antioxidants, SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide (He *et al.* 2008). Catalase (CAT) and ascorbate peroxidase (APX) detoxifies H_2O_2 to water and oxygen. These defence systems protect the plant cells against lipid peroxidation. On the other hand, plants have other non-enzyme based antioxidants such as ascorbate, glutathione and secondary pigments. Carotenoids play an important role in the protection of chlorophyll pigments under stress conditions (Gupta and Sinha 2009). α-amylase is an important enzyme for germination period of seeds. This enzyme breaks the α-1→4 glycosidic bonds in the polysaccharides and release six-carbon contained monomers for energetic purposes.

Zea mays is the third most important crop and also a major raw material of startch, glucose and oil in Turkey. The planted area of *Z. mays* has reached almost 600.000 ha in Turkey (Coşkun *et al.* 2006; Tanyolac *et al.* 2007). Since we have previously observed growth and antioxidant stimulation of *Z. mays* by seaweed extract (Cavas *et al.* 2010), we wanted to study in great detail in the present study. Hence, this study reports the growth parameters and antioxidant system of *Z. mays* which was germinated with *Caulerpa racemosa* var. *cylindracea* extract.

MATERIALS AND METHODS

Collection of seaweeds

C. racemosa var. cylindracea was collected by hand-picking from Seferihisar-İzmir in September, 2008. The geographical coordinates are 38^0 07' 58.61'' N, 26^0 50' 07.71'' E. The seaweeds were transported to the laboratory immediately and rinsed with tap water then distilled water to remove salt and epiphytes. After this treatment seaweeds were left onto the filter paper to remove excess water then separated to the polyethylene bags. The samples were stored at -20°C until used.

Preparation of seaweed fertilizer extract

The method of Sivasankari *et al*. (2006) was used to prepare the seaweed fertilizer. One kg of seaweed was homogenated first with mortar and pestle then homogenizator was used for further homogenization. The well homogenated seaweed sample was boiled with distilled water for an hour at 95°C and filtered. The filtered extract was named as stock solution. By using serial dilutions, different concentrations (5%, 10%, 15% and 20%) of seaweed extract were prepared using distilled water. The extracts were kept in refrigerator at + 4°C.

Preparation of seeds to germination

Z. mays seeds were obtained from Ege Tarımsal Araştırma Enstitüsü (Ege Agricultural Research Institute) İzmir, Turkey. For sterilization, each type of seeds was soaked in 1% H_2O_2 for 5 min. After 5 min seeds were washed with tap water then with distilled water. The experimental studies were carried out in two groups.

The abbreviations of the groups were defined below:
ESWT: Extract soaked-water treated (Experiment 1)
WSET: Water soaked- extract treated (Experiment 2)

For experiment 1 (ESWT), 200 seeds were soaked in different concentrations of seaweed extracts (5%, 10%, 15% and 20%) in sterile falcon tubes for 24 h in dark at growth chamber (Nuve ID 501). For the control group, seeds were soaked in distilled water. After soaking, the undamaged seeds were selected and placed in petri plates with filter paper. The seeds were watered with 10 mL distilled water every 24 h for 15 days. For the experiment 2 (WSET), 200 seeds were soaked in distilled water in sterile falcon tubes for 24 h in dark at growth chamber (Nuve ID 501). For the control group, seeds were soaked in distilled water. After soaking, the undamaged seeds were selected and placed in petri plates with filter paper. The seeds were watered with 10mL different

concentrations of seaweed extract every 24 h for 15 days. All the experiments were done in a growth chamber (NUVE ID501). The conditions of growth parameters were set as follows: temperature 25°C, humidity 55% and photo-period 16h light/dark. Growth parameters including germination percentage, root and shoot length were measured in *Z. mays*.

Preparation of supernatants for enzyme activities

0.05 g wet root, shoot and leaves were homogenized with pre-cooled mortar and pestle by adding 50 mM 1 mL phosphate buffer (pH 7.0). The homogenates were centrifuged in a refrigerated centrifuge (Universal 32R, Hettich Zentrifuggen) at +4 ^{0}C for 10 min at 12000 rpm**.** The supernatants were used in determination of enzyme activities and protein levels.

Protein Determination

Protein concentrations in the supernatants were measured according to the method of Bradford (Bradford 1976). Bovine serum albumin (BSA, Sigma) was used to obtain the standard curve of protein contents.

Catalase (CAT) assay

CAT activity was measured according to Aebi's method (Aebi 1985) at 25°C. CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm using with UV-VIS Spectrophotometer. The reaction mixture (1mL) contained 50 mM phosphate buffer (pH 7.0), 50 µL sample and 950 µL 10 mM H_2O_2. 1 unit of CAT activity was expressed as decomposition of hydrogen peroxide (10 mM) using reaction mixture and was normalised to per mg of protein.

Superoxide dismutase (SOD) assay

For the measurement of SOD activity superoxide dismutase kit (RANDOX, SD 125) was used. According to the kit manual, formazan dye is formed by xanthine-xanthine oxidase system in the presence of superoxide radical, and standard SOD inhibits the reaction. SOD activity in the samples was estimated by using a calibration curve which was obtained from standard SOD activities. The absorbance of the samples at 505 nm was measured with spectrophotometer at 37 ^{0}C against air as mentioned in its manual. One unit of SOD causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT).

Ascorbate peroxidase (APX) assay

The activity of APX was determined according to the oxidation of ascorbate in the presence of hydrogen peroxide (Nakano and Asada 1981; Choo *et al.* 2004). The reaction of ascorbate was obtained using 50 µL supernatant, 950 µL 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.5 mM ascorbate and 0.1 mM H_2O_2. The decrease of absorbance at 290 nm was measured with UV-VIS Spectrophotometer at 20°C. 1 IU APX was defined as oxidation of 1 µmol ascorbate in reaction mixture per min.

LPO determination

LPO was measured according to Zhu *et al*. (2004) with small modifications. 0.2 g fresh root, shoot and leaf were homogenized in 2 mL of 1.15% KCl with glass homogenizer. 1mL of homogenate was taken in falcon tubes containing 3 mL of 2% TBA (w/v) prepared in 20% TCA. The mixture was kept at 95°C for 30 min. After being cooled with tap water, samples were centrifuged at 12000 rpm for 10 min. Specific absorbance was measured at 532 nm and non-specific absorbance at 600 nm by using UV-Vis Spectrophotometer.

Chlorophyll a-b and total carotenoid determinations

Chlorophyll a-b and total carotenoid levels were determined Lichtentaler and Wellburn (1985) and Dere *et al*. (1998) methods. For each seed, 1cm^2 leaf samples were homogenized by using glass homogenizator. Well-homogenized samples were placed in a falcon tube and then 5 mL 100% acetone was added. The homogenates were centrifuged in 2500 rpm at 20^0C in a refrigerated centrifugator for 5 min (Universal 32R, Hettich Zentrifuggen). The absorbances at 470, 645 and 662 nm helped to estimate the total carotenoids, chlorophyll a and chlorophyll b levels of the leaf samples. The formulas below were used:

Chlorophyll a = 11.75 A_{662} - 2.350 A_{645}

Chlorophyll b = 18.61 A_{645} - 3.960 A_{662}

Total Carotenoids = ((1000 A_{470} - 2.270 Chlorophyll a - 81.4 Chlorophyll b)) / 227

Determination of α-amylase

The reduced groups liberated from starch by the reduction of 3,5-dinitrosalicylic acid were measured according to Bernfeld (1951) method. 0.02 M phosphate buffer (pH 6.9) containing 0.006M NaCl was used to prepare 1% starch solution. 1 g dinitrosalicylic acid color reagent was prepared in 50 mL distilled water then 30 g sodium potassium tartarate tetrahydrate and 20 mL of 2N NaOH were added slowly. The final

volume was diluted with distilled water. Standard curve was obtained using maltose solutions at different concentrations. For the measurement, 250 µL homogenate was put in a sterile falcon tube. 250 µL starch solution was added then incubated for 3 min at 25 ^{0}C. After incubation, 500 µL dinitrosalicylic acid color reagent was added into each tube. Then, all tubes were incubated in a boiling water bath for 5 min. After that they were cooled to room temperature and were added 10 mL distilled water and mixed well. The absorbance was measured at 540 nm spectrophotometrically.

Statistical analysis

MiniTAB (13.0) was used for statistical analysis. One way ANOVA and Tukey test were used to see the differences between seaweed fertilizer and growth parameters. For the correlation Pearson correlation test was used to evaluate the data. The results are the means of three different experiments (For SOD and α-amylase two different experiments). The error bars in the figures show the standard deviations. The statistical significance was considered as $p<0.05$.

RESULTS

In the present study, the effects of *C. racemosa* var. *cylindracea* extract on growth parameters of *Z. mays* seedlings were investigated in two experiments (water soaked and extract soaked). The maximum germination percentage and root length were observed at 10% WSET group when compared to control group. 20% seaweed extract affected the shoot length of *Z. mays* (7.2 ± 3.3 cm/seedling, $p<0.05$) positively. According to the statistical analysis, there were significant differences between the control group and 5%, 10% and 20% WSET groups. The highest germination was observed at 5% and 15% ESWT group *Z. mays* seedlings. The root length (3.2 ± 1.0 cm/seedling) of *Z. mays* at 10% ESWT group was higher than the control group (1.8 ± 1.3 cm/seedling).

The chemical parameters of *C. racemosa* var. *cylindracea* was analysed by Hıfzısıhha Enstitüsü in İzmir and was presented in Table 1. According to the results, seaweed extract contains high levels of potassium, magnesium, sodium, phosphorus, iron, chloride, sulphate and silicon.

Table 1. Chemical parameters of *Caulerpa racemosa* var. *cylindracea.*

Parameters	Results
Cu	0.06 mg/kg
Zn	0.4 mg/kg
Fe	4.9 mg/kg
P	16.12 mg/kg
K	57.71 mg/kg
Si	43.28 mg/kg
Na	856.7 ppm
Mg	26.03 ppm
SO_4	41.74 mg/kg
NO_3	7.7 mg/kg
Cl	1300 mg/kg
PO_4	30.4 mg/kg

The protein contents of *Z. mays* root, shoot and leaf were observed in Figure 1 and 2. When compared to Figures (1-2), pre-treatment with seaweed extract (ESWT) affected the protein contents of *Z. mays* seedlings positively. Maximum values were observed in the root, shoot and leaf of ESWT group *Z. mays* seedlings (0.13 ± 0.01 mg /ml supernatant, 0.77 ± 0.06 mg /ml supernatant 0.52 ± 0.06 mg /ml supernatant, respectively, $p<0.05$, Figure 2). Among WSET and ESWT groups, different concentrations of seaweed extract affected the protein contents of ESWT *Z. mays* shoot positively.

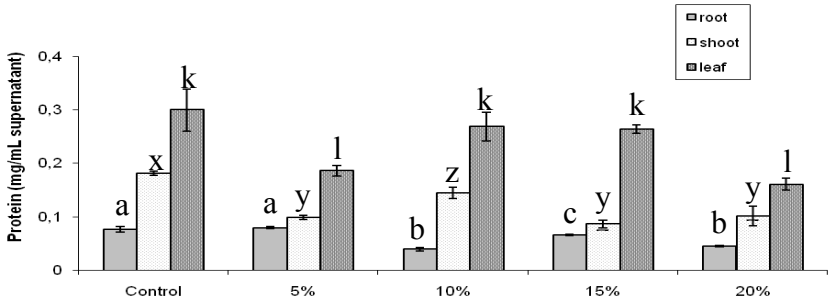

Figure 1. Protein contents of *Z. mays* root, shoot and leaf (WSET). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

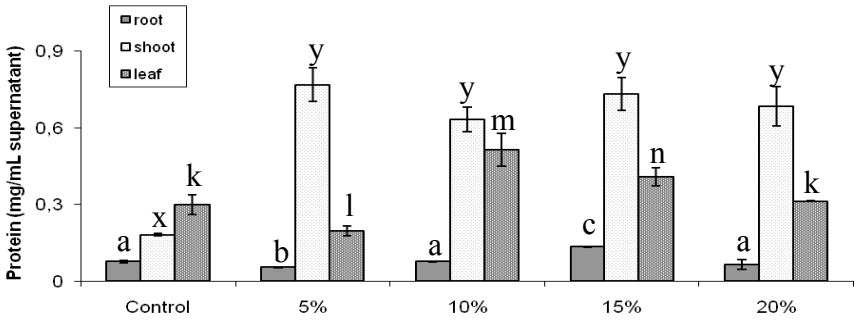

Figure 2. Protein contents of *Z. mays* root, shoot and leaf (ESWT). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

10%, 15% and 20% WSET *Z. mays* root showed positive response to CAT activity compared to the control group. Among WSET group, the highest CAT activity was observed at 10% WSET *Z. mays* root (135.7 ± 45.2 IU/mg protein, $p<0.05$, Figure 3).

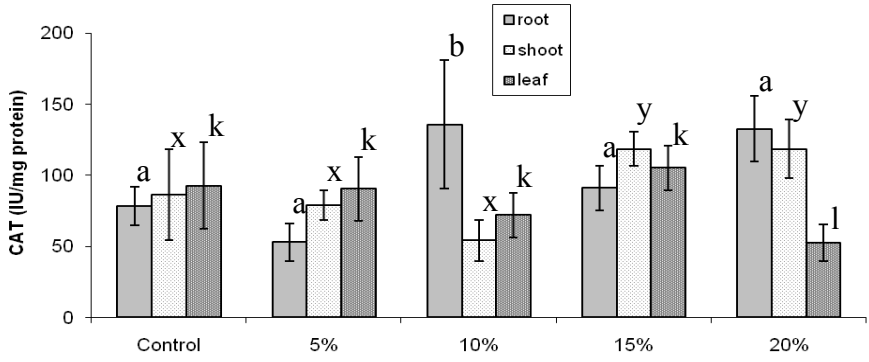

Figure 3. CAT activities of *Z. mays* root, shoot and leaf (WSET). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

According to Figure 4, seaweed extract affected CAT activities of *Z. mays* root positively. Maximum CAT activities were observed at 15% ESWT *Z. mays* root and shoot (1092.7 ± 165.9 IU/mg protein, 189.4 ± 35.9 IU/mg protein, respectively, $p<0.05$). However, there was no statistical difference between ESWT *Z. mays* shoot and CAT activity ($p>0.05$, Figure 4).

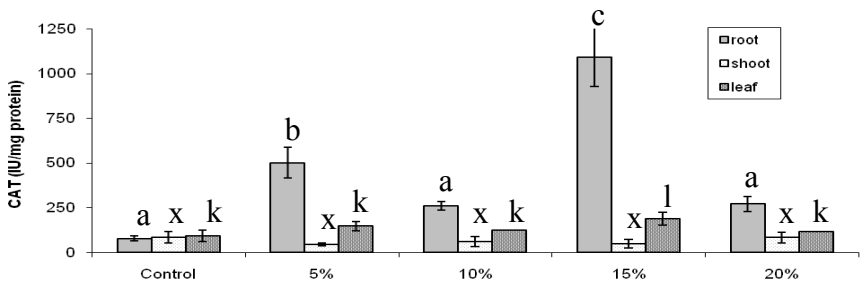

Figure 4. CAT activities of *Z. mays* root, shoot and leaf (ESWT). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

APX activities were higher at WSET groups than the control group (Figure 5). In the root of *Z. mays* maximum APX activity was found as 37.0 ± 3.4 IU/mg protein at 5% ESWT group ($p<0.05$, Figure 6). The highest APX activity at 5% ESWT and the lowest at 15% ESWT were observed in the shoot of *Z. mays*.

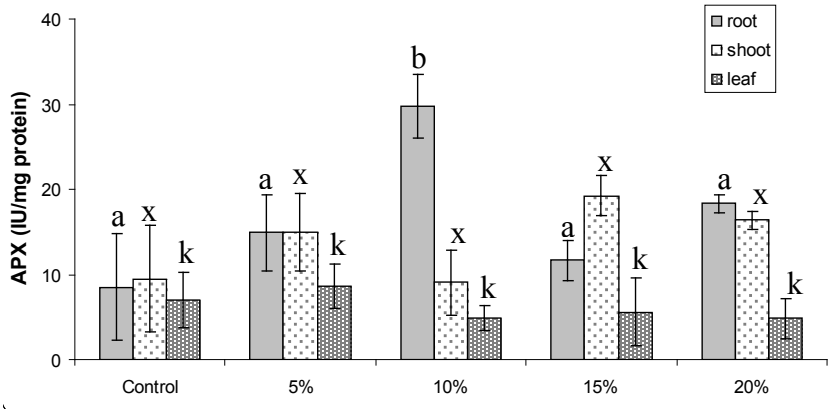

Figure 5. APX activities of *Z. mays* root, shoot and leaf (WSET). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

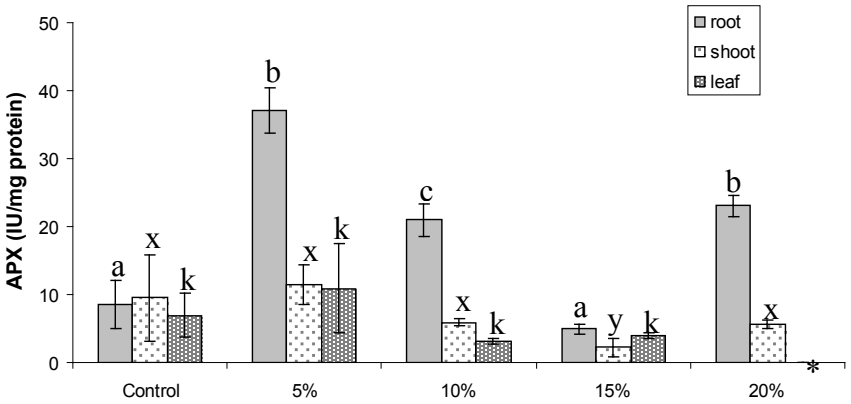

Figure 6. APX activities of *Z. mays* root, shoot and leaf (ESWT). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.
* There is not enough leaf to analyze.

When compared to the control group, maximum SOD activity was found at 20% WSET root and leaf of *Z. mays* ($p<0.05$, Figure 7). The extract effect showed higher SOD activity at 5% WSET *Z. mays* shoot (31.8 ± 0.1 IU/mg protein, $p<0.05$, Figure 7).

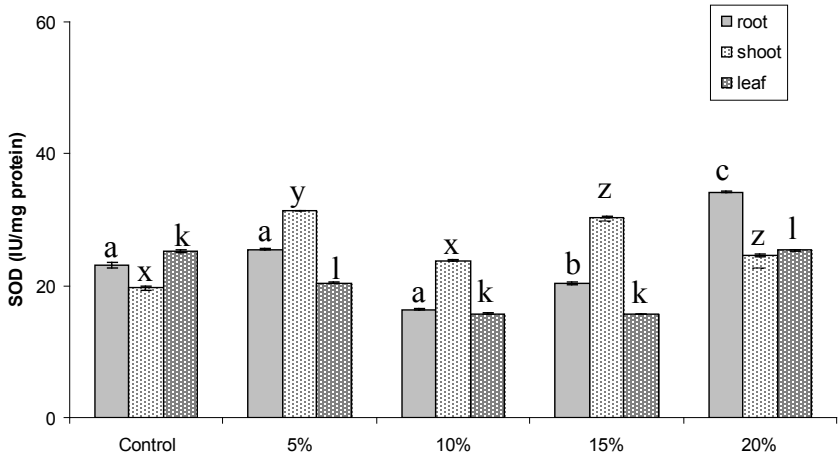

Figure 7. SOD activities of *Z. mays* root, shoot and leaf (WSET). Values are the means of two different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

In Figure 8, control group gave better results than ESWT group in the root and shoot of *Z. mays*. However, the effect of seaweed extract was

higher at 5% ESWT *Z. mays* leaf (24.6 ± 0.1 IU/mg protein, $p<0.05$, Figure 8).

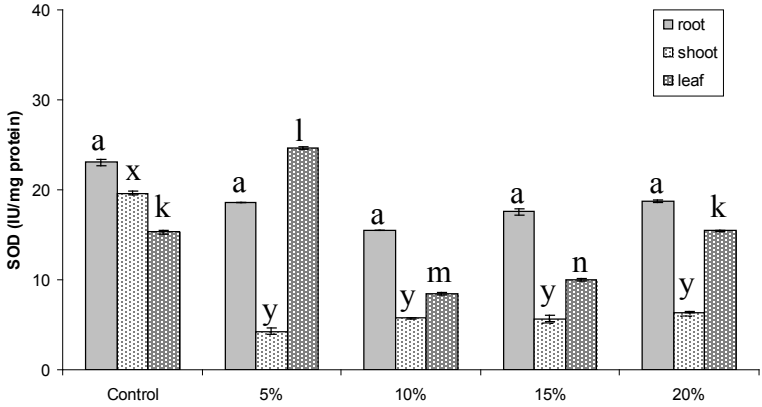

Figure 8. SOD activities of *Z. mays* root, shoot and leaf (ESWT). Values are the means of two different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

The root and leaf of *Z. mays*, maximum α-amylase activity was found at 5% WSET group (20.8 ± 0.0 µmol maltose/mg protein.min, 5.9 ± 0.4 µmol maltose/mg protein.min, respectively, $p<0.05$, Figure 9).

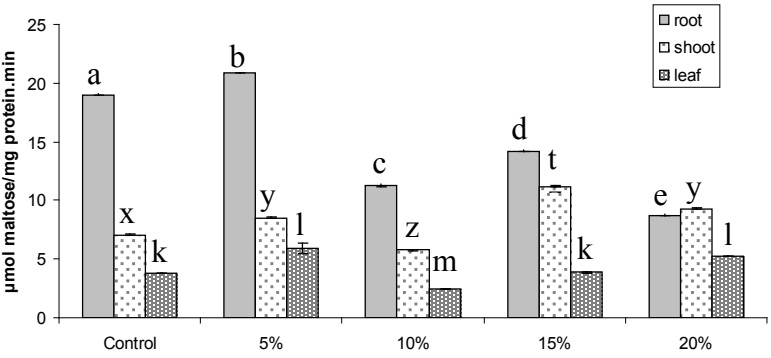

Figure 9. α-amylase activities of *Z. mays* root, shoot and leaf (WSET). Values are the means of two different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

Among WSET group, α-amylase activity was found at 15% WSET as 11.2 ± 0.0 µmol maltose/mg protein.min in the shoot of *Z. mays* ($p<0.05$, Figure 9). The negative effect of seaweed extract was observed in root and shoot of *Z. mays* (Figure 10). However, positive responses to α-amylase activities were observed at 15% ($p>0.05$) and 20% ESWT ($p<0.05$) *Z. mays* leaves (Control: 3.8 ± 0.0 µmol maltose/mg protein.min,

15%: 3.9 ± 0.0 µmol maltose/mg protein.min, 20% 5.5 ± 0.0 µmol maltose/mg protein.min.

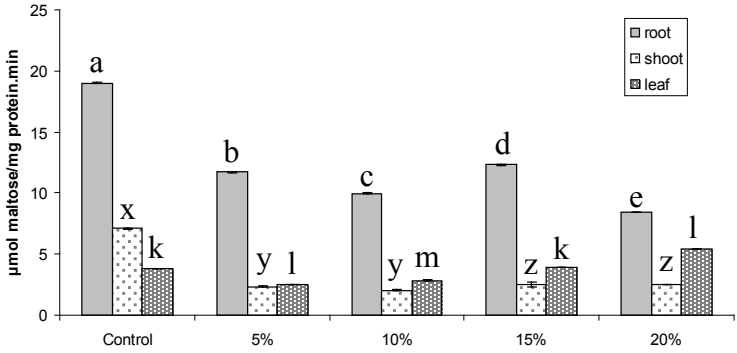

Figure 10. α-amylase activities of *Z. mays* root, shoot and leaf (ESWT). Values are the means of two different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

According to Figure 11, when seaweed extract was added, MDA levels were significantly tend to decrease in the shoot and leaf of WSET *Z. mays* ($p<0.05$). In the root of *Z. mays* MDA levels also decreased in ESWT group ($p<0.05$, Figure 12).

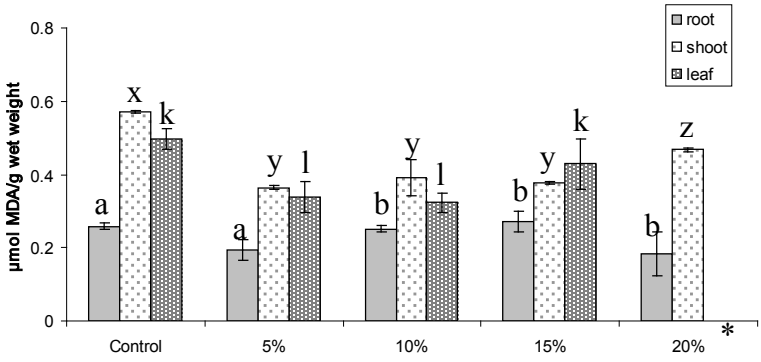

Figure 11. MDA contents of *Z. mays* root, shoot and leaf (WSET). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.
* There was not enough leaf to analyze.

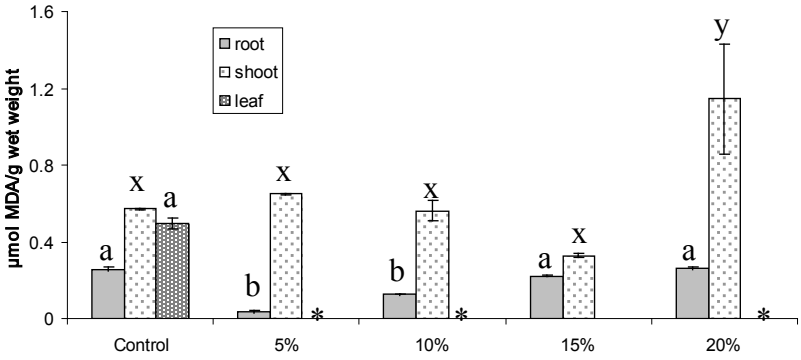

Figure 12. MDA contents of *Z. mays* root, shoot and leaf (ESWT). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.
* There was not enough leaf to analyze.

When the extract concentration increased, chlorophyll-a level increased as well (Figure 13). High chlorophyll-a level was found as 5.9 ± 0.01 µg/cm^2 wet weight ($p<0.05$, Figure 13). Among WSET group, maximum chlorophyll-b and total carotenoid levels were observed at 20% concentration of seaweed extract (1.8 ± 0.0 µg/cm^2 wet weight, 1.2 ± 0.0 µg/cm^2 wet weight, $p<0.05$).

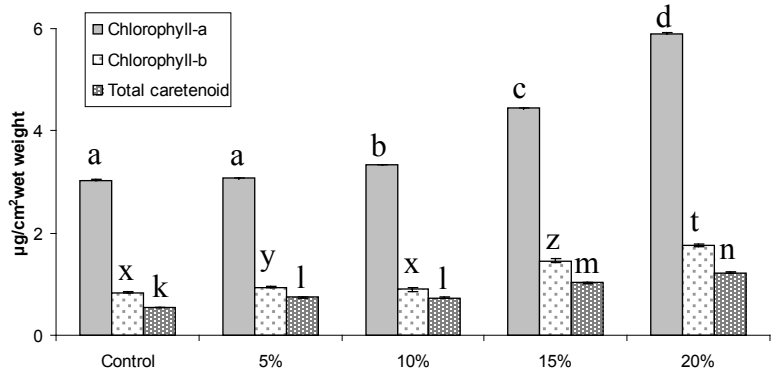

Figure 13. Chlorophyll-a, b and total caretenoid levels of *Z. mays* root, shoot and leaf (WSET). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.

When compared to the control group the highest chlorophyll-a, b and total carotenoid levels were found at 5% ESWT group (4.5 ± 0.0

µg/cm^2 wet weight, 1.6 ± 0.0 µg/cm^2 wet weight, 1.2 ± 0.0 µg/cm^2 wet weight, $p<0.05$, Figure 14).

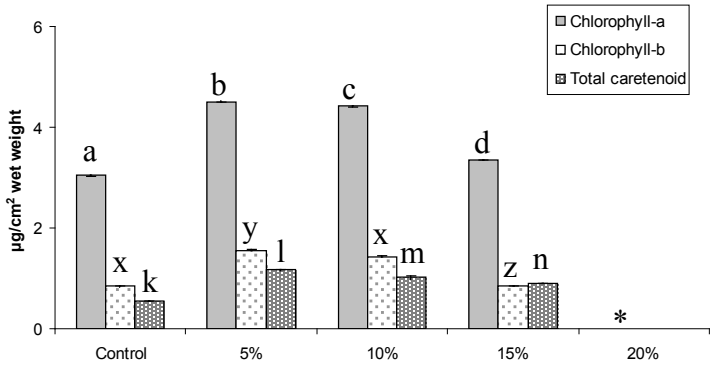

Figure 14. Chlorophyll-a, b and total caretenoid levels of *Z. mays* root, shoot and leaf (ESWT). Values are the means of three different experiments. Different letters above the error bars indicate significant differences at $p<0.05$.
* There was not enough leaf to analyze.

DISCUSSION

ROS are produced in plants as toxic byproducts of aerobic metabolisms (Apel and Hirt 2004). ROS are in balance under normal conditions. However, under stress conditions (abiotic and biotic) the production of ROS is accelerated in plants. Both biotic stresses (infection and/or competition by other organisms) and abiotic stresses (chilling, drought, UV, salt, exposed to heavy metal etc.) induce plants to produce reactive oxygen species. The balance breaking effects of ROS damage lipids, proteins, DNA, carbohydrates and other compounds of plants. The formation of ROS which provide oxidative damage in plants is prevented by defence systems (antioxidants) (Alscher *et al.* 2002; Blokhina *et al.* 2003). Over-produced ROS due to the environmental factors causes physiologic parameters, quality and yield of plants to be decreased (Burke 1990). The well known reactive oxygen species such as superoxide radical anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) cause lipid peroxidation as a result of this, increased membrane permeability, denatured proteins, inactivated enzymes and decreased pigments occur in plant cells (Fridovich 1986; Liebler *et al*. 1986; Davies 1987; Imlay and Linn 1988).

The various commercial products obtained from seaweeds are increasing day by day. Seaweeds are used as food and fertilizers due to their good source of vitamins, aminoacids, minerals, trace metals, proteins and bioactive substances (Kaliaperumal 2003). Previous reports also proved the positive effects of seaweed fertilizers in plants. In a prior report, Jeannin *et al*. (1991) studied the effect of seaweed extract (Goemar GA 14) as a folair spray onto the maize seedlings. Increasing production

of maize seedlings was observed compared to the control group (Jeannin *et al.* 1991). The favorable effect was analyzed by Crouch and Staden (1993) in the growth of tomato seedlings when used *Ecklonia maxima*. Their results showed that seaweed fertilizer increased the maturation and fruition ratio in tomato seedlings. Kowalski *et al.* (1999) studied the effect of a seaweed (Kelpak) concentrate on the in vitro growth and acclimatization of potato plantlets. According to their results, the improved plantlet quality in plants was observed when 0.25% of seaweed concentrate was applied. Sivasankari *et al.* (2006) studied the effect of seaweed fertilizers -*Sargassum wightii* and *Caulerpa chemnitzia*- on the growth and biochemical constituents of *V. sinensis* seeds. Their results showed that *S. wightii* extract was more effective than *C. chemnitzia* on the improvement of the crop. In another research, brown seaweed *Ascophyllum nodosum* extract was used as a bio stimulant to promote growth and productivity in barley seed halves. *A. nodosum* extract induced amylase activity independent of GA_3 and enhanced germination and seedling vigor in barley (Rayorath *et al.* 2008).

Organic agriculture has been improving to protect the natural life and to cultivate healthy crops in limited areas. In addition to these, the scarce agricultural areas have also been decreasing due to the high consumption of chemical fertilizers. The negative effects of these chemicals have been also damaging the soil quality. In Turkey, the use of organic fertilizers has not been developed enough in organic agriculture. However, global warming affects the living organism including plants negatively and lead to insufficent water and nutrient uptake from soil in Turkey (Yurttagül 2001; Evrensel 2001; Türk 2001).

The wide range of these positive effects motivated us to investigate the effect of invasive marine alga *C. racemosa* var. *cylindracea* as an eco-friendly biostimulator on *Z. mays*.

Different concentrations of seaweed extract improved the germination percentage, root and shoot lenghts of WSET *Z. mays* seedlings compared to the control group. In ESWT group, higher germination percentage was observed at 5%, 15% and 20% concentrations of seaweed extract. The high rate of germination percentage in *Z. mays* seedlings (ESWT group) may have been due to the presence of growth promoting substances such as gibberellins, cytokinins, micronutrients, vitamins and amino acids of *C. racemosa* var. *cylindracea* extract (Sivasankari *et al.* 2006). On the other hand, Sivasankari *et al*. (2006) reported that the higher concentrations of *S. wightii* and *C. chemnitzia* extracts inhibited the germination of *V. sinensis*. According to our biochemical analysis, when applied seaweed fertilizer, increased activities of CAT, APX, SOD, α-amylase (for SOD and α-amylase at ESWT group) and chlorophyll-a, b and total carotenoid levels were observed in *Z. mays*

root, shoot and leaf in both groups. At WSET *Z. mays* seedlings enhanced the SOD, CAT, APX and α-amylase activities whereas MDA levels decreased. Over-production of superoxide radicals are converted to H_2O_2 by SOD. CAT and APX enzymes detoxify H_2O_2 to water and molecular oxygen in peroxisomes of plants (Halliwell and Gutteridge 2003). High SOD, CAT and APX activities might show scavenging effect in *Z. mays* seedlings to prevent oxidative damage (Posmyk *et al.* 2005). These results indicate that the biological functions of membranes might have been protected in *Z. mays* seedlings by seaweed extracts.

A previous report showed that the activity of photosynthesis is obstructed by drought stress in plants (Foyer and Noctor 2000). The photochemical changes in plant chloroplasts induce the increase in reactive oxygen species (Peltzer *et al.* 2002). Our results showed that *C. racemosa* var. *cylindracea* extract changed the chlorophyll-a, b and total carotenoid levels in *Z. mays* leaves positively compared to the control group. The seaweed extract might have been increased tolerance against oxidative stress by stimulating the antioxidants.

CAT and APX activities in ESWT *Z. mays* seedlings showed increased trend but SOD activity did not. The high rates of superoxide anion radical induce the oxidative damage due to the lack of enough SOD enzymes. However, increased chlorophyll-a, b and total caretenoid levels were found in ESWT groups.

Silicon plays a major role in plant growth and development. Our experimental analysis showed that *C. racemosa* var. *cylindracea* extract contains trace metals such as silicon (Si). Antioxidant enzyme activities increased with the addition of Si to wheat and cucumber which were exposed to salinity and freezing (Zhu *et al.* 2004; Liang *et al.* 2008). High antioxidant activities might have protected *Z. mays* seedlings from the harmful effects of ROS. Cu is an important micronutrient for plant growth; however, higher concentrations of Cu inhibit plant production, damage photo systems and reduce the pigment content (Tanyolac *et al.* 2007). In agricultural studies plants are generally exposed to environmental pollutants such as heavy metals. However, plants require small amounts of heavy metals as copper, iron, manganese and zinc for their metabolisms (Labra *et al.* 2006). Our chemical parameters showed that *C. racemosa* var. *cylindracea* extract has trace amount of copper and zinc which can be useful for plant metabolisms. In addition to this, it has been reported by Cavas *et al*. (2009) that *C. racemosa* var. *cylindracea* has not been exposed to heavy metal pollution. The well known effect of heavy metals is reducing the productivity of the plants. It has been reported that soils containing Ni-Cu induce the decrease in chlorophyll contents of *Empetrum nigrum* (Monni *et al.* 2001). On the other hand, *Z. mays* cultivated in the presence of *C. racemosa* var. *cylindracea* extract

showed high chlorophyll-a, b and total caretenoid levels. Nitrogen is one of the important foodstuff in plant development which is taken in the forms of NO_3 and NH_4. Polesskaya *et al*. (Polesskaya *et al.* 2004) studied the changes of the antioxidant activities of wheat seedlings grown in a medium with nitrate treatment. The NO_3 treatment induced the rise in the activities of SOD, APX and CAT in leaves of *Z. mays* (Polesskaya *et al.* 2004). In the present study, the positive results in *Z. mays* defence system may be related to the presence of NO_3 in *C. racemosa* var. *cylindracea* extract. The response of *Z. mays* defence system to oxidative stress has been reported in previous studies (Coşkun *et al.* 2006; Labra *et al.* 2006; Ekmekçi *et al.* 2008; Neto *et al.* 2006; Yan *et al.* 1996). However, no study has been existed in the literature related to the response of antioxidative systems of *Z. mays* pre-treated with *C. racemosa* var. *cylindracea* extract.

In conclusion, the best effect of *C. racemosa* var. *cylindracea* was observed at WSET *Z. mays* seedlings. The positive effects suggest that *C. racemosa* var. *cylindracea* extract might stimulate antioxidant activities and may lead to productivity of the *Z. mays* with the presence of essential elements. Therefore, *C. racemosa* var. *cylindracea* extract can be used as an eco-friendly fertilizer in organic agriculture and might contribute to healthy crop improvement. Further analyses on other commercial seeds are also strongly recommended.

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The Effects of two Different Fe Forms (Fe^{+2} and Fe^{+3}) on the Growth of *Spirulina platensis* (Cyanophyta)

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ABSTRACT

The aim of the present study was to investigate the effects of two different Fe forms (Fe^{+2} and Fe^{+3}) on the growth parameters of *Spirulina platensis*. During the experimental period (12 day), pH, dryweight (g L^{-1}), chlorophyll a (µg mL^{-1}) and phycocyanin concentration (µg mL^{-1}) were measured on daily basis. The highest amount of chlorophyll a and the highest dry weight amount were observed in the second group respectively as 5.16±0.64 µg mL^{-1}, 0.055±0.014 g L^{-1}, while the lowest corressponding levels were found in the fifth group respectively as 1.70±0.32 µg mL^{-1} and 0.037±0.01 g L^{-1} in all experiments. The highest phycocyanin content was detected as 2.78±0.1 µg $mL^{-1}$for the group containing 0.2mM Fe^{+2}. Results indicated that algal growth of *S. platensis* were significantly effected by Fe^{+3} and Fe^{+2} forms.

Keywords: *Spirulina platensis*, iron, form, growth

INTRODUCTION

Low availability of bioactive trace metals may directly limit physiological processes of phytoplankton (Mckay *et al*. 2001). The presence of trace metals further plays a role in shaping the composition of the endemic phytoplankton assemblage (Landry *et al*. 2000). Among other bioactive trace metals is Fe required in the greatest amount. It is commonly used as a redox catalyst in enzymes or in proteins associated with the electron transfer pathways found in mitochondria and chloroplasts (Raven *et al*. 1999). Although Fe is the most abundant transition metal in the Earth crust, Fe chemistry in oxic waters is dominated by both inorganic and organic complex formation. In addition, dissolved inorganic Fe (II) and Fe(III) species generally occur in very low concentrations in aquatic systems.

Bioavailability of iron depends upon each aspect of Fe chemistry (solubility, complexation, thermodynamics, kinetics of ligand exchange)

in addition to phytoplankton uptake mechanisms and kinetics. There is still no conclusive agreement on describing and quantifying ‘‘bioavailable iron’’ (Wells *et al*. 1995). Some of the operationally defined iron forms may have strong correlations with bioavailability of iron to phytoplankton (Wells and Mayer 1991).

Iron is undoubtedly the most important transition metal in seawater, partly because of the demand for iron in the metabolic cycles of phytoplankton cells, and also because of the relatively low abundance of iron in the surface water. The biological uptake of iron depends strongly on its chemical speciation. Iron occurs in extremely low concentrations in oxygenated seawater at pH~8 primarily as thermodynamically stable iron (III), which is highly reactive with respect to hydrolysis, adsorption and complex formation.

Inorganic iron (III) exists in solution as mononuclear iron hydrolysis species that are sparingly soluble and precipitate as ferric hydroxide aggregates (Sunda 2001). As a result, iron in the form of oxides and hydroxides is biologically unavailable for algal uptake.

The cyanobacterium *Spirulina platensis* has a high nutritional value and has been used for many years as human food, animal feed and biofertilizer. *Spirulina platensis* is a photosynthetic, filamentous, spiral-shaped multicellular blue-green microalgea. Its chemical composition includes proteins (55%-70%), carbohydrates (15%-25%), fatty acids (18%) vitamins, minerals and pigments like chlorophyll "a" 1.7%, carotenoide and xanthophylls 0.5% of the dry weight (Ciferri 1983). *Spirulina platensis* has been reported as a plant growth activator by producing growth promoting regulators (GA, IAA and ABA), vitamins, amino acids, polypeptides, antibacterial and antifungal substances as well as exopolysaccharides that improve soil structure, plant growth and productivity. *Spirulina* alga has been effectively used in combination with other fertilizers for increasing the yield of tomato, carrot and sugar beet (Mahmoud *et al.* 2007).

The bulk of the evidence supporting the iron limitation hypothesis is derived not only from numerous studies of individual marine algae species (Paczuska and Kosakowska 2003, Kosakowska *et al*. 2004), but also from mesoscale experiments in the ocean, particularly in the HNLC areas (Boyd and Law 2001; Öztürk *et al*. 2002; Tsuda *et al*. 2003).

We focused on the brackish phytoplankton species *Spirulina platensis*, one of many important cultural microalgae in Algal Biotechnology. The present study aims to investigate the effects of two different Fe forms (Fe^{+2} and Fe^{+3} on the growth parameters (dry biomass, chlorophyll-a, pH, phycocyanin concentration) of *Spirulina platensis* (Cyanophyta).

MATERIALS AND METHODS

Cyanobacteria *Spirulina platensis*-M2 obtained from the Plankton Culture Laboratory at the Faculty of Fisheries, Mustafa Kemal University was used in this study. It was grown at 26±2°C in Zarrouk's medium (Zarrouk 1966) for 12 days under white flourescent light (90 µmol photon m-2 s-1) with 24 h illumination. The medium was sterilized at 121°C for 30 min. All the chemicals were of analytical grade. All the procedures were performed under aseptic conditions using triplicates.

To examine the effect of metal form on the test organism, (*S. platensis*-M2), Fe^{+3} ($FeCI_3.6H_2O$) and Fe^{+2} ($FeSO_4.7H_2O$) were added to the growth medium (Zarrouk's medium) in calculated amount to obtain the final concentration of 30mg L^{-1}. Each treatment had to setup series, with EDTA **(***Ethylenediaminetetraacetate).* Stock solutions were prepared in trace metal free distilled water (TMF-DW) (Isik *et al.* 2007). Each test was carried out in 5000 ml Polycarbonate (PC) bottles. PC culture carboys of 5 L were used for the experiments. The vessels were cultivated in a little space of a special culture laboratory. They were covered with PE-sheets to create a dust- free environment, and maintained at the 18±2°C under continuous elimination with fluorescent lights (Philips). pH measurement was carried out daily with a WTW-330 pH meter (Wissenschaftlich- Technische Werkstätten, Germany), calibrated with WTW buffer solutions prior two measurement. Samples from culture bags were collected using a peristaltic pump with an acid-washed during 15 days. The composition of the test medium, initial pH values and the chemical form of iron were used as shown in Table 1.

Table 1. The Composition of test mediums.

Groups	Fe^{+2}(mM)	Fe^{+3}(mM)	EDTA(µM)	Initial pH
1		0,2	451,3	9.46
2		0,3	451,3	9.44
3	0,2		451,3	9.70
4	0,3		451,3	9.50
5			451,3	9.76
C(Zarrouk Medium)	0,1		451,3	9.70

During the experimental period, optical density, pH, dry weight (g L^{-1}), chlorophyl *a* (µg mL^{-1}) and phycocyanin concentration (µg mL^{-1}) were measured every day. Chlorophyl *a* values were determined according to the method of Parson and Strickland (1963). The amounts of cellular chl *a* were calculated using the highest cell abundance and chl *a*

values (chlorophyll-*a* concentration; μ gL^{-1}) divided by cell abundance (cell L^{-1}) (Guillard 1973).

Optical density values were obtained according to the procedure reported by Boussiba and Vonshak (1992). The samples were measured at 680 nm absorbans value with *spectrophotometer***.** The sample containing 10 ml algal suspension was filtered through a Ø 47 mm filter (Whatman GF/C) that was dried in a microwave oven (105°C in 8 min) and weighed prior to filtration. The filter was put in a glass petri dish in the oven under the conditions. After cooling the filter in a desiactor (20 min.), it was weighed again (Boussiba and Richmond 1979).

Phycocyanin concentration (PC) was defined according to Bennett and Bogorad [21]. Growth rates were determined with the help of following equation: μ= log (N1/N0) x (3.322 /t), where N_1 and N_0 were the OD at the end and beginning of the period of time (t) expressed in days (Guillard 1973).

In the present study, we intended to investigate the effects of two different Fe forms (Fe^{+2} and Fe^{+3}) on the growth parameters of *Spirulina platensis* (Cyanophyta). Since we didn't intend to detect iron limitation, but to study high iron concentration, iron concentrations were not determined here.

Data were analyzed statistically using one way analysis of variance (ANOVA). When significant treatment effects were detected, Duncan's multiple range test was used to identify specific differences among treatment means at a probability level of 5%.

RESULTS AND DISCUSSION

The effects of two different forms of iron on such parameters as pH, dry weight (g L^{-1}), chl a (μg mL^{-1}), phycocyanin concentration (μg mL^{-1}) of *S .platensis* cultures for 12 days are shown in Table 2.

Table 2. Chlorophyl a (μg mL^{-1}), dry weight (g L^{-1}), pH, phycocyanin concentration (μg mL^{-1}) of *S.platensis* cultures in all experiments.

	Parameters			
Groups	**chl a (μg mL^{-1})**	**dry weight (g L^{-1})**	**pH**	**phycocyanin con. (μg mL^{-1})**
1	4.71 ± 0.74^{b}	0.041 ± 0.010^{a}	9.90 ± 0.069^{a}	0.72 ± 0.14^{a}
2	5.16 ± 0.64^{b}	0.055 ± 0.014^{b}	10.03 ± 0.011^{b}	0.56 ± 0.1^{a}
3	4.40 ± 1.06^{b}	0.051 ± 0.032^{a}	9.90 ± 0.023^{a}	0.97 ± 0.47^{a}
4	2.77 ± 0.89^{ab}	0.053 ± 0.005^{a}	9.84 ± 0.020^{a}	2.78 ± 0.1^{b}
5	1.79 ± 0.32^{a}	0.037 ± 0.01^{a}	9.91 ± 0.012^{a}	0.28 ± 0.06^{a}
C(Zarrouk Medium)	2.87 ± 0.18^{ab}	0.051 ± 0.003^{a}	9.90 ± 0.036^{a}	0.57 ± 0.4^{a}

1: 0.2mM Fe^{+3} ; 2: 0.3mM Fe^{+3} ; 3: 0.2mM Fe^{+2}; 4: 0.3mM Fe^{+2} ; 5: - ; 6: Control. There is a difference among the means, single- factor ANOVA. *x* ± S.E. (mean± standard error), Values are means and different letters in the same line denote significant differences ($p<0.05$).

The maximum chl *a* values (4.71±0.74 µg mL^{-1}, 5.16±0.64 µg mL^{-1}, 4.40±1.06 µg mL^{-1}) were detected in the first (0.2mM Fe^{+3}), second (0.3mM Fe^{+3}) and third (0.2mM Fe^{+2}) groups (Table 2), while the lowest chl *a* value was observed with the cultures supplemented without iron ($p<0.05$).

Hutchins (1995) reported that in all cases, cyanobacterial Fe requirements are higher than those of eukaryotic algae. These high requirements are commonly attributed to cyanobacterial evolution, since the availability of iron would have been significantly higher in the anaerobic conditions under which these organsims evolved. However, the higher iron requirements of cyanobacteria relative to eukaryotic plankton may also be attributed to reduced amounts of iron-poor structural material in the cyanobacteria, which would lead to a proportional increase in the cellular iron requirements (Trick and Wilhelm 1995).

The highest dry weight amount (0.055±0.014 g L^{-1}) was found in the second group enriched with Fe^{+3}, (Table 2) ($p<0.05$). Dry weight amounts in other groups were as follows: in the first, tirth, fourth and fifth groups (0.041±0.010 g L^{-1}, 0.051±0.032 g L^{-1}, 0.053±0.005 g L^{-1}, 0.037±0.01g L^{-1} and 0.051±0.003 g L^{-1}).

Many researchers indicated that under iron-limitation condition a large decrease in the amount of chl *a* is accompanied by structural alterations of the thylakoid membranes, and the number of iron-containing proteins within the photosynthetic apparatus is reduced (Kudo *et al.* 2000). Xing *et al*. (2008) indicated that the second group (0.3mM Fe^{3+}), had high chl *a* value. Moreover, the 0.3mM (Fe^{3+}), treatment group had the highest dry weight amount. Our findings in both our present study and in the previous one (Isik *et al*. 2007) showed an increase in chl *a* content with an increase in iron amount and iron form.

In situ iron-enrichment experiments and bottle incubations (Frank *et al*. 2000) consistently show an increase in primary production, phytoplankton biomass and nutrient uptake after iron addition in the Southern Ocean.

The maximum pH value (10.0 3±0.011) were determined in cultures enriched with 0.3 mM Fe^{+3} (Table 2). Differences were considered significant at $P<0.05$ when other pH values were compared. Boyd (1979) reported that in parallel with the rise in growth of cultivated algae, pH values of the medium increasedHe also pointed out that pH values became higher during phytoplankton bloom. The results showed that in parallel with rise in growth of *Spirulina platensis*, pH values of the

culture medium.This study also indicated that pH rose in accord with the higher growth.

The highest phycocyanin content was detected in the fourth group as 2.78±0.1 µg mL-1 while the lowest value was 0.28±0.06 µg mL-1 in the fifth group. Although, higher chl a values and dry weight amount were determined in the cultures grown with initially 0.3 nM Fe+3 (second group) than other cultures, the phycocyanin was decreased in this group Presumably, the phycocyanin declined with changing iron form in the medium ($P<0.05$) (Table 2).

Many investigations in the laboratory on freshwater and oceanic phytoplankton demonstrated that iron limitation inhibited chlorophyll and phycobilin pigment biosynthesis. In addition, it decreased photosynthetic efficiency and electron transport rate, and also restrained nitrate assimilation (Boyd *et al*. 2000; Gress *et al*. 2004).

CONCLUSION

The bioavailability and uptake strategies of iron species, Fe (II) and Fe (III), by S*pirulina platensis* were studied in this study.

The fact that chl a and dry weight amount appeared to be higher in Fe^{+3} (0.3mM) added second group than in other groups revealed that such addition contributed to an increase in biomass. The results pointed out that Fe^{+3}(0.3mM) addition enhanced the growth of *Spirulina platensis* than other Fe additions group. On the other hand, the lowest values for these parameters detected in the fifth group, which was not added with Fe confirmed that Fe addition enhanced biomass addition. In our experiment,

the increase in the iron amounts and the integration of different iron forms in to the cultures had significant effect on growth parameter.

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Evaluation of Different Microalgae Strains for Biomass Production

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ABSTRACT

Microalgae play an important role in marine biological ecosystems, because of their high value added metabolite products such as astaxanthin, phycocyanin and omega 3 fatty acids and etc. The growth of nine different microalgae strains (*Geitlerinema* sp., *Oscillatoria agardhii, Synechococcus* sp., *Dunaliella salina, Chlorella* sp., *Nannochloropsis* sp., *Ochrosphaera* sp., *Nitzschia* sp., and *Cymbella* sp.) were investigated for 23 days of cultivation period. The experiments were performed under the same growth conditions. After 23 days of cultivation period, the maximum specific growth rates of 0.10 day^{-1} and 0.10 day^{-1}, which correspond to the doubling times of 7.33 day and 6.87 day, were obtained for *Chlorella* sp. and *Dunaliella salina*, respectively, at the light intensity of 100 μmol photons m^{-2} s^{-1}.

INTRODUCTION

In the last decades the attention of the natural product researchers has been focused at the marine environment: Indeed it is a wealthy source of plants, animals and micro-organisms, which due to their adaptation to this unique habitat, produce a wide variety of primary and secondary metabolites that have demonstrated significant biological activities in e.g. antitumor, anti-inflammatory, analgesia, immunomodulation, allergy, and antiviral assays (Sennet 2001).

Microalgae play an important role in marine biological ecosystems, because of their photosynthetic activity. They are the major producers of biomass and organic compounds in the oceans. Marine microalgae are separated into five major divisions: Chlorophyta (green algae); Chrysophyta (goldenbrown, yellow algae and diatoms); Pyrrhophyta (dinoflagellates); Euglenophyta, and Cyanophyta (bluegreen algae). The last division Cyanophyta is widely classified and termed as cyanobacteria and not included in the category of algae, because of their prokaryotic characteristics, despite the fact that the organisms are photosynthetic and share many algal characteristics with their eukaryotic counterparts (Shimizu 1996).

The aim of this study is to evaluate the cultivated different microalgae strains for biomass production in long-term period.

MATERIALS AND METHODS

Microalgae growth conditions for 2-L sterile bottle productions;

The Aegean microalgae species were monoalgal (non-axenic) and cultured in appropriate media (Table 1) at 22±2 °C under continuous illumination (100 µmol photons m^{-2} s^{-1}) in 2-L sterile bottle for 23 days. Some species were obtained from the Culture Collection of Microalgae at the University of Ege, Izmir, Turkey. The temperature was controlled by air conditioner. Illumination was provided by standard cool white fluorescent lamps (18 W) from one side of the bottle. Irradiance was measured in the center of the bottle with a quantum meter (Lambda L1-185). Air was supplied to the culture at a flow rate of 1 L min^{-1} (1.25 vvm) by air pump continuously.

Table 1. The culture medium of different microalgae strains.

Cultivated Strains	Medium Culture
Geitlerinema sp.	Sea Water-BG11
Oscillatoria agardhii	Sea Water-BG11
Synechococcus sp.	Sea Water-BG11
Dunaliella salina	IMK Medium
Nannochloropsis sp.	Enrichment Seawater Medium ESM
Chlorella sp.	Enrichment Seawater Medium ESM
Ochrosphaera sp.	F/2 Medium
Nitzschia sp.	F/2 Medium
Cymbella sp.	F/2 Medium

Measurement of algal growth for 2-L sterile bottle productions;

These investigations were carried out by the measurements of absorbance and pH values. The optical density was measured at different absorbance for all species. The specific growth rate (µ) of the cells was calculated from the initial logarithmic phase of growth for at least 48 h, as $\mu = \ln X_2 - \ln X_1/dt$, where X_2 is the final cell concentration, X_1 is the initial cell concentration and dt is the time required for the increase in concentration from X_1 to X_2. Doubling time (DT) was also calculated as $DT = \ln 2/\mu$. All media were prepared then autoclaved separately. If necessary, the vitamins were added aseptically to the final medium after autoclaving. All components (*Merck Co.*) were used analytical grade.

RESULTS AND DISCUSSION

Biomass production for different microalgae strains

The growth of nine different microalgae strains (*Geitlerinema* sp., *Oscillatoria agardhii, Synechococcus* sp., *Dunaliella salina, Chlorella* sp., *Nannochloropsis* sp., *Ochrosphaera* sp., *Nitzschia* sp., and *Cymbella* sp.) were investigated for 23 days of cultivation period. The experiments were performed under the same growth conditions.

As shown in Figure 1, the maximum optical density, 2.114, was obtained at 540 nm for *Chlorella* sp., and the lowest for *Cymbella* sp. (0.166) under the light intensity of 100 μmol photons $m^{-2}s^{-1}$. The optical density decreased by only 1% for *Nannochloropsis* sp. in comparison with *Chlorella* sp. During the cultivation, the growth increased 22.83 times in terms of the initial optical density for *Dunaliella salina* at 100 μmol photons m^{-2} s^{-1}. The slow growths were reported for *Cymbella* sp., *Synechococcus* sp., *Ochrosphaera* sp., *Geitlerinema* sp.

The values of pH were given in Table 2 for 23 days of cultivation period of nine different microalgae strains.

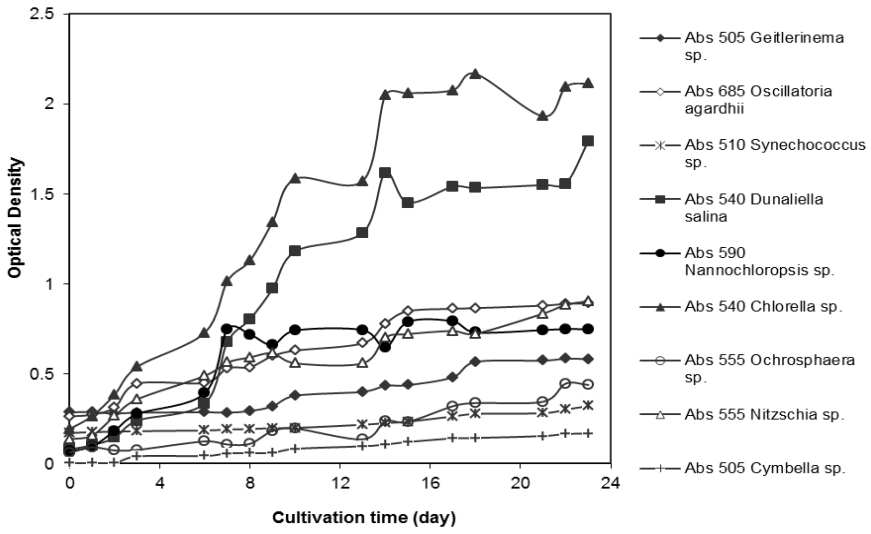

Figure 1. The turbidity data of different microalgae strains at different absorbance values during cultivation period

Table 2. The pH data of different microalgae strains during cultivation period.

pH

Day	Geitlerinema sp.	Oscillatoria agardhii	Synechococcus sp.	Dunaliella salina	Nannochloropsis sp.	Chlorella sp.	Ochrosphaera sp.	Nitzschia sp.	Cymbella sp.
0.00	8.79	8.70	8.66	7.62	8.11	8.35	7.71	7.58	8.65
1.00	8.32	8.62	8.30	7.62	8.11	8.35	7.71	7.58	8.54
2.00	8.45	8.66	8.46	7.76	8.00	8.15	7.79	8.04	8.58
3.00	8.63	8.80	8.52	7.81	8.04	8.15	7.70	8.24	8.70
6.00	8.73	8.83	8.57	7.97	8.12	8.20	8.00	8.12	8.69
7.00	8.79	8.93	8.69	7.88	8.14	8.15	7.80	8.03	8.70
8.00	8.73	8.95	8.66	8.05	8.18	8.17	7.72	8.02	8.59
9.00	8.72	9.02	8.68	8.16	8.17	8.26	7.77	8.08	8.58
10.00	8.80	8.95	8.62	8.10	8.24	8.19	7.65	8.01	8.68
13.00	8.79	8.95	8.68	8.18	8.23	8.28	7.76	8.04	8.74
14.00	8.79	8.89	8.66	7.89	8.22	8.19	7.42	7.68	8.66
15.00	8.75	8.88	8.66	8.11	8.13	8.16	7.66	7.87	8.70
17.00	8.69	8.85	8.68	8.17	8.15	8.23	7.69	8.00	8.68
18.00	8.74	8.88	8.64	8.35	8.21	8.28	7.75	8.05	8.66
21.00	8.82	8.90	8.72	8.25	8.09	8.19	7.80	7.34	8.68
22.00	8.85	8.87	8.70	8.24	8.20	8.30	7.56	8.01	8.66
23.00	8.68	8.90	8.71	8.18	8.19	8.21	7.97	8.05	8.68

As shown in Table 3, after 23 days of cultivation period, the maximum specific growth rates of 0.10 day^{-1} and 0.10 day^{-1}, which correspond to the doubling times of 7.33 day and 6.87 day, were obtained for *Chlorella* sp. and *Dunaliella salina*, respectively, at the light intensity of 100 μmol photons m^{-2} s^{-1}.

Table 3. Results of obtaining kinetic parameters of different microalgae strains during cultivation period.

Cultivated Strains	OD	pH	Spesific growth rate (μ, day^{-1})	Doubling Time (DT, day)
Geitlerinema sp.	0.58	8.68	0.03	20.44
Oscillatoria agardhii	0.89	8.90	0.06	11.69
Synechococcus sp.	0.32	8.71	0.03	23.18
Dunaliella salina	1.79	8.18	0.10	6.87
Nannochloropsis sp.	0.75	8.15	0.05	13.85
Chlorella sp.	2.11	8.21	0.10	7.33
Ochrosphaera sp.	0.44	7.97	0.08	9.30
Nitzschia sp.	0.90	8.05	0.06	11.88
Cymbella sp.	0.17	8.68	0.05	14.50

After the optimization studies of *Nannochloropsis oculata* growth, the maximum growth rate was achieved with the value of 0.0359 h^{-1} (Spolaore *et al*. 2006). Giordano (2000) reported that the obtained growth rate was 0.69 d^{-1} of *Dunaliella salina* cells cultured at high (5 %) CO_2 concentration in the presence of 10 mmol/LNH_4^+. On the other hand, the addition of 5 g L^{-1} glucose resulted in a growth rate of 0.60 day^{-1} for *Haematococcus pluvialis* and 0.59 day^{-1} for *Chlorella zofingiensis* (Oncel *et al*. 2010).

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Antioxidant Activities of 13 Marine Macroalgae Collected from the Coast of Urla and Effects of *Padina pavonica* on Cell Growth of MCF-7 Breast Cancer Cell Line

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ABSTRACT

Marine pharmacology research directed towards the discovery and development of novel antitumour agents. A significant number of in vitro cytotoxicity data generated with murine and human tumor cell lines has been reported. Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. These free radicals could cause damages that may lead to cancer. Antioxidants interact with free radicals and stabilize them and may prevent this damage. In this study 13 marine macroalgae collected from the coast of Urla were performed methanol extractions. Macroalgal methanolic extracts were evaluated for antioxidant and antitumoural activity. Antioxidant activities of extracts were determined using total phenolic content assay and DPPH free radical scavenger activity assay. The highest total phenolic content was observed in *Gracilaria gracilis* and the highest DPPH free radical scavenger activity in *Laurencia pinnatifida*. In terms of antioxidant capacities, extract of *Padina pavonica,* was selected for cell growth assay. Breast cancer cell line, MCF-7, was incubated with different concentrations of *Padina pavonica* methanolic extract (PPME; 1µg ml^{-1}, 10µg ml^{-1}, and 100µg ml^{-1}). PPME inhibited cell growth at the dose of 100µg ml^{-1}.

INTRODUCTION

Marine seaweeds are important sources of many potentially bioactive new pharmaceutical agents (Cardozo *et al*. 2007; El Gamal 2010) and researches achieved to date have reported that marine algae have many important properties such as antioxidant (Zhang *et al*. 2007; Lee *et al*. 2011) antimicrobial (Chattopadhyay *et al*. 2007; Genovese *et al*. 2012; Inbaneson, *et al.* 2012), anticoagulant (Mayer *et al*. 2011), hypoglycemic (Cherng and Shih 2006; Kang *et al*. 2010; Lee *et al*. 2010), hypolipidemic (Gad *et al*. 2011; Zha *et al*. 2012), hypotensive (Godard *et al.* 2009;

Schultz Moreira *et al.* 2011; Qu *et al.* 2010), anti-tumoural (Zubia *et al.* 2009) and anti-cancer (Shilabin and Hamann 2011).

By donating an electron, antioxidants neutralize free radicals that would otherwise oxidize biomolecules leading to cell death and tissue damage (Halliwell 1999). In shallow coastal habitats, macroalgae can be exposed to a combination of ultraviolet light and air that readily leads to the formation of free radicals and other reactive oxygen species (ROS). Despite their exposure to harmful ROS, algae lack oxidative damage in their structural components and resist oxidation during storage, indicating the presence of protective antioxidant defense systems in their cells (Mallick and Mohn 2000). Accordingly, interest in the search for natural antioxidants from algae has been increasing in recent years (Zhang and Omaye 2001; Yuan and Walsh 2006; Zubia *et al*. 2009; Gad *et al*. 2011).

Recently, the most noticeable macroalgal compound is Kahalalide F derived from different species of *Bryopsis* and researchers have showed that it could control to lung, colon and prostate tumors (El Gamal 2010) and its derivative Iso KF is currently undergoing phase II development in liver, melanoma and nonsmall cell lung cancer (Martín-Algarra *et al*. 2009). SargA, a potential antiangiogenic and antitumoral compound extracted from the brown marine alga *Sargassum stenophyllum*, caused to markedly decrease on B16F10 melanoma cell tumours without evident signs of toxicity (Dias *et al*. 2005). It has been reported that porphyran is the main component of *Porphyra yezoensis* and induces cancer cell death in a dose-dependent manner (Kwon and Nam 2006). Dioxinodehydroeckol isolated from *Ecklonia cava* inhibited the proliferation of MCF-7 human breast cancer cells (Kong *et al*. 2009). In addition, phlorotannin extract derived from brown algae *Laminaria japonica* has also shown anti-proliferative activity in the human hepatocellular carcinoma cell line (BEL-7402) and on murine leukemic cell line (P388) in a dose dependant manner and researchers have observed the morphologic features of tumor cells treated with phlorotannin extract and 5-fluorouracil (a commercial chemotherapy drug) are markedly different from the normal control group (Yang *et al*. 2010). The anti-tumor and immunomodulatory effects of the acidic polysaccharides isolated from *Gracilaria lemaneiformis* were evaluated in ICR mice transplanted H22 hepatoma cells and determined a significant inhibition on the growth of tumor, promoted splenocytes proliferation and macrophage phagocytosis, and increased the level of IL-2 and CD8+ T cells in blood of tumor-bearing mice (Fan *et al*. 2012).

MATERIALS AND METHODS

Sample collection

All of marine macroalgae were collected in approximate 0.5- 1kg wet weight quantities from the coast of Urla, İzmir, Turkey, transferred to laboratory in labelled plastic bags placed in cooled containers. Sample groups traditionally known as Chlorophyta, Rhodophyta and Phaeophyta. They were identified to genus or species levels based on their morphology and kept at -20°C until experiments.

Extraction procedure

Wet tissues from each of the 13 collected species were initially lyophilized, and 1g of each extracted with 10 ml of methanol for four days at +4°C. The obtained extracts were centrifuged at 4000 rpm, and supernatants were filtered by Whatman No1. Rotary evaporator was used for removal of solvent. Crude extracts stored at - 20°C and dissolved in methanol (1 mg crude extract per ml) for antioxidant activity assay or DMSO (1, 10 and 100ug ml^{-1}) for cell growth assay.

Determination of Total Phenolic Content

The total phenolic contents of macroalgae were determined according to the method of Marinova *et al*. (2005) with minor modifications. The Folin- Ciocalciteu assay was modified for 96- well plate. Methanol extracts were introduced into 96-well plate containing 90 µl of deionised water, added 10 µl of 2N Folin–Ciocalteu's reagent. Plates were incubated for 5 minutes at room temperature. After incubation, 100 µl of sodium carbonate (7%) was added. Plates were was mixed and allowed to stand for 90 min in the dark at room temperature. The absorbance was measured at 750 nm using UV–vis spectrophotometer. All samples were repeated three times in each plate and average absorbance values were used to evaluate results. A calibration curve of gallic acid (ranging from 0.010 mg ml^{-1} to 200 mg ml^{-1}) was prepared, and the results were determined by the regression equation of the calibration curve, were expressed as gallic acid (GA) mg equivalents.

DPPH scavenging activity assay

The free radical scavenging activity was measured by using the method of Sharma and Bhat (2012). 100 µl of 50 µM DPPH (2,2-diphenyl-1-picryl-hydrazyl) solution was added into 96- well plates including 100 µl of sample solutions and 100 µl of standard solutions. The decline in absorbance was recorded at 520 nm against a methanol blank over a

period of 1 h. 0.010 mg ml^{-1} of ascorbic acid was used a positive control. Percentage of inhibition DPPH free radical scavenging activity was expressed as percentage inhibition from the given formula:

$$\% \text{ inhibition of DPPH free radical} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100$$

Cell Culture

MCF-7 (a widely used breast cancer cell line) cells were grown in RPMI-1640 medium with l-glutamine 10% fetal bovine serum and 1% penicillin/streptomycin supplementation, and incubated in 37°C with 5% CO_2 and humidified atmosphere.

Cell Growth Assay

Trypan blue cell viability assay was used to determine the effect of extracts on cancer cell growth. Briefly $5x10^4$ cells (MCF-7) were seeded onto each well of 6-well plates. After 48 hours of incubation for both cell attachment and growth, three different concentrations (1µg ml^{-1}, 10 µg ml^{-1} and 100µg ml^{-1}) of PPME extract were applied to cells (maximum DMSO concentration was 1%). After 24 hours of incubation, treated and untreated cells were collected by trypsin digestion, treated with trypan blue (1:1) and counted by using neubauer cell counting chamber. Each sample was studied in duplicate and each duplicate were counted at least for 4 times. Untreated cell viability was considered as 100 % and treated cell viabilities were calculated accordingly.

RESULTS

Sample Collection and Extraction procedure

Macroalgal species and their extraction efficiency were listed in Table 1.

Table 1. Species and extraction efficiency.

Phylum	Species	Efficiency (Crude extract mg per lyophilized tissue g)
Brown macroalgae	*Scytosiphon lomentaria*	15.66
	Dilophus spiralis	11.58
	Petalonia fascia	8.00
	Padina pavonica	9.00

Table 1. Continued.

Green macrolgae	*Chaetomorpha aerea*	7.86
	Ulva rigida	7.10
	Enteromorpha sp.	9.50
	Codium fragile	40.45
Red macroalgae	*Laurencia pinnatifida*	23.33
	Acanthaphora nayadiphormis	12.50
	Gracilaria gracilis	8.69
	Polysiphonia variegata	24.59
	Gelidium pulchellum	10.00

Determination of Total Phenolic Content

The total phenolic contents of 13 macroalgal extracts investigated in this study are presented in Figure 1. The content of phenolic compounds varied from 42.5 (*Codium fragile*) to 215.2 mg (*Gracilaria gracilis*) gallic acid equivalent (GAE) per gram dry macroalga powder. The gallic acid equivalence of the extracts of *Gracilaria gracilis, Polysiphonia variegata, Padina pavonica, Scytosiphon lomentaria, Petalonia fascia* and *Laurencia pinnatifida* were higher than 109 mg GAE per gram crude extract. These species are the members of Phaeophyta and Rhodophyta.

DPPH scavenging activity assay

The stability of DPPH in the absence of ascorbic acid gave no significant change in absorbance at 520nm. The system showed a total stability within first 30 min, with some samples assayed for a longer time (120 min). But all samples and ascorbic acid dilutions practically showed the activity properly at the end of the first 60- min incubation. For longer incubations than 120 min, ascorbic acid did not show any change in absorbance. *Laurencia pinnatifida, Padina pavonica* and *Scytosiphon lomentaria* showed the higher DPPH free radical scavenger activities than 0.010mg ml^{-1} of ascorbic acid (70.9, 70.1 and 57.6, respectively). It is noteworthy that *Padina pavonica* exhibits very high free radical scavenging activity as well as total phenolic content.

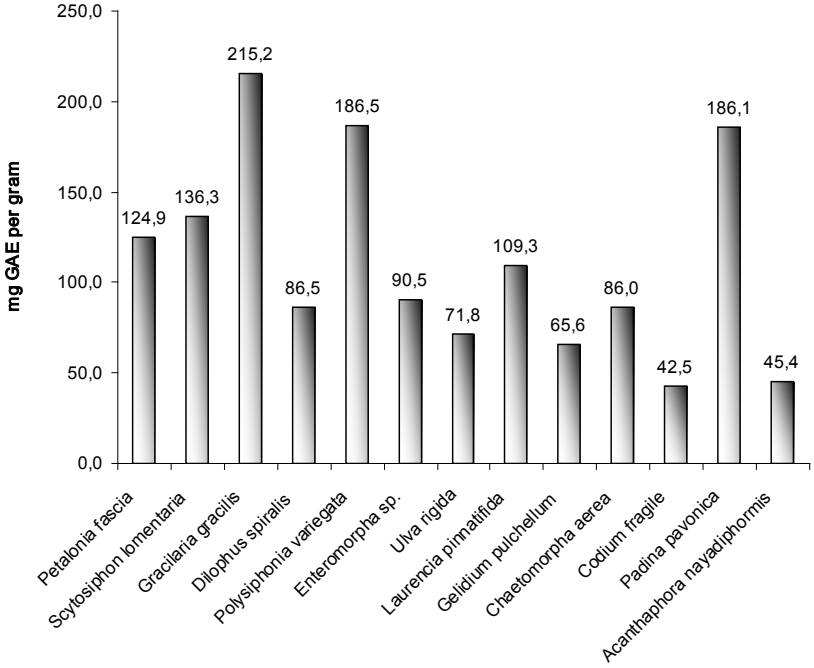

Figure 1. Total phenolic content of macroalgae.

Inhibiton of MCF-7 Cell Growth

100µg ml^{-1} PPME extract inhibited ~30% cell growth in a period of 24 hours. On lower concentrations extract did not show any growth inhibitory effects. On the contrary 1µg ml^{-1} of the extract induced ~25% increase in growth. Microscopic observations suggest no visible change in MCF-7 cell morphology (i.e.: no apoptotic cells were observed) which hints growth arrest instead of cell death.

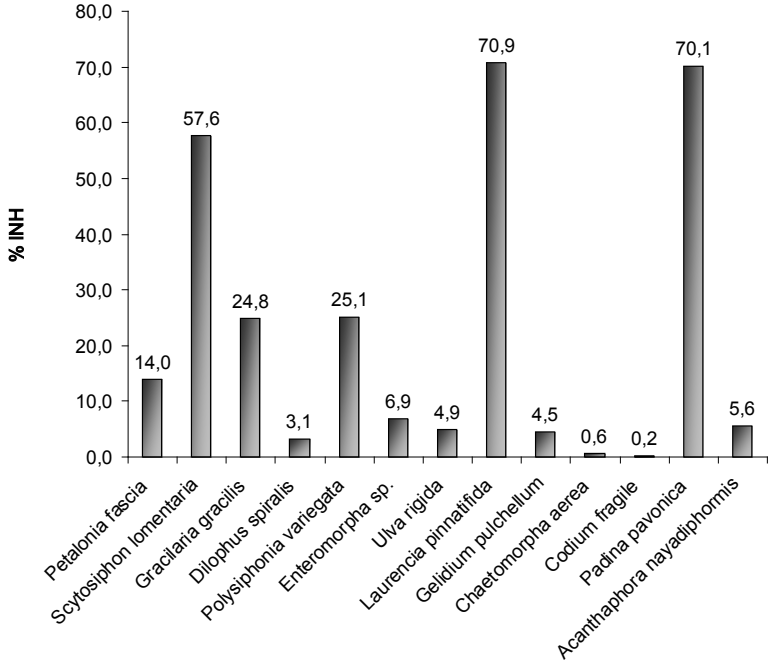

Figure 2. Inhibition of DPPH radical.

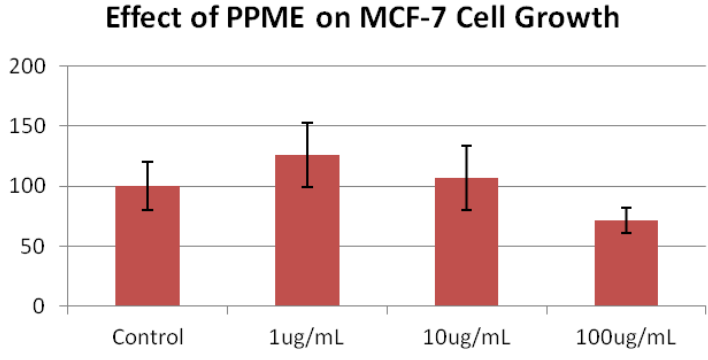

Figure 3. Dose dependent growth inhibitory effect of PPME extract on MCF-7 cells. Each column represents the average value of all cell counts. Error bars represent calculated standard deviation.

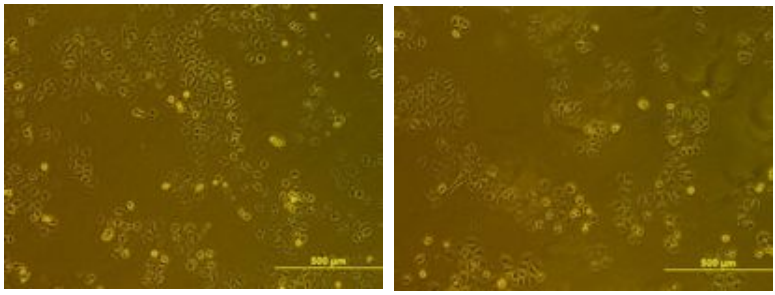

Figure 4. Effect of PPME on MCF-7 cells. PPME extract reduced the number of total cells without effecting the morphology of cells which points out a growth slowing effect rather than cell death.

DISCUSSION

Investigation of aquatic habitats has led to the discovery some new antioxidant- rich sources such as marine algae (Pangestuti and Kim 2011; Sokolova *et al*. 2011; Ngo *et al*. 2011). In addition to species- based researches (Yuan *et al.* 2005; Sokmen *et al*. 2005; Wang *et al.* 2010; Ananthi *et al*. 2010), many studies have include a multi-species set or a group belong to certain taxonomical level (Heo *et al.* 2005; Yuan and Walsh 2006; Zubia *et al.* 2007; Zubia *et al*. 2009; Costa *et al*. 2010; O'Sullivan *et al*. 2011). Because of this major importance, in the present study, we focused on antioxidant activities of some marine macroalgae from Urla. Methanol extraction is very suitable and commonly used method to extracts macroalgae. Due to its features as a solvent, also suitable for antioxidant activity assays (Matanjun *et al*. 2008; Koz *et al*. 2009; Kang *et al*. 2010; O'Sullivan *et al*. 2011). As a beginning study, we preferred to carve out a small experiment set.

All macroalgae subjected to total phenolic content assay showed high GAE ratios. When results evaluated in order to taxonomical grouping of macroalgae, the brown algae was the group which had the highest mean total phenolic content among collected macroalgae, followed by the red algae, and green algae. Same situation was observed for DPPH free radical scavenger activity. Despite the presence of high variations in the same taxonomical group, the averages of DPPH free radical scavenger activity for the brown algae, red algae and green algae showed the same with the averages of the result of total phenolic content assay. It is suggested the effect of the extraction conditions varies from group to group, due to their cell wall structures such as polyscchacharides or these groups already produce different substances in different amounts. Given

the knowledge about the anatomy and biochemistry of macroalgae to date, both cases could be valid.

Antioxidants are protective molecules against oxidant damage and shown to be protective potential against various clinical conditions such as obesity (Lee *et al*. 2011), diabetes (Pasupathi, Chandrasekar and Kumar 2009) and cancer (Mayer and Gustafson 2008; Martín-Algarra *et al*. 2009; Shilabin and Hamann 2011). When antioxidant is in question, two situations are very important. The first is the prevention of healthy cells, and the second is the inhibition of cancerous cell growth. For the prevention of cancer, it has been reported that antioxidant levels are extremely important as they represent direct removal of free radicals (pro-oxidants), thus providing maximal protection for biological sites. In addition, a good antioxidant should: (i) specifically quench free radicals; (ii) chelate redox metals; (iii) interact with (regenerate) other antioxidants within the "antioxidant network"; (iv) have a positive effect on gene expression; (v) be readily absorbed; (vi) have a concentration in tissues and biofluids at a physiologically relevant level; (vii) work in both the aqueous and/or membrane domains (Valko *et al*. 2006), so this is a good defense mechanism, but not express a therapeutic feature. The same antioxidants can be harmful for cancerous cells. This is because, some of the chemotherapeutic drugs induces ROS levels and lead to apoptosis in cancer cells (Brea-Calvo *et al*. 2006). Studies in last years regard as an antioxidant effect (prevention) is as if a kind of inhibition potential (Zha *et al*. 2012; Athukorala *et al.* 2006; Valko *et al*. 2006; Yuan and Walsh 2006; Zubia *et al*. 2009). Researches must be considering antioxidant and anti-cancer properties, individually. This tendency probably derived from the organism selection for an antiproliferative experiment in order to their antioxidant activity, or these antioxidant substances neutralize free radicals which are produced during progressive cell growth, and act as a protector for healthy cells. This nature of the oxidative stress mechanism is not clearly understood.

In our experiments, *Padina pavonica* had the high total phenolic content (186.1 mg GAE per gram) and DPPH free radical scavenger activity (inhibited 70%). These results indicated the antioxidant substances which neutralize free radicals. In the cell growth assay, 100ug ml^{-1} of PPME inhibited the growth of MCF-7 cells up to 30%. These effects can be intensified by increasing the incubation time. The most important handicap of cell growth assays extracts is the DMSO and the solubility of crude extract. Higher doses of crude extracts could have been shown higher inhibition ratios, but the solubility of crude extracts especially is a limit, even for DMSO, and using the suspensions is illusive. As expected, on lower concentrations extract did not show any

growth inhibitory effects. Microscopic observations suggest no visible change in MCF-7 cell morphology (i.e.: no apoptotic cells were observed) which hints growth arrest instead of cell death. Although it is necessary that re-evaluated and re-fined the treatment dose of macroalga extracts, these results is still promising.

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Chlorella vulgaris Continuous Culture and Lipid Content in Photobioreactor System

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ABSTRACT

The reason why the desired industrial production was not achieved across the world in using microalgae as a source of lipid is that, despite its other advantages the cost of algae production is high. This project aimed to produce *C. vulgaris,* which is easy to culture with not much cost and whose outdoor culture was proved practical, by increasing its lipid content. The study was conducted at the Algal Biotechnology Laboratory Pilot Plant of Fisheries Faculty, Cukurova University. Outdoor production was realised in the photobioreactor at the pilot plant.

In this study, *C. vulgaris* was cultured continuously at photobioreactor in the medium nitrogen-free with flow rate of 0.3m/s and inoculation quantity of 50%. At the end of the study, lipid and protein contents and biomass productivity were determined.

At the end of the experiment, the optical density and dry weight were determined as 0.9138±0.004 and 0.236gL^{-1}, respectively. The highest total lipid ratio of 21.65±0.7% and 15.85±0.6% protein content was recorded. At the same time, in the study, N limitation was caused to fall the optical density and biomass quantity.

INTRODUCTION

Microalgae, photosynthetic microorganisms, are able to use the solar energy combining water with carbon dioxide to create biomass. Many countries study about microalgae lipid for the biodiesel sources in recent years. Microalgae can be cultured throughout the year, have a simple reproducing system, use the water most effective, do not need rich soil, as a source of lipif for biofuels has increased the interest in.

Chlorella species are eukaryotic, unicellular, non-motile freshwater green algae that belong to the Division Chlorophyta (Kay 1991) *Chlorella* cells have hemicellulotic cell walls and are spherical with a diameter ranging from 2 to the 10 μm (Kay 1991; Becker 2007).

Beginning in the late 1940's, *Chlorella* was investigated for its possible wide-scale production and use for nutritional purposes, including as a source of protein, lipids, carbohydrates, vitamins, and minerals to help fill the "protein gap" and feed an ever expanding world population (Becker 2007).

Hundreds of microalgal strains capable of producing high content of lipid have been screened and their lipid production metabolism have been characterized and reported (Sheehan *et al*. 1998).

Several studies have shown that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions, such as temperature and light intensity, or nutrient media characteristics, concentration of nitrogen, phosphates, and iron (Illman *et al*. 2000; Liu *et al*. 2008).

The study was conducted at the Algal Biotechnology Laboratory Pilot Plant of Fisheries Faculty, Cukurova University. Outdoor production was realised in the photobioreactor at the pilot plant. In this study, *C. vulgaris* was cultured continuously in the photobioreactor in non-nitrogen nutritive medium and lipid, protein and biomass rates were determined at the end of the experiment.

MATERIALS AND METHODS

Microalga *Chlorella vulgaris* cultures were kept at a constant room temperature of 18±2 °C and illuminated with fluorescent lamps (Phlips TLM 40W/54RS) at an irradiance level of 80 μmol/m^2/s. The irradiance was measured by a Radiation Sensor LI-COR (LI-250). The microalgae stock culture were grown in 8 L glass jar in a batch culture system and the culture was continuously stirred by air. For inoculation to the photobioreactor, 140 liters of algae were cultured in laboratory.

The cultures were grown in Jaworsky medium. The content of the medium consists of the following composition (g/200 ml):4 $Ca(NO_3)_2.4H_2O$, 2.48 KH_2PO_4, 10 $MgSO_4.7H_2O$, 3.18 $NaHCO_3$, 0.45 EDTA FeNa, 0.45 EDTA Na_2, 0.496 H_3BO_3, 0.278 $MnCl_2.4H_2O_2$ 0.2 (NH_4) $6Mo7O_{24}.4H_2O$, 16 $NaNO_3$, 7.2 $Na_2HPO_4.12H_2O$ were added 1 ml to 1L.

Large volumes of culture were produced in the photobioreactor. Tubular photobioreactor system volume is 280 liters. Photobioreactor system, were installed horizontally. The diameter of the transparent acrylic tubes was 2.6 cm. The culture flow rate was 0.3m/s. The collection tank of about 300 L was formed for culture collecting chamber. CO_2 gas inlet was provided with flowmeter. pH and temperature was mesaured continuously, light intensity was measured 3 times a day. *C. vulgaris* cell concentration was determined daily by optical density measurements at

500 nm by a UV–vis spectrophotometer (Liu *et al*. 2008). Dry weight was determined by the filtering of different volumes of algal culture through Whatman GF/C glass fibre. Algae biomass was dried at 105 °C for two hours and weighed (Boussiba *et al*. 1992). Chlorophyll *a* (chl a) content of the algae was determined spectrophotometrically after extraction with acetone (Parsons and Strickland 1963). All measurements were made with three replications.

For lipid and protein analyses, samples of microalgae were collected from stationary growth phase. *C. vulgaris* cells were separated from the medium by centrifugation at 7500 rpm for 10 min, using the centrifuge model Hereaus. Biomass was dried at 55 °C for 2 h, pulverized in a mortar and stored at -20°C for later analysis.

Dry extraction procedure according to Zhu *et al*. (2002) as a modification of the wet extraction method by Bligh and Dyer (1959) was used to extract the lipid in microalgal cells. Typically, cells were harvested by centrifugation at 7500 rpm for 10 min. After drying the samples using overnight, the samples were pulverized in a mortar and extracted using mixture of chloroform: methanol (2:1, v/v). About 120 mL of solvents were used for every gram of dried sample in each extraction step. The solid phase was separated carefully using filter paper (Advantec filter paper, no. 1, Japan) in which two pieces of filter papers were applied twice to provide complete separation. The solvent phase was evaporated in a rotary evaporator under vacuum at 60ºC. The procedure was repeated three times until the entire lipid was extracted. The effect of solvents having different polarities for extracting the lipid, as well as the effect of drying temperature and ultrasonication time were investigated in this study.

Total protein was determined by Kjeldahl method (AOAC 1998). Depending on the results obtained in the evaluation of research data, the comparison of averages, the t-test was conducted using the program package SPSSX 10.0 (Zar 1999).

RESULTS AND DISCUSSION

It was provided that the maximum optical density was reached to use the *C. vulgaris* continuous culture in photobioreactor system in Jaworsky nutrient medium. The experiment was created as a control group. Culture was reached the maximum optical density of 8 days. While the initial optical density 0.3336 ± 0.001, last day of optical density 1.1341 ± 0.006. Likewise, while the initial and last day dry weights were 0.074 ± 0.002, 0.304 ± 0.04 gL^{-1}, respectively. At the end of the trial, total lipid and protein ratio were determined as 12.34±0.4 and 50.79±0.8 %, respectively (Table 1).

After reaching the desired density of culture, the continuous culture study was carried out with 50% inoculation and %10 dilution ratio in nitrogen free medium. Daily during the trial period the same amount of 28.8 L of culture medium were harvested and the addition of nitrogen-free medium are made. Harvested culture daily, in both total lipid and protein percentages were determined by drying. The experiment continued to 11 days. To the experiment was started with 1.1341±0.006 of optical density and 0.304±0.04 gL^{-1} of dry weight. However, optical density and dry weight values declined. At the end of the experiment, the values of 0.9138± 0.004 optical density and dry weight of 0.236 ± 0.01 gL^{-1} were determined (Table 1).

Table 1. The total lipid, biomass, optical density and protein contents of *C. vulgaris*.

	Control group	Continuous culture with N-free medium
Biomass (gL^{-1})	0.304±0.04	0.236±0.01
Optical density	1.1341±0.006	0.9138±0.004
Total lipid (%)	12.34±0.4	21.65±0.7
Total protein (%)	50.79±0.8	15.85±0.6

While the starting chlorophyll *a* and total carotene were 1.5220±0.06 and 0.9577±0.05 µg/ml respectively, these amounts declined. At the end of the experiment, chlorophyll *a* and total carotene amounts were determined as 0.3217± 0.009 and 0.4462±0.007 µg/ml, respectively (Figure 1).

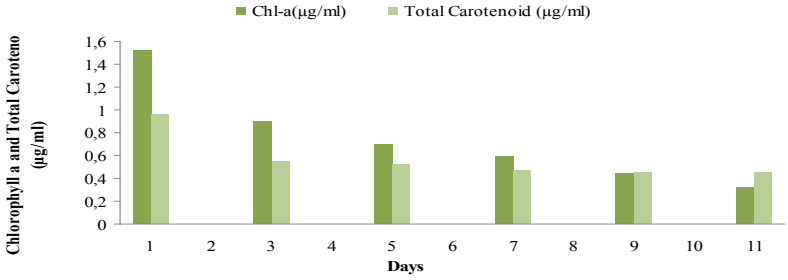

Figure 1. The chlorophyll *a* and total carotene values of *C. vulgaris* continuous culture in tubular photobioreactors.

The temperature of 28-31 ºC and light intensity of 1024-1297 µmol $m^{-2}s^{-1}$ photon were recorded in continuous culture in tubular system. On the last day of the experiment, the highest total lipid ratio of %21.65±0.7

was recorded. When the last day with 15.85±0.6 protein content was identified (Figure 2 and 3).

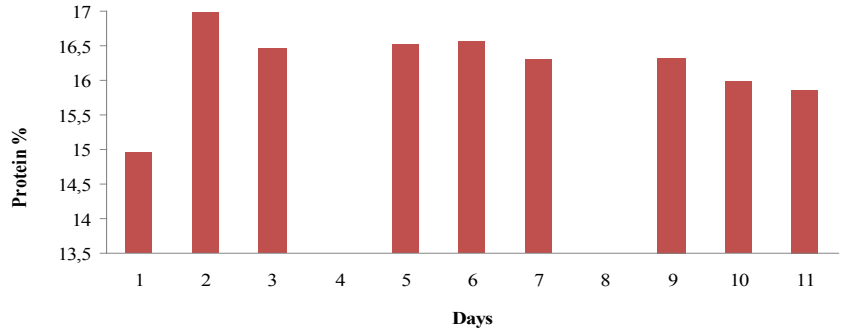

Figure 2. Protein contents of *C.vulgaris* cultured continuously in tubular photobioreactor system.

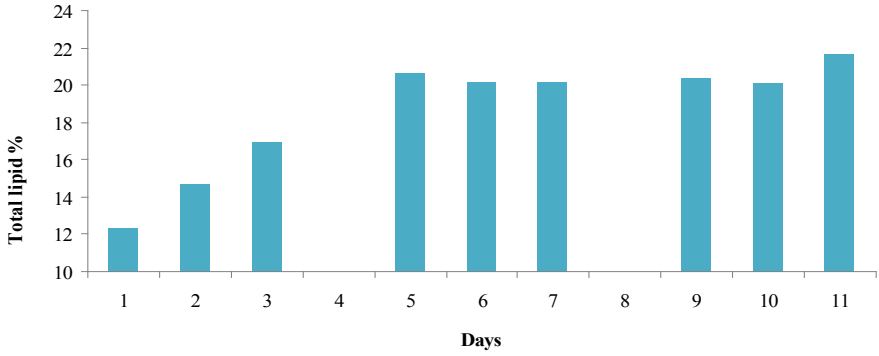

Figure 3. Total lipid contents of *C. vulgaris* culture continuously in tubular photobioreactor system.

There are various types of difficulties in microalgae species cultures although the developments of algal biotechnology. The main purpose of the production of phototrophic organisms in a continuous culture in general is to provide an optimal optical density. During an algal culture was carried out outdoor, to the environment factors showed great changes in both daily and seasonally it is necessary that the cells in the culture to react and suit. Growth factors as environmental factors, nutrient medium, temperature, salinity, pH, light can affect the biochemical composition of the biomass (Sukenik 1991; Cohen *et al*. 1988; Brown *et al*. 1989; Roessler 1990; Lourenco *et al*. 2002; Hu 2004). In this study, *C. vulgaris*

was cultured continuous in nitrogen-free media in photobioreactor on a large scale at the pilot plant of Algal Biotechnology Laboratory, Fisheries Faculty, Cukurova University.

It is known that different N sources and concentrations are effective on the growth and biochemical structure of microalgae (Gökpınar 1991; Fidalgo *et al*. 1995). Depending on the lipid content of algae amount of biomass obtained is also important. The biomass decrease in the cultures to applied nitrogen deficiency, generally. Bulut Mutlu *et al*. (2011) were cultured *C. vulgaris* in the medium N is enough and deficient. The researchers reported that the biomass amount was the lower at the medium N deficient. In similar studies it was reported that the nitrogen limitation was caused to decrease of optical density and biomass (Kilham *et al*. 1997; Pruvost *et al*. 2009). In this study, N limitation was caused to fall the optical density and biomass quantity.

The application of nitrogen limitation, increase the lipid ratio of many species. The nitrogen limitation slowed down the growth. However increased the lipid ratio were determined to many microalgae species as *Dunaliella tertiolecta*, *N. doeabundas*, *I. affinis galbana*, *B. braunii*, *D. salina*, *Nannochloropsis* sp, *N. oculta, C. vulgaris*, *P. tricornutum, Chaetoceros* sp., *I. galbana, P. lutheri, N. atomus, Tetraselmis* sp., *Gymnodinum* sp, *H. pluvialis* and *S. platensis* (Fabregas *et al*. 1989; Tornabene *et al*. 1983; Sukenik and Wahnon 1991; Zhila *et al*. 2005; Weldy and Huesemann 2007; Rodolfi *et al*. 2009; Converti *et al*. 2009; Reitan *et al*. 1994; Damiani *et al*. 2010; Bulut *et al*. 2011; Uslu *et al*. 2011). In this study, nitrogen limitation slowed down the growth rate, however increased the cellular lipid, similarly. But the important thing, to evaluate the increased lipid content economically is to obtain the reasonable biomass amounts.

In another study at which the effects of nitrogen limitation on the metabolits were studied, the decreased of optical density and chlorophyll *a* quantity were determined while the increasing of organic carbon compounds as lipid was recorded. However, yellowing of colors was observed due to increasing carotene quantity in the cultures (Shifrin and Chisholm 1981; Sukenik *et al*. 1989). In this study, chlorophyll *a* amount decreased and yellowing of colors were determined.

This study aims to increase the amount of both oil and biomass. The lipid values showed increase from 12.34% to 21.65%. However, it wasn't observed important decrease of algae biomass. It is recommended that industrial scale of cultures should be tried considering the economic analysis.

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Applications of Microalgae for Tissue Engineering

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ABSTRACT

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences to the development of biological substitutes that restore, maintain or augment tissue function. Biocompatible scaffolds are among the main components of tissue engineering. They provide three dimensional mechanical support and reinforcement for the cells while maintaining a surface for cell attachment and regulating the interactions of the cells with their extracellular environment.

Nanofibers originating from natural polymers have recently drawn increasing attention for their applications in the biomedical sector including tissue engineering scaffolds. Natural polymers possess biocompatibility and may contain domains that regulate cellular functions and development. Although microalgae have great potential for use in tissue engineering scaffolds both by themselves or as a reinforcement material in other biopolymers, there is very limited literature on their use in this field. In this presentation, we will give a brief overview of current state of art in the area, and give examples of our experience with use of microalgae in tissue engineering scaffolds, including their effects on cell viability and cell surface interactions.

Keywords: Tissue engineering, scaffold, microalgae, biocomposites.

INTRODUCTION

Spirulina, an important alkali water alg, was first discovered by Aztecs. Mayans had cultivated it in irrigation lines at 300-900 AD (Capelli *et al.* 1993). It was found that Calcium Spirulan, a polysaccharide isolated from *Spirulina* inhibits replication of enveloped viruses like *Herpessimplex* type 1, human cytomegalovirus, measles, mumps, influenza A and HIV-1(Hayashi *et al.* 1996, Conk-Dalay *et al.* 2008; Ozdemir *et al.* 2004).

It is a new strategy to deliver growth factors and cytokines within tissue engineering scaffolds to improve cell-scaffold interactions, tissue integration, proliferation, differentiation, etc. in tissue engineering applications (Hammond *et al.* 2011, Miyagi *et al.* 2010). Crude *Spirulina platensis* extract at 1μg.ml^{-1} and higher concentrations were proved to be cytocompatible with cell lines like L-929, MDCK, SW-480 (Deliloğlu-Gürhan *et al*. 2007). Also, it has been shown that microalgae like *Chlorella*, *Scenedesmus* and *Spirulina*, when added to the culture media, reduces the serum requirements of several human cell lines from 10% to 1%. In a very recent work by *Spirulina* LEB 18 biomass in different concentrations have been added to polyethylene oxide solutions, and highly porous, nanofibrous surfaces suitable for tissue engineering have been produced by electrospinning (Morais *et al.* 2010). All these results show that microalgae have great potential in improving cell-surface interactions and cellular viability of tissue engineered constructs as a supplement to different growth factors, or even alone as tissue engineering scaffolds.

We have used chitosan, a natural polysaccharide produced from chitin (Klekkevold *et al.* 1999, Madihally *et al.* 1999, nettles *et al.* 2002, Kim *et al.* 2008, Feng *et al.* 2007), as the scaffold material in which different concentrations of *Spirulina* were added. Cell – scaffold interactions, cellular viability and mechanical properties were tested.

MATERIALS AND METHODS

Spirulina production

Spirulina platensis (cc27) cells were provided from Ege MACC (Ege Mikroalg Culture Collection), and cultured at Zarrouk media. Spirulina cultures were seeded in 200 mL media in 250 mL flasks at a concentration of 0.014 g.L^{-1} (dry weight). Spirulina flasks were placed in shakers at 90 rpm for 360 hours.

Production of tissue engineering scaffolds

Scaffolds were produced by freeze drying. 2% chitosan was dissolved in 0.2 M acetic acid. Given concentrations (0, 2, 4, 8, 10%) of microalgae were added to chitosan solutions and vortexed. Prepared solutions were molded in 5 mm diameter, 10 mm legth cylinders, frozen at -20^{o}C, and lyophilized for 3 days (Sendemir Urkmez *et al.* 2007).

Mechanical characterization of scaffolds

Mechanical characterization was performed by uniaxial tension test in physiological PBS buffer (Xia *et al.* 2008).

Culture of epithelial cells

Epithelial cells were provided from HÜKÜK (Turkish Institute of FMD Cell Culture Collection), and cultured in minimum essential medium (MEM) supplemented by 10% FBS.

Cell seeding and evaluation of cell viability

Cell were seeded onto scaffolds by drop-on technique. Cell viability were characterized by spectrophotometric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] technique.

RESULTS AND DISCUSSION

Increase of tensile strengths and elastic moduli were observed by increased microalg amounts (Figures 1 and 2).

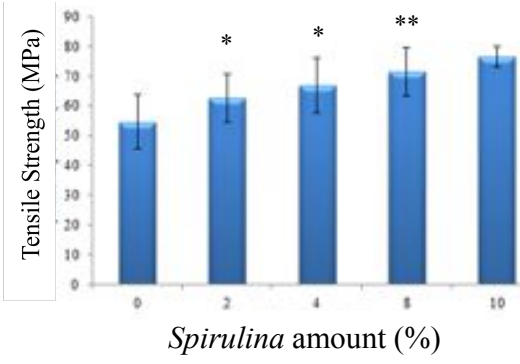

Figure 1. Tensile strength of scaffolds ($p<0.05$)

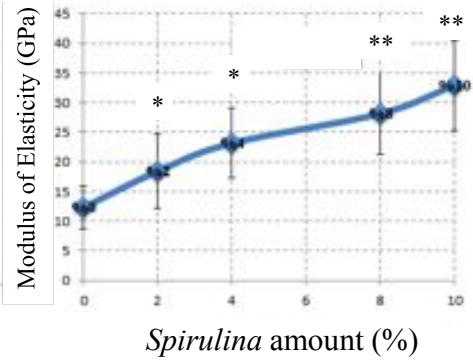

Figure 2. Elastic moduli of scaffolds ($p<0.05$)

2 and 4 % *Spirulina* addition was shown to improve cell viability on scaffolds (Figure 3).

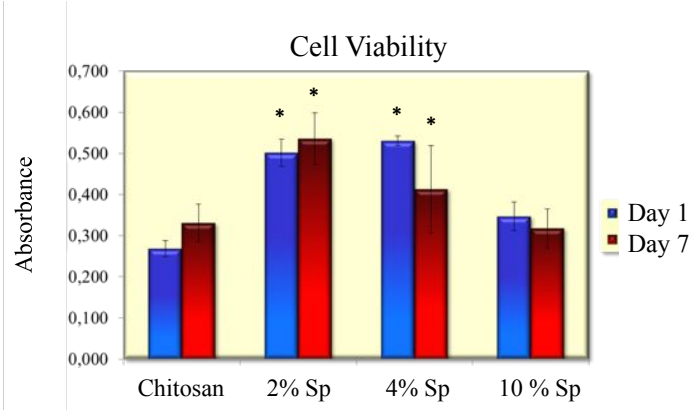

Figure 3. Cell viability on scaffolds measured by MTT ($p<0.05$).

There is no "golden standard" material for tissue engineering scaffolds, although several materials like hyalouronic acid, polylactide, hydroxyapatite are still being investigated. One of the main problems faced is mechanical stability within the body, among biocompatibility issues. Our preliminary results show that microalgae addition is a promising method for improving mechanical properties as well as cell attachment and viability in scaffold materials. Various other matrices and microalgae will be tested in different combinations to facilitate microalgae for tissue engineering applications.

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Biochemical and Pharmacological Researches on Turkish Marine Algae

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ABSTRACT

Investigations on systematic, chemistry and pharmacology of marine macroalgae in Turkish coasts have been conducted by various Turkish authors. In this review, the research on marine algae was listed as chemical, pharmacological and the use of algae in food and medicine and monitoring of pollution.

INTRODUCTION

Marine macroalgae in Turkish coast were investigated systematical, chemical and pharmacological by various Turkish authors. The first systematical study was made by Diratzuyan in 1894-1895 (Öztığ 1971), later Fritsch in 1899 and Handel-Mazetti in 1909. A long period after by Zeybek in 1966, Ünal in 1968-1970, Güven in 1962-1970 (Güven 1970), Aysel and Güner in 1983 and 1986. Reviews on Turkish marine algae were published by Güven and Öztığ (1971); Güven *et al*. (1991), and in book of FAO /CEE contributed by Güven (1987).

The first chemical study was made by Güven *et al.* in 1969 and pharmacological Güven and Aktin 1961 (Güven and Aktin 1962).

In this paper, the research on marine algae was listed in three parts as:

1. Chemical
2. Pharmacological
3. Use of algae in food and medicine and monitoring of pollution

Chemical Research
Alkaloids

Hordenine (*4-(2-dimethylaminoethyl)phenol*)
The first marine alkaloids isolated from *Phyllophora nervosa* in 1969 (Güven *et al.* 1969, 1970) and *Gelidium crinale* (Yalçın *et al*. 2007). The amount of Hordenine was determined also in *Phyllophora nervosa* (Percot *et al.* 2007).

Phenylethylamine

Phenylethylamine was isolated from the algae are (Güven *et al.* 2010): *Desmerestia aculeata*, *D. viridis*, *Ceramium rubrum*, *Cystoclonium purpureum*, *Delesseria sanguine*, *Dumontia incrassata*, *Polysiphonia urceolata*, *Polyides rotundus*, *Gelidium crinale*, *Gracilaria bursa-pastoris*, *Halymenia floresii*, *Phyllophora crispa*, *Polysiphonia morrowii*, *P.tripinnata*.

Loliolide

Loliolide was isolated from the algae are (Percot *et al.* 2009): red; *Phyllophora crispa*, *Boergeseniella fruticulosa*, *Polysiphonia morrowii*, *Gelidum crinale*, *Hypnea musciformis*, *Corallina granifera*, *Halymenia floresii*, brown; *Taonia atomaria*, *Cutleria multifida*, *Dictyota dichotoma*, *Cystoseira mediterranea*, *Sporochnus pedunculatus*, green; *Enteromorpha compressa.*

Protein and Amino acid

Isolated protein and determined amino acids content of algae are: *Cystoseira barbata* (Güven and Bergişadi 1973; Çetingül *et al.* 1995; Aysel *et al.* 1992), *Sargassum vulgare* (Güven and Bergişadi 1973), *Cystoseira corniculata* (Güven and Güven 1975), *Phyllophora nervosa* (Güven and Bergişadi 1980), *Sypocaulon scoparium*, *Colpomenia sinuosa*, *Dictyopteris membranacea* (Aysel *et al.* 1992); *Rytiphloea tinctoria*, *Digenea simplex*, *Laurencia papillosa*, *Peyssonnelia crispata*, *Hypnea musciformis* (Çetingül *et al.* 1994); *Gracilaria verrucosa*, *Acanthophora najadiformis*, *Hypnea musciformis*, *Halopitys incurvus*, *Halopteris scoparia* (Çetingül *et al.* 1995), *Pterocladia capillacea* (Güven and Güler 1979).

Vitamin B_{12}

Vitamin B_{12} determined algae are: *Corallina rubens* (Güven *et al.* 1975), *Gelidium capillaceum* (Güven *et al.* 1976), *Cystoseira crinita* (Güven *et al.* 1980), *Enteromorpha linza, Cystoseira corniculata*, *C. crinita*, *Dictyopteris membranacea*, *Halopteris scoparia*, *Acanthophora delilei*, *Digenea simplex*, *Gigartina teedii*, *Gracilaria confervoides*, *Grateloupia dichotoma*, *Halophytis incurvus*, *Liagora farinosa*, *Polysiphonia subulifera*, *Rytiphlaea tinctoria* (Güven *et al.* 1982).

Sterols

Sterols isolated algae are: *Cystoseira barbata* (Güven and Bergişadi 1973), *Corallina rubens* (Güven *et al.* 1975), *Cystoseira corniculata* (Güven and Hakyemez 1975), *Corallina granifera* (Güven and Bergişadi 1975), *Pterocladia capillacea* (*Gelidium latifolium*) (Güven and Güler

1979), *Cystoseira crinita* (Güven *et al.* 1980), *Dictyopteris membranecea* (Güven and Kızıl 1983), *Gracilaria verrucosa* (Aydoğmuş *et al.* 2008).

Bromo compounds
Bromo compounds isolated from the algae are: *3-bromo-4,5-dihydroxybenzaldehyde* from *Halopitys incurvus* (Wagner *et al.* 1981), *Laurencia obtusa* (Imre *et al.* 1981), polybrominated nonterpenoid C15 from *Laurencia paniculata* and *L. obtusa* (Imre *et al.* 1995), secondary metabolite from *Laurencia obtusa* (Imre and Aydoğmuş 1997; Aydoğmuş *et al.* 2004).

Fatty acids of lipids
Fatty acids of lipids obtained from algae are: *Ulva rigida*, *Enteromorpha linza*, *Codium fragile*, *Chaetomorpha linum*, *Cystoseira barbata*, *Colpomenia peregrina*, *Zanardinia prototypus*, *Peyssonnelia squamaria*, *Rhodymenia corallicola*, and *Phyllophora nervosa* (Yazıcı *et al.* 2007).

Volatile oils
Volatile oils obtained from algae are: *Phyllophora nervosa* (Güven *et al.* 1972), *Cystoseira barbata* (Güven and Bergişadi 1973), *Corallina rubens* (Çetingül *et al.* 1995), *Pterocladia capillacea* (Güven and Güler 1979), *Halopytys incurvus* (Güven and Kızıl 1985).

Cellulose
Cellulose content and paper preparation from algae are: *Chaetomorpha linum*, *Ceramium rubrum*, *Hypnea musciformis*, *Cystoseira barbata*, *Phyllophora nervosa* (Kıran *et al.* 1980).

Algal polysaccharides

Agar
Agar obtained algae are: *Phyllophora nervosa* (Güven *et al*. 1966; 1971; 1972; 1975; Güven and Güler 1979).

Alginic acid
Alginic acid obtained algae are: *Cystoseira barbata* (Güven and Bergişadi 1973), *Cystoseira corniculata* (Güven and Hakyemez 1975), *Cystoseira crinita* (Güven *et al.* 1980), *Dictyopteris membranacea* (Güven and Kızıl 1983).

Carrageenan
Carrageenan obtained algae are: *Acanthophora delilei* and *Grateloupia dichotoma* (Güven *et al.* 1984).

Identifications of algal polysaccharides types were made by metachromatic method for agar, alginic acid and carrageenan (Güven and Güvener 1985a, b), (Iota-Kappa-Lambda) Carrageenans (Güven and Güvener 1985b), agar and carrageenan type *LC* (Sur and Güven 2004).

Succinic acid

Succinic acid was obtained from *Acanthophora delilei* and *Grateloupia dichotoma* (Güven *et al.* 1984).

Pharmacological Research

Antitumor activity

Antitumor activity examined algae are: *Sargassum vulgare*, *Cystoseira barbata*, *Phyllophora nervosa* (Güven and Bergişadi 1985), *Enteromorpha compressa, Ulva lactuca, Sargassum linifolium, Ceramium rubrum, Corallina mediterranea, Grateloupia dichotoma, Pterocladia capillacea, Vidalia volubilis.* The highest KB cell line activities were found in the algae: *U. lactuca, C. barbata, G. dichotoma, and P. nervosa* (Ünlü *et al.* 1995). *Caulerpa racemosa, Codium bursa, Cystoseira barbata, C. crinita, Corallina granifera, Jania rubens, Ceramium rubrum, Gracilaria verrucosa, Dasya pedicellata, Gelidium crinale* (Süzgeç-Selçuk *et al.* 2011).

Antilipaemic, anticoagulant and antiaggregant activities

The examined algae are: *Phyllophora nervosa* (Güven and Aktin 1962b; *Güven et al.* 1972), *Cystoseira barbata* (Güven and Aktin 1964; Güven *et al.* 1974)*, Polysiphonia denudate, Corallina granifera* (Güven and Aktin 1964), *Sargassum vulgare, Polysiphonia subulifera* (Güven and Aktin 1965), *Corallina rubens* (Güven *et al.* 1973; Güven *et al.* 1975), *Pterocladia capillacea* (Güven *et al.* 1979), *Halopytys incurvus* (Güven and Kızıl 1986), *Gelidium capillaceum* (Akçasu *et al.* 1978), *Gracilaria verrucosa*, *Gelidium latifolium* (Aktin and Güven 1969).

The anticoagulant activity of carrageenan, alginic acid types and *Grateloupia dichotoma* were examined. Carrageenan showed anticoagulant activity but alginic acid not (Güven *et al.* 1991b).

Prostaglandin

Prostaglandin activity was examined in *Halopteris filicina* (Güven *et al.* 1985). A review was published on pharmacological activities of marine algae by Güven *et al.* (1990a).

APPLICATION OF ALGAL POLYSACCHARIDE IN INDUSTRY, FOOD AND MEDICINE

In food

Sources of algal polysaccharides used in food.

Agar

Agar obtained algae are: *Acanthopeltis japonica, Ahnfeltia plicata, Ceramium* sp*., Gelidium* sp*., Gracilaria* sp*., Pterocladia* sp. Manufacture of agar is manufactured by hot-water extraction followed by freezing and filtration. The freezing allows removal of the soluble salts. In summary, extraction, filtration, gelation, freezing, thawing, filtration, drying, bleaching, washing and drying.

Application in food: Icings, frozen foods, fruit juices, candies, dessert gels, canned meat, fish, poultry, cheeses, yogurt.

Alginic acid

Alginic acid isolated algae are listed below: *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Laminaria* sp., *Ecklonia cava*, *Eisenia bicyclis*, *Ectocarpus* sp., *Dictyota dichotoma*, *Dictyopteris polypodioides*, *Desmerestia aculeata*, *Cystoseira barbata*, *Sargassum linifolium*.

Manufacture of alginate were algae heat in water (alkali media), clarification, precipitation with calcium chloride solution, removal calcium alginate, treatment with acid, alginic acid precipitate, sodium carbonate addition, and separation of sodium alginate.

Application of alginate: frozen foods, pastry fillings, bakery icings, frozen desserts, gelling, emulsifying, salad, meat, stabilizing beer, fruit juice, fountain syrups, sauces, milkshakes, tomatoes, macaroni and cheese, food gels, dessert gels, cold water gels, cold milk gels,

Commercial use: Thickeners, formulation of paints, paper coatings, textile printings, textile dyeing, air-freshener gel, antifoams, ceramics, filling forming, dressing.

Carrageenan

Carrageenan obtained algae are: *Chondrus crispus*, *C. canaliculatus*, *Gigartina* sp., *Gymnogongrus norvegicus*, *Gloiopeltis coliformis*, *Iridaea* sp.

Manufacture extraction with water in alkaline medium, filtration. Carrageenans are separated with acid, after addition of isopropyl alcohol and carrageenan precipitate and separated and drying. Application: in chocolate, ıce cream and sherbet, cottage and cream cheese products, canned milk, whipping cream, yogurt, imitation milk, pizza, syrups, fish gels, puddings.

In medicine

Agar
Agar is most used in microbiology, agar is used as a suspending agent for barium sulfate in radiology, in prosthetic dentistry, dental molds, criminology, tool making, laxative.

Alginic acid
Alginate is used in dentistry, as impression material, fillers.

Carrageenan
Carrageenan water gels
Alginate and carrageenan can be used for clarification and retention of zinc and manganese from and coagulation of suspended matter in raw water (Güven *et al*. 1992a; 2006).

Monitoring of pollution
Marine algae were used for monitoring of sea water pollutions.

Metal pollution
Metal uptake in marine algae were investigated in Black Sea water (Güven and Topçuoğlu 1991; Güven *et al.* 1992b; 1992c; 1993a; Güven and Akyüz 1995; Güven *et al.* 1998; Topçuoğlu *et al.* 2001; Güven *et al.* 2007; Topçuoğlu *et al*. 2010).

Radioactivity pollution
Radionuclides accumulate from marine algae were also investigated in Black Sea and Sea of Marmara (Güven *et al.* 1990b; Topçuoğlu *et al.* 1990; Güven *et al.* 1993b; Küçükcezzar *et al.* 1994; Topçuoğlu *et al.* 1999).

Oil pollution
Oil pollution investigated marine algae were (Binark *et al*. 2000; Erakın and Güven 2008).

Phthalate
Phthalate pollution of algae are listed (Güven *et al.* 1990c; Gezgin *et al*. 2001).

CONCLUSION

In this paper were listed the research made on Turkish marine algae. The sources of algae are found in Turkish coast for preparation of agar, alginate, and carrageenan. *Gracilaria verrucosa* collected at İzmir and

İzmit Bays for agar production exports to Japan for agar production. The author hopes the industrial preparation of these algal polysaccharides in Turkey.

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Composition of Fat and Fatty Acids of the Mediterranean Gastropods and usage for Human Health

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ABSTRACT

Seasonal variations of lipids and fatty acids profiles (FA) of three Gastropods species (banded dye-murex *Hexaplex trunculus*, purple dye-murex *Bolinus brandaris,* and Persian conch *Conomurex persicus)* from the Mediterranean Sea were investigated. The results showed the fatty acid compositions of each species ranged from 26.89–32.84% saturated (SFA), 8.00–15.16% monounsaturated (MUFAs) and 43.53–53.11% polyunsaturated fatty acids (PUFAs). The high proportions of EPA were obtained from *B. brandaris* (13.96%) in summer, *H. trunculus* (13.67%) in spring and also *C. persicus* (11.28%) in summer. The high proportions of DHA were obtained from *B. brandaris* (10.68%) in summer, *H. trunculus* (12.43%) in spring and also *C. persicus* (18.55%) in autumn. The results showed that from a quality point of view, all species were suitable food for humans, since muscle lipids are rich in EPA+DHA for all seasons.

Keywords: gastropods, season, species, fatty acids, lipid

Abbreviations: AI, atherogenicity index; TI, thrombogenicity index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; FID, flame ionization detector; GC, Gas Chromatography

INTRODUCTION

All marine products, not only marine animals but also marine algea, characteristically have been well known that they accumulate considerable amount of *n*-3 polyunsaturated fatty acids (PUFA) in their cell membranes; especially, docosahexaenoic acid DHA (C22:6 *n*-3) and eicosapentaenoic acid (EPA, C20:5 *n*-3) are the main PUFA in their tissues (Ackman 1989; Morris and Culkin 1989; Kamal-Eldina and Yanishlieva 2002). These fatty acids have great importance for humans because there are positive health effects to consumption of them such as

infant development, arthritis, cardiovascular disease, platelet aggregation, auto-immune disease, hypertension, hyperlipidemia, cancer, dementia and Alzheimer's disease, depression, and inflammation (Hornstra 1999; McManus *et al*. 2009; Riediger *et al*. 2009; Ruxton and Derbyshire 2009). It has been reported that the mortality from coronary heart disease was also less in subjects who ate at least two servings of fish per week in comparison to people who did not eat fish (Dolecek and Grandits 1991; Kromhout *et al*. 2005; Kelley 1996). *n*-3 PUFA are also important for the development of the eye and brain (Kelley 1996; Haumann 1997). The beneficial effect of *n*-3 PUFA is mainly due to its effect on the fatty acid composition of cell membranes (increased levels of EPA acid at the expense of arachidonic acid) and the effect of this on the production of prostaglandins and leukotrienes (Okuyama *et al*. 2000; Riemersma *et al.* 2001).

Gastropod are one of the most important seafoods in the world. Eight marine gastropod species from the littoral zone of the Red and Mediterranean Seas and four freshwater gastropods from the Sea of Galilee were investigated by Go *et al.* (2002). Neutral and polar lipids in the soft parts of a gastropod species, *Ifremeria nautilei*, collected from deep sea hydrothermal vents were determined by Saito and Hashimoto (2010). The distribution of fatty acids and fatty aldehydes of total lipids in mantle, foot and digestive gland of two prosobranch gastropod mollusks, *Bellamya bengalensis* and *Pila globosa*, from India were studied (Misra *et al.* 2002). Proximate composition, fatty acid and amino acid analysis of two gastropod molluscs (*Littorinalittorea* and *Patella coerulea*) were also determined (Zlatanos *et al.* 2009). Kawashima *et al.* (2002) investigated sexual differences in gonad fatty acid compositions in dominant limpets species from the Sanriku coast in northern Japan. However, little information is available on the effects of season on the lipid and fatty acid compositions of most popular three Mediterranean gastropods; banded dye-murex-*Hexaplex trunculus*, purple dye-murex-*Bolinus brandaris*, and Persian conch-*Conomurex persicus*. Therefore, it is important to determine seasonal level of lipid and fatty acid profiles of these species from the nutritional point of view.

MATERIALS AND METHODS

Materials

Three different gastropods species were caught by bottom trawling from the Mediterranean Sea in all seasons in 2011. Gastropods species were; banded dye-murex (*Hexaplex trunculus* Linnaeus 1758), purple dye-

murex (*Bolinus brandaris* Linnaeus 1758), and Persian conch (*Conomurex persicus* Swainson 1821). In the every season, 20 individuals of each species were caught and kept in polystyrene boxes with ice and transferred in ice to the laboratory.

Sample preparation

Meat of mantle which is the main edible portion of gastropods was homogenized and chemical analyses were carried out on this part of fresh samples. The analyses were performed in triplicate.

Lipid analysis

Lipid content by the Bligh and Dyer (1959) method.

Fatty acid methyl ester analyses

Fatty acid profiles of fat extracted from the gastropod samples were determined by gas chromatography (GC). Methyl esters were prepared by transmethylation using 2 M KOH in methanol and *n*-heptane according to the method described by Ichihara *et al.* (1996) with minor modification. Extracted lipids (10 mg) were dissolved in 2 mL *n*-heptane followed by 4 mL of 2 M methanolic KOH. The tube was then vortexed for 2 min at room temperature. After centrifugation at 4,000 rpm for 10 min, the heptane layer was taken for GC analyses.

Gas chromatographic condition

The fatty acid composition was analyzed by the GC Clarus 500 with autosampler (Perkin Elmer, Shelton, CT, USA) equipped with a flame ionization detector and a fused silica capillary SGE column (30 m 0.32 mm, ID 0.25 mm, BP20 0.25 UM; SGE Analitic Science Pty Ltd, Victoria, Australia). The oven temperature was 140 ^{o}C, held for 5 min, raised to 200 ^{o}C at a rate of 4 ^{o}C/min and to 220 ^{o}C at a rate of 1 ^{o}C/min, while the injector and the detector temperature were set at 220 ^{o}C and 280 ^{o}C, respectively. The sample size was 1 µL and the carrier gas was controlled at 16 ps. The split used was 1:100. Fatty acids were identified by comparing the retention times of fatty acid methyl esters with a standard 37-component fatty acid methyl ester mixture (catalog no 18919; Supelco). Triplicate GC analyses were performed and the results were expressed in GC area % as the mean value±standard deviation.

Atherogenicity Index (AI) and Thrombogenicity Index (TI)

The atherogenicity index (AI) and thrombogenicity index (TI) linked to the fatty acid composition were calculated according to Ulbricht and Southgate (1991).

IA=[(a*12:0)+(b*14:0)+(c*16:0)]×[d*(PUFAsn-6+n-3)+e*(MUFAs)+f*(MUFAs-18:1)]

IT=[g*(14:0+16:0+(18:0)]×[(h*MUFAs)+i*(MUFAs-18:1)+(m*n-6)+(n*n-3)+(n-3/n-6)]

a, c, d, e, f=1, b=4, g=1, h, i, m=0.5 and n=3

Statistical analysis

Prior to the analyses all data were checked for outliers (Z values were checked) and homogeneity of variance (Duncan test was used) was also tested. Statistical analysis of data was carried out with the SPSS statistical program. ANOVA (Analysis of Variance) was used to evaluate the effects of species and season on the lipid and fatty acid composition.

RESULTS AND DISCUSSION

Lipid analyses

Table 1 shows the seasonal variations in the lipid levels of gastropods. Significant seasonal variation in lipid content was observed in all species ($p<0.05$).

Table 1. The effect of species and season on lipid levels of gastropods (%).

Latino name	Autumn $\overline{X} \pm S_X$	Winter $\overline{X} \pm S_X$	Spring $\overline{X} \pm S_X$	Summer $\overline{X} \pm S_X$	Common name
Hexaplex trunculus	0.64 ± 0.02^{ab}	0.58 ± 0.03^{a}	0.70 ± 0.02^{b}	0.79 ± 0.02^{c}	Banded dye-murex
Bolinus brandaris	0.56 ± 0.10^{a}	0.62 ± 0.11^{a}	0.90 ± 0.01^{b}	0.88 ± 0.01^{b}	Purple dye-murex
Conomurex persicus	0.69 ± 0.02^{a}	0.76 ± 0.02^{b}	0.71 ± 0.01^{a}	0.79 ± 0.01^{b}	Persian conch

Values in same lines with different letters are significantly different ($p<0.05$).
$\overline{X} \pm S_X$: Average±Standard deviation

The lipid contents of species were found to be very low. The lowest lipid content was observed for *B. brandaris* in autumn (0.56%) and *H. trunculus* in winter (0.58%) whereas *B. brandaris* gave the highest level of lipid (0.90%) in spring and (0.88%) in summer. In general, lipid contents of gastropods were observed to increase in summer and spring. This may be due to the fact that nutrients in summer and spring are more abundant than in other seasons.

Fatty acids composition

Tables 2-4 show seasonal variations in fatty acid compositions of gastropod species. The results of the fatty acid composition show that

marine gastropods are very rich in n-3 fatty acids. The fatty acid compositions of each species ranged from 26.89–32.84% saturated (SFA), 8.00–15.16% monounsaturated (MUFAs) and 43.53-53.11% polyunsaturated fatty acids (PUFAs).

Table 2. The effects of species and season on saturated fatty acids (SFA) of the gastropods (%).

Fatty acid $\overline{X} \pm S_x$	Autumn	Winter	Spring	Summer	Species
C12:0	0.25±0.02^{a}	2.23±0.16^{c}	1.29±0.09^{b}	2.83±0.09^{d}	**HT**
	ND	ND	0.03±0.00^{a}	0.12±0.01^{b}	**BB**
	2.90±0.14^{b}	1.39±0.08^{a}	3.70±0.02^{c}	3.95±0.07^{c}	**CP**
C14:0	3.34±0.47^{ab}	3.81±0.19^{b}	3.83±0.23^{b}	3.06±0.10^{a}	**HT**
	2.69±0.18^{b}	5.45±0.14^{d}	2.23±0.02^{a}	4.31±0.08^{c}	**BB**
	2.18±0.06^{a}	3.11±0.16^{c}	2.78±0.09^{b}	3.12±0.16^{c}	**CP**
C15:0	0.49±0.04^{b}	0.55±0.06^{b}	0.17±0.00^{a}	0.68±0.03^{b}	**HT**
	0.46±0.10^{b}	0.41±0.04^{b}	0.45±0.01^{b}	0.27±0.05^{a}	**BB**
	0.46±0.02^{a}	0.45±0.01^{a}	0.81±0.04^{b}	1.16±0.06^{c}	**CP**
C16:0	6.81±0.05^{a}	6.92±0.11^{a}	8.09±0.13^{c}	7.56±0.30^{b}	**HT**
	9.47±0.47^{c}	6.60±0.06^{a}	10.18±0.07^{c}	7.67±0.15^{b}	**BB**
	9.05±0.06^{a}	8.82±0.09^{a}	10.11±0.15^{b}	10.27±0.09^{b}	**CP**
C17:0	1.07±0.03^{a}	1.17±0.11^{a}	2.05±0.06^{b}	1.30±0.04^{a}	**HT**
	0.92±0.14^{a}	1.09±0.03^{a}	1.08±0.08^{ab}	1.27±0.06^{b}	**BB**
	1.40±0.09^{b}	1.05±0.06^{a}	1.35±0.01^{b}	1.85±0.09^{c}	**CP**
C18:0	8.65±0.17^{b}	8.89±0.16^{bc}	7.67±0.15^{a}	8.97±0.05^{c}	**HT**
	9.16±0.15^{a}	9.15±0.12^{a}	9.18±0.12^{a}	9.26±0.17^{a}	**BB**
	7.02±0.03^{a}	7.05±0.06^{a}	7.37±0.06^{b}	7.56±0.21^{b}	**CP**
C20:0	6.17±0.23^{bc}	6.28±0.08^{c}	5.58±0.18^{b}	4.38±0.24^{a}	**HT**
	5.39±0.83^{ab}	5.86±0.25^{b}	5.56±0.46^{b}	4.61±0.23^{a}	**BB**
	3.11±0.16^{a}	3.04±0.05^{a}	3.56±0.11^{b}	2.83±0.24^{a}	**CP**
C22:0	0.13±0.00^{b}	0.03±0.00^{a}	0.12±0.01^{b}	ND	**HT**
	ND	ND	ND	ND	**BB**
	3.12±0.16^{b}	3.02±0.19^{b}	3.19±0.06^{b}	2.11±0.16^{a}	**CP**
ΣSFA	**26.89±0.87^{a}**	29.86±0.49^{c}	28.78±0.55^{b}	28.77±0.37^{b}	**HT**
	28.08±0.16^{b}	28.55±0.01^{c}	28.79±0.90^{c}	27.49±0.01^{a}	**BB**
	29.22±0.10^{b}	27.90±0.01^{a}	32.86±0.42^{c}	**32.84±0.15^{c}**	**CP**

Values in same lines with different letters are significantly different ($p<0.05$).

$\overline{X} \pm S_x$: Average±Standard deviation ND: not detected

HT, Hexaplex trunculus; BB, Bolinus brandaris; CP, Conomurex persicus

Table 2 presents seasonal variations in the total saturated fatty acids (SFA) of gastropod species. The saturated (SFA) fatty acid compositions of each species were in the range of 26.89-29.86% for banded dye-murex, 27.49-28.79% for purple dye-murex, and 27.90-32.86% for Persian conch, Significant differences ($p<0.05$) in SFA contents were observed in terms of seasons. The highest proportions of SFA fatty acids in gastropod species were lauric acid (C12:0, 0.03-3.95%), myristic acid (C14:0, 2.18-5.45%), palmitic acid (C16:0, 6.60-10.27%), heptadecanoic acid (C17:0,

0.92-2.05%), stearic acid (C18:0, 7.02-9.26%) and arachidic acid (C20:0, 2.83-6.28%). The proportion of these fatty acids changed significantly throughout the seasons (Table 2).

Table 3. The effects of species and season on monounsaturated fatty acids (MUFA) of the gastropods (%).

Fatty acid $\overline{X} \pm S_x$	Autumn	Winter	Spring	Summer	Species
C14:1	1.84±0.10^{a}	1.96±0.06^{a}	2.49±0.09^{b}	3.11±0.16^{c}	**HT**
	1.73±0.02^{b}	1.22±0.28^{a}	1.70±0.06^{b}	1.83±0.08^{c}	**BB**
	0.31±0.01^{d}	0.14±0.00^{b}	0.18±0.00^{c}	0.08±0.01^{a}	**CP**
C15:1	0.17±0.00^{a}	0.16±0.01^{a}	0.21±0.02^{b}	0.45±0.03^{c}	**HT**
	0.11±0.03^{a}	0.18±0.02^{b}	0.18±0.06^{b}	0.33±0.00^{c}	**BB**
	0.12±0.01^{b}	0.22±0.02^{d}	0.17±0.01^{c}	0.09±0.00^{a}	**CP**
C16:1	1.08±0.07^{a}	1.19±0.08^{ab}	1.33±0.14^{b}	2.43±0.09^{c}	**HT**
	1.42±0.10^{b}	1.06±0.16^{a}	1.42±0.10^{b}	4.52±0.11^{c}	**BB**
	2.89±0.16^{c}	2.94±0.09^{c}	2.58±0.04^{b}	2.04±0.05^{a}	**CP**
C17:1	0.23±0.02^{a}	0.23±0.02^{a}	0.25±0.01^{a}	0.76±0.02^{b}	**HT**
	0.22±0.03^{a}	0.25±0.01^{a}	0.22±0.03^{a}	0.41±0.05^{b}	**BB**
	0.21±0.01^{b}	0.35±0.02^{c}	0.17±0.00^{a}	0.18±0.01^{a}	**CP**
C18:1*n9*	3.13±0.18^{b}	3.34±0.13^{b}	2.31±0.11^{a}	3.89±0.16^{c}	**HT**
	6.69±0.12^{b}	5.25±0.20^{a}	6.89±0.16^{b}	5.64±0.35^{a}	**BB**
	6.05±0.07^{b}	6.39±0.09^{c}	5.72±0.07^{a}	5.91±0.18^{ab}	**CP**
C18:1*n7*	1.16±0.23^{a}	1.85±0.03^{b}	0.95±0.08^{a}	2.16±0.23^{b}	**HT**
	ND	ND	ND	2.22±0.16^{a}	**BB**
	1.73±0.25^{a}	1.73±0.06^{a}	1.73±0.06^{a}	1.59±0.13^{a}	**CP**
C20:1	0.22±0.03^{b}	0.33±0.01^{c}	0.12±0.01^{a}	0.13±0.01^{a}	**HT**
	0.10±0.01^{a}	ND	0.21±0.01^{b}	ND	**BB**
	0.20±0.03^{a}	0.21±0.02^{a}	0.31±0.01^{b}	0.20±0.00^{a}	**CP**
C22:1*n9*	0.53±0.03^{c}	0.56±0.01^{c}	0.36±0.02^{b}	0.04±0.01^{a}	**HT**
	0.47±0.09^{c}	0.38±0.01^{b}	0.54±0.01^{c}	0.23±0.01^{a}	**BB**
	0.41±0.01^{a}	0.39±0.03^{a}	1.67±0.05^{c}	0.45±0.01^{b}	**CP**
ΣMUFA	8.35±0.26^{b}	9.60±0.00^{c}	**8.00±0.07^{a}**	12.96±0.03^{d}	**HT**
	10.73±0.04^{b}	8.33±0.06^{a}	11.15±0.25^{c}	**15.16±0.28^{d}**	**BB**
	11.90±0.18^{b}	12.35±0.23^{c}	12.51±0.05^{c}	10.53±0.04^{a}	**CP**

Values in same lines with different letters are significantly different ($p<0.05$).

$\overline{X} \pm S_x$: Average±Standard deviation ND: not detected

HT, *Hexaplex trunculus*; BB, *Bolinus brandaris*; CP, *Conomurex persicus*

There were significant differences ($p<0.05$) in the level of MUFA between species. The monounsaturated (MUFA) fatty acid compositions of each species ranged from 8.00-12.96% for banded dye-murex, 8.33-15.16% for purple dye-murex, and 10.53-12.51% for Persian conch (Table 3). Among monounsaturated fatty acids, the percentages of myristoleic acid (C14:1, 0.08-3.11%), palmitoleic acid (C16:1, 1.06-4.52%), oleic acid (C18:1n9, 2.31-6.89%), vaccenic acid (C18:1n7, 0.95-2.22%) were found to be higher than the other MUFAs throughout all seasons.

There were significant differences ($p<0.05$) in the level of PUFA between species. The polyunsaturated (PUFA) fatty acid compositions of each species ranged from 48.95-53.11% for banded dye-murex, 43.53-49.65% for purple dye-murex, and 45.21-49.28% for Persian conch (Table 3). Among polyunsaturated fatty acids, the percentages of linoleic acid (C18:2n-6, 2.06-6.26%), arachidonic acid (C20:4n6, 10.64-15.64%), cis-5,8,11,14,17-eicosapentaenoic acid (EPA, 20:5n-3, 8.17-13.96%), cis-13, 16- docosadienoic (C22:2 cis, 4.09-6.45%), and cis-4, 7,10,13,16,19-docosahexaenoic acid (DHA, C22:6 n-3, 10.28-18.55%) (Table 4). These results are in agreement with previous studies on fatty acids of other marine species (Ozogul *et al.* 2008; 2009)

Table 4. The effects of species and season on polyunsaturated fatty acids (PUFA) of the gastropods (%).

Fatty acid $\overline{X} \pm S_x$	Autumn	Winter	Spring	Summer	Species
C18:2*n6*	3.39±0.35^{b}	3.77±0.18^{b}	4.12±0.16^{c}	2.84±0.23^{a}	**HT**
	6.26±0.06^{c}	2.27±0.13^{a}	6.22±0.01^{c}	5.95±0.08^{b}	**BB**
	3.04±0.10^{b}	3.84±0.23^{c}	2.87±0.14^{b}	2.06±0.08^{a}	**CP**
C18:3*n6*	0.32±0.01^{b}	0.23±0.01^{a}	0.46±0.02^{c}	0.40±0.06^{c}	**HT**
	0.22±0.02^{a}	ND	0.22±0.02^{a}	0.39±0.08^{b}	**BB**
	0.14±0.01^{c}	0.16±0.01^{d}	0.12±0.01^{b}	0.04±0.00^{a}	**CP**
C18:3*n3*	1.55±0.17^{c}	0.32±0.04^{a}	0.39±0.01^{b}	0.35±0.04^{ab}	**HT**
	0.51±0.04^{b}	0.41±0.00^{a}	0.78±0.01^{c}	0.41±0.00^{a}	**BB**
	0.35±0.03^{b}	0.44±0.00^{c}	0.32±0.02^{b}	0.24±0.01^{a}	**CP**
C20:2*cis*	1.49±0.01^{b}	1.57±0.13^{b}	1.12±0.01^{a}	1.06±0.08^{a}	**HT**
	0.09±0.00^{a}	0.39±0.01^{b}	0.09±0.00^{a}	0.38±0.01^{b}	**BB**
	0.21±0.01^{b}	0.11±0.00^{a}	0.31±0.03^{c}	0.12±0.01^{a}	**CP**
C20:4*n6*	15.40±0.14^{c}	15.64±0.20^{c}	14.39±0.12^{b}	13.33±0.18^{a}	**HT**
	13.27±0.38^{b}	12.54±0.19^{a}	13.77±0.33^{c}	12.84±0.23^{a}	**BB**
	11.90±0.15^{b}	10.64±0.26^{a}	11.57±0.32^{b}	11.16±0.22^{b}	**CP**
C20:5*n3*	12.76±0.08^{b}	12.57±0.34^{a}	**13.67±0.47^{c}**	12.94±0.18^{b}	**HT**
	11.88±0.88^{a}	13.50±0.58^{bc}	12.75±0.35^{ab}	**13.96±0.06^{c}**	**BB**
	9.16±0.40^{b}	10.21±0.29^{c}	8.17±0.24^{a}	**11.28±0.38^{d}**	**CP**
C22:2*cis*	6.06±0.15^{ab}	6.31±0.21^{b}	6.45±0.30^{b}	5.77±0.33^{a}	**HT**
	5.03±0.91^{ab}	4.09±0.12^{a}	5.34±0.47^{b}	4.31±0.19^{a}	**BB**
	5.94±0.09^{b}	5.93±0.06^{b}	5.75±0.31^{b}	4.78±0.31^{a}	**CP**
C22:6*n3*	12.16±0.22^{ab}	11.72±0.40^{a}	**12.43±0.17^{b}**	12.29±0.30^{ab}	**HT**
	10.28±0.23^{a}	10.35±0.61^{abc}	10.50±0.08^{b}	**10.68±0.15^{c}**	**BB**
	18.55±0.77^{b}	17.83±0.24^{b}	16.12±0.02^{a}	16.28±0.23^{a}	**CP**
ΣPUFA	**53.11±2.54^{b}**	52.12±0.63^{b}	53.02±0.93^{b}	48.95±0.44^{a}	**HT**
	47.53±1.97^{b}	**43.53±1.63^{a}**	49.65±0.28^{c}	48.89±0.33^{bc}	**BB**
	49.28±0.01^{b}	49.14±0.50^{b}	45.21±1.05^{a}	45.94±0.11^{a}	**CP**

Values in same lines with different letters are significantly different ($p<0.05$).
$\overline{X} \pm S_x$: Average±Standard deviation ND: not detected
HT, *Hexaplex trunculus*; BB, *Bolinus brandaris*; CP, *Conomurex persicus*

Go *et al.* (2002) reported that the major acids in marine gastropods (*Nassa serata*, *Nassarius albescens*, *Nodilittorina subnodosa*, and *Planaxis sulcata)* species were C18:3n-3 (7.46% to 13.75%), C18:3n-6 (10.21% to 13.87%) and C20:4n-6 (7.05% to 9.36%). Low amounts of C20:5n-6 (0.27-0.41%) and C22:6n-3 (2.90-3.80%) acids compared to the current study were reported. They also indicated that marine gastropods, differ substantially from freshwater gastropod species belonging to the same class. It is possible to assume that they could accumulate from microalgae or synthesis from exogenous fatty acids.

Braza˜o *et al*. (2003) evaluated the effects of season and spatial distribution on the fatty acid composition of gastropoda (*Patella depressa* gonads and *Patella* spp.) soft body tissue. They reported that most important fatty acids were the saturated fatty acids (SFA) C16:0, C14:0 and C18:0; the monounsaturated fatty acids (MUFA) C18:1(n-7), C18:1(n-9), C16:1(n-7) and C20:1(n-9) and the polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA C20:5(n-3)), and arachidonicacid (ARA C20:4(n-6)) as reported in this study. Significant differences between sexes were also observed. Males showed significantly higher percentages of PUFA, highly unsaturated fatty acids (HUFA), (n-3) fatty acids and ARA, while in females significantly higher proportions of MUFA were found. Some variability was also reported to occur due to shore location and seasons.

In this study, significant differences were found ($p<0.05$) in the levels of EPA and DHA among season and species (Table 4). The highest proportions of EPA were obtained from banded dye-murex and purple dye-murex (11.88-13.96%) for all seasons whereas the lowest value was obtained from Persian conch (8.17-11.28%). Persian conch had the highest DHA in all seasons especially in autumn (18.55%) and winter (17.83%). Although gastropods contained low level of lipids, EPA and DHA were found to be high in all species for all seasons. The proportions of n-3 PUFAs (ranging from 22.67% for purple dye-murex in autumn to 28.48% for Persian conch in winter) were higher than those of n-6 PUFAs (ranging from 14.55% for Persian conch in spring to 20.20% for purple dye-murex in spring). It was recommended that a maximum dietary ratio was n6/n3 of 4.0 (HMSO, 1994). In this study, n-6/n-3 was found to be lower (1.19 for purple dye-murex in spring and 1.95 for Persian conch in winter) than recommended value. Moreira *et al*. (2001) indicated that values higher than the maximum value are not healthy, causing cardiovascular diseases. A recommended minimum value of PUFA/SFA ratio is 0.45 (HMSO, 1994) which is lower than those obtained from all species throughout all seasons (0.51-0.73) (Table 5). These results demonstrated a lower change in the ∑n3 and ∑n6 PUFA contents within a season for the species investigated.

Table 5. The effects of species and season on fatty acids index of the gastropods.

Season	PUFA/SFA	∑ω6	∑ω3	ω3/ω6	DHA/EPA	AI	TI	Species
Autumn	1.98	19.11	26.46	1.38	0.95	0.38	0.20	**HT**
	1.69	19.74	22.67	1.15	0.87	0.38	0.25	**BB**
	1.68	15.08	28.06	1.86	2.03	0.38	0.18	**CP**
Winter	1.74	19.63	24.61	1.25	0.93	0.45	0.22	**HT**
	1.52	14.80	24.26	1.64	0.77	0.60	0.25	**BB**
	1.76	14.63	28.48	1.95	1.75	0.41	0.19	**CP**
Spring	1.84	18.96	26.49	1.40	0.91	0.46	0.21	**HT**
	1.72	20.20	24.02	1.19	0.82	0.35	0.24	**BB**
	1.37	14.55	24.60	1.69	1.97	0.48	0.23	**CP**
Summer	1.70	16.56	25.57	1.55	0.95	0.41	0.21	**HT**
	1.77	19.17	25.04	1.31	0.77	0.42	0.23	**BB**
	1.39	13.25	27.79	2.10	1.44	0.52	0.22	**CP**

HT, *Hexaplex trunculus*; BB, *Bolinus brandaris*; CP, *Conomurex persicus*

British Nutrition Foundation (1992) recommended that people who has balanced and healthy diet consume 0.2 g of EPA+DHA daily. The American Heart Association also recommends about 1.0 g/day of EPA+DHA, or two servings of fatty fish per week are required to reduce the risk of death from coronary heart disease.

Ulbricht and Southgate (1991) have proposed the term atherogenic index (AI) for the lipids as nutritional indices for the risk of cardio-vascular diseases. The atherogenic index is calculated on the basis of the content of the middle chain fatty acids C12:0, C14:0 and C16:0, and the groups MUFA and PUFA. In this study, AI values were in the range of 0.35 in spring and 0.60 in winter for purple dye-murex whereas TI values ranged from 0.18 for Persian conch in autumn to 0.25 for purple dye-murex in autumn.

The results of fatty acid compositions indicated that from the nutritional view of point gastropods species contained low level of lipid and they were rich in *n*-3 fatty acids, especially EPA and DHA. The results also showed that the seasons affected lipid content and the fatty acid composition of gastropod species.

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The Effects of Natural Zeolite on Fatty Acids Profile of Mediterranean Sardine Fillets

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ABSTRACT

The effect of natural zeolite at different doses (1% and 5%) on fatty acids of sardine (*Sardinella aurita*) fillets were investigated during 19 days at 4 ± 1 °C. In all groups, the major fatty acids were found to be palmitic acid (16:0, 20.93-23.03%), palmitoleic acid (C16:1, 13.97-15.02%), eicosapentaenoic acid (EPA, 20:5n3, 13.18-14.88%), myristic acid (C14:0, 12.27-13.54%), oleic acid (18:1n9, 5.10-7.00%) and docosahexaenoic acid (DHA, 22:6n3, 5.00-6.96%). The total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) content of fish fillets were in the range of 37.68-40.02%, 25.05-27.03% and 23.53-26.28%. Significant differences in SFA and MUFA were found among groups ($P<0.05$) at 8th and 12th days of storage, whereas zeolite application did not have any effect on the PUFA content of sardine fillets except for 8 days of storage in which fish fillets treated with 5% zeolite had slightly higher PUFA content than other groups.

INTRODUCTION

Zeolites are defined as crystalline, hydrated aluminosilicates of alkali and alkaline earth cations, having a potentially infinite, three-dimensional structure. Each zeolite species has its own unique crystal structure, size, shape of cavities, porosity and hardness of mother rock and, hence, its own set of physical properties and chemical composition and properties (Pond and Lee 1984). Such diverse properties thus had made use of zeolite in many industries possible (Shariatmadari 2008) Natural zeolites of sedimentary origin, which are present on all the continents of our planet as minable deposits, find extremely wide application in the solution of pressing problems in various areas of industry and agriculture due to their unique adsorptive, ion-exchange, catalytic, molecular-sieve properties as well as the recently discovered detoxication capacities (Tsitsishvili *et al.* 1992; Mupton 1999; Andronikashvili *et al*. 2009). Zeolite is an important mineral group for human health since they have a positive effect on detoxification of the body, removal of pollutants and capture of free

radicals, increasing the stability of the immune system, controlling mineral metabolism, increasing mental and physical performance, inhibition of the aging process and stress reducing effect (Hecht 2010).

Natural zeolites are the main absorptive and low cost material used in agriculture and industry (Sakadevan and Bavor 1998; Hrenovi *et al*. 2003). Zeolites show remarkable selectivities for removing ammonia from water. There are potential applications of zeolite in industrial and agricultural wastewater purification, aquaculture, animal feeding, agriculture, and horticulture (with use of natural zeolites as nitrogen fertilizers) (Colella 1996). Zeolites are already in use in the food industry especially as food packaging materials (Pavelic and Mirko Hadzija 2003).

Sardine is an important food species in Turkey. It is generally consumed as fresh, canned or salted and also utilised as fish meal and oil. Sardine is known to be high protein and oil fish (Erkan and Özden 2008). Fish oil contains PUFA such as EPA (C20:5n3) and DHA (C22:6n3) that are considered to have a number of health benefits (Ozogul *et al*. 2010). Marine oil n-3 fatty acids are useful for suppressing increases in plasma cholesterol (Suzuki *et al*. 1995), preventing cardiovascular diseases (Iso *et al*. 2001), and improving learning ability (Suzuki *et al*. 1998) and visual function (Birch *et al*. 2000). It has been recently suggested that an increased of n–3 fatty acid intake and/or increase of n–3/n–6 PUFA ratio in the diet is associated with a lower breast cancer risk, especially in premenopausal women (Goodstine *et al*. 2003).

Sardine (*Sardina pilchardus*) lipids have important nutritional characteristics because of their high level of n3 fatty acids. Total lipids ranged between 1.2% and 18.4% (w/w). They were both high in n3 polyenoic fatty acids mainly EPA and DHA (Bandarra *et al*. 1997). There is limited study regarding effect of zeolite on quality and compositions of fish and fish products. The lipid contents and fatty acid compositions (% of total fatty acids) of rainbow trout fed with four different ratios of clinoptilolite were studied by Danabas (2011). The study results showed that fatty acid compositions of the groups depend on feed, age, environmental conditions, and effects of feed additives like zeolite. The aim of the study was to investigate effects of natural zeolite on fatty acids profile of mediterranean aardine fillets.

MATERIALS AND METHODS

Sample preparation

Sardine (*Sardinella aurita*) was caught by gill net in Mersin Bay, Turkey. The average weight and length of fish were 20.03±2.88g and 13.40±0.81cm, respectively. They were stored in boxes with ice when fish

were on board after catching and delivered to the laboratory. The fish were immediately filleted. The fish fillets were divided into three groups. Two groups were immersed in a 1L of distilled sterile water containing 10g (1% zeolite group) or 50 g zeolite (5% zeolite group) for 4 min. The control was not treated with zeolite and was only immersed in distilled sterile water. The control and the treated groups were packaged in pouches of polyamide film (Polinas, Manisa, Turkey) using a vacuum packaging machine (Reepack RV50, Italy). The thickness of the film was 90 µm and water and oxygen permeability were 8.5 g m^{-2} 24 h and 160 cm^3 m^{-2} 24 h, respectively. All samples were stored at 4±1ºC.

FAME analyses

Lipid extraction was done according to the Bligh and Dyer method (1959). Methyl esters were prepared by transmethylation using 2 M KOH in methanol and n-hexane according to the method described by Ichihara *et al.* (1996) with minor modification; 10 mg of extracted oil were dissolved in 2 ml hexane, followed by 4 ml of 2 M methanolic KOH. The tube was then vortexed for 2 min at room temperature. After centrifugation at 4000 rpm for 10 min, the hexane layer was taken for GC analyses.

Gas chromatographic conditions

The fatty acid composition was analysed by a GC Clarous 500 with autosampler (Perkin–Elmer, USA) equipped with a flame ionization detector and a fused silica capillary SGE column (30 m · 0.32 mm ID · 0.25 lm BP20 0.25 UM, USA). The oven temperature was 140 ºC, held 5 min, raised to 200 ºC at the rate 4 _C/ min and held at 220 ºC at 1 ºC/min, while the injector and the detector temperatures were set at 220 and 280 _C, respectively. The sample size was 1 ll and the carrier gas was controlled at 16 ps. The split used was 1:100. Fatty acids were identified by comparing the retention times of FAME with the standard 37 component FAME mixture. Two replicate GC analyses were performed and the results were expressed in GC area % as mean values ± standard deviation.

Statistical analysis

The mean value and standard deviation were calculated from the data obtained from the three samples for each treatment. One way ANOVA was used to determine the significance of differences at $P<0.05$. All statistics were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL. USA).

RESULTS AND DISCUSSION

Fatty acid composition of sardine treated with zeolite at doses of 1% and 5% is given in Tables 1-3. The fatty acid compositions of sardine ranged from 37.68% to 40.02% SFA, from 25.05% to 27.03% MUFA and from 23.53% to 26.28% PUFA. Özogul and Özogul (2007) reported that SFA, MUFA and PUFA content of sardine (*Sardinella aurita*) were 38.7%, 17,6% and 31.02%, respectively. Fatty acid profiles of sardines (*Sardina pilchardus*) with different fat contents were 27-29.86% SFA, 6.44-35.15% MUFA and 28.88-63.74 PUFA (Bandarra *et al*. 2003).

Table 1. Changes in fatty acids profile of vacuum packaged sardine treated with zeolite at different doses.

Fatty acids	Storage Days						Groups
	0	4	8	12	15	19	
C14:0	13.03±0.35	13.09±0.05a	12.27±0.14a	12.87±0.10a	12.72±0.11b	13.20±0.11a	Control
	-	12.31±0.14b	12.65±0.06a	13.08±0.29a	13.06±0.33ab	13.31±0.13a	1% Zeolite
	-	13.23±0.21a	12.74±0.37a	13.24±0.24a	13.54±0.18a	13.24±0.10a	5% Zeolite
C16:0	22.84±1.22	22.22±0.04a	23.03±0.18a	21.53±0.24ab	21.86±0.62a	22.23±0.21a	Control
	-	22.26±0.24a	20.93±0.25b	21.38±0.04b	20.82±0.48a	21.69±0.25a	1% Zeolite
	-	22.10±0.74a	21.50±0.43b	22.13±0.30a	21.99±0.59a	22.67±0.60a	5% Zeolite
C17:0	0.08±0.00	0.09±0.00a	0.10±0.00a	0.09±0.00a	0.10±0.01a	0.10±0.01a	Control
	-	0.09±0.00a	0.09±0.00a	0.09±0.01a	0.10±0.01a	0.08±0.00a	1% Zeolite
	-	0.09±0.01a	0.10±0.00a	0.10±0.01a	0.10±0.01a	0.10±0.01a	5% Zeolite
C18:0	3.92±0.30	3.82±0.05a	4.08±0.06a	3.70±0.05a	3.87±0.11b	3.76±0.07a	Control
	-	3.88±0.03a	3.85±0.02ab	3.77±0.08a	4.29±0.14a	3.71±0.04a	1% Zeolite
	-	3.73±0.15a	3.78±0.14b	3.90±0.21a	3.75±0.06b	3.86±0.04a	5% Zeolite
C20:0	0.32±0.01	0.33±0.02a	0.34±0.02a	0.15±0.02a	0.41±0.02a	0.32±0.01a	Control
	-	0.38±0.02a	0.43±0.03a	0.39±0.00a	0.32±0.06ab	0.34±0.01a	1% Zeolite
	-	0.36±0.02a	0.32±0.01a	0.36±0.04a	0.28±0.02b	0.30±0.01a	5% Zeolite
∑SFA	40.01±1.17	39.37±0.06a	39.63±0.10a	38.34±0.39b	39.52±0.71a	39.43±0.18a	Control
	-	38.70±0.13a	37.68±0.29b	38.47±0.15b	38.71±0.63a	38.94±0.35a	1% Zeolite
	-	39.29±0.68a	38.28±0.21b	39.50±0.27a	38.45±0.95a	40.02±0.55a	5% Zeolite

Different lowercase letters (a–c) in a column indicate significant differences ($P < 0.05$) among groups.

Table 2. Changes in fatty acids profile of vacuum packaged sardine treated with zeolite at different doses.

Fatty acids	Storage Days						Groups
	0	4	8	12	15	19	
	0.18±0.01	0.19±0.04a	0.19±0.01a	0.21±0.01a	0.20±0.00a	0.17±0.03a	Control
	-	0.18±0.00a	0.18±0.03a	0.21±0.01a	0.17±0.05a	0.20±0.00a	1% Zeolite
C14:1	-	0.21±0.02a	0.20±0.01a	0.23±0.03a	0.18±0.03a	0.17±0.04a	5% Zeolite
	14.47±0.04	14.51±0.11a	14.02±0.03a	14.64±0.11a	14.68±0.50a	14.27±0.18a	Control
	-	13.97±0.00b	14.58±0.37a	15.02±0.06a	14.40±0.43a	14.76±0.19a	1% Zeolite
C16:1	-	14.77±0.13a	14.53±0.43a	14.20±0.46a	14.71±0.43a	14.39±0.08a	5% Zeolite
	0.30±0.04	0.29±0.04a	0.30±0.01b	0.39±0.00a	0.32±0.04a	0.34±0.01ab	Control
	-	0.31±0.01a	0.43±0.02a	0.40±0.03a	0.36±0.02a	0.36±0.00a	1% Zeolite
C17:1	-	0.34±0.04a	0.38±0.04a	0.31±0.02b	0.34±0.01a	0.31±0.01b	5% Zeolite
	6.05±0.50	6.06±0.35b	6.42±0.37a	6.12±0.16a	6.15±0.06a	6.24±0.46a	Control
	-	7.00±0.11a	6.31±0.19a	6.04±0.11a	5.27±0.82a	6.22±0.06a	1% Zeolite
C18:1n9	-	6.11±0.06b	5.60±0.43a	6.87±0.46a	5.10±0.45a	6.48±0.24a	5% Zeolite
	4.03±0.10	3.93±0.01a	3.83±0.06c	4.35±0.08b	4.15±0.16a	4.29±0.08a	Control
	-	4.14±0.04a	4.62±0.03a	4.55±0.04a	4.68±0.16a	4.06±0.34a	1% Zeolite
C18:1n7	-	4.12±0.15a	4.07±0.06b	3.93±0.03c	4.00±0.34a	3.82±0.40a	5% Zeolite
	0.15±0.01	0.15±0.00a	0.15±0.00a	0.36±0.01a	0.15±0.00b	0.15±0.00a	Control
	-	0.15±0.00a	0.16±0.01a	0.16±0.01b	0.18±0.01a	0.16±0.00a	1% Zeolite
C20:1n9	-	0.15±0.00a	0.15±0.00a	0.15±0.01b	0.15±0.01b	0.15±0.00a	5% Zeolite
	0.40±0.01	0.41±0.01a	0.41±0.01a	0.41±0.00a	0.39±0.01b	0.44±0.01a	Control
	-	0.41±0.01a	0.43±0.00a	0.43±0.01a	0.42±0.01ab	0.43±0.01a	1% Zeolite
C22:1n9	-	0.41±0.00a	0.43±0.01a	0.40±0.01a	0.45±0.01a	0.43±0.00a	5% Zeolite
	25.74±0.49	25.70±0.35a	25.49±0.42b	26.47±0.03b	26.28±0.80a	26.06±0.33a	Control
	-	26.38±0.09a	26.97±0.17a	27.03±0.05a	25.60±1.23a	26.36±0.19a	1% Zeolite
∑MUFA	-	26.30±0.27a	25.50±0.04b	26.29±0.08c	25.05±0.36a	25.89±0.27a	5% Zeolite

Different lowercase letters (a–c) in a column indicate significant differences ($P < 0.05$) among groups.

Table 3. Changes in fatty acids profile of vacuum packaged sardine treated with zeolite at different doses.

Fatty acids	Storage Days						Groups
	0	4	8	12	15	19	
	1.26±0.01	1.29±0.03a	1.25±0.02b	1.23±0.03a	1.27±0.01b	1.25±0.01a	Control
	-	1.27±0.02a	1.33±0.01a	1.26±0.00a	1.43±0.05a	1.14±0.11a	1% Zeolite
C18:2 n6	-	1.27±0.00a	1.28±0.01b	1.32±0.08a	1.26±0.01b	1.16±0.13a	5% Zeolite
	0.14±0.01	0.14±0.01a	0.13±0.00a	0.12±0.00a	0.11±0.01b	0.13±0.00a	Control
	-	0.14±0.00a	0.13±0.00a	0.13±0.01a	0.12±0.00a	0.10±0.02a	1% Zeolite
C18:3 n6	-	0.14±0.00a	0.13±0.00a	0.12±0.00a	0.12±0.00a	0.10±0.03a	5% Zeolite

Table 3 Continued.

	0.05±0.01	0.99±0.01a	1.02±0.03b	0.93±0.04a	0.90±0.01b	0.96±0.07a	Control
	-	0.98±0.04a	0.96±0.00c	0.89±0.01a	0.88±0.04b	0.91±0.01a	1% Zeolite
C18:3n3	-	0.97±0.01a	1.11±0.00a	0.93±0.09a	1.07±0.02a	0.99±0.02a	5% Zeolite
	2.11±0.16	2.06±0.08a	2.10±0.04a	1.96±0.01b	2.22±0.07a	1.99±0.03a	Control
	-	1.96±0.03a	2.03±0.00a	2.11±0.01a	2.29±0.12a	2.05±0.03a	1% Zeolite
C20:2cis	-	2.05±0.06a	2.04±0.04a	1.95±0.01b	2.10±0.03a	2.07±0.03a	5% Zeolite
	0.47±0.01	0.49±0.01a	0.47±0.00c	0.49±0.01a	0.47±0.01b	0.52±0.01a	Control
	-	0.49±0.01a	0.52±0.01a	0.50±0.01a	0.51±0.00a	0.51±0.01a	1% Zeolite
C20:4 n6	-	0.50±0.01a	0.50±0.01b	0.48±0.02a	0.53±0.01a	0.51±0.00a	5% Zeolite
	13.72±0.81	14.34±0.18a	13.18±0.20b	14.52±0.28a	14.01±0.58a	14.46±0.20a	Control
	-	13.86±0.02a	14.33±0.30a	13.92±0.20a	14.48±0.09a	14.88±0.19a	1% Zeolite
C20:5n3 EPA	-	14.01±0.52a	14.24±0.18a	13.75±0.47a	14.65±0.66a	14.50±0.02a	5% Zeolite
	0.10±0.03	0.12±0.01a	0.13±0.00a	0.10±0.00a	0.13±0.01a	0.12±0.00a	Control
	-	0.12±0.01a	0.12±0.01b	0.11±0.01a	0.15±0.02a	0.12±0.00a	1% Zeolite
C22:2 cis	-	0.11±0.00a	0.12±0.00b	0.11±0.01a	0.13±0.03a	0.12±0.00a	5% Zeolite
	5.69±0.27	6.04±0.09a	6.96±0.59a	5.61±0.13a	5.73±0.21a	5.00±0.24a	Control
	-	6.11±0.69a	5.55±0.10b	5.13±0.10b	6.44±0.74a	5.03±0.07a	1% Zeolite
C22:6n3 DHA	-	5.37±0.18a	6.69±0.00a	5.13±0.08b	5.78±0.49a	5.19±0.06a	5% Zeolite
	23.53±0.37	25.44±0.34a	25.24±0.49ab	24.95±0.23a	24.82±0.44a	24.43±0.15a	Control
	-	24.91±0.77a	24.95±0.18b	24.04±0.32a	26.28±0.83a	24.72±0.18a	1% Zeolite
∑PUFA	-	24.41±0.30a	26.10±0.23a	23.78±0.56a	25.62±0.20a	24.63±0.08a	5% Zeolite
	0.59	0.65	0.64	0.65	0.64	0.62	Control
	-	0.64	0.66	0.62	0.68	0.63	1% Zeolite
PUFA/SFA	-	0.62	0.68	0.60	0.65	0.62	5% Zeolite
	19.46	21.36	21.16	21.05	20.63	20.42	Control
	-	20.94	20.84	19.94	21.80	20.81	1% Zeolite
∑n3	-	20.35	22.04	19.81	21.49	20.67	5% Zeolite
	1.87	1.91	1.85	1.84	1.84	1.90	Control
	-	1.89	1.97	1.88	2.06	1.75	1% Zeolite
∑n6	-	1.90	1.90	1.91	1.90	1.77	5% Zeolite
	0.10	0.09	0.09	0.09	0.09	0.09	Control
	-	0.09	0.09	0.09	0.09	0.08	1% Zeolite
n6/n3	-	0.09	0.09	0.10	0.09	0.09	5% Zeolite
	0.41	0.42	0.53	0.39	0.41	0.35	Control
	-	0.44	0.39	0.37	0.44	0.34	1% Zeolite
DHA/EPA	-	0.38	0.47	0.37	0.39	0.36	5% Zeolite

Table 3 Continued.

	10.72	9.50	9.65	10.25	10.20	10.10	Control
	-	10.02	10.41	10.48	9.67	9.99	1% Zeolite
Unidentified	-	10.01	10.13	10.44	9.82	9.47	5% Zeolite

Different lowercase letters (a–c) in a column indicate significant differences ($P < 0.05$) among groups.

In all groups, the major fatty acids were found to be palmitic acid (16:0, 20.93-23.03%), palmitoleic acid (C16:1, 13.97-15.02%), eicosapentaenoic acid (EPA, 20:5n3, 13.18-14.88%), myristic acid (C14:0, 12.27-13.54%), oleic acid (18:1 n9, 5.10-7.00%) and decosahexaenoic acid (DHA, 22:6 n3, 5.00-6.96%), which is in agreement with findings of Özogul and Özogul (2007). Zotos and Vouzanidou (2012) found that the sum of EPA and DHA ranged from 1.18 to 2.76 g/100 g edible portion of sardine caught from different season. During storage periods, significant differences were observed in myristic acid (C14:0), oleic acid (C18:1n9), linoleic acid (C18:2 n6), γ-linolenic acid (C18:3n6), linolenic acid (C18:3n3) and docosahexaenoic acid (DHA, 22:6n3) content of fish ($P<0.05$). There were also significant differences in palmitic acid (C16:0), cis-11-octadecenoic acid (C18:1n7), linolenic acid (C18:3n3) and cis-11-eicosenoic (C20:1n9) among groups.

Palmitic acid (C16:0) was the main fatty acids found in sardine (>20.8%). Lower palmitic acid content was found for sardine, with corresponding value of 15-20.5% (Bakara *et al*. 1997; Özogul and Özogul, 2007). Palmitic acid content of control group was the highest compared to other groups at 8 days, whilst zeolite at dose of 5% resulted in higher palmitic acid content in fish at 12 days. Danabas *et al*. (2011) did not observe significant changes in palmitic acid content of rainbow trout fed with zeolite at different concentrations. Linolenic acid (C18:3n3) composition of sardine fillet treated with 5% zeolite was the highest at 8 days and 15 days of storage. Although zeolite statistically did not affect cis-11-octadecenoic acid (C18:1n7) composition of fish at 4, 15 and 19 days of storage, zeolite application at doses of 1% induced slightly increase in cis-11-octadecenoic acid (C18:1n7). Danabas *et al.* (2011) found that rainbow trout fed with 1% zeolite had low levels of arachidonic acid (C20:4 n6%) and cis 13,16 –docosadienoic acid (C 22:2) compared to control group. In the current study, zeolite application did not effect arachidonic acid and cis 13,16–docosadienoic acid content of fish, apart from 8 days of storage.

Significant differences in SFA and MUFA were found among groups ($P<0.05$) at 8th and 12th days of storage, whereas zeolite application did not have any effect on the PUFA content of sardine fillets

except for 8 days of storage in which fish fillets treated with 5% zeolite had slightly higher PUFA content than other groups. The UK Department of Health recommends that a minimum value of PUFA/SFA ratio is 0.45 (HMSO, 1994), which was obtained in sardine with value of between 0.59 and 0.65. Õzogul *et al*. (2007) reported PUFA/SFA value for sardine as 0.8. The PUFA/SFA ratio was found as 0.63% for eel, 1.02% for bream, 1.34% for herring, 1.69% for roach, 1.85% for perch, and 2.04% for pikeperch (Polak-Juszczak and Komar-Szymczak 2009).

The ratio of n–3 PUFAs to n–6 PUFAs in freshwater fish mostly varies between 0.5 and 3.8, whereas for marine fish, the ratio is 4.7–14.4 (Uysal *et al*. 2011). The UK Department of Health recommends a maximum n-6/n-3 dietary ratio of 4.0 (HMSO, 1994). In the present study, the ratios of n–3 PUFAs to n–6 were in range 0.08 and 0.10. Similarly, Özogul *et al*. (2007) found n-6/n-3 in freshwater fish within the range of 0.21-1.0 and for marine fish of 0.009-0.59. Zotos and Vouzanidou (2012) found that the ratios of n3 to n6 in sardine caught from different season ranged from 7 and 11.

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DNA Barcoding the Scyphozoa

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ABSTRACT

Species from the class Scyphozoa of the Cnidaria phylum were referred as the true jellyfishes. Scyphozoans are the most important jellyfish species that are fished commercially and used as seafood products. Identification of Scyphozoan species is difficult because of their fragile anatomy (no durable hard parts like head or skeleton) and complex life stages. In this study we used DNA barcoding approach to identify 19 Scyphozoan species. In order to generate unique DNA barcodes we amplified 654 base pair long fragment of the mitochondrial cytochrome c oxidase I (COI) gene. This region of mitochondrial COI has been used as a universal DNA barcode for a wide range of taxa including Cnidaria. Nucleotide composition and nucleotide pair frequencies were analyzed together with codon usage to see the general pattern of the Scyphozoa class. Average nucleotide composition ratios were calculated as A=26,3%; C=18,7%; G=19,3%; and T=35,7%. Genetic pairwise distances were calculated using Kimura 2-parameter (K2P) model with 1000 bootstrap replicates. Interspecific K2P variation among species was ranged from 0.072 to 0.427 (mean 0.256). A Neighbor Joining (NJ) tree was constructed with K2P model (bootstrap replicates=1000) to analyze the evolutionary relationship among 19 species. Interspecific variation determined in mtCOI gene of Scyphozoan species was found suitable for his fragment to be used in species level identification.

Keywords: DNA barcoding, jellyfish, mtCOI, molecular identification, Scyphozoa

INTRODUCTION

Identification of Scyphozoan Species Class Scyphozoa of the phylum Cnidaria covers about 200 different jellyfish species with a distribution from brackish waters to the epipelagic and benthic depths of oceans (Arai 1997; Bayha *et al*. 2010). They have important impact on fisheries, both economically and ecologically, in terms of establishing aggregations, blooms and swarms; damaging swimmers; preventing industrial water intakes; invading ecosystems and causing dysoxic conditions (Hay 2006; Graham and Bayha 2007; Purcell *et al*. 2007; Richardson *et al*. 2009; West *et al*. 2009).

Scyphozoan species also have an interesting life cycle in which, meroplanktonic species having benthic and planktonic medusae phases and holoplanktonic species having only the planktonic medusae phase (Ramsak *et al*. 2012). Taxonomy of Scyphozoan polyps and medusae is a complicated issue as they have restricted morphological characters and the plasticity of ecological populations is typical for all the species (Dawson 2003).

Constructing a reliable phylogeny of the Scyphozoa has been prevented by lack of morphologically definable characters among species of these anatomically fragile Cnidarians (Dawson 2003). Only subsets of some taxa have been successfully resolved in the way of constructing a reliable evolutionary relationship using conventional taxonomy and morphology based phylogenetic frameworks (Bayha *et al*. 2010). Consequently, lots of different phylogenetic assumptions still exist regarding to systematic alignment of taxonomic levels from class to species (Kramp 1955, 1961; Werner 1975; Mills *et al*. 1987; Marques and Collins 2004). As the usage of new approaches in taxonomy, such as molecular tools to determine the phylogenetic relationships becomes widespread, identifying species or families, discovering new and cryptic species and resolving phylogeographic patterns among scyphozoans is getting more uncomplicated (Ramsak *et al*. 2012).

Molecular tools have influenced this systematic confusion by revealing certain evidences in favor of one or another taxonomic assumption at different levels of Scyphozoan systematic alignment. Although recent advances in molecular biology, some taxonomic relationships remain unresolved or partially analyzed. Besides, phylogenetic relationship among Scyphozoan families has never been the main target of any study despite they have never been resolved completely using morphological characters (Bayha *et al*. 2010). Possible reasons lying behind this lack of resolution in molecular systematics among Scyphozoan families may be explained with insufficient samplings, selection of improper molecular markers or the molecular methods and models used in analyzing (Regier *et al*. 2008; Seo and Kishino 2008).

DNA Barcoding

DNA sequence analysis could be used for fast, reliable and incisive identification of species from all metazoan groups. These nucleotide sequences are useful especially for identification of species without divergent body parts or species that are rare, fragile or small which makes them unavailable for morphological identification. Particularly, DNA barcodes will lead to a fast and accurate molecular based analysis of samples for known species through fast growing DNA barcode libraries (Bucklin *et al*. 2011).

The term DNA barcoding, a short fragment of mitochondrial DNA to discriminate species, is compromising certain empirically determined standards. For metazoan animals, approximately 650 base pair region from the 5' part of mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene has been used as a DNA barcode (Hebert *et al*. 2003). As it is expected, *COI* barcodes should have higher divergence levels among different species than populations of the same species. This condition was explained as levels of intraspecific variation should be significantly lower than interspecific variation with no overlap between intraspecific and interspecific genetic distance leaving a barcoding gap (Meyer and Paulay 2005). An optimal application of DNA barcoding should result with a phylogenetic tree in which same species of different populations cluster together excluding other species based on genetic distance analyzed from nucleotide diversity (Ortman *et al*. 2010).

The aim of this study was to use 654 base pair of mitochondrial *cytochrome c oxidase subunit I* gene to determine whether the DNA barcoding approach will allow discrimination of genetic variability within and between species from the Cnidarian class Scyphozoa and construct a reliable relationship among family and order levels.

MATERIAL AND METHODS

Sampling

Scyphozoan specimens were collected from the Mediterranean Sea (*Aurelia aurita*, *Cassiopea andromeda*, *Marivagia stellata*, *Pelagia noctiluca*, *Phyllorhiza punctata* and *Rhopilema nomadica*), Gulf of Mexico (*Cassiopea frondosa*), Caribbean Sea (*Cassiopea xamachana*), Bering Sea (*Chrysaora melanaster*), Red Sea (*Crambionella orsini*, *Cyanea annaskala*), North Sea (*Nausithoe atlantica*), East China Sea (*Nemopilema nomurai*), Gulf of Alaska (*Aurelia labiata* and *Cyanea capillata*), Japan (*Aurelia limbata*) and Australia (*Catostylus mosaicus* and *Cyanea rosea*) during 2010-2011. Tissue samples were preserved in 95% ethanol and stored at -20°C. In total, 54 specimens were collected from 18 species, representing all three orders (Coronatae, Rhizostomae and Semaeostomae) of Scyphozoa. Every species were represented with 3 individuals.

DNA extraction, PCR amplification and Sequencing

Genomic DNA's were extracted using Qiagen DNeasy Blood & Tissue Kit according to the supplier's protocol and DNA concentrations were all set to 50 ng/µl and stored at -20°C. A 655 base pair long portion of the

mitochondrial *cytochrome c oxidase subunit I* gene was amplified using using universal HCO(5'-TAAACTTCAGGGTGACCAAAAAATCA-3')-

LCO(5'GGTCAACAAATCATAAAGATATTGG-3') primer pair (Folmer *et al*. 2005). Each PCR reaction was set in volume of 50 µl, containing: 5 µl of 10X Taq Buffer with KCl (100 mM Tris-HCl, 500 mM KCl, pH 8.8), 4µl of MgCl2 (25mM), 1 µl of dNTPs (10 mM), 1.5 µl of each primer (10 pM/µl), 3 U of Taq polymerase (5U/µl) and 4 µl of DNA (50 ng/µl). Reaction conditions were 95°C for 2 minutes for an initial denaturation, followed by 30 cycles at 95°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute, with a final extension step at 72°C for 10 minutes.

Promega Wizard SV Genomic DNA Purification System was used in purification of PCR amplified DNA fragments, according to the supplier's protocol and checked via agarose gel electrophoresis. DNA sequencing reactions were conducted on an ABI 377 automated sequencer using ABI Prism Dye Terminator Cycle Sequencing Reaction Kit.

Analyses of DNA Barcodes

Nucleotide sequences were initially aligned using Sequencher 5.0 and checked by eye, then compared to the reference sequences gathered from BOLD and GenBank databases to confirm the validity of the sequences and detect possible insertions, deletions or numts. Nucleotide sequence belonging to *Atolla vanhoeffeni* (GQ120085) was taken from GenBank database and analyzed with our dataset to support the order Coronatae, as it is represented with only one species (*Nausithoe atlantica*).

Statistical data such as nucleotide composition, nucleotide pair frequencies and codon usage were calculated. Maximum likelihood estimates of substitution matrix and transition/transversion bias were performed. Estimates of evolutionary divergence between sequences were conducted using Kimura 2-parameter substitution model. Evolutionary relationships of taxa were generated on a Neighbor Joining tree constructed with Kimura 2-parameter model. Bootstrap tests with 1000 replicates were performed to determine the robustness of the clades. The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) was calculated using a Codon-based Test of Neutrality for analysis between sequences using the Nei-Gojobori method. The variance of the difference was computed using the bootstrap method (1000 replicates). Analyses were conducted in MEGA5 (Tamura *et al*. 2011).

RESULTS AND DISCUSSION

Results

A total of 54 uniform (654 bp) mtCOI sequences were generated for 18 species of Scyphozoans. Statistical analyses of the nucleotide sequences pointed out 345 variable sites, of which 316 were parsimony informative (Table 1). Nucleotide pair frequencies within orders were given in Table 1. Average nucleotide frequencies of the complete dataset were: A=26,3%; C=18,7%; G=19,3%; and T=35,7%. Average nucleotide compositions of orders were given in Table 2.

Table 1. Nucleotide pair frequencies according to complete data set and orders.

	Dataset	Coronatae	Rhizostomae	Semaeostomae
Length (bp)	654	654	654	654
Conserved Regions	309/654	505/654	378/654	403/654
Variable Regions	345/654	149/654	276/654	251/654
Pi	316/654	-	271/654	245/654
Singleton Sites	29/654	149/654	5/654	6/654
ii	515/654	580/654	527/654	539/654
si	73/654	25/654	65/654	69/654
sv	67/654	50/654	62/654	47/654
R	1.09	0,51	1,06	1,46

All frequencies are averages (rounded) over all samples.
Pi = parsimony informative; ii = identical pairs; si = transitional pairs; sv = transversional pairs; R = si/sv.

Kimura 2-parameter distances based on sequence divergence among individuals of the same species ranged from 0 to 0.063 (mean 0.011, s.d. 0.002). Interspecific variation among congeneric species ranged from 0.155 to 0.210 for *Aurelia*, 0.171 to 0.198 and 0.146 to 0.175 for *Cassiopea* and *Cyanea* respectively (mean range 0.146-0.210, mean 0.175, s.d. 0.002). Barcoding gap (Meyer and Paulay 2005) was observed for all taxa as interspecific variation was always higher than the intraspecific variation and no overlap detected among them. No cryptic species were discovered from DNA barcode analysis of Scyphozoan species, as individuals of the same species always cluster under the same branch.

When we consider the whole data set, pairwise distance (Kimura 2-parameter) among species of the same family ranged from 0.191 to 0.298 (mean 0.243, s.d. 0.012), and found out to be lower than the pairwise

distance between species of the same order (0.191-0.313; mean 0.263, s.d. 0.002) as expected. Genetic distance within the order Coronatae was estimated using nucleotide sequence of *Atolla vanhoeffeni* (GQ120085), which was gathered from GenBank to be used in comparison with *Nausithoe atlantica.* Minimum genetic distance between groups did not increase with higher taxonomic levels of orders, despite the maximum and mean genetic distance. The maximum and minimum mtCOI divergence was observed among 0 to 0.427 with variation rates of 0% and 42.7% respectively (Supplementary data available as requested).

Table 2. Nucleotide composition of Scyphozoan orders.

	T(U)%	C%	A%	G%	A-T%
Coronatae	38.0	16.1	28.1	17.8	66.1
Rhizostomae	35.8	18.4	26.8	19.0	62.6
Semaeostomae	35.2	19.5	25.5	19.8	60.7

Estimation (maximum likelihood) of substitution pattern and rates were calculated using Kimura 2-parameter model (Table 3). Each substitution rate given in matrix represents the probability of substitution from one base to other. Transitional substitutions were: 12.85% for A/G; 26.25% for T/C; 13.77% for C/T and 9.41% for G/A. Transversional substitution rates were given in Table 3. Transition/transversion bias estimated for the dataset was calculated as R=1.58. Nucleotide frequencies of 26.33%, 35.69%, 18.72% and 19.27% were used for A, T, C and G respectively, during the calculations.

Table 3. Maximum likelihood estimate of substitution matrix.

	A	T/U	C	G
A	-	6.73	3.53	**9.41**
T/U	4.97	-	**13.77**	3.63
C	4.97	**26.25**	-	3.63
G	**12.85**	6.73	3.53	-

Numbers indicate the probability of substitution (*r*) from one base (row) to another base. Rates of different transitional substitutions are shown in bold.

Codon based test of neutrality was estimated using Nei-Gojobori model. Number of synonymous (dS) and nonsynonymous (dN) substitutions per site were calculated as test statistic value (dN-dS). P

values lower than 5% level (0.05) was considered significant. P values higher than 5% levels were detected only among individuals of the same species and considered as non-significant (Supplementary data available as requested).

The Neighbor Joining tree (Figure 1) constructed using Kimura 2-parameter model assigned individuals of the same species under the same cluster. There is no case that a species was clustered together with a different species. Also three orders of the class Scyphozoa were well separated from each other and clustered into three different groups. Species of the same family also clusters under the same branch except the Semaeostomaen family Pelagiidae. *Chrysaora* and *Pelagia* were supposed to cluster under the same branch but species of the family Cyanea was also clustered with them.

Neighbor joining tree of mitochondrial COI sequences revealed two main branches: first branch includes two species from the order Coronatae; *Nausithoe atlantica* and *Atolla vanhoeffeni*. This cluster was well separated from the second main branch including the other two orders; Rhizostomae and Semaeostomae.

They were clustered under the same main branch, but well separated from each other. Second main branch was divided into two branches. First branch includes only the species *Rhopilema nomadica* of the family Rhizostomatidae. Second branch includes three sub branches. First two sub branches include the rest of Rhizostomaen families and completing the order Rhizostomae with *Rhopilema nomadica*. Genus *Crambionella* and *Catostylus* clustered together at the first sub branch, as they are both members of the family Catostylidae; and the genus *Nemopilema* was clustered under the same branch but well separated from Catostyldiae. Second sub branch includes three well separated clusters: species from genus *Cassiopea*, *Phyllorhiza* and *Marivagia*. Remaining sub branch, the third one includes the species of the order Semaeostomae. Four taxa belonging to the order Semaeostomae clustered under four different branches. Three *Aurelia* species in first cluster, *Pelagia* species at next, then *Cyanea* species under the same branch with *Chrysaora* species, but significantly separated from them. The only result incongruous with the current taxonomy was separation of the members of the family Pelagiidae. Instead of clustering together, *Pelagia noctiluca* and *Chrysaora melanaster* clustered under the same branch but separated and both closer to the members of Cyaneidae family.

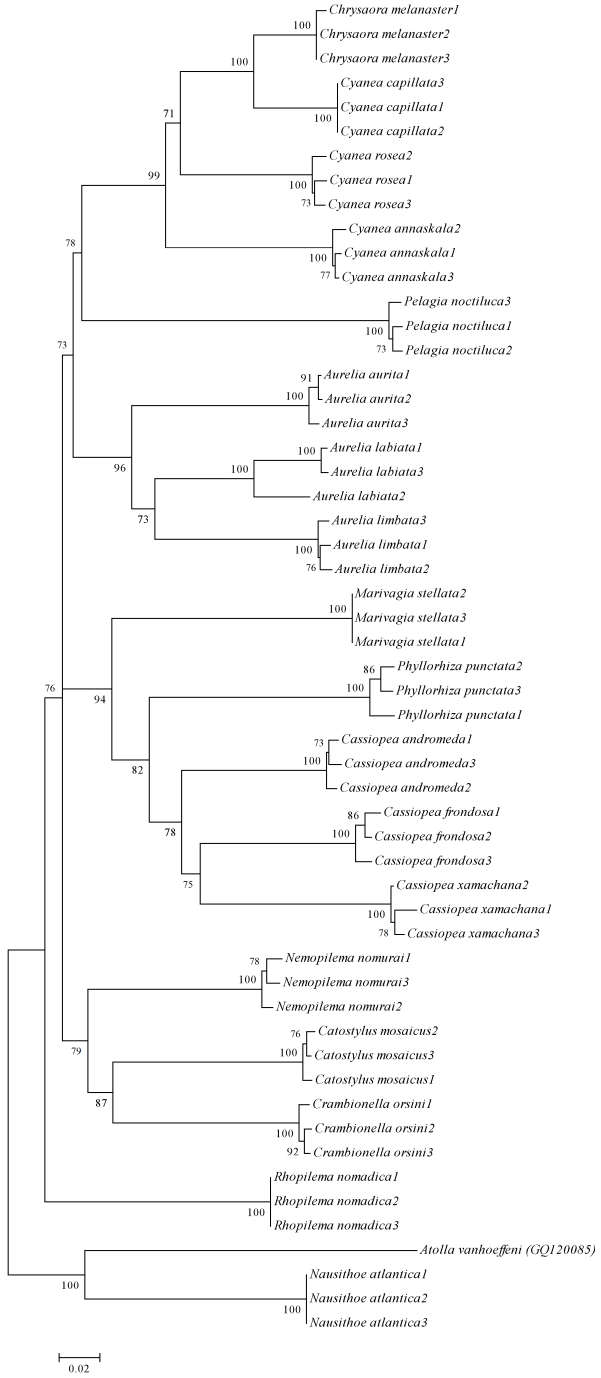

Figure 1. Neighbor Joining (Kimura 2-parameter) tree of 19 Scyphozoan species.

Bootstrap tests resulted in strong support for the clades representing 18 species from 3 orders (71-100%). Bootstrap supports for intraspecific nodes were also high (minimum 71%) as it is not always possible to calculate high bootstrap values among individuals of same species with low genetic distance.

DISCUSSION

DNA barcoding can be an important molecular approach in identification of Scyphozoan species. Yet, it has to be used together with morphological taxonomy as it is useless until a reference sequence for comparison exist in DNA library. A DNA barcode should be used to identify species based on sequence similarities in DNA libraries, not to describe an unknown species; therefore DNA barcoding approach needs a comprehensive database representing wide range of a taxonomic group for accurate identification of the barcoded species.

DNA barcoding has been used to uncover the existence of cryptic species belonging to Medusozoa (Dawson and Jacobs 2001; Dawson and Martin 2001; Holland *et al*. 2004). Mitochondrial *COI* sequences were also used to associate different life stages. Polyps were successfully matched up with corresponding medusa stages in previous studies (Govindarajan *et al*. 2005a, 2005b) and synonymous species names were corrected (Alroy 2002).

Even though *COI* barcodes has been shown to contain a phylogenetic signal among closely related taxa (Bucklin *et al*. 2010), it is hard to talk about a sufficient amount of phylogenetic signal at deeper taxonomic levels (Hajibabaei *et al*. 2007). However this lack of phylogenetic information does not reduce the effectiveness of DNA barcodes as molecular identification tools.

There was a significant tendency towards C/T transitions (26.25%), which explains the T consistency in nucleotide compositions. This A-T rate seems characteristic for all Scyphozoan species without any dependency to the order or family. Except intense C/T transitions, nucleotide composition seems to be in balance in terms of transition/transversion bias.

Among all of the Scyphozoan families, only Pelagiidae seems to be not congruent with the taxonomic classification, as genuses *Pelagia* and *Chrysaora* was not clustered under the same node. These two taxa require increased taxon samplings together with robust phylogenetic data to investigate deeper relationships. Apart from that *COI* barcodes seems to be effective to identify Scyphozoan species and cluster them correctly according to higher levels of taxonomic classification like families and orders.

CONCLUSION

Nucleotide sequences from 5' region of mitochondrial *cytochrome c oxidase subunit I* gene was found to be an effective tool to characterize patterns of intraspecific and interspecific variation for Scyphozoan species. Totally, 55 *COI* barcodes (one from GenBank) analyzed for 19 species and significant distance were calculated among different taxonomic levels. Genetic distance was found out to be ascending as the taxonomic levels becomes larger. *COI* barcode can be used to identify Scyphozoan species and in the mean time helping to construct basic phylogenetic relationships. For sure it is only possible to identify and/or classify a species when there is a close nucleotide sequence in the DNA library available to compare. It will soon be possible to construct better trees for every Scyphozoan species identified as COI barcode libraries like BOLD and GenBank becomes more comprehensive. Moreover, individuals from broader ranges should be sampled to resolve cryptic species and phylogeographic distribution of the species.

This study compromised approximately %11 of known Scyphozoan species. Further studies, including expanded datasets of Scyphozoan barcodes will give us a better understanding of phylogenetic relationship of these invertebrates.

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Construction of Prs416g Vector for use as Feed Additive of B-1,3-Glucanase in Culture of Marine and Fresh Water Fish

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ABSTRACT

After the restriction of antibiotics as feed additive in marine and inland water fisheries hatchery, many researchers have focused on natural ingredients as an alternative to use of antibiotics. In addition to many natural additives, β-glucans from different sources such as yeast, bacteria, fungi, oats, rye, barley are thought to be effective product on immunity and so, improving the health of animals. With this study, a recombinant vector pRS416G was formed by transferring β-1,3-Glucanase to pRS416, a new replicative shuttle vector developed for *E.coli* and *Saccharomyces cerevisiae*. By transferring pRS416G to competent *E. coli* DH5-α cells, it is thought to lead for further studies.

Keywords: β-1,3-Glucanase, pRS416, cloning, *Saccharomyces cerevisiae*

INTRODUCTION

It is well known that the usage of antibiotics as feed additive have positive effects on utilization of feed and growth performance. The unnecessary usage of antibiotics in feeds, antibiotic residues may cause food poisoning for human. Because of the development of multi-drug resistance of pathogenic microorganism like *Salmonella* and *Campylobacter* in poultry. Therefore, the usage of antibiotic growth factors has been banned by European Union dating from 1 January 2006 with the concern of threatening human health by using antibiotics in feeds causing bacterial resistance and leaving the residues in animal by products. (Keser *et al.* 2008) The studies about getting under control enteric bacterial illnesses protecting digestive system ecology healthy and development of feed additives as alternative to antibiotics for strengthening immune system of animals have gained speed upon this banning. Recently, as alternative to feed additives, usage of enzymes have gradually become widespread.

Various enzymes such as xylanase, β-glucanase, pectinase, cellulase, protease, lipase, phytase, α-galaktosidase, β-mannanase have been used in feed industry by means of being added to compound feed separately or combined. Usage of enzyme and immune stimulated product such as β Glucans increase digestion degree of feed and rates of benefiting from feed for animals (Karademir and Karademir 2003).

The most important input of specifying affectivity and cost in cultivation of animal is feed raw materials containing high level of protein have been intensely used in feeds. The high level of protein, having balanced amino acid composition being found in fish flour in fish feeds indispensable protein resource. However, decreasing fish stocks generally directly used in human nutrition, decreased production of fish flour recently and feed producers have started import fish flour. In consequence of this, the price of fish flour have increased the cost of feed and, brought into question replacement of fish flour with any other sources. In this context, with the aim of decreasing feed cost, determining alternative protein sources and terms of use instead of fish flour, various studies have been performed by fish nutritionist (Akiyama *et al*. 1995; Webster *et al*. 1992; Wu *et al.* 1995). It is indicated that enzymes have been used in various countries to obtain the increasing of digestion and so, live weight gain and feed evaluation in feeds based on plantal raw materials (Karademir and Karademir 2003).

Raa (2000), has informed that with the usage of immunostimulant β-glucans in culture fishing, fish have obtained resistance to various bacterial, viral and parasitary illnesses, mortality has decreased because of opportunistic pathogen in larva period, increasing has been seen in the effectiveness of antimicrobial materials and growth and negative effects of stress has decreased.

Dalmo *et al.* (2008), shows that increase of growth performance depending on glucan level in fish, feed timing, ambient temperature and species under study. It has been informed that development of feeding strategies is required for each fish species related to glucan dose. Beta glucan stimulated system of animals. The immune systems animals have been weakining due to being exposed to ambient stress factors every day and so rendering sensitive of animals to get infectious and other diseases. Serum immunoglobulin levels of animals, being nourished with feeds including beta glucan, have risen and so they are getting resistant to infectious and other diseases. But, adding of this enzyme outwardly has been increased the feed cost. Being inadequate of raw materials, constitutive of fundamental of prepared feeds, and increasing in price seem important problems of culture fishing. (Arıman and Aras 2002). In this regard, adding of enzymes products directly to feed the decrease gene low

cost. Nowadays, commercial enzymes are being sold for using feed of poultry but, enzymes produced for fish feed have just started to take part in marketplace. (Yiğit and Koca 2011).

With this study, pRS416G recombinant vector has been created by transferring β-1,3-Glucanase enzyme to pRS416 for strengthening both immune system of fish and decreasing the cost of feed additive. Thus, replicative vector has been developed for yeast (*Saccharomyces cerevisiae*). It is thought that transferring competent pRS416G to *E.coli* DH5-a cells will lead for further studies.

MATERIALS AND METODS

We supplied pRS416 vector (Figure 1), from Cukurova University, Animal Biotechnology and Genetic Engineer Laboratory, Adana Turkey, β-1,3-glucanase gene of *C. Cellulans in E.coli (*pETG11 (puC18+ β-1,3-glucanase) vector) from B.Devrim ÖZCAN (Osmaniye Korkut Ata University, Osmaniye Turkey), *Escherichia coli* DH5α strain from Stratagene, restriction enzymes, marker DNAs and other chemicals from Fermentas and Sigma.

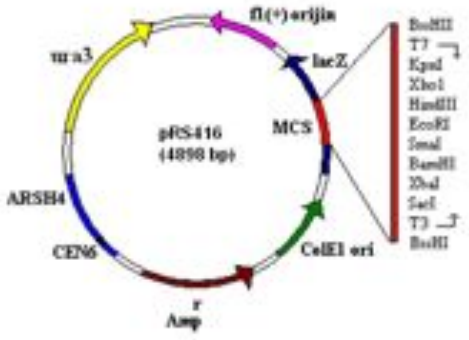


Figure 1. Structure of pRS416 plasmid (4898 bp).

Growth Mediums and enzyme assays

E. coli strains DH5α was grown on Luria Bertani (LB/amp) medium (10 g bacto tryptone, 5 g yeast extract and 10 g NaCl per l, 50 µg/ml amp pH 7.5) and LB-agar-amp-Xgal (1.5% w/v agar, 50 µg/ml amp, 40 µg/ml Xgal,) at 37°C and LB-amp-agar-laminarinase(1%w/v laminarinase, 1.5% agar, 50 µg/ml amp) at 37°C for determining enzyme activity was use

(Sambrook *et. al.* 1989). *E.coli* strain was stored at -20 ˚C using 15% v/v glycerol.

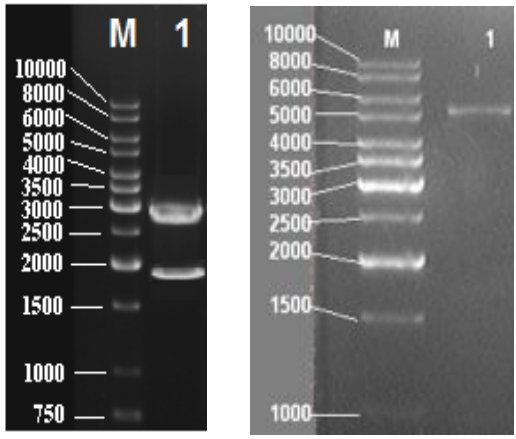
Extraction of β-1,3-glucanase gene (1900 bp) from pETG11 vector
pETG11 plasmid DNA was isolated from *E. coli* as described by Birnboim ve Doly (1979). Digestion with SacI of pETG11were performed by standart methods (Sambrook et. al., 1989). Thus β-1,3-glucanase gene was separated on agarose gel electrophoresis and extracted with using purification method from agarose gel.

Isolation and Transformation of pRS416 plasmid
pRS416 plasmid DNA was isolated from *E. coli* according to Birnboim ve Doly (1979). After pRS416 plasmid DNA was purificated from RNA, DNA was digested with SacI. β-1,3-Glucanase gene was ligated into the *E. coli*-yeast shuttle vectors pRS416 to create the yeast *Saccharomyces cerevisiae vector* pRS416G. pRS416G harbouring the β-1,3 glucanase was transfered into *E.coli* DH5α by $CaCl_2$ methods Hanahan, (1985). All recombinant techniques were performed according to Sambrook et. al. (1989). *E.coli* cells harbouring pRS416 recombinant plasmids were selected on LB/X-gal/Amp/Agar (40 μg/ml Xgal, 1.5% agar, 50 μg/ml amp) plates.

Detection of activities on laminarinase plates of E. coli colonies expressing β-1,3 glucanase
β-1,3 glucanase-positive *E. coli* cells harbouring recombinant plasmid pRS416G were selected on LB/Laminarinase/Amp/agar (1%w/v laminarin, 1.5% agar) by staining with 0.1 w/v Congo Red for 15 minutes followed by destaining with 1M NaCl for 15 minutes.

RESULTS AND DISCUSSION

pRS416 and pETG11 were digested with SacI and digested plasmids were subjected to electrophoresis on a 0.8% agarose gel. Then digested products were imaged using the Gel Documentation System (Figure 2). β-1,3-glucanase (laminarinase) extracting with purificating method from agarose gel was ligated into pRS416 plasmid DNA and then constructed pRS416G harbouring β-1,3-glucanase was then transfered into *E.coli*. (Figure 3)



A B

Figure 2. A. Agarose (0.8%) gel electrophoretic profile of SacI restriction enzyme digested pETG11 (pUC18/β-1,3-glucanase) plasmid DNA (M: marker, 1: pETG11/*Sac* I). **B**. Agarose (0.8%) gel electrophoretic profile of SacI restriction enzyme digested pRS416 plasmid DNA (M: Marker 1:pRS416/*Sac*I)

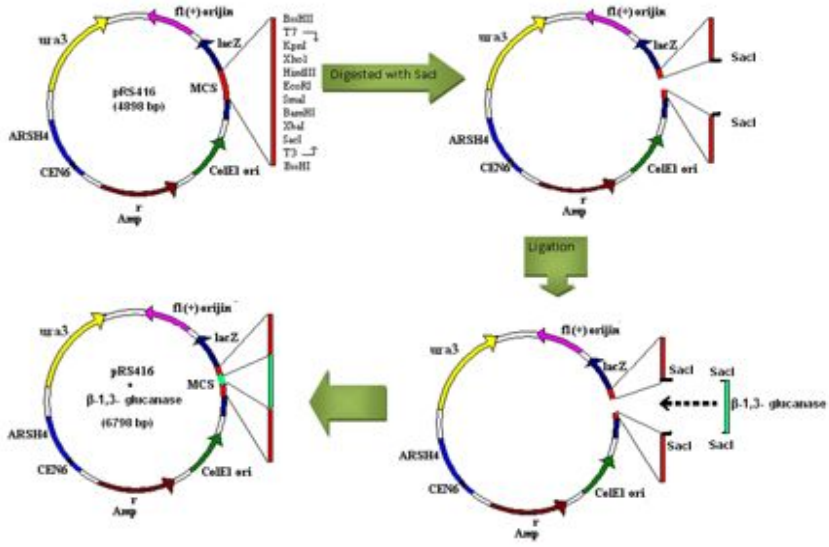


Figure 3. Constituting of recombinant pRS416G plasmid (6798 bp).

Recombinant *E.coli* cells harbouring pRS416G recombinant plasmids were determinated on X-gal plates (Figure 4). pRS416G recombinant plasmid DNA harbouring the β-1,3 glucanase (pRS416G) was then isolated from recombinant *E. coli* and digested with SacI. digested plasmid (pRS416G/S*ac*I) were subjected to electrophoresis on a 0.8% agarose gel. Then digested pruducts were imaged using the Gel Documentation System (Figure 5).

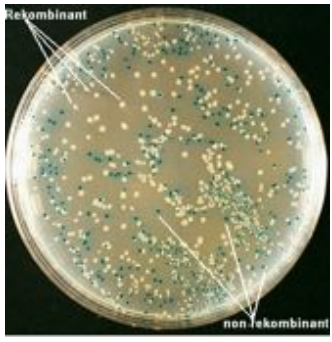


Figure 4. Recombinant colonies and non-recombinant colonies growing transformation plates.

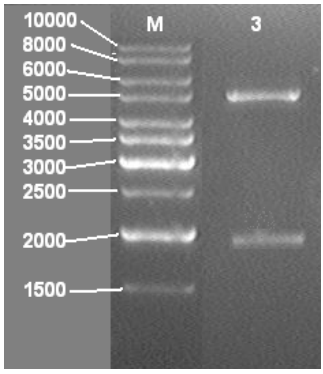


Figure 5. Agarose (0.8%) gel electrophoretic profile of SacI restriction enzyme digested pRS416G plasmid DNA (M:Marker 1:pRS416G/*Sac*I).

β-1,3 glucanase activity of transformed bacteria on laminarin plates was detected with Congo Red staining procedure and clear zones were appeared around the recombinant colonies (Figure 6).

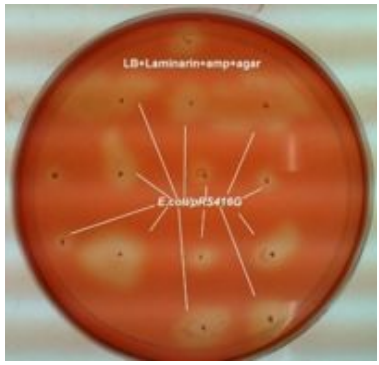


Figure 6. Determination of laminarinase activity of *E.coli*/pRS416G bacteria on LB/laminarinase/amp/agar plates.

In this study, we have cloned β1,3-glucanase gene into *E. coli*-yeast shuttle vectors pRS416 to create pRS416G for finally expressing in the yeast *Saccharomyces cerevisiae*. pRS416G first transfered into competent *E. coli* DH5-α cells. With the same purpose, two new plasmids, pEC3 and pECkan, were constructed and their use in yeast transformation described by Bardazzi and Casalone (2004). Both plasmids are derivative of the pRS416 vector, in which the URA3 auxotrophic marker was replaced by the LEU4* gene (pEC3) or the kanMX4 gene (pECkan). Özcan (2001) cloned both an α-amylase gene of *Bacillus subtilis* RSKK246 and a gene encoding β−glucanase from *B. subtilis* RSKK243 and expressed in both *E. coli* XL1-Blue MRF and *B. subtilis* YB886 by using the vectors pUC18 and pUB110 respectively.

He also cloned these genes into the *E. coli*-yeast shuttle vectors pRS406 and pRS416 for transfer into the yeast *Saccharomyces cerevisiae*. Then he expressed these constructs carrying α-amylase and β-glucanase, genes which were cloned into the pRS406 vector, by integration into the yeast chromosome. He also expressed these genes in the yeast by autonomous replication of the ARS/CEN plasmid pRS416. He demonstrated that genes belonging to *B. subtilis* can replicate in both *E. coli* and in the yeast *S. cerevisiae*. On the other hand Peng and et.al. (2011) isolated the exo-β-1,3-glucanase (WsEXG1gene) structural gene from both the genomic DNA and cDNA of the marine yeast *Williopsis saturnus* WC91-2 by inverse PCR and RT-PCR. They over-expressed to the WsEXG1 gene in *Yarrowia lipolytica* Po1h and purified and characterized to recombinant WsEXG1. Therefore They showed that the purified rWsEXG1 had high exo-β-1,3-glucanase activity and the recombinant β-1,3-glucanase may have highly potential applications in food and pharmaceutical industries.

pRS416G vector created in our study will lead to further studies and will be able to be used in yeast biotechnology. Due to the fact that adding these enzymes or enzyme related β−glucans to fish feed outwardly reduce the costs much, these additives will decrease feed cost and at the same time, it will increase immune system of fish and resistant to diseases.

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A New Tool: Phage Therapy Against Fish Pathogens in Marine Aquaculture

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ABSTRACT

Bacteriophages are candidates as therapeutic and prophylactic agents for bacterial infections. The application of phages prevention and treatment of human bacterial infections were studied in the 1980s. Over the past years, phage therapy in aquaculture increasingly gained interest and attraction. Cultured fish and sellfish were affected by microbial attacts. Although chemotherapy, vaccination, usage of probiotics are methods to treat or prevent bacterial infections, phage therapy can provide an alternative and an enviromental friendly approach in treating diseases in marine aquaculture. Phages are highly host specific that they only infect target organism without affecting the normal marine microflora.

Here, we describe a new biotechnological tool to treatment of *Vibrio anguillarum* and *Vibrio harvei* pathogens in cultured marine fish. In our preliminary studies, we isolated and characterized host-specific phages by looking their genomic sizes and efficency against on experimental infection with these bacterial strains.

Keywords: Phages, *Vibrio anguillarum, Vibrio harvei,* marine fish.

INTRODUCTION

Aquaculture is the cultivation of fresh water or sea water animals/plants for food purpose. The aquaculture animal includes fish, prawn, squid, crabs, mollusks. Vibriosis is a major pathogenic disease in the aquaculture animals with high mortality rate (Muroga 1997). Vibriosis is caused by the different species of *Vibrio,* includes *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelaginus*, *V. ordalii*, and *V. parahaemolyticus* (Austin and Austin 1993; Akaylı *et al.* 2002). Vibrio is a gram negative, comma shaped bacteria that are usually present in water in very low count and sudden changes in physiochemical conditions of water may lead to the rapid proliferation of the bacterium (Don *et al*. 2005).

The infectious bacteria can be controlled by the use of antibiotics, chloramphenicol, natural therapeuic agents or vaccines (Vinod *et al*. 2006;

Yang *et al*. 2007; Jayasudha *et al*. 2011). Although these methods are ideal methods to prevent infectious diseases, the frequent use of antibiotics has resulted in increasing drug resistant pathogenic bacteria.
Bacteriophage treapy has been used for controlling and treating bacterial infection human and animal diseases as an alternative approach. Bacteriophages are viruses consisting of DNA or RNA genome contained within a protein coat that are extremely abundant in nature and believed to be important in controling bacterial infection. They infect bacteria and multiply inside of host cell before releasing phage by actively lysing the cells.

Phage therapy has advantages over other therapies; 1) it is effective against multidrug-resistant pathogenic bacteria 2) higly specific for target bacteria 3) the cost of developing a phage system is cheaper that that of developing a new antibiotic 4) phages do not effect eukaryotic cells.

In literature, isolation of phages of some fish or crustaceans pathogen bacteria such as *Aeromonas salmonicida* (Rodgers *et al*. 1981), *A. hydrophila* (Merino *et al*. 1990), *Yersinia ruckeri* (Stevenson and Airdrie 1984), *Lactococcus garviae* (Nakai *et al*. 1999), *Flavobacterium psychrophilum* (Stenholm *et al*. 2008) have been reported. But there were no mentioned application of phages or their products as a biological control of fish pathogens (Matsuzaki *et al*. 2005; Sanmukh *et al*. 2012).
In this study, we worked with *Vibrio anguillarum* and *V. harvei* which are a common luminous bacterium occuring marine form of life such as fish or crustaceans (Vinod *et al*. 2006). We design two stages of experiments for using phage therapy tool for Vibriosis treatment. In the first stage experiment, we focused on growing reference phages and reference bacteria. Then we designed in vitro experiements to show the effect of bacterophage against host *Vibrio anguillarum* and *V. harvei*. In addition to this, we developed genotypic characterization methods for second stage isolation and identification of field isolate of phages. In the second stage, we will develop isolation and identification methods for Vibrio phages and pathogen Vibrio spp. from environment and aquatic animals. We will look in vitro and in vivo survival of isolated Vibrio bacteriopages. Finally we will stard chalenging the potential of lytic infection for *Vibrio anguillarum* and *V. Harvei* in infected fish.

MATERIALS AND METHODS

Bacterial strains

Two field isolates and two ATCC bacterial cultures were used, consisting of *Vibrio harveyi* 33867 ATCC, *V. anguillarum* 43305 ATCC, *V. harveyi*

isolate 1 and *V. anguillarum* isolate 2. Strains were cultured on both nutrient medium and tryptic soy agar (TSA) with marine salt and Thiosulfate citrate bile salts sucrose (TCBS) agar at 28 ^{o}C for 2-3 days. Each culture was stored with %30 glycerol at -20 ^{o}C freezer for further work.

Growing phages

Bacteriophages of *Vibrio spp.* ATCC 51589-B1 and ATCC 11985-B1 were used in this study as reference strains. We modified Vinod *et al*. (2006) and Sugimoto *et al*. 1999 phage isolation procedure as our phage gowing procedure. One milliliter of phage suspension was added to 12 and 18 h old *V. harveyi* and *V. anguillarum* cultures grown in 50 ml tryptone soya broth with 1 % marine salt (TSBS) and incubated at 28 ^{o}C for 12-24 h. 5 ml of mixture was centrifuged at 10,000 ×*g* for 15 min at 4 °C. The supernatant was filtered through 0.45 µm filter and the filtrate tested for the presence of phage by inoculating 100 µl of the filtrate on lawns of *V. harveyi* and *V. anguillarum* prepared on tryptone soya agar with 1 % NaCl. The plates were left at 28 ^{o}C temperature for 18 to 48 h and inspected for zones of clearing. Areas of clearing were examined for confirmation of phage activity, as described above.

Bacteriopages of *Vibrio spp.* ATCC 51589-B1 and ATCC 11985-B1 are also grown in TSA liquid medium. Grown phages (1 ml) were added to 50 ml of Trypticase broth medium containing 1 % marine salt. Phages and bacterial suspesion were grown in shaking incubator for 48 h at 28 ^{o}C. Then their absorbance were measured.

Purification of bacteriopage DNA

Phage DNA was extracted from filtrated infected cultures (Bastias *et al*. 2010). This filtrate was precipitated with polyethyleneglycol (PEG-8000) and NaCl at a final concentration of 10% and 1.5 M respectively. The precipitated phage was then centrifuged at 11 000 *g* for 20 min. For DNA extraction, samples were incubated with DNase (2 mg ml-1) and RNase (100 mg ml-1) for 1 h at 37°C. These samples were subsequently treated with 500 mg ml-1 proteinase K for 15 min at 65°C. Sodium dodecyl sulfate (SDS) was added at a final concentration of 0.5% (w/v). After incubation at 65°C for 45 min, the solution was extracted twice with phenolchloroform. Finally, the DNA was precipitated by adding 1/10 volume 3 M sodium acetate (pH 5.0) and two volumes of absolute ethanol at -20°C. After the pellet was washed with 70% ethanol, it was dissolved in TE buffer (0.01 M Tris, 0.001 M EDTA, pH 7.5).The DNA yields from both extracts was determined using a Nanodrop spectrophotometer (Nanodrop Technologies, USA) with DNA yields ranging between 3 and 100 $\mu g^{-1}g$.

Bacterial DNA extraction

Genomic DNA from both reference and field isolates of *Vibrio* species were extracted by modification of methods of Cagatay and Hickford (2005). Bacterial suspension was harvested from agar plates into a 1.5 mL eppendorf tubes containing TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Samples were preheated with lysozyme (2 mg/mL in 50 mM EDTA) at 37 ^{o}C for 60 min. Genomic DNA was extracted with Qiagen Bacterial Extraction Kits (Qiagen, USA) according to the manufacturer's instruction. The extracted DNA was then treated with ml of SET (Sodium-EDTA-Tris) solution (75 mM NaCl, 25 mM EDTA and 20 mM Tris, pH 7.5). Ten percent SDS and 200 mg/ml of proteinase K were added and the tubes were inverted gently and incubated at 55 ^{o}C for at least 2 h with occasional inversion. Subsequently, 1.2 ml of 5 M NaCl and 4 ml of phenol/chloroform (1:1) were added and the contents mixed. The tubes were shaken for 50 sto lyse the bacterial cells. The lysates were then centrifuged at 13.000xg for 2 min and the aqueousphase containing the DNA was collected. The crude lysates were purified with equal volumes of phenol: chloroform followed by ethanol treatment. Purified DNA was eluted in 50 µL of TE buffer. The DNA yields from both extracts was determined using a Nanodrop spectrophotometer (Nanodrop Technologies, USA) with DNA yields ranging between 3 and 100 $\mu g^{-1}g$.

RESULTS

Determination of lytic activity of Vibrio phages on agar medium

Both phages tested against *V. harveyi* and *V. anguillarum* showed lytic activity in semisolid medium. As shown in plate pictures, clear zones proved lysis effect on top of plate (Figure 1).

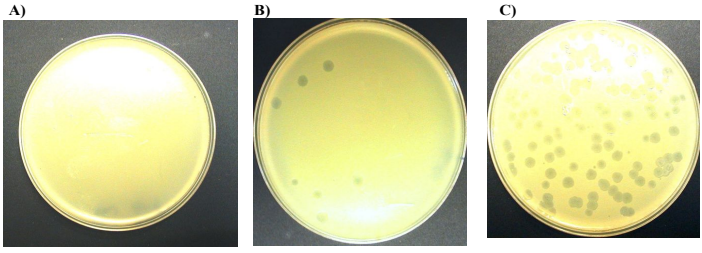

Figure 1. Phage ATCC 51589-B1 plaques with *V.harveyi*. **A.** First day **B.** 12 hours **C)** 24 hours

Effect of phages on broth culture of Vibrio spp.

The lytic activity of two phages in broth medium was also tested by looking growth rate and absorbance changes during incubation period

(Figure 2). We measured those bacterial and phages suspensions every four hours until 48 hours completed. Vibrio medium changed color and totaly become clear after 48 hour of incubation with phages.

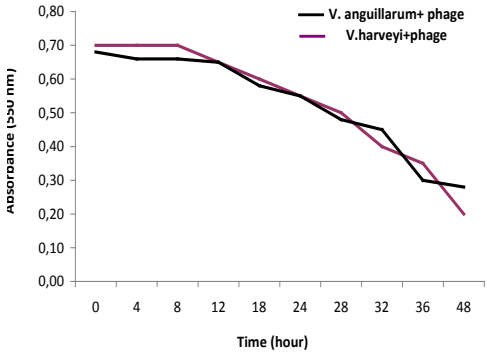

Figure 2. Vibrio species treated with phages for 48 hour. **Lane 1.** *V. anguillarum*+ phages *Vibrio spp.* ATCC 51589-B1 **Lane2.** *V. harveyi*+ phages *Vibrio spp.* ATCC 51589-B1.

DNA extraction results of phages and host bacteria

We developed quick DNA extraction protocols for douple stranded phage DNA and bacterial DNA for isolation field strains. Extracted DNA products were electrophoresed to show presense of genomic materials on agarose gels (Figure 3).

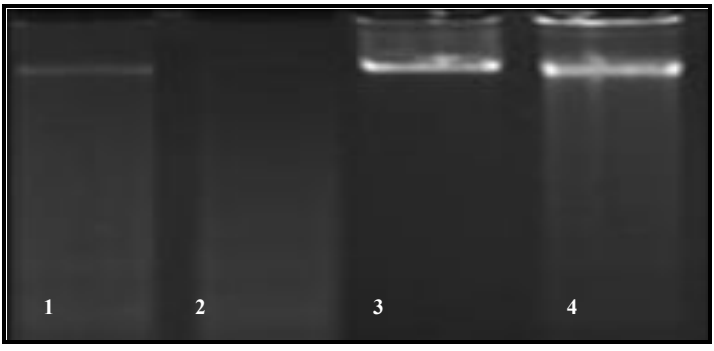

Figure 3. Electrophoretic analysis of DNA from extracted phages and thier host. **1.** DNA extracts od *Vibrio spp.* ATCC 51589-B1 **2.** DNA extracts of *Vibrio spp*. ATCC 11985-B1 **3.** DNA of *V. harveyi* **4.** DNA of *V. anguillarum*.

DISCUSSION

Outbreak of diseases in marine aquaculture results in big economic losses. Vibrio species are causative agents of vibriosis in aquatic animal like

shrimp and fish (Karunasagar *et al*. 1994; Choudhury and Debnath 2012).Treating and protecting animals from bacterial diseases has been managed by different applications like using antibiotic or vaccines for many years (Clark and March 2006). However, phage treapy involves to kill pathogenic bacteria as an alternative to the thetapeutics (Pereira *et al*. 2011; Stoms *et al*. 2010).

In this study, before isolation phages from environment to treath vibriosis in aquatic animals, we designed and showed lytic activity against in reference strains of *Vibrio harveyi* and *V. anguillarum*. In our finding, Vibrio phage ATCC 51589-B1 and Vibrio phage ATCC 51589-B1 had produced clear plaques in solid medium after 24 hour incubation. We have seen also a total discoloration in liquid medium. We measured absorbance changes at 550 nm between starting incubation time 0 h and 48 hours incubation (Figure 3). Our observation results were further supported by the findings of Nakai and Park (2002). Payne *et al*. (2004) has reported *V. harveyi* phages could lyse different Vibrio species.

In conclusion, our primary finding of positive phage and host interreaction can be used for second stage in vitro and in vivo challenge experiment of animals. Further studies should be developed to select the effective phage strains to threat marine fish bacterial enfection, or effective combination of phages with therapetic agents together as a new tool for marine fish pathogens.

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A Preliminary Study on Induction of Triploidy by Caffeine Treatment in the Trout

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ABSTRACT

To allow the development of aquaculture activities, chromosome manipulation techniques are necessary for the genetic improvement of the trout. In the present paper suitable conditions were determined for caffeine treatment that is safe and inexpensive for the induction of triploid trout. To suppress the first meiotic division, fertilized eggs were exposed to three different concentrations (5- 10 and 15-mM) caffeine solution for 10 min beginning at 15 min after fertilization. After that, the eggs were incubated at ambient temperature until hatching. Incidence of triploid fry was determined from flow-cytometric analysis. There was no significant difference in the mean triploid rates between 5- and 10-mM caffeine treatments and not observed successful results in these groups. The best proportion of triploids were obtained with a 15 mM caffeine treatment at 10 °C for 10 min duration initiated 15 min after fertilization.

Keywords: Trout, *Oncorhynchus mykiss*, Triploid, Flow cytometry, Caffeine

INTRODUCTION

Chromosome set manipulation techniques have been developed and applied to the genetic improvement of various fish species over the past few decades (Pandian and Koteeswaran 1998 or Arai, 2001). Especially, the induction of triploids may be beneficial to salmonid aquaculture. Triploids are organisms with three sets of chromosomes in their somatic cells instead of the usual two in diploids. If the general assumption that the three sets of homologous chromosomes of triploids cannot synapse in meiosis is true, then triploids should be sterile. The importance of triploid animals in aquaculture arises from this predisposition for sterility. Sterility means that energy otherwise used for gonadal development is channeled into somatic growth. Adult triploids should therefore grow faster than their diploid counterparts. Sterility also implies that triploids are likely to have an extended market season in that they should not suffer reduced meat quality associated with spawning activity. A bonus of using sterile triploid animals in aquaculture is that they eliminate potential risks of genetic pollution posed by animals escaping from farms. Use of triploids

could also enable farming in high conservation value areas and even 'ranching' of exotic genotypes in non-indigenous habitats of particular species (Liu *et al*. 2004).

For the mass production of triploid rainbow trout in commercial hatcheries, large quantities of eggs must be treated simply, cheaply, and safely because the treatment must not damage either humans or the natural environment (Thorgaard *et al*. 1986). Diaz *et al*. (1993), reported that triploid rainbow trout *Oncorhynchus mykiss* have a higher mean weight than diploid fish and especially, have observed in faster growth in triploid females of rainbow trout after the normal age of sexual maturation. Also, the triploid rainbow trout *Oncorhynchus mykiss* × *coho salmon Oncorhynchus kisutch* hybrids showed a higher resistance against IHN virus, which is responsible for severe losses in salmonid species aquaculture (Parsons *et al*. 1986). Cold (Sun *et al*. 1992; Pandian and Koteeswaran 1998; Yang *et al*. 1997) and hot (Pandian and Koteeswaran 1998) treatments for triploid induction are safe because no chemicals are used. However, these treatments require an optimal treatment temperature to be maintained in a large volume in order to treat a large quantity of eggs, and expensive large-scale equipment to control water temperature is needed. Pressure treatment (Pandian and Koteeswaran 1998) is also considered safe, but specific equipments required and it is usually difficult to treat many eggs on a commercial scale because the pressure cell has limited volume. Chemical treatments such as cytochalasin B (CB) (Sun *et al*. 1992; Liu *et al.* 2004; Stepto and Cook 1998; Maldonado *et al*. 2001) and 6-dimethylaminopurine (6-DMAP) (Yan and Chen; 2002; Liu *et al*. 2004; Zhang *et al*. 1998; Norris and Preston 2003) are simpler than physical treatments because specific equipment is not required. However, CB is highly toxic, and careful handling is necessary. Moreover, both CB and 6-DMAP are very expensive and not realistic for large scale treatment in commercial hatcheries. Caffeine, a chemical that is a recognized food constituent in many countries, is safer and cheaper than either CB or 6-DMAP. Therefore, caffeine is a promising agent for the mass production of triploids (Okumura *et al*. 2007). But, triploid induction by caffeine treatment have been only reported exclusively in bivalves by Scarpa *et al*.1994 and abalon by Okumura *et al*. (2007).

Therefore, in the present study we intended to ascertain whether induction of triploidy by caffeine treatment in the trout *Oncorhynchus mykiss*.

MATERIALS AND METHODS

Origin of fish stock and induced reproduction

The experiment was carried out in Kahramanmaraş trout farm of Kılıç Holding Co. in Turkey. The one female and one male were manually stripped for release of eggs and sperm that were gently mixed. Water was then added to achieve fertilization.

Induction of triploidy

Given that no previous protocols of caffeine treatment have been established to induce triploids in trout, abalone triploidy induction protocols were used as a baseline to set the parameters for this species (Okumura *et al*. 2007). A preliminary trial was conducted so that the variables would be adjusted. To suppress the first meiotic division, fertilized eggs were exposed to three different concentrations (5- 10 and 15-mM) caffeine solution for 10 min beginning at 15 min after fertilization. (Table 1). Diploid controls were originated from the same parents but not subjecting them to caffeine treatment

Experimental design and egg incubation

A pool of fertilized eggs from different spawners was distributed in triplicate for each trial and one control group. The temperature and dissolved oxygen were monitored with an oxygen and temperature meter (YSI 5750, YSI, Yellow Springs, Ohio). The fertilization was carried out in water at a temperature of 10.0 ±2.4 °C. Approximately 2500 eggs for each treatment were shocked by caffeine treatment and then incubated in hatching cabinet with constant water flow to ensure gentle movement of eggs. During incubation, dead eggs were regularly removed to prevent *Saprolegnia* spp. growth.

Survivals and ploidy evaluation

The survival rate was evaluated after hatching (d.p.h). Survival rate (expressed as percentage) was calculated as the number of live fry in relation with the number of total fry. The larvae were sampled from each treatment group at 3 day after hatching (d.p.h) and fixed with 70% ethanol and stored at -20°C until analysis (Nomura *et al*. 2004). For measurement of the relative DNA content, flow cytometry (FCM) was conducted using a BD FACS Canto flow cytometer (Beckton Dickinson Immunocytometry Systems San Jose, CA, USA). The method of FCM analysis followed the protocol described by Çakmak, (2011).

Statistical analysis

Results are presented as mean±standard deviation of the mean and statistical testing to verify differences between the groups was carried out using a one-way analysis of variance (ANOVA). Differences were accepted as significant when $P<0.05$ (Norusis, 1993).

RESULTS AND DISCUSSION

Treatments for caffeine treatment triploidy induction and their corresponding survival and triploidy rates are shown in Table 1. The survival rate was ranged from 83.24% to 77.54%, and there was no statistical difference between experimental and control groups (Table 1).

Table 1. Treatments for caffeine treatment triploidy induction and their corresponding survival and triploidy rates.

Treatment	Time after fertilization (min)	Shock duration (min)	Survival rate*	Ploidy**	
				Percent Diploid (2n)	Percent Triploid (3n)
Control	15	10	83.24±4.51[a]	100	-
5 mM	15	10	77.54±5.93[a]	87.79	12.21
10 mM	15	10	82.51±5.20[a]	85.29	14.71
15 mM	15	10	82.31±3.26[a]	54.60	45.40

*Values (mean ± S.D. of triplicate) with same superscripts in each line indicate not significant differences ($P<0.05$).
**Based on the relative DNA content measured by flow cytometry

There was no adverse influence of caffeine treatment on survival fryin the present study. While the best triploidy rate was obtained as 45.40% at a 15 mM caffeine treatment at 10 °C for 10 min duration initiated 15 min after fertilization (Figure 1), the lowest rates were respectively determined as 12.21% and 14.71% at 5 ve 10 mM caffeine treatment at 10 °C for 10 min duration initiated 15 min after fertilization, respectively (Table1, Figure 2).

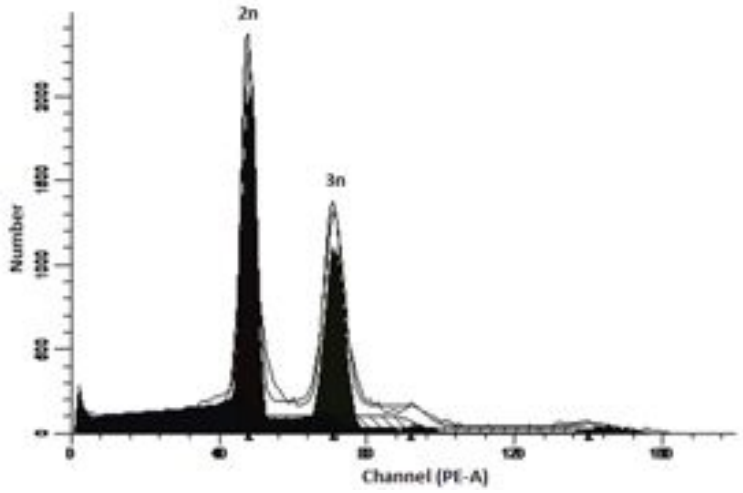

Figure 1. Flow cytometry analysis histogram of the DNA content in the 2n/3n mosaic specimen from 15 mM caffeine-treated fry. 2n, diploid and 3n triploid.

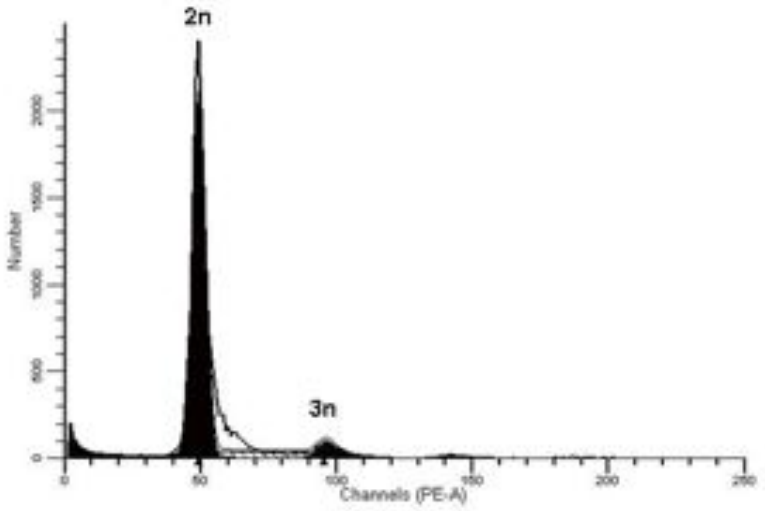

Figure 2. Flow cytometry analysis histogram of the DNA content in the 2n/3n mosaic specimen from 5 mM caffeine-treated fry. 2n, diploid and 3n triploid.

Data obtained from this study indicated that caffeine concentration had significant effect on triploidy rates of trout fry. Until recently, there was no published information on triploidy induction by caffeine treatment in trout. Triploidy induction protocols applied in the present study were best guesses based on available information on bivalves and other abalone

species (Scarpa *et al*. 1994; Okumura *et al*. 2007). Triploid percentages in fry were 45.40% in 15 mM caffeine treatment. This percentage may be low but strongly indicate that caffeine treatment on triploidization of trout is effective. Thorgaard (1981) and Doğankaya (2004) reported that the most effective timing and shock duration for triploid induction in rainbow trout is generally for 10 min duration initiated 15 min after fertilization in rainbow trout.

Therefore, we used similar timing and shock duration for triploid induction in present study. Results of this study showed that 15 mM caffeine treatment is more effective concentration than other caffeine concentrations. However, further detailed investigation is required to examine the effect of caffeine treatment in order to realize a 100% triploid population.

This is a first report to our knowledge regarding the potential of caffeine treatment on induction of triploidy in rainbow trout. As described previously, caffeine treatment is simple, safe, and inexpensive. Therefore, this method can be applied for commercial production of triploid trout in aquaculture. In the near future, aquaculture performance of triploid trout induced by caffeine treatment should be examined and characterized for commercial use.

Acknowledgements

The study was supported by the BAP project (08 M 0205). We thank to Cenk ARZUMAN and Taner SEKER from the KILIÇ HOLDING Co. for allowing this experiment in their farm. Also, we thank the İzmir İnstitute of Technology for its support.

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Triploidy Induction by Combination of Caffeine-Thermal Shock Treatments in the Trout

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ABSTRACT

Triploid is one of the biotechnology methods in trout culture that uses genetic manipulation which alters chromosome structure during fish breeding. Sterile fish may spend a greater part of the nutrients absorbed on body weight gain and therefore may attain a higher growth rate and a more efficient feed conversion. However, the production needs proper optimization in terms of induction parameters in order to obtain higher triploid yield. With the goal to optimize the production of triploid trout, we have begun to pursue the use of combined heat shock with caffeine treatment. To suppress the first meiotic division, fertilized eggs were exposed to combined thermal shock (28°C) with 15 mM caffeine solution for 10 min beginning at 15 min after fertilization. After that, the eggs were incubated at ambient temperature until hatching. Triploidy was estimated by measuring DNA content in cells from fry with flow-cytometric analysis. The rate of triploids at combined thermal shock (28°C) with 15 mM caffeine treatment was 59.21%, but observed survival rate (52.15%) was lower than control group (83.24%). It is the first report on triploidy induction by caffeine-thermal shock treatments in the trout.

Keywords: Trout, *Oncorhynchus mykiss*, Triploid, Flow cytometry, Thermal shock, Caffeine

INTRODUCTION

Chromosome manipulation is considered a practical means of rapid genetic improvement of cultured fish, and is generally not so risky for the environment because it can be regarded as an extension of conventional selective breeding. Worldwide, trout aquaculture is a multi-billion pound industry that has led to the emergence of novel breeding and rearing techniques, including the culture of triploid fishes. Triploids have three sets of chromosomes instead of two sets in diploids. Triploids are generally sterile due to irregular meiotic division of chromosomes resulting in reduced gonadal development and aneuploid gametes. Therefore, in such fish, the energy consumption for sexual maturation

may be avoided and more biological effort may be directed towards improving flesh quality and somatic growth (Basant *et al*. 2004). Especially in rainbow trout, *Oncorhynchus mykiss*, biological characteristics and aquaculture performances of induced triploids have been intensively studied for commercial applications by various researcher (Okada 1985; Suresh and Sheehan 1998; Muller-Belecke *et al*. 2006). Diaz *et al*. (1993), reported that triploid rainbow trout *Oncorhynchus mykiss* have a higher mean weight than diploid fish and especially, have observed in faster growth in triploid females of rainbow trout after the normal age of sexual maturation. Also, the triploid rainbow trout *Oncorhynchus mykiss* × coho salmon *Oncorhynchus kisutch* hybrids showed a higher resistance against IHN virus, which is responsible for severe losses in salmonid species aquaculture (Parsons *et al*. 1986).

Triploid fish are produced through a number of techniques; chemical, thermal or mechanical methods. Both the thermal and mechanical methods are commonly used in production facilities. Thermal (heat) shock is the current standard for production of triploid rainbow trout (*Oncorhynchus mykiss*) and has been used at a production level since the last ten years. Although the process is viable, the triploid rate is variable and the survival is often poor (Couture *et al.* 2008). Pressure treatment (Pandian and Koteeswaran 1998) is also considered safe, but specific equipments required and it is usually difficult to treat many eggs on a commercial scale because the pressure cell has limited volume. Chemical treatments such as cytochalasin B (CB) (Liu *et al*. 2004 Maldonado *et al*. 2001) and 6-dimethylaminopurine (6-DMAP) (Yan and Chen 2002; Liu *et al*. 2004) are simpler than physical treatments because specific equipment is not required. However, CB is highly toxic, and careful handling is necessary. Caffeine (1,3,7-trimethylxanthine), a chemical that is a recognized food constituent in many countries, occurring naturally in many plants (*e.g.* coffee beans (Coffee Arabica), tea leaves (Commelia thea)), is safer and cheaper than either CB or 6-DMAP. Caffeine is a promising agent for the mass production of triploids (Okumura *et al.* 2007). But, triploid induction by caffeine treatment have been only reported exclusively in bivalves by Scarpa *et al*. (1994) and abalon by Okumura *et al*. (2007). Also, in the recent investigations were observed that triploid trout obtained via genome engineering do not always present favourable traits. Among triploid hybrids of rainbow trout and salmon trout, the survival rate was very low (Blanc and Maunas 2005). A similar undesirable event occurred among hybrids of .Siberian sturgeon *Acipenser baerii* and bester (*Huso huso* x *A. ruthenus)* (Fopp-Bayat *et al*. 2007). Based on this point, to increase both triploid rate and survival rate in rainbow trout, we intended to ascertain whether induction

of triploidy by caffeine-thermal shock treatments in the trout *Oncorhynchus mykiss*.

MATERIALS AND METHODS

The experiment was carried out in Kahramanmaraş trout farm of Kılıç Holding Co. in Turkey. The one female and one male were manually stripped for release of eggs and sperm that were gently mixed. Water was then added to achieve fertilization. Given that no previous protocols of caffeine-thermal shock treatments have been established to induce triploids in trout, triploidy induction protocols in abalone and oyster were used as a baseline to set the parameters for this species (Durand *et al*. 1990; Mao *et al*. 2000). To suppress the first meiotic division, fertilized eggs were exposed to combined thermal shock (28°C) with 15-mM caffeine treament for 10 min beginning at 15 min after fertilization. Diploid controls were originated from the same parents but not subjecting those to caffeine-thermal shock treatments. The temperature and dissolved oxygen were monitored with an oxygen and temperature meter (YSI 5750, YSI, Yellow Springs, Ohio). The fertilization was carried out in water at a temperature of 10.0 ±2.4 °C.

Approximately 5000 eggs for this experiment and then incubated in hatching cabinet with constant water flow to ensure gentle movement of eggs. During incubation, dead eggs were regularly removed to prevent *Saprolegnia* spp. growth. The survival rate was evaluated after hatching (d.p.h). Survival rate (expressed as percentage) was calculated as the number of live fry in relation with the number of total fry. The larvae were sampled from each treatment group at 3 day after hatching (d.p.h) and fixed with 70% ethanol and stored at -20°C until analysis (Nomura *et. al.* 2004). For measurement of the relative DNA content, flow cytometry (FCM) was conducted using a BD FACS Canto flow cytometer (Beckton Dickinson Immunocytometry Systems San Jose, CA, USA). The method of FCM analysis followed the protocol described by Cakmak (2011). The results were analyzed statistically using Student's t-test. The differences were considered significant at $P< 0.05$ (Norusis 1993).

RESULTS AND DISCUSSION

For caffeine-thermal shock triploidy induction, survival and triploidy rates were 52.15±3.90 (mean ± S.D.) and 59.21% respectively. The survival rate of control group was 83.24±4.51 and there was significant statistical difference between experimental and control groups ($P<0.05$). It is clearly observed that triploidy was induced (59.21%) at a 15 mM caffeine

treatment-thermal shock (28°C) for 10 min duration, initiated 15 min after fertilization (Figure 1). However, survival rate of experimental group was very low compared to control group. In a previous study negative effect of caffeine treatment on rainbow trout fry was not observed. Therefore, the reason of the mortality in triploid fry may be the heat shock at 28°C in comparison to control group.

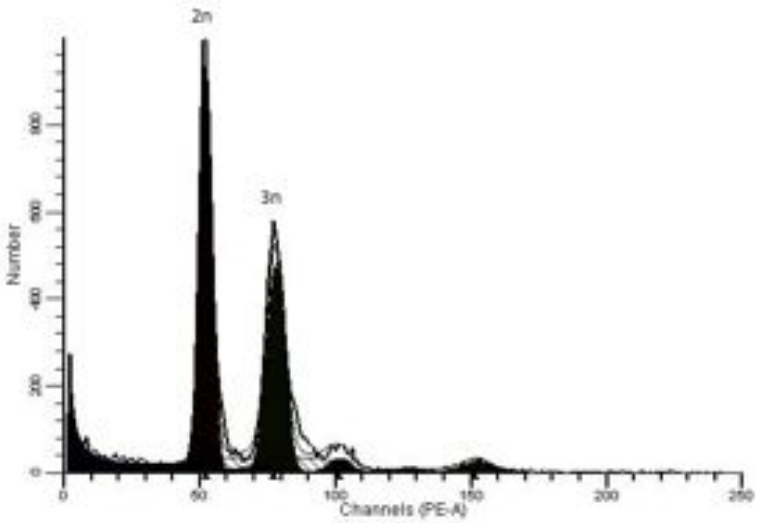

Figure 1. Flow cytometry analysis histogram of the DNA content in the 2n/3n mosaic specimen from 15 mM caffeine-thermal shock (28°C)-treated fry. 2n, diploid and 3n triploid.

The results of this study are the first report on triploidy induction by caffeine-thermal shock treatments in the trout. Data obtained from this study indicated that 15 mM caffeine treatment-thermal shock (28°C) had significant effect on triploidy rates of trout fry. However, the rate of survival rate was lower than control group. Similarly, Okumura *et al*. (1998) reported that caffeine treatment without combined thermal treatment was effective for the induction of triploid abalone. Until recently, there was no published information on triploidy induction by caffeine-thermal shock treatments in trout. Triploidy induction protocols applied in the present study were best guesses based on available information on oyster and abalone species (Durand *et al.* 1990; Mao *et al.* 2000). Therefore, further detailed investigation is required to examine the effect of caffeine-thermal shock treatments in order to achieve 100% triploid population.

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Genetic Variation between Wild and Cultured Sea Bass (*Dicentrarchus labrax*) Populations in Turkish Marine Waters

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ABSTRACT

Sequence of mitochondrial *12S rRNA, Cytochrome b, Cytochrome oxidase II* genes were used to deduce genetic diversity and genetic relationship between wild and cultured populations of sea bass (*Dicentrarchus labrax* L., 1758) in Turkish coastal waters. Data set was analyzed with Neighbor Joining (NJ) and Minimum Evolution (ME). The bootstrap analyses (1000 replicates) were performed and the relationships between samples are given through phylogenetic trees. Genetic diversity among wild and cultured stocks was estimated using gene diversity, number of haplotypes and nucleotide diversity. There were no significant differences in variation of three gene sequences. The maximum genetic variation degree observed in *12S rRNA*. All findings support the current classification with lack of subdivisions and no differentiation between wild and culture stocks.

Keywords: *Dicentrarchus labrax*; genetic diversity; mtDNA; *12S rRNA, Cytochrome b; Cytochrome oxidase II*

INTRODUCTION

The sea-bass, (*Dicentrarchus labrax* L., 1758) has a widespread distribution in Mediterranean and Atlantic coasts. As a commercially valuable fish, it feels important to know the population structure for fisheries and aquaculture management. Also it is important to know the identity of stocks in terms of biodiversity.

Sea bass is the most popular cultural fish species in Turkey and has of great commercial importance. The total production in 2010 was 50796 tones (Anonymous 2010).

In accordance with the high commercial value of this species, researches on brood stock management, genetic and biotechnology have been performed by institutions, universities and private sector. In Mediterranean aquaculture escape events of fish are known due to accidents or carelessness (Innocentiis *et al.* 2005) but very limited data is available. Although, rearing large individuals in cages which are potential

spawners may have a risk of genetic influence on wild stocks (Dimitriou *et al.* 2007). The potential threat on wild stocks may have more importance if non-local brood stocks or juveniles take place in culture operations.

Ergüden and Turan (2005), studied the genetic and morphological characteristics of sea bass from Black Sea, Marmara, Mediterranean and Aegean Sea and examined 9 loci. But out of this study very limited data is available on genetics of sea bass populations in Turkish marine waters.

In the present study, we used the DNA sequence analysis of mitochondrial *12S rRNA*, *Cytochrome b* and *Cytochrome oxidase II* genes to estimate the level of genetic divergence and to survey the existence of any subdivisions in Turkish marine waters.

MATERIALS AND METHODS

Fish samples and DNA extraction

Specimens were collected both from fish farms and wild. All fish were taken to the laboratory in thermo-isolated packages with ice. Liver tissue samples were taken from each individual and stored with 95% ethanol in labeled tubes at -20°C until DNA extraction. All specimens were given a code according to sampling site and stock type for further use (Table 1).

Table 1. Short-codes for sample fish.

Sampling site		**Wild/culture**	***12S rRNA***	***Cytochrome oxidase II***	***Cytochrome b***	**Example**
Adana	A	W / C	*12S*	*COII*	*Cytb*	AW12S-1 *12S rRNA* for the first fish from wild in Adana sampling site
Mersin	M					
Antalya	AN					
Bodrum	B					
Izmir	I					
Western Black Sea	WB					
Eastern Black Ssea	EB					

DNA extraction from liver tissue was performed by Promega Wizard Genomic DNA Purification Kit. DNA concentration and integrity were checked by NanoDrop ND – 1000 spectrophotometer and 1% agarose gel electrophoresis. Following the dilution of DNA to 200 ng/µl, all samples stored at -20°C.

DNA amplification

Mitochondrial gene regions were amplified with polymerase chain reaction (PCR) by using primers L1091 (5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3') and H1478 (5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3') for 12S rRNA (Kocher et al., 1989); D. labrax COII F (5'-GGCACATCCCCTGCAA-3') and D. labrax COII R (5'-CTATGATTGGCGCCACA-3') for Cytochrome oxidase II; DLcytb F (5'-TCTTCACGCCAATGGTGCA-3') and DLcytb R (5'-TTTGTTCGGGATCGAGCGAA-3') for Cytochrome b. PCR reactions carried out as follows: *12S rRNA* –an initial denaturing step of 94°C for 2 min, followed by denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min (35 cycles) and the final extension was at 72°C for 5 m. *Cytochrome oxidase II* –an initial denaturing step of 94°C for 2 min, followed by denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min (35 cycles) and the final extension was at 72°C for 5 m. *Cytochrome b* –an initial denaturing step of 94°C for 5 min, followed by denaturation at 94°C for 40 sec, annealing at 50°C for 1 m, extension at 72°C for 2 min (40 cycles) and the final extension was at 72°C for 5 m. All results were confirmed by gel electrophoresis.

DNA sequencing

Double-stranded PCR products were purified by Promega Wizard SV Gel and PCR Clean-Up System (Promega A9282). Amplified products were sequenced with Beckman Coulter CEQ 8000 Genetic Analysis System using Beckman Coulter CEQ Dye Terminator Cycle Sequencing Kit. Primers used were the same as those for PCR.

Sequence analysis

The sequences were aligned in Sequencer 4.9 and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.* 2007). The consensus sequences have been recorded to GenBank database with accession numbers of GU902247 (*12S rRNA*), GU902248 (*Cytochrome b*) and GU902249 (*Cytochrome oxidase II*). Phylogenetic trees were constructed with neighbor joining (NJ) and minimum evolution (ME) algorithms (Apostolidis *et al*. 2001). Reliability of the inferred trees was assessed by bootstrap tests with 1000 replicates (Iwi *et al.* 1999). Genetic diversity among wild and cultured stocks was estimated using gene diversity, number of haplotype and nucleotide diversity in DnaSP v 5.0 (Rozas *et al.* 2003).

RESULTS AND DISCUSSION

Individuals from each sampling site of wild and cultured stocks were sequenced in a total fish number of 93. Haplotype sequences were used for further analysis.

The average nucleotide composition for *12S rRNA* gene is A=24.8%, T=24.5%, G=23.0%, C=27.7% with an average A+T content of 49,3%; for *Cytochrome oxidase II* A=27.3%, T=26.8%, G=18.8%, C=27.1% with an average A+T content of 54,1% and for *Cytochrome b* A=26.2%, T=29.0%, G=13,5%, C=31.3% with an average A+T content of 55,2%. While close rates were observed from *Cytochrome b* and *Cytochrome oxidase II* genes, *12S rRNA* region showed higher values for the haplotype number, haplotype diversity and nucleotide diversity (Table 2).

Table 2. Genetic diversity parameters.

	Number of haplotypes (*N*)	**Haplotype diversity (Hd)**	**Nucleotide diversity (π)**
12s rRNA	12	0,7174	0,006
Cytochrome oxidase II	4	0,5000	0,001
Cytochrome b	7	0,6571	0,003

A total of 17 sites (9 in *12S rRNA*, 3 in *COII* and 5 in *Cytb*) were variable among all sequences and 5 of these (3 in *12S rRNA* and 2 in *COII*) were phylogenetically informative. The percentage of parsimony informative sites calculated as 33,3% (*12S rRNA*), 66,6% (*COII*) and 0% (*Cytb*).

Genetic divergence between haplotypes generated from *12S rRNA* sequence of *D. labrax* populations varies among 0-0,019. Average genetic distance was calculated as 0.006, which has the highest divergence ratio among three analyzed genes (0.6%). There is no specific pattern detected among haplotypes of wild and farmed populations in terms of geographic region, being sampled from wild or farmed populations. No genetic distance was detected among haplotypes of wild and cultured populations from Adana, Antalya, Western Black Sea, Bodrum and Mersin.

Genetic divergence between haplotypes generated from *COII* sequence of *D. labrax* populations varies among 0-0.006. Average genetic distance was calculated as 0.001, which has the lowest divergence ratio among three analyzed genes (0.1%). There is no specific pattern detected among haplotypes of wild and farmed populations in terms of geographic region, being sampled from wild or farmed populations. No genetic

distance was detected among haplotypes of wild and cultured populations from Antalya, Western Black Sea, Bodrum, Eastern Black Sea, Izmir and Mersin. A genetic distance of 0.002 was detected among wild and farmed populations of Adana.

Genetic divergence between haplotypes generated from *Cytb* sequence of *D. labrax* populations varies among 0-0.008. Average genetic distance was calculated as 0.003, with a divergence ratio of 0.3%. There is no specific pattern detected among haplotypes of wild and farmed populations in terms of geographic region, being sampled from wild or farmed populations. No genetic distance was detected among haplotypes of wild and cultured populations from Adana, Antalya, Bodrum and astern Black Sea populations. Mersin haplotype was represented by culture population only. A genetic distance of 0.004 and 0.007 was detected among wild and farmed populations of Western Black Sea and İzmir respectively. Mean transition-transversion rates were 0,25; 0,73 and 0,22 for *12S rRNA*, *Cytochrome oxidase II* and *Cytochrome b* respectively.

Nucleotide divergence value was about two fold higher in *12S rRNA*. The highest genetic variation was observed in *12S rRNA*. Two common methods of analysis Neighbor Joining (NJ) and Minimum Evolution (ME) were performed to generate molecular trees separately for each gene. *12S rRNA* (Figure 1), *COII* (Figure 2) and *Cytb* (Figure 3) trees as inferred by two methods were congruent.

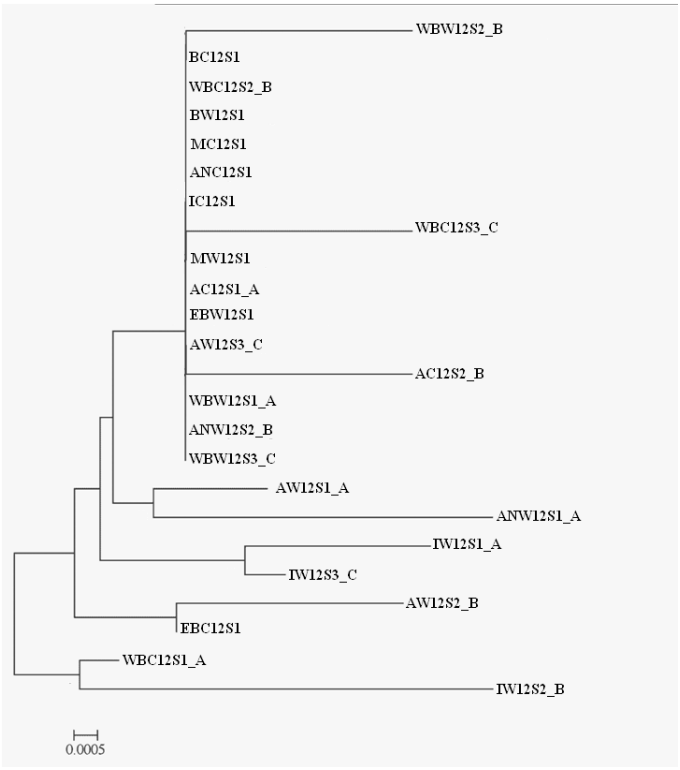

Figure 1. *12SrRNA* Neighbor Joining (NJ) tree of sea bass.

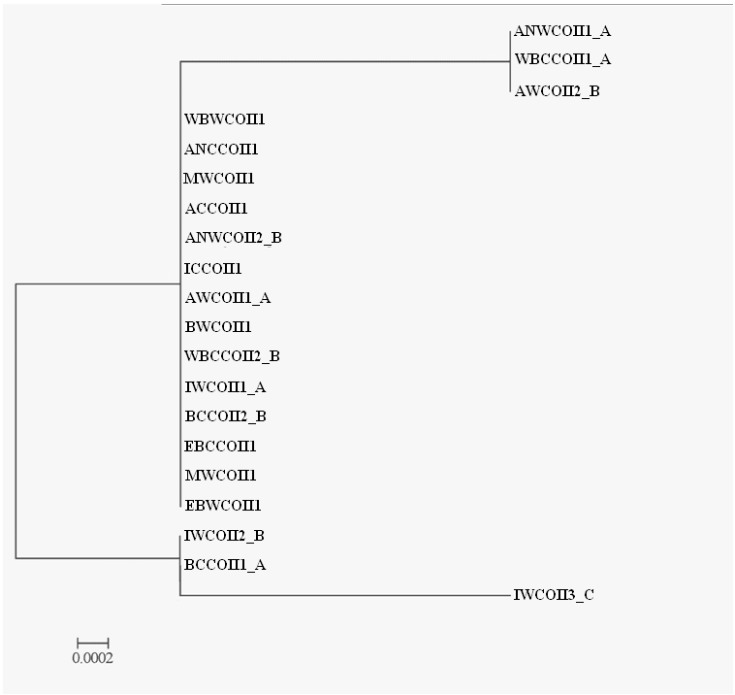

Figure 2. *Cytochrome oxidase II* Neighbor Joining (NJ) tree of sea bass.

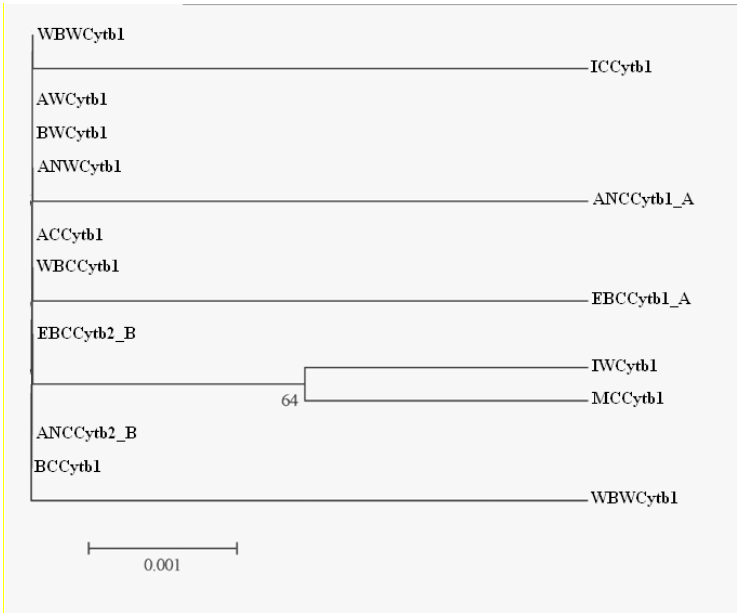

Figure 3. *Cytochrome b* Neighbor Joining (NJ) tree of sea bass.

Patarnello *et al.* (1993) used mitochondrial DNA sequence variation to determine the haplotype distribution in wild and farmed populations. They fixed 5 different haplotypes which one of them was not present in farm samples while two were not found in farmed populations. By using Arlequin version 3.5 we detected 12, 4 and 7 haplotypes for *12S rRNA*, *Cytochrome oxidase II* and *Cytochrome b* respectively. Despite analyzing a shorter fragment of 268 bp, our haplotype number was higher for *Cytb*.

This indicates that the haplotype number in *Cytb* region is not only associated with the length of the partial sequence but also changes with the selection of the more variable fragment of the gene. Higher genetic variation of the population in our study than the one in Patarnello *et al.* (1993)'s examination would be another answer for this difference in haplotype number. Also our findings demonstrated that the distribution pattern among haplotypes does not follow a specific pattern in means of wild and farmed populations.

Allegrucci *et al.* (1999) sequenced the entire Cytb gene (1140 bp) to understand its utility as a marker in describing the pattern of genetic differentiation among *D. labrax* populations and explore its phylogenetic utility at different hierarchical levels. They found very low levels of genetic differentiation (average D: 0.005). The percentage of A+T composition ranged from 53,7% to 57.3%. Our findings on A+T composition (55.2%) are in accordance with Allegrucci *et al.* (1999)'s. This shows that the A+T ratio is not strongly related with the length of the amplified region. When compared the average genetic distance between *D. labrax* populations our results (03%) are lower but close with the prior study (0.5%). This difference occurs from the change of the amplified region. While we used a partial sequence, the study involves a complete sequence of 1140 bp.

Ergüden and Turan (2005) studied genetic and morphologic structure of the sea-bass, Dicentrarchus labrax, in Turkish coastal waters. They sampled 120 individuals from the Black, Marmara, Aegean and North-eastern Mediterranean and analyzed four-enzyme systems (G3PDH*, ME*, MDH*, PGI*) representing 9 loci. They point out that there were no genetic differences between populations using 9 loci and the difference was 0.0001. in our study using mtDNA sequences the genetic difference between populations for *12S rRNA*, *Cytochrome oxidase II* and *Cytochrome b* were 0.006; 0.001 and 0.003 respectively. The difference between findings of the two studies is to be due to more determinant mtDNA sequences.

Our findings indicate that the wild and culture populations of European sea bass (*Dicentrarchus labrax*) in Turkish marine waters are not genetically differentiated. We observed low diversity rates and

similarly with former studies on Mediterranean populations. However this is not meaning that there is no difference. Therefore complete mtDNA sequences and additional SNP, microsatellite analysis will serve more accurate data on genetic differentiation between wild and culture stocks and different origin populations of sea bass.

Acknowledgements
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Monitorization of Aquaculture Farm by Devoloping New Software

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ABSTRACT

In this study, *production control software was developed to* control total biomass, daily feed consumption and statistical fish mortality, and calculate feed conversion rate (FCR). In this software, monitorization of aquaculture farm can be controlled and the user can notice for some critical points such as the time of harvest or high mortality ratio. This system also provides some information about organoleptic index, yield of carcass, calculation of FCR according to feed values, and cost and income of fish farm.

INTRODUCTION

The use of computer software is *extremely important* for food and other *industry*. Computer software is also used as auxiliary equipment since it eliminates various types of errors occurred as a result of man-made. People must use sophisticated computer software. Businesses that do not use computer software often make many mistake especially critical calculations. Therefore, most businesses will focus on to use software industry which is suitable for their work.

Aquaculture is the world's fastest growing food sector (FAO 2002). The marine and inland aquaculture sector in Turkey is developing rapidly. The number of aquaculture farm in Turkey is 1748 according to data of Turkish Statistical Institute (2008). The number of rainbow trout farm among them is 1342. Among marine fish, the numbers of sea bream and sea bass farm are 305, whereas there is more than 9 tuna farm in Turkey. Total annual trout, sea bass and sea bream production in Turkey were 68.649, 49.270 and 31.670 tons in 2008, respectively (*Deniz et al.* 2009). These values provide approximately 350 million dollars contribution to the national economy (Aydın *et al.* 2007). In our country, there are significant improvements in fish-breeding and production since the 1970s.

In a prediction study conducted between 1995 and 2014, it is estimated that total fish production would be 49 124 tons in 2005, 54 036 tons in 2006 and 59 440 tonnes in 2007, although total aquaculture

production reached 128 943 tons in 2006 and reached 139 873 tons in 2007 (Atay *et al.* 1995).

As a result of the necessity of critical calculations, there is a need to develop software for fish farm which is one of the most important problems of this sector. Recently fish farms need various software to carry out their production calculations. In order to meet the needs of farms, many people generally use Microsoft Excel-based systems which is simple. However, such systems do not have sophisticated structure and a virtual intelligence to minimize user errors. Therefore, this system is not very useful for the fish sector. Total production in cages among inland trout production was 44% in 2007 (Emre *et al.* 2008).

Similarly, almost all marine fish farming is made in cages. One of the biggest problems of the fish production in cages is very difficult to control due to distance of cage systems to land. Therefore, the aim of the study was to develop *production control software to* control total biomass, daily feed consumption and statistical fish mortality, and calculate feed conversion rate (FCR).

MATERIAL AND METHODS

Microsoft Visual Basic6 program was used in writing a computer program to plan the daily work to be done and to control financial affairs in fish farm. Microsoft Access program was also used as a database. In software, fish cages, the number of fish, current weight and target weight of fish is firstly defined as computer data from the "Information Input" menu. Based on these definitions, the program can calculate the amount of feed which is must be given according to the desired feed conversion ratio in the cages. Medicine applied to fish, feed intakes and hatchery records is entered in the introduction menu. In the following days, reporting of information for a single cage or all cages is provided. Moreover, the control of feed stock and the table of financial analysis in business flow are provided and reported the user.

It is possible to see feed costs as daily, weekly, monthly and yearly. At any time, the calculation of FCR according to feed values, carcass yield, organoleptic index and, diagnosis of disease based on symptoms, daily mortality input and analysis, the control of personnel and companies that product is sold, and the calculation of current move such as buying and selling is also made by program.

Cage Fisheries Program (CFP)

Cages information input

The data such as the number of fish recorded in individual cages, responsible personnel, the amount of feed, current and target weights of fish provide to keep business records and to perform the software making calculations by using the entered information. Figure 1 shows cages information input.

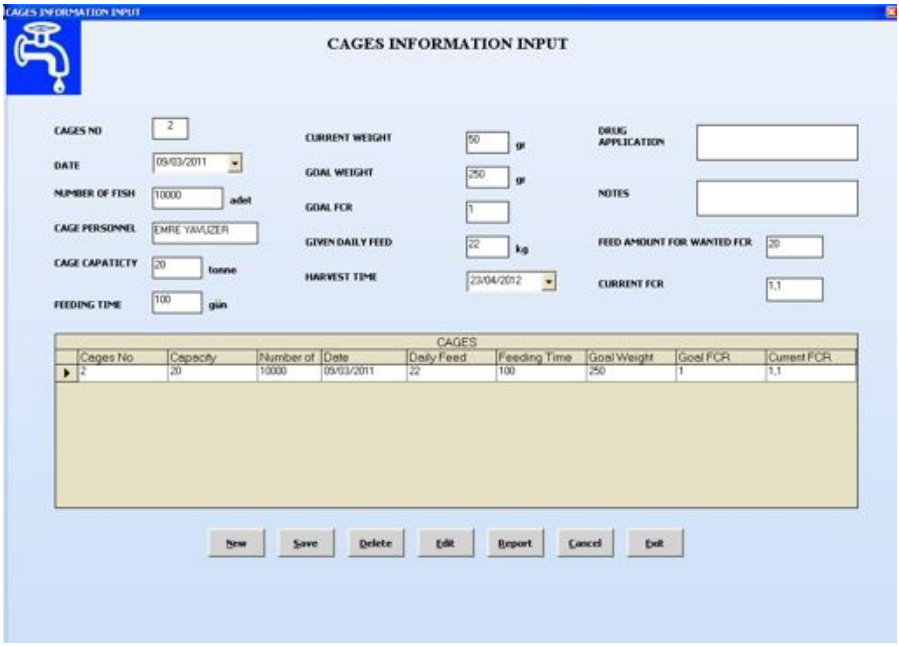

Figure 1. Screen for cages information input.

Feed Purchase Process

It informs the user or farm about where feed is provided, amount of feed and price of feed. Therefore, it is recorded the dates of feed taken and can be calculated feed stock situation. It also shows forms of feed and unit costs of the feed. This situation offers the opportunity to choose more economical diets.

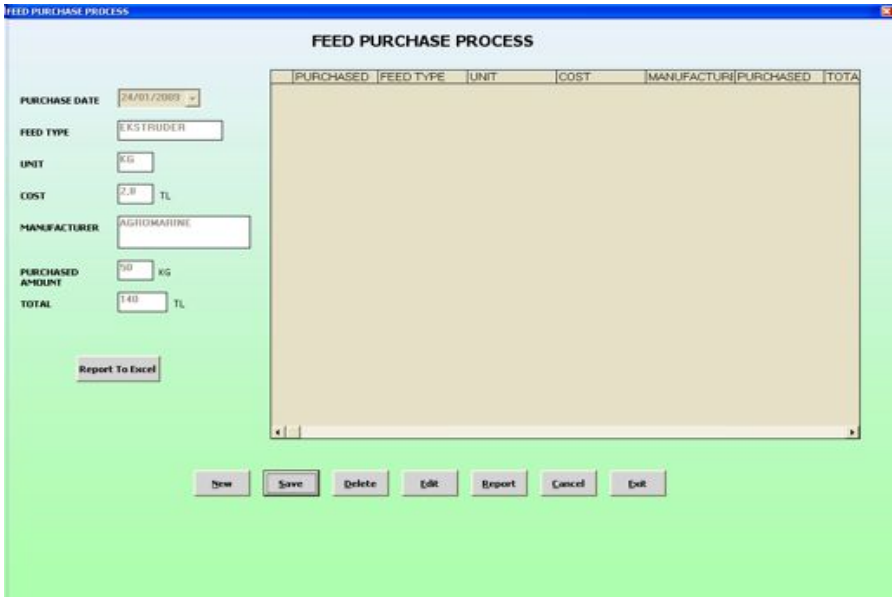

Figure 2. Feed purchase process.

Hatchery Information Input

In this menu, the current number of fish in the hatcheries and feeding duration is entered and the feeding rates given are calculated.

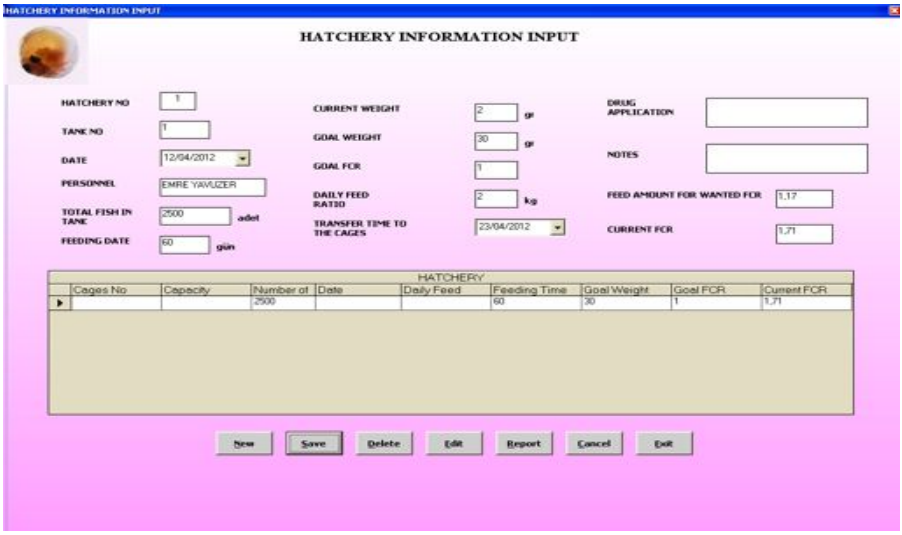

Figure 3. Hatchery information input.

Feed Costs Page

This menu displays the daily, weekly, monthly and yearly calculations of total feed costs of fish in cages and hatchery ask.

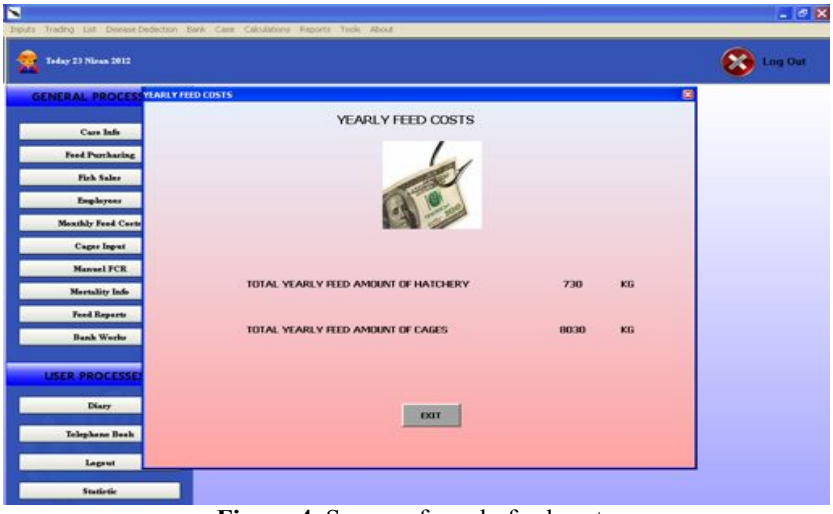

Figure 4. Screen of yearly feed costs.

Quick Calculations Page

The menu can be used for quickly calculating of various values such as FCR, condition factor, carcass yield, organoleptic index and liver index.

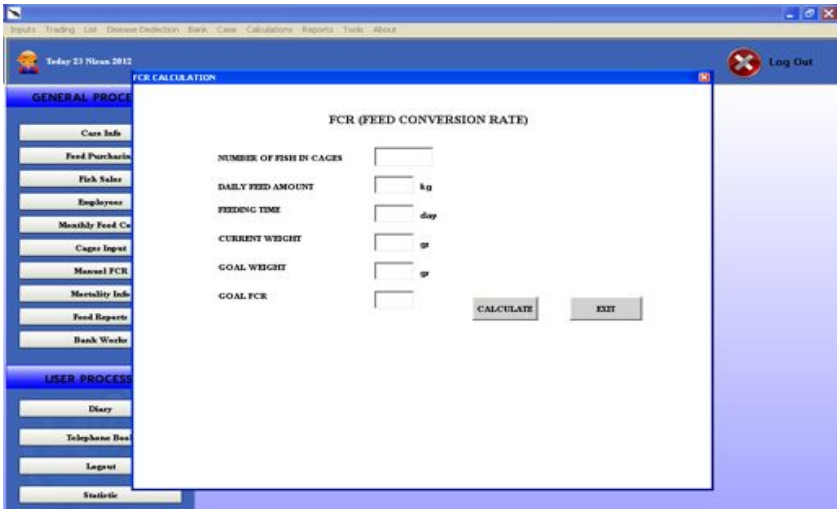

Figure 5. Screen for calculation of FCR.

Case Status

The menu is for following income and outcome of farm. For this purpose, it is important in determining the profitability of the business with the creation of financial analysis reports. Fish sales, cost of personnel and feed, transactions, checks, promissory notes are also controlled by user in this menu.

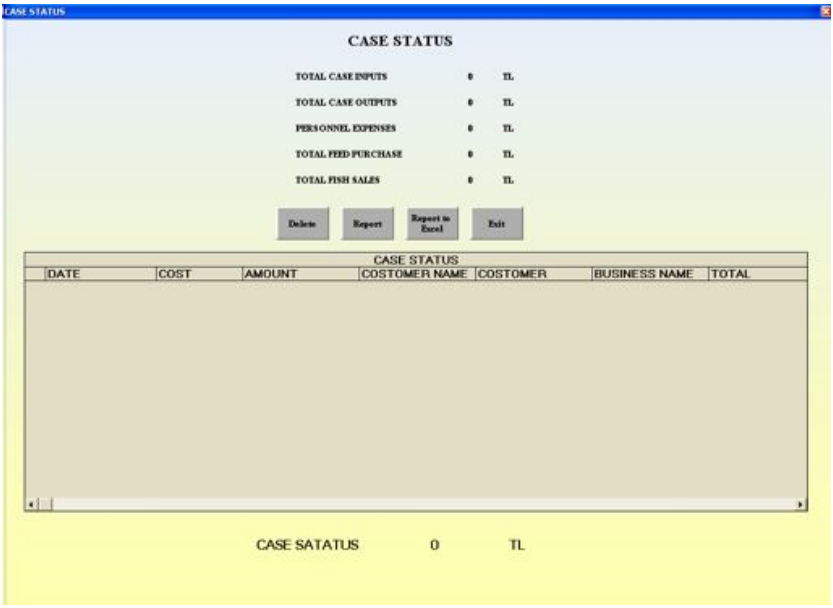

Figure 6. Case Status.

Reporting daily work to be done

The user is alerted in the main screen at the time of harvesting of fish in fish cages or transfer fish from hatchery into fish cages. Clicking on any warning alert informs the user about the content of the daily work.

In conclusions, thanks to this software, fish farms will be control using necessary data for businesses, fish numbers in cages or tanks, mortality rates, estimation of disease based on symptoms, feed ratio, calculation of FCR, as well as current accounts such as case status, check and voucher. This software to be generated by scientific data provides optimum profit to fish farm due to minimizing feed ratio given and producer error.

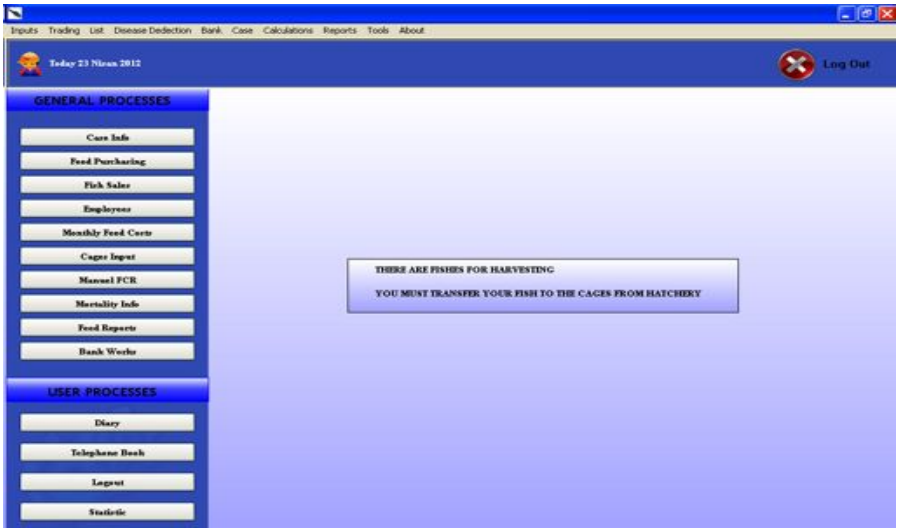

Figure 7. Reporting daily work to be done

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