

ARTICLE

Seaweeds agarophytes and associated epiphytic bacteria along Alexandria coastline, Egypt, with emphasis on the evaluation and extraction of agar and agarose

Agarófitas marinas asociadas con bacterias epifitas de la costa de Alejandría, Egipto, con énfasis en la evaluación y extracción de agar y agarosa

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Resumen.- Se realizó un estudio de algas marinas en la costa de Alejandría para seleccionar una agarófitas adecuada para la extracción y evaluación de las sustancias de agar y agarosa, además de estimar e identificar las bacterias epifitas asociadas con estas algas capaces de secretar paquetes de enzimas hidrolíticas degradantes de paredes celulares de algas. Un total de 13 muestras de algas se recolectaron durante primavera del 2012. Del total se obtuvieron 2 especies de Rhodophyta; *Gelidium crinale* y *Pterocladia capillacea*, las cuales pueden ser explotadas para la producción de agar y agarosa. Se encontraron un total de 9 géneros de aislados bacterianos dominantes asociados con las diferentes muestras de algas. Estos fueron identificados como; *Alcaligenes* sp., *Bordetella* sp., *Acinetobacter* sp., *Pseudomonas* sp.1, *Pseudomonas* sp.2, *Pseudomonas* sp.3, *Flavobacterium* sp., *Vibrio* sp. y *Vigribacillus* sp. La especie con mayor capacidad de secretar un paquete de enzimas degradantes fue *Vigribacillus* sp. que fue aislada de *P. capillacea* cosechado en Western Harbor. Los hidratos de carbono, lípidos y proteínas totales se determinaron tanto en *P. capillacea*, como en el agar extraído, y agar Oxoid (material comercial de referencia). Además se estimaron algunos metales (cobre, cadmio, plomo, zinc, níquel, cromo, hierro, manganeso y arsénico) en las 3 muestras mencionadas. La resistencia de gel, temperatura de gelificación y fusión, además de la claridad y viscosidad de los extractos y agares Oxoid mostraron diferentes patrones. El contenido de sulfato del agar extraído fue ligeramente mayor que la de agar Oxoid, mientras que el contenido de piruvato mostró patrón inverso. El espectro FTIR del agar extraído indicó grupo metilado, lo que sugiere que el agar extraído de *P. capillacea* es un polisacárido altamente metilado por tanto de una alta gelificación.

Palabras clave: Bacterias epifitas, agarofitas, agar, agarosa

Abstract.- A survey of seaweeds along Alexandria coastline was carried out to choose a suitable agarophyte for extraction and evaluation of the agar and agarose substances, in addition to estimate and identify the epiphytic bacteria associated with these seaweeds which are capable of secreting package of hydrolytic enzymes and hence degrading algal cell walls successfully. A total of 13 algal samples were collected during spring 2012. There were 2 Rhodophytes species; *Gelidium crinale* and *Pterocladia capillacea*, which can be exploited for agar and agarose production. Nine dominant bacterial isolates were associated with the different algal samples; they were identified at genus level as; *Alcaligenes* sp., *Bordetella* sp., *Acinetobacter* sp., *Pseudomonas* sp.1, *Pseudomonas* sp.2, *Pseudomonas* sp.3, *Flavobacterium* sp., *Vibrio* sp. and *Vigribacillus* sp. The most superior species able to secrete a package of degrading enzymes was *Vigribacillus* sp. that was isolated from *P. capillacea* harvested from Western Harbor. The total carbohydrate, lipid and protein contents were determined in both *P. capillacea* and its agar, and in Oxoid agar (a reference commercial material). Some metals (copper, cadmium, lead, zinc, nickel, chrome, ferrous, manganese and arsenic) were also estimated in the 3 mentioned samples. The gel strength, gelling temperature and melting temperature besides the clarity and viscosity of extracted and Oxoid agars showed different patterns. The sulphate content of the extracted agar was slightly higher than that of Oxoid agar, while the pyruvate content showed inverse pattern. The extracted agar FTIR spectrum indicated methylated group, which proved that the agar extracted from *P. capillacea* is a highly methylated polysaccharide resulting in high gelation.

Key words: Epiphytic bacteria, agarophyte, agar, agarose

INTRODUCTION

Seaweed surfaces provide a protected and nutrient-rich 'hot spot' for opportunistic bacteria that are abundant wherever organic material is available (Armstrong *et al.* 2001). Marine macroalgae generally associate with specific bacterial communities that differ significantly from those occurring in the surrounding seawater (Lachnit *et al.* 2009). Algal cell wall components and secondary metabolites can trigger specific interactions between seaweeds and beneficial bacteria (Lachnit *et al.* 2010). Seaweed-associated bacteria are important sources of fixed nitrogen and detoxifying compounds (Goecke *et al.* 2010). Besides being epiphytic on algal surfaces, bacteria also live inside the thallus. On the other hand, seaweed grazers or epiphytic bacteria capable of degrading algal cell walls can damage algal thalli and provide an entrance for pathogenic and opportunistic bacteria (Wang *et al.* 2008).

The Egyptian Mediterranean coast is highly diversified in seaweeds belonging to the 3 classes; Chlorophyceae, Phaeophyceae and Rhodophyceae. Aleem (1993) recorded 244 species at Alexandria shores, while there are many sites along the Mediterranean coast still unexploited although they sheltered large number of seaweeds (Shams El-Din & El-Sherif 2012). However, the Rhodophyte *Pterocladia capillacea* is the most abundant agarophyte growing along Alexandria shores. Huge masses of this species drift throughout the year (Rao & Bekheet 1976). The alga grows profusely along the sub-littoral belt of the rocky coast of Egypt (Nasr *et al.* 1966) and is the raw material for agar manufacture in Alexandria (Rao & Bekheet 1976). In general, the chemical composition of seaweeds reveals their richness in polysaccharides, minerals and vitamins (Mabeau & Fleurence 1993). However, the major polysaccharides of seaweeds are either structural (contributing to the fiber content), which are analogous to terrestrial plants and are mainly celluloses, hemicelluloses and xylans or storage components, including alginates (in brown algae), agars, and carrageenans (in red seaweeds) (MacArtain *et al.* 2007). Agars consist of alternating D- and L-galactopyranose units (MacArtain *et al.* 2007). The species used in agar production give rise to products with difference in their properties. For this reason, it is customary to indicate the origin of raw material, such as *Gelidium* agar, *Gracilaria* agar, *Pterocladia* agar, etc. In *Pterocladia capillacea*, agar accumulates in the cell walls of the alga. It is used primarily as a gelatin in food, and can also be used in laboratory settings as a

culture medium to support the growth of cells in experiments (Freile-Pelegrin *et al.* 1996, Sebaaly *et al.* 2012). Made up of 80% fiber, it is an excellent laxative agent (Glicksman 1983). It is also used to prepare dietetic formulas and food for diabetics (Armisen & Galatas 1987). According to Rao & Bekheet (1976), *Pterocladia* is an excellent seaweed for agar-agar production, and it has been observed that the gel strength of the Egyptian agar is higher than that of the known agar samples collected from other countries, including Japan and the United States. However, the available data concerning the production of agar from this agarophyte or other ones in Egypt are still not satisfactory and much information is lacking about the process and only very few studies have been conducted (Rao & Bekheet 1976).

The aim of the current study was to investigate the seaweeds occurring along the Alexandria coastline to choose a suitable agarophyte for extraction and evaluation of the agar and agarose substances, in addition to estimate and identify the epiphytic bacteria associated with these seaweeds which are capable of secreting package of hydrolytic enzymes and so degrading algal cell walls successfully.

MATERIAL AND METHODS

STUDY AREA

A survey was conducted during 2012 covering 6 sites along Alexandria coast with depths (0.5-1m), namely: Abu-Qir Bay (St. I), El Silsila (St. II), Eastern Harbor (St. III), El Anfoushy (St. IV), Western Harbor (St. V) and El Dekheila Harbor (St. VI) extending from latitude 31°16'N and longitude 30°03'E to 29°47'N and 31°10'E (Fig. 1). All these stations are subjected to different types of land-based activities and/ or freshwater input (Shams El-Din & Dorgham 2007, Abdallah 2008), in addition to shipping activities and fisheries in the 3 harbors (St. III & St. V & St. VI) (Zaghloul 1994, Zaghloul *et al.* 1995, Shams El-Din *et al.* 2007).

SAMPLES COLLECTION

A total of 13 algal samples were collected during spring (6 April, 2012) at these sites. Afterwards, another trip was conducted to collect only the agarophyte during early summer (11 June, 2012). The samples were collected by hand at the depths (0.5-1.0 m), washed with sea water at the sampling sites to remove the adhered sediments, impurities and epiphytes, separated in polyethylene bags

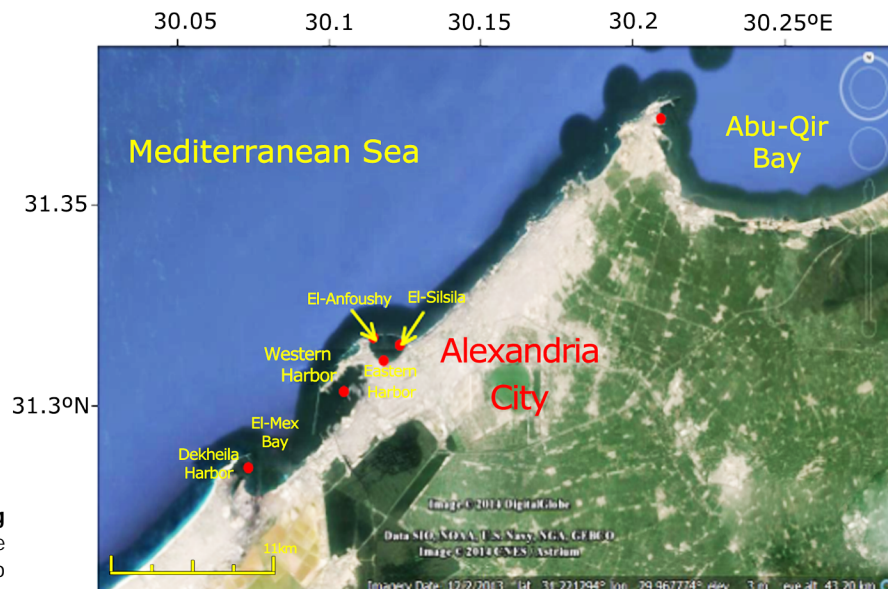


Figure 1. The study area and sampling stations during spring 2012 / Área de estudio y estaciones del muestreo durante la primavera del 2012

and stored under refrigeration 4°C. Quick rinsing of the algae with tap water was carried out in the laboratory on the same day to get rid of the remaining impurities and epiphytes. Herbarium sheets with a preliminary identification of separated species were done and/or preserved in 4% formalin. Microscopic identification of the investigated algae was carried out according to Aleem (1993) and Braune (2008).

COUNTING, ISOLATION AND IDENTIFICATION OF MARINE BACTERIA

Sea water agar medium according to Zobell (1946) was used for the enumeration and isolation of viable aerobic heterotrophic bacteria associated with algae. Serial dilutions from 10^{-2} through 10^{-4} were made using filtered sterilized sea water. A portion (0.1 ml) from each appropriately diluted sample was used to inoculate plates, which were incubated at 30°C for 24-48 h and later were counted. The pure dominant colonies obtained were transferred to fresh slants. Dominant bacteria were stained for Gram reaction, and then examined microscopically. Colony morphology characters such as size, appearance, configuration, and elevation were examined on nutrient agar plates after 24 h incubation. Cell morphology was microscopically examined after Gram staining of 24 h old cultures. Gram reaction was confirmed by potassium hydroxide as follows: a loopfull of bacterial colony was mixed with a drop of 3% potassium hydroxide on a clean glass slides. The absence of slimy substance confirms a

Gram-positive reaction. Each of the selected bacterial strains was examined for several characters; catalase, oxidase, indole production, methyl red and Voges-Proskaur tests, H_2S precipitation, nitrate reduction, citrate utilization, acid production, etc. Degradation of gelatin, casein, starch, chitin, etc., was also achieved. Finally, these isolates were identified according to tests in Cheesbrough (1985), Williams *et al.* (1989) and Cowan & Steel (1993).

AGAR EXTRACTION

A total of 300 g of the air-dried agarophyte (*Pterocladia capillacea*) was washed twice with hot water at 40°C and then boiled for 2 h at 30 lb/in² using 4.5 liters of water (weed-to-water ratio, 1:15). The residue after 2 h of extraction was separated from the solution by filtering through a 'double cloth' and squeezing the cloth as much as possible to remove liquid. The filtrate was collected in trays, allowed to set to a gel, and then kept in a cold chamber for 24 h. After thawing, the gel was washed several times, bleached with 1.0% $NaClO_2$ solution, and then treated with dilute H_2O_2 solution. The sheets were then washed several times with water before being dried (Rao & Bekheet 1976). Samples were extracted within 3 weeks to prevent any possible agar degradation upon prolonged seaweed storage according to Villanueva *et al.* (2010). The yield of dried agar was calculated from the original dried sample weight.

DETERMINATION OF EXTRACTED AGAR PROPERTIES

CARBOHYDRATES, PROTEINS AND LIPIDS ESTIMATION

Algal sample was dried at room temperature (25°C) to a constant weight and then ground to fine powder. For each component, 1 g dry weight was taken. Total carbohydrates (TCHs) content was determined according to the phenol-sulphuric acid method investigated by Strickland & Parsons (1972) using double-beam Shimadzu spectrophotometer (visible-UV model 150-02) at 490 nm. The accuracy of the method for the determination of TCHs was measured by triplicate analysis of blanks, Standard (D- Glucose) and samples. The results of quality control measurements represented 10% of the total analytical load. The limit of detection was 0.1 µg L⁻¹. Total proteins were estimated using the Biuret method of Raymont *et al.* (1964) using a double beam UV/V Spectrophotometer (SPEKOL 1300 Analytikjena) at 546 nm. Total lipids (TL) were estimated according to Bligh & Dyer (1959) based on mixing 1:1 (v/v) chloroform/ methanol, which allows extraction of both polar and nonpolar lipids. The lipids in the chloroform layer were isolated and measured using double-beam Shimadzu spectrophotometer (visible-UV model 150-02) at 525 nm. The results of the 3 components were expressed as mgg⁻¹ dry weight. The same procedures were applied for dry extracted agar at 70°C and manufactured Oxoid agar (used as a reference commercial material). The accuracy of the method for the determination of proteins and lipids was measured by triplicate analysis of blanks and samples. The results of quality control measurements represented 10% of the total analytical load.

MAJOR ELEMENTS CONTENT

For major elements (calcium, magnesium, sodium and potassium) analysis, dried algal sample to a constant weight (60°C), dried extracted agar and Oxoid agar were homogenized by crushing each sample in a porcelain pestle and mortar, and kept away from metallic materials and dusty conditions to avoid contamination. One gram dry weight of each sample was acid digested in 5 ml concentrated HNO₃ in a Teflon cups. Digested samples were filtered through an acid-washed filter (Whatman GF/C) and diluted to 25 ml with double distilled water. All glassware, plastic and Teflon devices were thoroughly acid washed according to Haritonidis *et al.* (1983) and Mohamed & Khaled (2005). Moreover, Na and K were measured using a Flame Photometer (JENWAY PEP7). Calcium and Mg were determined volumetrically using EDTA standard solution Erio-Chrome T and Murexide

indicators (APHA 1975). The concentrations were expressed as mgg⁻¹.

TRACE METALS

The concentrations of the trace metals: Cu, Cd, Zn, Ni, Cr, Pb, Fe and Mn were determined according to Haritonidis *et al.* (1983) and Mohamed & Khaled (2005) using Atomic absorption AAS/flame mode (Shimadzu AA-6800) for all trace metals except As. Hydride/ASS (HVG-1; Shimadzu AA-6800) was used for determination. Working standards (Merck, Germany) of the major and trace elements were prepared by diluting concentrated stock solutions with deionized water. To monitor the quality of chemical analysis and examine the accuracy of the data, reference materials SD-M-2/TM and IAEA/356 were analyzed with the digested seaweeds solutions during the course of analysis. The measured concentrations of trace metals were within the range of certified values with a recovery within ± 10% (Table 1), whereas precision was agreed to be within 10% for triplicate measurements.

PHYSICAL AND RHEOLOGICAL PROPERTIES OF AGAR

Extracted agar was dried at 70°C and then was grounded in a grinder to fine powder. Both extracted and Oxoid agars (Oxoid Co., UK) were reconstituted into 1.5% w/v solutions to measure rheological properties.

Moisture content: One gram of dried extracted agar and Oxoid agar was heated 12 h at 37°C to constant weight and the moisture content was calculated as the percentage of the lost weight to the initial weight.

Table 1. Results of validation study for heavy metals concentrations (µg g⁻¹) in reference material (SD-M-2/TM) analyzed together with samples / Resultados del estudio de validación para las concentraciones de metales pesados (µg g⁻¹) en material de referencia (SD-M-2 / TM) analizado junto con las muestras

Element	Wave length (nm)	LOD	Certified values	Found values	Recovery %
Cu	324.7	0.008	32.7	31.1	95.11
Cd	228.8	0.005	0.11	0.12	109.09
Zn	213.9	0.002	74.8	78.0	104.28
Ni	232	0.02	56.1	53.9	96.08
Cr	357.9	0.015	77.2	74.9	97.02
Fe	248.3	0.025	27.1	26.46	97.64
Mn	279.5	0.01	12	11.4	95.00

LOD= the detection limit of the trace elements

Ash content: One gram of dried extracted agar and Oxoid agar was heated at 40°C for 48 h and the remained material was weighted again and the ash content was calculated as percentage of the remained dried weight to the initial weight.

pH: The pH meter (Model HANNA PHep) was adjusted to 7.4 and then applied to the 1.5% w/v solution of both extracted agar and Oxoid agar and the pH was determined at room temperature (25°C).

GEL STRENGTH MEASUREMENT

The gel strength was measured in dry extracted agar and Oxoid agar after gelling overnight at room temperature (25°C) by measuring the load causing the cylindrical plunger (1 cm² cross-section) to break a standard gel (Armisen & Galatas 1987, Freile-Pelegrin *et al.* 1996). The weight was added sequentially to the plunger, beginning with 100 g, until the plunger broke the gel surface. The time between the addition of the last weight and the point where the plunger reached the bottom of the beaker was determined using 3 replicates, for each sample (Praiboon *et al.* 2006). The gel strength for the dry extracted agar and Oxoid agar was also measured at temperature of 85 and 90°C. The gel strength was expressed as the gcm⁻² that the gel resisted for 20 s.

GELLING AND MELTING TEMPERATURE

Gelling temperature was obtained by the addition of 10 ml hot agar solution into a test tube (2.3 cm diameter, 6 cm height). A glass bead (5 mm diameter) was placed in the test tube. The tube was tilted up and down in a water bath at room temperature until the glass bead ceased moving. The gel temperature in the tube was immediately measured introducing a precision thermometer (0.1°C divisions). Melting temperature of the gel in a test tube (2.3 cm diameter, 16.5 cm height) was measured by placing an iron bead (9 mm diameter) on the gel surface. The test tube was clamped in a water-bath and the temperature rose from 50 to 100°C; melting point was recorded with a precision thermometer when the bead sank into the solution (Freile-Pelegrin *et al.* 1996).

VISCOSITY

An Ostwald's Viscometer measured apparent viscosity. Spindle No. 1 at 1500 rpm (round per minutes) was used for measuring apparent viscosities of agar samples (1.5% in deionized water) at 80°C (Praiboon *et al.* 2006).

CLARITY

The clarity of the gel solution was determined according to Tang *et al.* (2001). Sodium chloride (20 - 450 mM) was added to the prepared gel solutions of extracted agar and manufactured Oxoid agar. Each solution was continuously stirred for 2 to 3 min and poured into 6 polystyrene shape cuvettes of 4.5 ml 1 x 1 cm². All cuvettes containing the gels were cooled in room temperature (25°C) for 24 h. Light absorbance of the gel solutions was measured at λ 490 nm with a spectrophotometer (Model Japanese Spekoll 11), using water as the reference. The mean absorbance at λ 490 nm of gels cooled in air was determined from 3 replicates. Small absorbance represents little turbidity and good clarity of the gels (Tang *et al.* 2001). The results of clarity were expressed as nepholes.

SULPHATE AND PYRUVATE

The percent of sulphate was determined by hydrolyzing 1 g of agar powder in 10 ml HNO₃ in 100 ml Kjeldahl flasks, resulting in complete hydrolysis of the ester sulphate followed by quantitative precipitation with barium chloride of the liberated sulphate. The precipitates were collected on ash-free gravimetric filters, dried, ignited and weighed on a precision balance (0.0001 g). The weight of the obtained barium sulphate, multiplied by 0.4116, gave the equivalent of sulphate (Freile-Pelegrin *et al.* 1996). According to Freile-Pelegrin *et al.* (1996), the percent of pyruvate was determined by hydrolyzing 0.5 g of agar powder in oxalic acid 0.02 M following the spectrophotometric method based on lactic dehydrogenase (Duckworth & Yaphe 1970).

3, 6-ANHYDROGALACTOSE

The percentage of 3, 6-anhydrogalactose (3, 6-AG) was determined by the resorcinol-acetal method (Yaphe & Arsenault 1965), using D-fructose as standard. Absorbance was read spectrophotometrically at 555 nm.

PREPARATION OF AGAROSE BY ADSORPTION (REMOVAL OF SULPHUR)

One hundred grams of agar were suspended in 5 L of 0.02M EDTA, then stirred for 2 days, and followed by filtration through nylon cloth. Afterwards, particles were re-suspended in 5 L of 0.01 M EDTA and EDTA was removed by filtration and washing with deionized water for 1 h (3 times subsequent). The agar particles were easily dried with acetone. Solution of 1.5% (w/v) was prepared with the aid of a boiling water bath. When the solution

was completely clear (1/4 vol), Al (OH)₃ -gel containing 4% AL₂O₃ was added and mixed with the hot agar solution for at least 10 min. The AL(OH)₃- gel was removed from the hot solution by centrifugation (10 min at 2500 rpm) or by filtration under suction on a preheated Buchner funnel with a piece of nylon under filter paper in order to have an optimal filtrating surface. After gelatinization, the completely clear supernatant was cut into small pieces which were freeze-thawed and washed twice with deionized water. After then, agarose was dried with acetone (Barteling 1969).

IR ANALYSIS OF EXTRACTED AGAR AND AGAROSE

Infra-Red spectroscopy was recorded to reveal their functional groups on Shimadzu- Pc 8601 FT-IR spectrometer. The samples were analyzed as KBr pellet. Both extracted agarose and purchased from Sigma Co., (USA) were analyzed comparatively.

THE CALORIFIC CONTENT OF EXTRACTED AGAR

The calorific content of the extracted agar was calculated to evaluate its nutrition value for human being, using the following known conversion values to convert the organic content into calorific values: fats 9.45, carbohydrates 4.10 and protein 5.65 K cal g⁻¹ (Brody 1945). The calorific content of Oxoid agar was also calculated for comparison.

STATISTICAL ANALYSIS

The Pearson's correlation coefficient matrix of various chemical and physical properties of agar including agar yield%, gel strength, gelling and melting temperature, sulphate, pyruvate, 3,6 anhydrogalactose, viscosity and clarity of agar solution was calculated at confidence limit 90% ($P \geq 0.1$), using Excel© program.

RESULTS

ALGAL SPECIES COMPOSITION

The results of the survey comprised a total of 13 algal samples representing 8 species namely: *Ulva compressa* (Linnaeus) Nees, *Ulva fasciata* Delile, *Ulva lactuca* (Linnaeus), *Colpomenia sinuosa* (Mertens ex Roth) Derbes et Solier, *Corallina officinalis* Linnaeus, *Gelidium crinale* (Turner) Gaillon, *Pterocladia capillacea* (S.G. Gmelin) Santelices & Hommersand and *Grateloupia doryphora* (Montage) M.A. Howe, which is recently

introduced to the Egyptian Mediterranean Sea (pers. comm. Nihal Shams El Din) (Table 2).

BACTERIA ASSOCIATED WITH ALGAE

The average of triplicate count of marine bacteria associated with the algal samples collected from each site in the study area was calculated. However, the standard deviation between the 3 replicates of total bacterial count was negligible. *P. capillacea* collected either from Abu Qir (I) or from Western Harbor (V) recorded the highest associated bacterial counts. However, it was clear that, *P. capillacea* AQ (I) had the highest bacterial count (10000 cfu g⁻¹), followed by *P. capillacea* WH (V) (8200 cfu g⁻¹) and subsequent by *U. lactuca* from Eastern Harbor (III) (7800 cfu g⁻¹). *C. officinalis* collected from Anfoushy (IV) had (6250 cfu g⁻¹), while the count of associated bacteria with *U. compressa* collected from El Dekheila (VI) was (6000 cfu g⁻¹). The count of associated bacteria with the other algal samples ranged from 1200 to 4600 cfu g⁻¹.

Table 2. Occurrence of the different algal species along Alexandria Coastline, during spring (2012) / Ocurrencia de las diferentes especies de algas a lo largo de la costa de Alejandria, durante la primavera (2012)

Algal group	Sampling sites					
	AQ (I)	Sil (II)	EH (III)	Anf (IV)	WH (V)	DK (VI)
Class: Chlorophyceae						
Order: Ulvales						
Family: Ulvaceae						
<i>Ulva compressa</i>	-	-	-	+	-	+
<i>Ulva fasciata</i>	+	+	-	+	-	-
<i>Ulva lactuca</i>	-	-	+	-	+	-
Class: Phaeophyceae						
Order: Scytosiphonales						
Family: Scytosiphonaceae						
<i>Colpomenia sinuosa</i>	+	-	-	-	-	-
Class: Rhodophyceae						
Order: Corallinales						
Family: Corallinaceae						-
<i>Corallina officinalis</i>	-	-	-	+	-	-
Order: Gelidiales						
Family: Gelidiaceae						
<i>Gelidium crinale</i>	-	-	+	-	-	-
<i>Pterocladia capillacea</i>	+	-	-	-	+	-
Order: Halymeniales						
Family: Halymeniaceae						
<i>Grateloupia doryphora</i>	-	-	+	-	-	-
N° of samples	3	1	3	3	2	1

AQ= Abu-Qir Bay (I), Sil= El-Silsila (II), EH= Eastern Harbor (III), Anf.= El-Anfoushy (IV), WH= Western Harbor (V) and DK= El Dekheila (VI)

Table 3. Gram stain and biochemical tests of dominant bacterial isolates associated to algal species / Tinción de Gram y pruebas bioquímicas de bacterias aisladas dominantes asociadas a especies de algas

Test	Bacterial isolate*								
	A1	A2	A3	A4	A5	A6	A7	A8	A9
Morphological and physiological tests									
Shape	R	R	R	R	R	R	R	R	R
Gram reaction	-	-	-	-	-	-	-	-	+
Motility	+	+	-	-	-	-	-	-	-
Aerobic growth	+ ^{Rs}	+ ^{Rs}	+ ^{Rs}	+ ^{Rs}	+ ^{Rs}	+ ^{Rs}	+ ^{Rs}	+ ^F	+ ^F
Anaerobic growth	-	-	-	-	-	-	-	+	+
Temperature range (°C)	20-40	30-37	20-37	20-40	20-40	20-40	20-40	20-45	20-45
pH	5-10	6-9	6-9	6-8	5-9	5-9	4-8	5-10	4-10
NaCl %	1-10	1-7	0-3	0-4	0-6	1-10	0-6	4-10	1-6
Biochemical tests									
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	+	+	+	+	-	+
Indole test	-	-	-	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-	-	-	+	-
ONPG	-	-	-	-	-	-	+	-	-
Citrate utilization	+	+	-	+	+	-	+	-	-
Nitrate reduction	-	+	-	+	+	-	+	-	+
H ₂ S production	-	-	-	-	-	-	+	-	-
Urease production	-	+	-	-	-	-	+	-	-
Haemolysis	-	+	-	-	-	-	-	-	-
Casein hydrolysis	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	+	+
Gelatin hydrolysis	-	-	-	-	-	+	+	-	-
Tween 20 hydrolysis	-	-	+	-	-	-	-	-	+
Tween 80 hydrolysis	-	-	+	-	-	+	-	-	+
Esculin hydrolysis	-	-	-	-	-	-	+	-	-
Carbohydrates, acid from									
Glucose	+	-	-	-	+	+	+	+	+
Arabinose	-	-	-	-	-	+	+	-	-
Ethanol	-	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	+	+	-
Mannitol	+	-	-	+	+	-	+	+	-
Sucrose	-	-	-	-	-	-	+	+	-
Xylose	-	-	-	-	-	+	+	-	-
Lactose	-	-	-	-	-	-	+	+	-

R= rods, ^F= facultative aerobe, ^{Rs}= restrict aerobe, ONPG= ortho-Nitrophenyl-β-galactoside, Bacterial isolate* were identified as: A1= *Alcaligenes* sp., A2= *Bordetella* sp., A3= *Acinetobacter* sp., A4= *Pseudomonas* sp.1, A5= *Pseudomonas* sp.2, A6= *Pseudomonas* sp.3, A7= *Flavobacterium* sp., A8= *Vibrio* sp. and A9= *Vigribacillus* sp.

From data shown in Table 3, the isolate A1 was characterized by rod shaped cells, Gram -ve bacterial strain, motile and capability of growing in aerobic conditions only with temperature ranged from 20-40°C. The growth was reported in pH ranging from 5-10 and NaCl 1-10%. They were able to produce catalase, oxidase and utilize citrate. It was negative to the other tests. Acid

production was detected from glucose and mannitol fermentations only and it was identified at genus level as *Alcaligenes* sp.

The cells of isolate coded A2 were rod shaped, Gram -ve, motile, restricted aerobe with growth temperature ranging from 30-37°C at pH ranging from 6-9 and NaCl 1-7%. It was positive to catalase, oxidase, citrate

utilization, nitrate reduction, urease and haemolytic ability. It was reported as negative acid production from fermentation of tested sugars. It was identified at genus level as *Bordetella* sp.

The isolate A3 was rod shaped, Gram -ve, non-motile and restricted aerobe. The growth temperature ranged from 20 to 37°C at pH 6-9 and NaCl 0-3%. It was positive to catalase and hydrolyzed tween 20 & 80. It was unable to produce acids from the tested sugars. The strain was identified at genus level as *Acinetobacter* sp.

The isolate A4 was shown as rod shaped cells, Gram -ve, non-motile, restricted aerobe with growth temperature range from 20-40°C at pH 6-8 and NaCl 0-4%. It was positive to catalase, oxidase, citrate utilization, nitrate reduction, and acid production only from mannitol. The strain was identified at genus level as *Pseudomonas* sp.1.

The isolate A5 was characterized by rod shaped cells, Gram -ve, non-motile restricted aerobe with growth temperature range from 20-40°C at pH 5-9 and NaCl 0-6%. It was positive to catalase, oxidase, citrate utilization, nitrate reduction and produced acids from glucose and mannitol fermentations. This strain was identified at genus level as *Pseudomonas* sp.2.

The cells of isolate coded A6 was characterized by rod shaped cells, Gram -ve, non-motile, restricted aerobe with growth temperature range between 20-40°C at pH 5-9 and NaCl 1-10%. It was positive to catalase, oxidase, gelatin hydrolysis and tween80 hydrolysis. It produced acids only from glucose, arabinose and xylose. It was identified

at genus level as *Pseudomonas* sp.3.

The isolate A7 appeared in rod shaped, Gram -ve, non-motile, restricted aerobe. The growth temperature range was between 20-40°C at pH 4-8 and NaCl 0-6%. It was positive to catalase, oxidase, ONPG, citrate utilization, nitrate reduction, H₂S production, urease production, gelatin hydrolysis and esculin hydrolysis. It produced acids from glucose, arabinose, maltose, mannitol, sucrose, xylose and lactose. It was identified at genus level as *Flavobacterium* sp.

The isolate coded A8 was rode shaped cells, Gram -ve, non-motile, aerobic strain and facultative anaerobe with growth temperature range between 20-45°C at pH 5-10 and NaCl 4-10%. It was positive to catalase and Voges-Proskauer test, starch hydrolysis. It produced acids from glucose, maltose, mannitol, sucrose and lactose. It was identified at genus level as *Vibrio* sp.

The isolate A9 was characterized by rode shaped cells, Gram +ve, non-motile, aerobe and facultative anaerobe with growth temperature range between 20-45°C at pH 4-10 and NaCl 1-6%. It was positive to catalase, oxidase, nitrate reduction, starch hydrolysis, tween 20 and 80 hydrolysis and acid production from glucose only. It was identified at genus level as *Vigribacillus* sp.

On the other hand, the hydrolytic enzyme activities of the 9 dominant isolates associated with algal species were estimated. The results represented in Table 4 illustrated that the most superior species had the property to secrete a package of degrading enzymes was *Vigribacillus* sp.,

Table 4. Hydrolytic enzyme activity of dominant bacterial isolates associated with algal species / Actividad de la enzima hidrolítica de las bacterias aisladas dominantes y asociadas con especies de algas

Bacterial isolate	Enzyme activity (AU)						Algal associated	Sampling site
	C ^{ase}	P ^{ase}	A ^{ase}	Ag ^{ase}	Ch ^{ase}	L ^{ase}		
<i>Alcaligenes</i> sp.	-	-	-	-	2.7	-	<i>U. fasciata</i>	Sil. (II)
<i>Bordetella</i> sp.	-	-	-	1.5	-	-	<i>U. compressa</i>	DK (VI)
<i>Acinetobacter</i> sp.	1.4	-	-	2.0	-	2.1	<i>C. officinalis</i>	Anf. (IV)
<i>Pseudomonas</i> sp.1	-	-	-	1.6	-	-	<i>U. lactuca</i>	EH (III)
<i>Pseudomonas</i> sp.2	-	1.5	-	1.2	-	-	<i>G. crinale</i>	EH (III)
<i>Pseudomonas</i> sp.3	1.8	-	-	1.7	1.5	-	<i>C. sinuosa</i>	AQ (I)
<i>Flavobacterium</i> sp.	-	1.2	-	1.5	-	-	<i>U. compressa</i>	DK (VI)
<i>Vibrio</i> sp.	-	-	1.8	-	1.7	-	<i>P. capillacea</i>	AQ (I)
<i>Vigribacillus</i> sp.	2.2	1.8	1.6	2.5	1.2	1.7	<i>P. capillacea</i>	WH (V)

C^{ase}= cellulase, P^{ase}= protease, A^{ase}= amylase, Ch^{ase}= chitinase and L^{ase}= lipase

which was isolated from *P. capillacea* harvested from Western Harbor (V). Such isolate had the highest absolute activity for cellulase (AU= 2.2), protease (AU= 1.8) and agarase (AU= 2.5). Whereas, it had a moderate absolute activity for amylase (AU= 1.6) and lipase (AU= 1.7) compared to the other producers, *Vibrio* sp. (AU= 1.8) and *A. lwoffii* (AU= 2.1), respectively. Moreover, *Vibrifacillus* sp. had the lowest absolute activity for chitinase (AU= 1.2), while higher activity was observed by *Pseudomonas* sp.3 (AU= 1.5) followed by, *Vibrio* sp. (AU= 1.7), attending a maximum by *Alcaligenes* sp. (AU= 2.7), where AU is the absolute unit and equals $\pi Y^2/\pi X^2$ where the (Y) is the radius of clearance zone around well, while (X) is the radius of the well itself (El-Masry *et al.* 2002).

AGAR PRODUCTION

However, the 2 Rhodophytes *G. crinale* and *P. capillacea* only can be exploited for agar and agarose production. But due to the scarcity of *G. crinale* and its presence in minor quantities in contrast to the large quantities of *P. capillacea*, the latter species was chosen for the extraction of agar. Large amounts of the alga (about 1.0-1.5 kg) were harvested from the most abundant site (ST. I), washed very carefully, let to be dried on sheets for few days (5-6 days) at room temperature (25°C) and then was used in dry form.

BIOCHEMICAL, MAJOR AND TRACE ELEMENTS CONTENTS OF EXTRACTED AGAR

The results of biochemical contents (total carbohydrates, total proteins and total lipids) of *P. capillacea*, extracted agar and Oxoid agar are shown in (Table 5). The total carbohydrates in the alga was 25.2%, whereas it attained in extracted and Oxoid agar (77.3 and 74.6%), respectively. The total protein content was relatively low in the 3 samples: *P. capillacea* (7.3%), extracted and Oxoid agar (0.5 and 2.7%), respectively. The total lipid content was 20.5% in the alga, whereas it attained in the extracted and Oxoid agar the percentage of 14.0 and 11.7%, respectively.

The concentration of calcium element in the algal sample far exceeded that in extracted agar (33.4 and 6.7 mgg⁻¹), respectively, whereas, it was undetectable in Oxoid agar. The results for magnesium showed low concentrations in the alga and extracted agar (12.2 and 15.8 mgg⁻¹), respectively and was undetectable in Oxoid agar. The sodium content was 1.9 mgg⁻¹ in the alga, whereas it attained 5.0 mgg⁻¹ in the extracted and 4.4 mgg⁻¹ in Oxoid agar. The

Table 5. Chemical analysis of *P. capillacea*, extracted agar and Oxoid agar / Analisis químico de *P. capillacea*, agar extraído y Oxoid

Analysis	<i>Pterocladiaella capillacea</i>	Extracted agar	Oxoid agar
Total carbohydrate (%)	25.2	77.3	74.6
Total protein (%)	7.3	0.5	2.7
Total lipids (%)	20.5	14.0	11.7
Ca (mg g ⁻¹)	33.4	6.7	N.D
Mg (mg g ⁻¹)	12.2	15.8	N.D
Na (mg g ⁻¹)	1.9	5.0	4.4
K (mg g ⁻¹)	0.7	1.3	N.D
Cu (ppm)	9.1	29.5	2.7
Cd (ppm)	0.9	0.9	0.7
Pb(ppm)	*	1.5	0.5
Zn (ppm)	76.5	60.3	26.2
Ni (ppm)	9.6	11.3	8.3
Cr (ppm)	6.1	7.2	7.1
Fe (ppm)	481.9	229.2	225.2
Mn (ppm)	78.2	41.9	12.9
As (ppm)	*	2.4	0.9
Calorific content (cal/28g)	95.2	126.0	120.4

ND means under the detection limit; *Means that not measured

potassium concentration was low in the alga and the extracted agar, whereas it was undetectable in Oxoid agar (Table 5).

Concerning the trace metals, they recorded comparable concentrations in extracted and Oxoid agar; except that of copper, lead, zinc, manganese and arsenic, which exceeded in extracted agar. On the other hand the alga recorded relatively high concentration of zinc (76.5 ppm) and ferrous (481.9 ppm) (Table 5).

THE PHYSICAL AND RHEOLOGICAL PROPERTIES OF EXTRACTED AGAR

As shown in Table 6, the moisture and ash content of extracted agar were higher than Oxoid agar; (6.8 ± 0.3 and $2.2 \pm 0.1\%$) for moisture and (3.8 ± 3.1 and $2.2 \pm 2.5\%$) for ash, respectively. The pH of the 1.5% w/v extracted agar solution was alkaline (8.5), whereas the pH of the Oxoid solution was almost neutral 7.5. The gel strength of extracted agar at different temperatures was lower than that of Oxoid agar, namely: (608 ± 30.1 and 1180 ± 80.0 gcm⁻²) at 25°C; (405.0 ± 40.0 and 648.3 ± 18.9 gcm⁻²) at 85°C and (386.7 ± 15.3 and 755.0 ± 40.9 gcm⁻²) at 90°C, respectively. The gelling temperature of extracted agar was higher than that of Oxoid one (37.0 ± 2.0 and $31.0 \pm 0.2^\circ\text{C}$), respectively. Whereas, the melting temperature of extracted and Oxoid agar showed inverse pattern. The sulfate content of the extracted agar was slightly higher

than that of Oxoid one (2.0 ± 0.2 and $1.3 \pm 0.2\%$), respectively. The same pattern was for and 3, 6 anhydrogalactose (24.4 ± 2.7 and $13.3 \pm 2.3\%$), respectively. Whereas, the results of viscosity, clarity and pyruvate content were lower in extracted agar than Oxoid agar.

THE CALORIFIC CONTENT OF EXTRACTED AGAR

The calorific content of the extracted agar was higher than that of Oxoid agar (126.0 and 120.4 cal/ounce), respectively. Whereas, the calorific content of *P. capillacea* was 95.2 cal/ounce, where one ounce equals 28 g (Table 5).

Table 6. Properties of extracted agar corresponding to Oxoid agar / Propiedades del agar extraído correspondiente al agar Oxoid

Property	Extracted agar	Oxoid agar
Moisture content (%)	6.8 ± 0.3	2.2 ± 0.1
Ash (%)	3.8 ± 3.1	2.2 ± 2.5
pH 1.5%	8.5 ± 0.0	7.5 ± 0.0
Gel strength at 25°C (g cm^{-2})	608.3 ± 30.1	1180 ± 80.0
Gel strength at 85°C (g cm^{-2})	405.0 ± 40.0	648.3 ± 18.9
Gel strength at 90°C (g cm^{-2})	386.7 ± 15.3	755.0 ± 40.9
Gelling temp. (°C)	37.0 ± 2.0	31.0 ± 0.2
Melting temp. (°C)	76.0 ± 1.5	86.8 ± 3.0
Viscosity 1.5% (cps)	3.9 ± 0.1	6.5 ± 0.3
Clarity (Nepheols)	60.7 ± 4.0	81.0 ± 7.0
Sulfate %	2.0 ± 0.2	1.3 ± 0.2
Pyruvate %	8.8 ± 1.1	14.4 ± 0.7
3, 6 anhydrogalactose %	24.4 ± 2.7	13.3 ± 2.3

The properties of native agar extracted from *Pterocladia capillacea* in this study are compared with native agar from the same alga of previous studies, which showed great differences (Table 7).

IR OF AGAR

Both types of agar were examined by IR spectrophotometer. The results of the agar spectrum (Fig. 2) showed a strong absorption band in Oxoid agar at 3471.98-3475.84 due to the stretching vibration of $\gamma(\text{O-H})$, a band at 1068.49 36-anhydro-galactose bridge vibration (typical agar peak), a band at 1401.19 peak with unknown meaning and band at 1644.20 (amine function deformation vibrations). On the other hand, the extracted agar showed a band at 1060.78 indicating the presence of ester sulphate link vibration. The first band indicated that methylated group; complete methylation was appeared at C6 position of D-galactopyranosyl units by the absence of C6 non-methylated D-galactopyranosyl single at 652. This indicated that the agar extracted from *P. capillacea* is highly methylated polysaccharide resulting in high gellation.

IR OF AGAROSE

The result of agarose from *P. capillacea* (Fig. 3) revealed the presence of a band at 3475.84 which is related to OH-stretching of hydroxyl group, a band at 1647.26 cm^{-1} assigned to an asymmetric stretching vibration of O-C-O and the characteristic absorption bands of the vibrations of sulphate groups at 1410.01, 1080.17 which is an important criterion in determination the quality of agarose.

Table 7. Comparison of properties of extracted agar with previous studies from *P. capillacea* / Comparación de las propiedades de agar extraído con estudios previos de *P. capillacea*

Locality and reference	Yield %	Gel strength (gcm^{-2})	Gelling T (°C)	Melting T (°C)	Viscosity (cps)	Clarity	Sulphate %	Pyruvate %	3,6 Anhydro %
Egypt, Present study	12	608.3	37.0	76.0	3.9	60.7	2.0	8.8	24.4
Egypt, Rao & Bekheet (1976)	14.0	725.0	-	-	-	-	-	-	-
Cote & Hanisak (1986), Florida	33-41	110-190	41-45	82-85	-	-	1.8-2.2	-	-
Venezuela, Lemus <i>et al.</i> (1991)	32.1	735.3	35.5	91.5	-	-	3.7	0.0	33.9
Agate, Spain, Freile-Pelegrin <i>et al.</i> (1996)	15-29.5	813-1428	34.6-36.3	88.9-95.5	-	-	2.12-3.43	0.14-0.47	-
Bocabarranco, Spain, Freile-Pelegrin <i>et al.</i> (1996)	16.7-29.8	912-1354	34.4-36.9	86.9-94.9	-	-	1.9-3.1	0.1-0.5	-

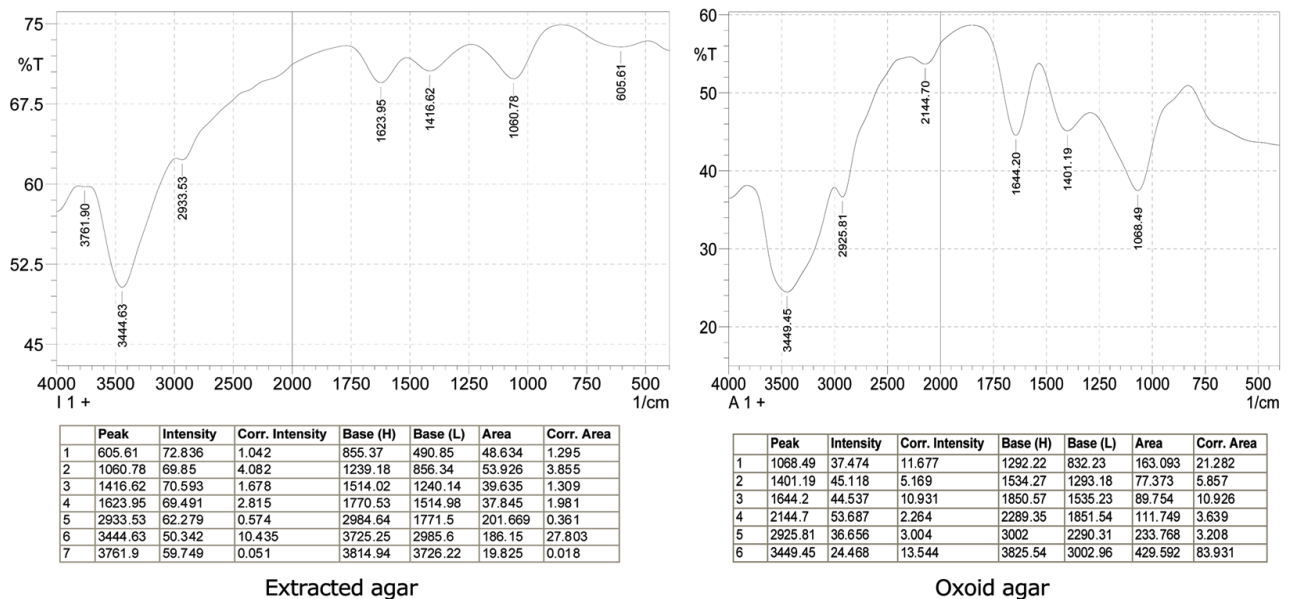


Figure 2. FTIR profile analysis for both extracted agar and Oxoid agar / FTIR análisis del perfil tanto para el agar extraído y Oxoid

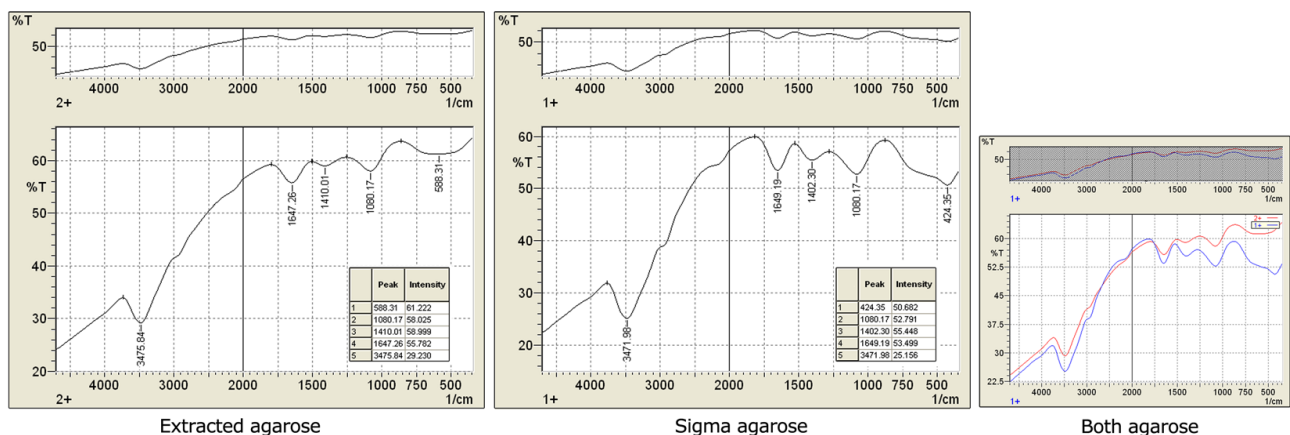


Figure 3. FTIR profile analysis for both extracted agarose and Sigma agarose / FTIR análisis del perfil tanto para agarosa extraída y Sigma

DISCUSSION

Although the ecological relevance of most bacterial associates on or within macroalgae remains unclear, a number of beneficial and detrimental functions have been postulated for particular bacterial species (Goecke *et al.* 2010). However, Chan & McManus (1969) reported that the number of seaweed-associated bacteria exceeds those in the surrounding seawater by 100-10000 times. Also, Largo *et al.* (1997) found that total viable count reached up to 10^7 bacterial cells per gram dry algal weight using

the agar spread plate method, a number that even increases by 2 orders of magnitude when applying direct enumeration techniques.

Many bacteria growing on seaweed surfaces are able to enzymatically decompose algal cell wall, making them key players in biotransformation and nutrient recycling in the oceans (Goecke *et al.* 2010). Also, beneficial bacterial-macroalgal interactions are based on the bacterial capacity to mineralize algal organic substrates and

subsequently supply the seaweed host with carbon dioxide, minerals, vitamins, and growth factors (Singh *et al.* 2011).

Herein, the dominant bacterial isolates detected in the association with the different algal samples were identified at genus level as; *Alcaligenes* sp., *Bordetella* sp., *Acinetobacter* sp., *Pseudomonas* sp.1, *Pseudomonas* sp.2, *Pseudomonas* sp.3, *Flavobacterium* sp., *Vibrio* sp. and *Vigribacillus* sp. As well as, the most superior isolate able to secrete a package of degrading enzymes was *Vigribacillus* sp. isolated from *P. capillacea* harvested from Egyptian Western Harbor. However, Goecke *et al.* (2010) mentioned that the algal cell wall degrading bacteria mainly belong to the Alphaproteobacteria, Gammaproteobacteria, and the CFB group. Especially *Alteromonas*, *Flavobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Vibrio*, and *Zobellia* species possess sugar-degrading enzymes like agarases, carrageenases, and alginate lyases (for an overview of macroalgal cell wall-degrading bacteria).

Currently, the species *P. capillacea* collected in the present study from Abu-Qir Bay appeared to be a rich source of carbohydrates (25.2%), lipids (20.5%), protein (7.3%) and minerals such as Ca (33.4 mg g⁻¹), Mg (12.2 mg g⁻¹), Na (1.9 mg g⁻¹), K (0.7 mg g⁻¹), Cu (9.1 ppm), Zn (76.5 ppm), Fe (481.9 ppm) and Mn (78.2 ppm), which were more or less comparable to the results of *Pterocladia* collected from Alexandria coast (Abdallah 2008) except remarkably higher Mg content (170.69 mg g⁻¹) in the previous study. However, Abdallah (2008) and El-Said & El-Sikaily (2013) recommended this species for human food consumption. In this trend, Sebaaly *et al.* (2012) confirmed the potential use of the red alga *Pterocladia* in the food industry as a source of highly nutritious ingredients. On the other hand, this species contains polysaccharide-type compounds such as sulfated galactans and carrageenans that exhibit a broad spectrum of biological activity.

However, few literatures were available on extraction of agar from this alga. The agar isolated from *P. capillacea* in this study recorded a yield of 12% dry weight of the alga. This was comparable to the results of Rao & Bekheet (1976). But lower than the results of Cote & Hanisak (1986), Lemus *et al.* (1991). On the other hand, Freile-Pelegrin *et al.* (1996) recorded wide range of agar yield during different seasons and from different regions. Meanwhile, the present yield was found to be considerably lower than that reported for almost other agarophytes (Kaliaperumal *et al.* 1992, Freile-Pelegrin & Murano 2005, Arvizu-Higuera *et al.* 2008). It was reported that agar yield

depends on gender and species of agarophytes, which have their specific genetic characteristics (Armisen & Galatas 1987). On the other hand, the variation in agar yield depends on the environmental conditions, the geographical distribution, the season of harvesting the alga (Freile-Pelegrin *et al.* 1996) and different life phases of algae (Whyte *et al.* 1981).

One of the most important factors that affected the yield of agar is the extraction method, which includes the pretreatment of the agarophytes with alkali or without treatment, concentration of the alkali, the nature of the alga (grounded or not) (Rao & Bekheet 1976, Villanueva *et al.* 2010), temperature and duration of extraction (Pereira-Pacheco *et al.* 2007). According to Montano *et al.* (1999) the low yield extraction of our native agar from the alga might be due to a high content of non-gelling water-soluble agar and leaching out during the extraction process. In this trend, Villanueva *et al.* (2010) reported that the agar extracted without alkali in the pretreatment solution makes the algal thallus relatively softer than those treated with alkali after the pretreatment step. Alkali could have a stiffening effect on the thallus, thereby preventing leaching out of agar from the seaweed during the pretreatment, but in low concentrations (Rath & Adhikary 2004). These findings may explain our results.

However, agar is built on a disaccharide-repeating unit of 3-linked D-galactose and 4-linked 3, 6-anhydro-L-galactose (3,6-AG) residues, with possible occurrence of sulphate, methoxyl, and/or pyruvate substituents at various positions in the polysaccharide chain (Rees 1969). The type, pattern, and degree of substitution are useful in classifying agar properties (Villanueva *et al.* 2010).

Gel strength of agar is considered as an important quality parameter distinguishing between the strong brittle gels used in bacteriological and biomedical applications, and soft elastic gels used in the food industry (Yaphe & Duckworth 1972). The gel strength of extracted agar was more or less comparable to gel strength of native agar of the same alga (Rao & Bekheet 1976, Lemus *et al.* 1991), higher than that of Cote & Hanisak (1986) and lower than that of Freile-Pelegrin *et al.* (1996). On the other hand, gel strength of *P. capillacea* was higher than the remarkably low gel strength recorded in *Gracilaria corticata* (12.0 g cm⁻²) (Kaliaperumal *et al.* 1992) and that of other native agarophytes (Lemus *et al.* 1991, Freile-Pelegrin & Murano 2005, Arvizu-Higuera *et al.* 2008 & Villanueva *et al.* 2010) except that of *Gracilaria floridanum* (1030.0 g cm⁻²) (Lemus *et al.* 1991) and *Gracilaria blodgettii* (750.0 g cm⁻²) (Freile-Pelegrin & Murano 2005). This result was associated

with low sulphate percent and high 3, 6-anhydrogalactose percent, which are considered as the gel regulating factors (Asare 1980). In this trend, Rath & Adhikary (2004) reported that higher contents of 3, 6-AG and lower levels of sulphation resulting in strong gels. However, Armisen & Galatas (1987) found that the common percentage of sulphate content is below 0.15% but sometimes higher content (5-8%) are registered. In the present study, these parameters were not limiting factors for gel strength and were insignificantly correlated with it. Our findings agreed with Yaphe & Duckworth (1972), and Freile-Pelegrin *et al.* (1996).

In fact, many authors reported that gel strength increases by alkali pretreatment, giving higher values than that of native agar. This is attributed to that during alkali treatment the L-galactose 6-sulfate can be converted to 3,6-anhydrogalactose (Villanueva *et al.* 2010). Furthermore, the variability in agar gel quality in the same species and/or in different agarophytes may be attributed to the different geographical origin and environmental conditions where the seaweeds grow, which can partly influence the quality of the agar from the alga (Rebello *et al.* 1997). On the other hand, Armisen & Galatas (1987) reported that the harvesting period of the algae is of great importance for the production of agarobioses (types of agar), and they considered that summer season is the most suitable as agar plants matured gradually through this season, which was the reason for harvesting the plant during this season in the present study.

However, Armisen (1995) set a value for different use of agar according to the gel strength. Agar with gel strengths $< 600 \text{ gcm}^{-2}$ can be used for traditional domestic use, whereas that with gel strength $> 600 \text{ gcm}^{-2}$ can be used as industrial food-grade type. On the other hand, agar with gel strength $> 700 \text{ gcm}^{-2}$ can be used for bacteriological purposes (Armisen & Galatas 1987). Accordingly, our extracted agar can be used in food industries.

The gelling temperature of extracted agar was comparable to that of Lemus *et al.* (1991) and Freile-Pelegrin *et al.* (1996) but lower than that of Cote & Hanisak (1986). Whereas, the gelling temperature of our agar was more or less comparable to that of other agarophytes (Lemus *et al.* 1991, Freile-Pelegrin & Murano 2005, Prasad *et al.* 2007), lower than that of Kaliaperumal *et al.* (1992) and Praiboon *et al.* (2006) and higher than that of Arvizu-Higuera *et al.* (2008) and Villanueva *et al.* (2010).

On the other hand melting temperature of extracted agar was lower than all the results of agar recorded in the previous studies (Cote & Hanisak 1986, Lemus *et al.* 1991, Freile-Pelegrin *et al.* 1996). Whereas, the melting temperature of our agar was lower than of the other agarophytes in the previous studies (Lemus *et al.* 1991, Prasad *et al.* 2007) and higher than that of Arvizu-Higuera *et al.* (2008) and Villanueva *et al.* (2010).

According to Armisen & Galatas (1987), gel temperature is an indicator to identify the agarophyte used to produce an agar. Armisen (1995) reported that the typical gel temperature of agar extracted from *Pterocladia* spp. ranging from (33-35°C), which differed in the other agarophytes. Our results agreed with these findings.

It was reported that the higher gelling temperature is indicative of higher methoxyl content in the polysaccharide chain constituting the agar (Guiseley 1970). Similarly, higher melting temperature is an indicator of greater molecular weight and size of the agar polymer (Selby & Wynne 1973). The longer polymers might be more capable of interacting with each other and forming a greater 3-dimensional lattice between water and the gel helices. On the other hand, Cote & Hanisak (1986) showed that increasing amounts of 36-AG and decreasing amounts of sulphate in agar lead to higher melting temperature. However, the amounts of 36-AG in our agar was lower than all the agarophytes of the previous studies (Lemus *et al.* 1991, Freile-Pelegrin & Murano 2005, Prasad *et al.* 2007, Villanueva *et al.* 2010), and higher than that of Rath & Adhikary (2004), who recorded a range from 16.0 to 22.2%. In agreement with Freile-Pelegrin & Murano (2005), a strong positive correlation was found between gelling and melting temperature ($r = 1.00$; $P < 0.1$). Moreover, both variables were correlated negatively with gel strength (r for both = -0.99 ; $P < 0.1$). This result contrasted with that of Freile-Pelegrin & Murano (2005). However, any correlation between gel strength and gelling and melting temperature could in part be explained by the methoxyl substitutions at various positions in the agar molecule (Yaphe & Duckworth 1972).

In fact, the difference between the gelling and melting temperatures known as hysteresis is a striking factor, which controls the agar gel strength. Armisen & Galatas (1987) recorded a gel hysteresis of 45°C in many agarophytes, which is considered the most common. This property depends on the presence of agarobioses originally in the agarophyte from where the agar extracted (Armisen & Galatas 1987), which may explain the difference in gel hysteresis in the present study (39°C)

and the previous ones. Currently, high gelling hysteresis is required to improve gel strength (Armisen & Galatas 1987).

The pyruvate content in the present study was remarkably higher compared with the extracted agar from the same alga (Lemus *et al.* 1991, Freile-Pelegrin *et al.* 1996). Also pyruvate content of our agar far exceeded that from other agarophytes; *Gracilaria floridanum* and *G. serulatum* (0.2 and 0.4%), respectively (Lemus *et al.* 1991). According to Freile-Pelegrin *et al.* (1996), the high pyruvate content decreases the gel strength, which may explain the lower gel strength of our agar in comparison with the other literatures (Lemus *et al.* 1991, Freile-Pelegrin *et al.* 1996). However, food grade agars are reported to be containing more agarose molecules that are substituted with sulfate or pyruvate (Duckworth & Yappe 1971).

Higher viscosity correspondence with low yield of agar is due to the difficulty of filtration of the gel solution (Istini *et al.* 1994). There was no available literature of viscosity in *P. capillacea* but Praiboon *et al.* (2006) recorded values of viscosity in *Gracilaria fisheri* and *G. edulis* (18.4 and 22.1 cps), respectively, which were higher than that of the present study. On the other hand, Prasad *et al.* (2007) recorded wide range of viscosity with different values in *Gledia acerosa* according to the geographical distribution and the harvesting period of the agarophyte (15.0-67.0 cps). These factors in addition to the extraction conditions and duration time of extraction process, measurement conditions of viscosity such as temperature of the gel solution, concentration of agar and (spindle and rpm) (Pereira-Pacheco *et al.* 2007), may explain the difference of these results than our result. In fact, Prasad *et al.* (2007) reported that agar samples having low gel strength and high visco-elasticity are preferred for food applications.

Another feature of an agar gel is the clarity, which is generally required in commercial agar used for various purposes, but is omitted from most of literatures. In fact, the only available literature was that of Rao & Bekheet (1976), where they only described the gel solution from the point of its color, transparency and presence or absence of insoluble particles. Thus, it was difficult to compare the present results with this descriptive study. On the other hand, Praiboon *et al.* (2006) approved that good clarity is required to have better rheological properties of agar but they didn't measure this parameter.

The use of infrared spectroscopy for the structural analysis of agars is well established (Rochas *et al.* 1986). The FTIR spectrum of the present phycocolloid sample showed the main characteristic absorption bands of agar (Murano 1995), thus confirming its identity as agar.

On the other hand, from the IR spectrometer we found that the spectrum of agar and agarose were not the same. However, agar is an intermediate form of low molecular weight and quite sulphated which is secreted in Golgi apparatus of the cell and once deposited in the cell wall and polymerizes and desulfates enzymatically being converted mostly in agarose that gives the agar its gelling power (Armisen & Galatas 1987). Thus, agarose contains low sulphate value and high 3,6-anhydrogalactose. Rao & Bekheet (1976) reported that low ash and sulphate contents and high gel strength lead to obtain agar richer in agarose of high quality. The acceptable value for sulphate content is 0.7% (Guiseley & Renn 1975).

As extracted agar can be potentially used in different purposes, including food for human being, determination of heavy metals in the extract was imperative. However, there are no available literatures that give information about concentrations of these elements in their produced agar.

Heavy metals can be toxic for humans when they are not metabolized by the body and accumulate in the soft tissues. It is noteworthy to mention that consumption of food with a high heavy metal concentration can cause acute or chronic poisoning, which can result in damaged or reduced mental and central nervous function as well as damage to blood composition, lungs, and other vital organs. Long-term exposure to heavy metals may result in slowly progressing physical, muscular, and neurological degenerative conditions as well as cancer (Ducos *et al.* 2010).

The German Institute for Standardization (DIN 2009)¹ enforces the DIN EN 15763 norm for the quantification of some heavy metals in foodstuffs such as Arsenic content ranging from 0.06 to 21.5 ppm dry weight, Cadmium (0.03-28.3 ppm) and Lead (0.01-2.4 ppm). Accordingly, extracted agar recorded lower values than the permissible range of the concentration of these metals. Also this seaweed species was of high quality and safety and might be used in the field of nutrition.

On the other hand, no available literatures that gives information about nutrition value of their extracted agar from different red algal species. According to Chew (2013)², 1-ounce serving size (28 g) of agar contains 0.9 g of total fat,

¹German Institute for Standardization (DIN). 2009. Foodstuffs - determination of trace elements - determination of tin by inductively coupled plasma mass spectrometry (ICP-MS) after pressure digestion; German version EN 15763:2009. DIN. Berlin

22.93 g of carbohydrate, 1.76 g of protein and 87 calories. The mineral content in this serving size includes 177.19 mg of calcium, 318.84 mg potassium, 6.07 mg iron and 28.92 mg of sodium. Thus, 1- ounce serving size of our agar contains 3.92g of total fat, 21.64 g of carbohydrate, 0.14 g of protein and 126 calories. Whereas, the mineral content of our agar includes 187.6 mg of calcium, 36.4 mg of potassium, 6.3 mg iron and 140.0 mg of sodium. However, these values corresponded with that of Oxoid agar, except higher values of calcium and sodium than that in Oxoid agar and much lower values of protein and potassium than reported by Chew (2013)². Accordingly, extracted agar can be used in food products for human being.

In conclusion, epiphytic bacteria were associating with algae and hence degrading algal cell walls successfully by secreting package of hydrolytic enzymes. *Pterocladia* have high nutritional value and can be considered as a major resource for economic and industrial development as well as a source of therapeutic and phycocolloidal substances like agar. The extracted agar might be used in the field of nutrition, especially that it recorded lower values of trace metals than the permissible dose. Finally, the improvement of rheological properties of agar gel is highly required such as improvement of agar gel strength, gelling and melting temperature and hysteresis, by applying suitable extraction methods to reach the optimum conditions to obtain higher agar quality.

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