Agar extraction from integrated multitrophic aquacultured *Gracilaria vermiculophylla*: Evaluation of a microwave-assisted process using response surface methodology

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ABSTRACT

Microwave-assisted extraction (MAE) of agar from *Gracilaria vermiculophylla*, produced in an integrated multitrophic aquaculture (IMTA) system, from Ria de Aveiro (northwestern Portugal), was tested and optimized using response surface methodology. The influence of the MAE operational parameters (extraction time, temperature, solvent volume and stirring speed) on the physical and chemical proper- ties of agar (yield, gel strength, gelling and melting temperatures, as well as, sulphate and 3,6-anhydro-L- galactose contents) was evaluated in a 2^4 orthogonal composite design. The quality of the extracted agar compared favorably with the attained using traditional extraction (2 h at 85 °C) while reducing drasti- cally extraction time, solvent consumption and waste disposal requirements. Agar MAE optimum results were: an yield of 14.4 ± 0.4%, a gel strength of 1331 ± 51 g/cm^2, 40.7 ± 0.2 °C gelling temperature,
93.1 ± 0.5 °C melting temperature, 1.73 ± 0.13% sulfate content and 39.4 ± 0.3% 3,6-anhydro-L-galactose
content. Furthermore, this study suggests the feasibility of the exploitation of *G. vermiculophylla* grew in IMTA systems for agar production.

Keywords:
Integrated aquaculture systems, *Gracilaria vermiculophylla*, Agar, Microwave-assisted extraction Response surface methodology

1. Introduction

Aquaculture is one of the fastest expanding agricultural indus- tries in the world with a three times faster sector growth com- pared with terrestrial farm animal meat production. As main fishing areas have reached their maximum potential, and with the expected increase of the global population demand for aqua- tic food, wild living resources provided by capture fisheries are clearly insufficient. However, the massive production of aqua feed resources brings some negative impacts caused by intensive nutrients (nitrogen and phosphorus) released to the environment due to animal excretion. This release may cause environmental and socio-economic problems, compromising aquaculture activity itself (Matos et al., 2006). In this context, integrated multitrophic aquaculture, which promotes economic and environmental sus- tainability, is rising. In this farming approach, seaweeds and other extractive organisms convert dissolved nutrients produced by fed aquaculture (e.g., finfish and shrimp), into additional crops (Abreu et al., 2009). Autotrophic plants like seaweeds, work as biofilters, using solar energy and the excess nutrients to photosynthesize new biomass, effectively stripping nutrients from aquaculture effluent prior to its release to the environment (Neori et al., 2004).

2. Methods

Agar, a cell-wall polysaccharide is extracted from selected mar- ine red algae including those of the genus *Gracilaria* and *Gelidium*. This biopolymer is extensively used in food and pharmaceutical industries as gelling and stabilizing agent. Agar is traditionally hot extracted with water for several hours. *Gracilaria* genus com- prises the greatest number of species in *Gracilariaceae* (Rhodo- phyta). Although *Gelidium* extracted agar has typically better quality (higher gel strength), the use of an alkali treatment prior to the extraction to enhance gelling properties, allows *Gracilaria* genus to be currently the major agar source worldwide (Marin- ho-Soriano et al., 2001; Freile-Pelegrín and Murano, 2005; Pere- ira-Pacheco et al., 2007).

Gracilaria vermiculophylla is a red algal species, originally de- scribed from Japan and recently established in European waters as an invasive species. One possible strategy to mitigate the impact of these invasive seaweeds, that threatens the ecological balance of coastal ecosystems, is its mechanical removal (harvesting) which would yield a tremendous biomass that can be utilized for various

applications. New populations of *G. vermiculophylla* seaweed are new established in Ria de Aveiro (northwestern Portugal), and con- sequently, intensive studies are being conducted to the best knowledge and potential use of this marine alga (Villanueva et al., 2009). Several studies were published concerning traditional extraction method of agar using *G. vermiculophylla* (Arvizu-Higuera et al., 2008; Mollet et al., 1998; Orduña-Rojas et al., 2008a,b; Villanueva et al., 2009). This species may also be used in IMTA sys- tems, which is in fact under study at the moment by Maria H. Abreu from CIIMAR and co-workers in Coelho & Castro Aquacul- ture, Rio Alto, Portugal, with promising results so far (unpublished data).

In the last decade, microwave-assisted extraction (MAE) has been successfully applied to various fields of analytical chemistry. This technique consists in using microwave energy to heat solvents (mostly organic solvents) in contact with a sample in order to par- tition analytes from the sample matrix into the solvent (Eskilsson and Bjorklund, 2000). The ability to rapidly heat the sample-sol- vent mixture is inherent to MAE and the main advantage of this technique (Srogi, 2006). By using closed vessels, the extraction can be performed at elevated temperatures accelerating the mass transfer of target compounds from the sample matrix. In most cases, reproducibility and extraction yields are improved com- pared to those reached by traditional methods, using less energy and solvent volume (Srogi, 2006; Portet-Koltalo et al., 2007; Her- bert et al., 2006; Castro et al., 2009). Only two works were found concerning extraction of biopolymer seaweed with some kind of microwave-based technique. Navarro and Stortz (2005) used a domestic microwave oven to study the alkaline modification of three different red seaweeds galactans. Uy et al. (2005) suggested a microwave procedure as a promising and efficient commercial method for extraction of carrageenan.

The main objectives of this work are the study and optimization of a new agar extraction process based on MAE, while providing fundamental information about the physical and chemical proper- ties of the extracted agar from *G. vermiculophylla* produced in an IMTA system along the northern coast of Portugal. As many factors can influence the characteristics of the resulting polymer, response surface methodology (RSM; Montgomery, 1991) was applied to fit and exploit mathematical models representing the relationship be- tween the responses (extraction yield, gel strength, gelling and melting temperatures, as well as, sulfate and 3,6-anhydro-L-galact- ose contents) and input variables (extraction time, temperature, solvent volume and stirring speed).

2.1. Agar extraction

G. vermiculophylla samples were composed by a mixture of algal biomass from an integrated multitrophic aquaculture system with a three week minimum period of cultivation. The biomass was sup-plied by Laboratório de Biodiversidade Costeira from CIIMAR and produced in IMTA located at Coelho & Castro Aquaculture, Rio Alto, Portugal. Biomass of different tanks (subjected to different nutri- ents amounts) was mixed, washed with freshwater and dried in an oven at 60 °C. The pretreatment step of agar extraction proce- dure from *G. vermiculophylla* (alkali treatment and acid neutraliza- tion) was performed according to the traditional method using optimum parameters obtained in previous work (Villanueva et al., 2009). Briefly, 1 g of dried sample was soaked in 100 ml alkali solution (6% (w/w) sodium hydroxide) at 85 °C for 3.5 h. The NaOH solution was then discarded and the algal material was washed with fresh water until the removal of the slimy feel. Then, the sam- ples were neutralized with 100 ml of 0.5% (v/v) acetic acid for 1 h at room temperature after which, the acid was discarded and sea- weed samples were again washed with freshwater. The algal mate- rial was then ready to the MAE.

Traditional extraction method of agar using *G. vermiculophylla* was performed according to Villanueva et al. (2009). Briefly, 4 g of dried sample were hot extracted at 85 °C during 2 h, after the pretreatment step followed by acid neutralization, previouslydescribed.

Microwave-assisted extractions were performed with a MARS- X 1500 W (Microwave Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA) configured with a 14 posi- tion carousel. One-gram of dried sample was transferred to the glass extraction vessels with the tested desionized water volume; then the vessels were closed. The operational parameters of the MAE apparatus applied were the followings: magnetron power 100% and time to reach settings 10 min. During operation, both temperature and pressure were monitored in a single vessel (con- trol vessel). Magnetic stirring in each extraction vessel and a sensor registering the solvent leaks in the interior of the microwave oven were also utilized.

After the extraction, the vessels were opened still warm because of the agar gelling properties. The mixture was filtered using filter cloth. Agar was recovered through freeze–thawing process after which it was washed and dehydrated with ethanol (96%, v/v) then oven dried at 60 °C. The agar yield (%) was calculated as percentage of dry matter.

2.2. Optimization strategy of agar microwave-assisted extraction

The optimization of agar MAE was made using RSM (Montgom- ery, 1991). It is a combination of mathematical and statistical tech- niques used to analyze problems where the response of interest is affected by several factors with complex interactions. The main objective of RSM is to optimize this response or determine the re- gion that satisfies the operating specifications. This procedure in- volves fitting a function to the experimental data and then using optimization techniques to obtain the optimum parameters (Garg et al., 2008).

Due to the lack of information related to agar MAE, the experi- mental domain was defined taking into account the operative lim- its of the instrument and all significant parameters in a typical
MAE process were chosen: extraction time (X_1; min), temperature (X_2; °C), solvent volume (X_3; ml) and stirring speed (X_4; four posi- tions are available in modern apparatus: turned off, minimum, medium and maximum speed). The response variables studied were, yield (Y_1; %), gel strength (Y_2; g/cm^2), gelling (Y_3; °C) and melting (Y_4; °C) temperatures, and sulfate (Y_5; %) and 3,6-anhy- dro-L-galactose (Y_6; %) contents. An orthogonal central composite design with four parameters, 2^4, was the approach made to the optimization problem. This design included 36 experiments to esti- mate the models coefficients: 16 points of a factorial design at lev- els $a = \pm 1.000$, eight axial points at a distance $a = \pm 2.000$ from the center, and a center point with 12 replications (Table 1). The 12 replicates at center point allowed estimating experimental error and checking the fit. The results in the initial set of experiments (runs 1–16 in Table 1) were fitted to a first order model and its ade- quacy was checked. If the lack of fit was not significant, steepest ascent method should be applied in order to move rapidly to the optimum region. On the contrary, if the first order model lack of fit reached significance, probably due to a quadratic effect, addi- tional runs were performed to improve model adjustment. Then, experimental data were fitted to the following second order model (Montgomery, 1991),

$$Y_i = b_0 + \sum_i b_i X_i + \sum_{ij} b_{ij} X_i X_j + \sum_i b_{ii} x^2 + e_i$$

where Y_i is the experimental response, X_i are the studied factors, b_0 is the average response, b_i are the average effects of the different factors, b_{ij} are the average effects of second interaction factors, b_{ii}

Table 1
Real values and coded levels for the experimental design 2^4 ($X1$ – extraction time; $X2$ – temperature; $X3$ – solvent volume; $X4$ – stirring speed) and results for all the responsevariables studied, yield ($Y1$; %), gel strength ($Y2$; g/cm^2), gelling temperature ($Y3$; °C), melting temperature ($Y4$; °C), sulfate ($Y5$; %) and 3,6-anhydro-L-galactose ($Y6$; %) contents.

Real and coded values					Response values					
Exp.	$X1$	$X2$	$X3$	$X4$	$Y1$ (%)	$Y2$ (g/cm^2)	$Y3$ (°C)	$Y4$ (°C)	$Y5$ (%)	$Y6$ (%)
2^4 factorial design with twelve replicates at the c.p.										
1	10 (−)	70 (−)	20 (−)	min. (−)	3.0	291.7	37.9	82.5	2.09	31.3
2	10 (−)	70 (−)	20 (−)	max. (+)	3.4	382.0	39.2	83.8	2.69	26.6
3	10 (−)	70 (−)	40 (+)	min. (−)	2.2	n.d.	n.d.	n.d.	n.d.	n.d.
4	10 (−)	70 (−)	40 (+)	max. (+)	3.0	83.5	30.0	78.3	5.07	14.4
5	10 (−)	90 (+)	20 (−)	min. (−)	9.2	1020.8	40.5	91.1	1.96	30.8
6	10 (−)	90 (+)	20 (−)	max. (+)	13.5	686.6	40.1	85.7	1.83	32.4
7	10 (−)	90 (+)	40 (+)	min. (−)	9.6	714.2	39.9	86.9	1.69	39.4
8	10 (−)	90 (+)	40 (+)	max. (+)	12.1	695.2	39.6	86.9	3.22	25.9
9	20 (+)	70 (−)	20 (−)	min. (−)	2.5	n.d.	n.d.	n.d.	n.d.	n.d.
10	20 (+)	70 (−)	20 (−)	max. (+)	10.7	765.2	40.7	89.1	1.87	34.9
11	20 (+)	70 (−)	40 (+)	min. (−)	2.5	n.d.	n.d.	n.d.	n.d.	n.d.
12	20 (+)	70 (−)	40 (+)	max. (+)	5.1	255.7	37.6	81.0	2.94	20.5
13	20 (+)	90 (+)	20 (−)	min. (−)	13.7	743.8	39.6	88.9	1.96	32.9
14	20 (+)	90 (+)	20 (−)	max. (+)	11.6	1032.9	39.5	90.2	1.80	33.6
15	20 (+)	90 (+)	40 (+)	min. (−)	11.4	677.8	38.6	87.5	1.99	34.2
16	20 (+)	90 (+)	40 (+)	max. (+)	10.9	823.0	39.6	88.8	1.72	36.6
17 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	6.2	814.4	39.6	92.7	2.14	28.9
18 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.5	597.4	37.5	85.5	2.69	25.3
19 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.4	786.2	38.6	87.7	1.65	26.8
20 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.0	593.9	37.4	84.9	2.49	25.0
21 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.9	608.0	37.8	87.9	2.58	23.2
22 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.7	706.9	38.0	87.0	1.58	28.5
23 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.5	698.0	38.5	88.2	2.00	28.7
24 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.3	612.1	37.0	86.2	2.45	25.0
25 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.0	717.4	38.6	89.2	1.82	30.2
26 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	6.2	698.4	37.6	87.7	1.92	26.4
27 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.3	749.2	38.3	88.6	1.72	34.9
28 (CP)	15 (0)	80 (0)	30 (0)	med. (0)	5.8	777.1	37.1	88.0	1.66	37.7
Additional runs – model expansion										
29	5 ($-2^{4/4}$)	80 (0)	30 (0)	med. (0)	5.3	1103.2	40.7	92.9	1.60	37.3
30	25 ($+2^{4/4}$)	80 (0)	30 (0)	med. (0)	7.0	911.6	39.9	92.4	1.91	34.4
31	15 (0)	60 ($-2^{4/4}$)	30 (0)	med. (0)	0.5	n.d.	n.d.	n.d	n.d.	n.d.
32	15 (0)	100 ($+2^{4/4}$)	30 (0)	med. (0)	11.0	717.2	39.1	87.3	2.34	33.6
33	15 (0)	80 (0)	10 ($-2^{4/4}$)	med. (0)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
34	15 (0)	80 (0)	50 ($+2^{4/4}$)	med. (0)	7.7	436.8	36.7	85.7	3.17	21.8
35	15 (0)	80 (0)	30 (0)	t.o. ($-2^{4/4}$)	4.0	810.6	39.7	89.0	2.28	29.4
36	15 (0)	80 (0)	30 (0)	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.a. – not available, the equipment does not have a stirring speed higher than the maximum one; n.d. – not determined; C.P. – center point; t.o. – turned off.

are the quadratic components and e is the experimental error. The lack of fit in the second order model is desired to be not significant and, if it persisted, steepest ascent method should be used.

All statistical analyses were made using the software Statistica version 6.0 (StatSoft, Inc., Tulsa, UK), namely, multifactor variance analysis (ANOVA) and response surface 3D plots. The two factors not represented by the horizontal axes were fixed at their 0 level values.

In order to validate a model, appropriate analysis of variance (ANOVA) must be carried out (Masmoudi et al., 2008). The total sum of squares of the mathematical model is divided into the sum of squares due to the regression (SS model in Table 2) and the residual sum of squares (SS residual in Table 2). The latter, can be divided in two parts: one part due to pure experimental er- ror and is computed as the sum of squared deviations (SS pure er- ror in Table 2) in the center point experiments, and the second part corresponds to the lack of fit (SS lack of fit in Table 2). The fitted models are considered adequate if they reach significance (p-va- lue < 0.05 for a 95% confidence level) and their lack of fit is not sig- nificant (p-value > 0.05 for the same confidence level).

Significance of each coefficient present in regression equations was determined by the Student's t-test and p-values. Optimum extraction conditions were obtained by surface 3D plots inspection and based on statistical information. The p-value at a 95% confi- dence level was also used to confirm the significance of the studied factors and their interaction effects. The relationship between two agar characteristics was determined by Pearson's correlation anal- ysis. All experiments were performed in randomized order to min- imize bias effect.

2.3. Agar physical and chemical properties

The gel strength determination was made using a texture ana- lyzer (Stable Micro Systems model TA-XT2, Surrey, England). This equipment has a cylindrical probe with a 10 mm diameter and penetrates at a 0.2 mm s^{-1} rate. Gel strength is defined as the stress required for breaking the gel surface.

Gel preparation was made as described in Marinho-Soriano and Bourret (2003). A 1.5% (w/w) agar solution was prepared with dis- tilled water. The solution was boiled and stirred until complete dis- solution of the biopolymer. Approximately 15 g of the hot solution was transferred to a cylindrical container with 30 mm diameter, covered with aluminum foil and allowed to set at room tempera- ture for 20 h. The gel depth was approximately 21–22 mm.

The gelling and melting temperatures of a 1.5% agar solution were studied through dynamic rheological measurements in a stress- controlled rheometer (AR2000, TA Instruments, USA). The experimental procedure was analogous to the described by Hilliou et al. (2006). Parallel plate geometry was used with a crosshatched acrylic geometry (4 cm diameter, 2 mm gap) to avoid slippage. The agar solution was loaded on the peltier plate (pre-heated to 80 °C) after being degassed for 5 min in a vacuum oven, at 80 °C, to min-

Table 2
Analysis of variance (ANOVA) for regression models.

Response	Source	SS	DF	MS	*F*-value	*p*
Yield, Y_1 (%)	Model	358.60	14	25.61	12.54	<0.0001
	Residual	38.80	19	2.04		
	Lack of fit	37.27	8	4.66	33.52	<0.0001
	Pure error	1.53	11	0.14	184.25	<0.0001
	Total	397.40	33			
	R^2	0.9024				
Gel strength, Y_2 (g/cm^2)	Model	1,188,405	14	84886	5.28	0.0014
	Residual	241,218	15	16081		
	Lack of fit	174,144	4	43536	7.14	0.0044
	Pure error	67,074	11	6098	13.92	<0.0001
	Total	1,429,623	29			
	R^2	0.8313				
Gelling temperature, Y_3 (°C)	Model	97.43	14	6.96	6.71	0.0004
	Residual	15.56	15	1.04		
	Lack of fit	9.32	4	2.33	4.11	0.0282
	Pure error	6.24	11	0.57	12.27	<0.0001
	Total	112.99	29			
	R^2	0.8623				
Melting temperature, Y_4 (°C)	Model	232.85	14	16.63	3.76	0.0078
	Residual	66.29	15	4.42		
	Lack of fit	22.55	4	5.64	1.42	0.2918
	Pure error	43.74	11	3.98	4.18	0.0111
	Total	299.14	29			
	R^2	0.7784				
Sulphate content, Y_5 (%)	Model	10.54	14	0.75	2.84	0.0270
	Residual	3.98	15	0.27		
	Lack of fit	2.22	4	0.55	3.46	0.0462
	Pure error	1.77	11	0.16	4.69	0.0070
	Total	14.52	44			
	R^2	0.7258				
3,6-AG content, Y_6 (%)	Model	636.20	14	45.44	2.02	0.0704
	Residual	309.18	15	20.61		
	Lack of fit	111.17	4	27.79	1.54	0.2568
	Pure error	198.02	11	18.00	2.52	0.0647
	Total	945.38	44			
	R^2	0.6730				

SS = sum of squares; DF = degree of freedom; MS = mean square; R^2 = quadratic correlation coefficient.

imize the air influence in the tests (the degasification time chosen guaranteed a negligible water evaporation percentage of the sam- ple solution). Excess sample was removed and its periphery was coated with paraffin oil to minimize evaporation. Hot solutions were cooled down to 25 °C at a rate of 2.33 °C/min, while small amplitude oscillatory shear strain with 1% amplitude was applied at 1 Hz, in order to probe the temperature evolution of linear vis- coelastic properties such as tan *d*, the tangent of the phase shift an- gle *d* between imposed sinusoidal strain and measured sinusoidal stress. The gelling temperature was defined as the point for which tan *d* = 1. The time evolution of the storage modulus G^0 and loss modulus G^{00} was followed at 25 °C (1% strain at 1 Hz) allowing the gel to equilibrate. Gels mechanical spectra were then measured in the linear regime by performing frequency sweeps at 1% strain. Finally, a heating scan (2.33 °C /min) to 95 °C was made with 1% strain at 1 Hz, enabling the determination of the melting tempera- ture defined as the point for which tan *d* = 1.

Sulfate concentrations were determined based on the method described by Matos et al. (2008), using a Dionex ion exchange chro- matography system (Dionex Corporation, USA) constituted by an ED 50 electrochemical detector, an Analytical AS9 (4 mm) column and an Anion Suppressor-ULTRA (4 mm). The mobile phase used was Na_2CO_3 9 m mol/L, pH 13, at a flow rate of 1 ml/min. Dried agar samples (20 mg) were hydrolyzed in 10 ml HCl 1 mol/L, by heating under reflux during at least 4 h. After that, the hydrolyzed solution was diluted to a final volume of 25 ml. Sulfate standards were prepared using Na_2SO_4 and an HCl solution with the same pH as the diluted hydrolyzed agar solution.

The 3,6-anhydro-L-galactose content (3,6-AG) was determined by the colorimetric method of Yaphe and Arsenault (1965) using the resorcinol–acetal reagent and with fructose as standard. Exper- iments were performed, at least, in triplicate.

3. Results and discussion

3.1. Yield

Yield results obtained in the first set of runs were adjusted to a first order model (runs 1–16 in Table 1) which revealed a very sig- nificant lack of fit ($p < 0.0001$), probably due to a quadratic effect. Therefore, additional runs were performed in order to achieve opti- mum conditions (runs 29–36 in Table 1). Due to experimental lim- itations, runs 33 (10 ml of solvent are not enough for immersing totally the sample) and 36 (the equipment does not have a stirring speed higher than the maximum one) were not performed nor sta- tistically considered by the software. The second order model showed high statistical significance ($p < 0.0001$; Table 2) however, its remarkable lack of fit persisted ($p < 0.0001$; Table 2) suggesting steepest ascent method should be applied. This apparent contra- diction may be due to the insufficient number of experimental observations to produce an appropriate analysis of the residues be- cause of the high number of parameters studied (Domingos et al., 2008). The second order model quadratic correlation coefficient, $R^2 = 0.9024$, can be considered acceptable for data of chemical nat- ure (>0.8; Lundstedt et al., 1998), advocating a good correlation be- tween observed and predicted values. Steepest ascent method

suggested operational parameters values impossible to apply, consequently, optimum yield was determined by 3D surface plots analysis and statistical information. Additional tests showed that higher temperatures, 100 and 110 °C (with remaining parameters set at center point values) produced an increase on yield, respec- tively, 10.6 ± 0.4% and 12.4 ± 1.5%, although gel strength decreased but still fulfilling industrial standards (>700 g/cm^2 in a 1.5% solu- tion (Pereira-Pacheco et al., 2007)). Complete polysaccharide deg-radation occurred at 120 °C due to heating excess. Low temperatures (<60 °C) resulted in poor polymer recovery (less than 1%). As reported in other MAE studies concerning polymers (Mar- cato and Vianello, 2000; Costley et al., 1997), temperature had the most significant influence on yield ($p < 0.0001$) with higher temperatures clearly improving results (Fig. 1 and Table 1). At high temperatures, the rate of extraction increases because the viscosity and the surface tension decrease, while solubility and diffusion rate into the sample increase. However, draconian extraction conditions usually affect negatively the extraction selectivity. There- fore, 110 °C was admitted as an optimum possibility.

Solvent volume showed a negative influence ($p < 0.05$) on yield (except on runs 5–7 where a slight increase in response was veri- fied when increasing solvent volume and runs 2–4, 9–11 which re- vealed equal yields, Table 1), contrary to its quadratic effect that positively influenced yield results ($p < 0.05$). 3D surface plots anal- ysis revealed that, enhanced yields were achieved with higher tem- peratures (100 °C or more) coupled with maximum solvent volume (50 ml). In order to ensure enough space in the vessels to promote solvent volatilization to attain the selected temperature (above solvent boiling point at atmospheric pressure), a 40 ml solvent vol- ume was chosen as the most appropriate to assure a reproducible and safe process.

Stirring speed interacted significantly with temperature ($p < 0.05$) with high temperatures needing lower stirring speed rates (minimum or without) in order to achieve better yields (Fig. 1). Stirring speed quadratic effect also reached negative signif- icance ($p < 0.05$). On the contrary, all the runs performed at 70 °C (runs 1–4 and 9–12, in Table 1), produced enhanced yields when increasing stirring speed. This behavior was also observed for runs with 10 min extraction time and 90 °C (runs 5–8 in Table 1). This pattern of variation inverted when high temperatures (P90 °C) and longer extraction times (P20 min) were applied (runs 13– 16, in Table 1) reaching the best yields. 3D surface plot analysis corroborated this information, where longer extraction times (20–25 min) and minimum speed of agitation seemed to produce the best results. Because extraction time was not an influent parameter in yield response ($p > 0.05$), 20 min was the extraction time chosen as optimum (Fig. 1). Maximum yields were obtained with runs 6 and 13 operational conditions, respectively, 13.5% and 13.7% (Table 1). Therefore, possible optimum yield conditions were studied at 110 °C, 20 min of extraction, 40 ml of solvent, and minimum/without stirring. Five replicates were done for each set of operational parameters. Runs 6 (13.2 ± 0.4%) and 13 (13.4 ± 0.3%) were also investigated and no significant difference ($p > 0.05$) was found among treatments. Regarding 110 °C opti- mum possibilities, the agar yields reached with the first set (with minimum stirring speed) were 14.8 ± 0.6% and 14.4 ± 0.4% with the second one (with no stirring). A Student's *t*-test was applied and no significant difference ($p > 0.05$) between both groups was detected, consequently 20 min, 110 °C, 40 ml of solvent and no agi- tation was chosen as best option. On the contrary, significant dif- ferences were observed when comparing runs 6 and 13 with 110 °C optimum, therefore, optimum yield conditions were defined as: 20 min extraction, 110 °C, 40 ml of solvent and no agitation.

The traditional agar extraction method (2 h at 85 °C; Villanueva et al., 2009) was also applied to the same biomass and the obtained yield, 8.5 ± 1.9%, was remarkably lower (40.9% less) than the value reached using MAE (14.4 ± 0.4%). In addition, the tradi- tional agar extraction method from *G. vermiculophylla* grown in IMTA systems originated significantly lower yields when com- pared with biomass from the same species harvested directly from Ria de Aveiro, Portugal: 29.4 ± 0.9% (Villanueva et al., 2009). Orduña-Rojas et al. (2008a,b) and Arvizu-Higuera et al. (2008) reported optimum yields of 9.6% and 16.5%, respectively, for *G. vermiculophylla* using 1.5–2 h of extraction in boiling water.

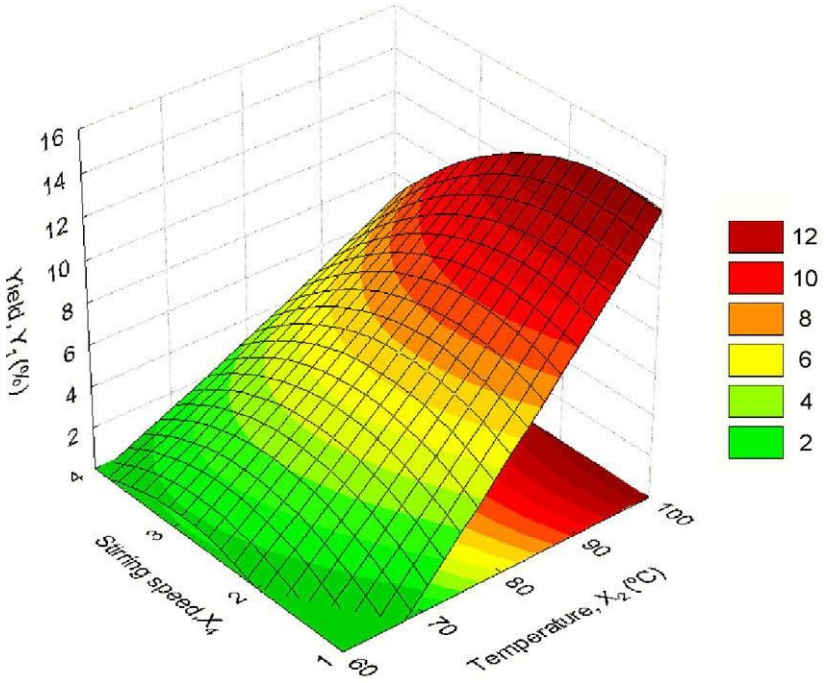

Fig. 1. Response surface of *G. vermiculophylla* agar MAE yield (*Y*1) as a function of temperature (*X*2) and stirring speed (*X*4) (extraction time (*X*1) = 15 min, solvent volume (*X*3) = 30 ml).

Meena et al. (2008) studied the effect of alkali treatment in prop- erties of agar extracted from different species, namely, *Gracilaria edulis*, *Gracilaria crassa*, *Gracilaria foliifera* and *Gracilaria corticata* using 1.5 h extraction time. *G. corticata* revealed lower optimum yield 12 ± 0.9% and the remaining species showed yields in the range of 15–18% (Meena et al., 2008). The diversity of reported yields are due to differences in the extraction methods used, but also, to the dependence of agar yield upon species, season, environmental parameters and stage of the life-cycle (Marinho- Soriano and Bourret, 2003). Although cultured *Gracilaria vermicul- ophylla* was used, the MAE results are in line with these reported studies concerning wild *Gracilaria* species. Furthermore, Marinho- Soriano and Bourret (2003) verified that environmental parame- ters, like nitrogen content, had a negative correlation with yield for species *Gracilaria bursa-pastoris*. The elevated nitrogen content in aquaculture systems environment, due to animal excretions, can explain the yield values obtained. Nevertheless, yields be- tween 15% and 25% are considered acceptable for industrial appli- cations and the optimum MAE value can be taken into account for this purpose (Pereira-Pacheco et al., 2007).

The extractions with yield values less than 3% were statistically ignored in terms of the other response variables.

3.2. *Gel strength*

In the initial set of experiments (runs 1–16 in Table 1), the lack of fit of gel strength first order model was not significant ($p > 0.05$) suggesting steepest ascent method should be applied, however, it suggested parameters values impossible to put in practice. There- fore, additional experiments were carried out to achieve optimum conditions (runs 29–36 in Table 1). Second order model lack of fit was very significant ($p < 0.01$), as well as its statistical significance ($p < 0.01$) (Table 2). Steepest ascent method could not be applied and gel strength second order model did not compute a satisfac- tory solution. Gel strength quadratic correlation coefficient was considered satisfactory, $R^2 = 0.8313$ (Lundstedt et al., 1998). ANO- VA results revealed that quadratic effect of extraction time reached positive significance in gel strength ($p < 0.01$) (runs 29 and 30 in Table 1). 3D surface plots analysis showed that temperatures in the range of 90–100 °C produced stronger gels for shorter times (5 min) (Fig. 2), as well as maximum stirring speed and the same extraction time. Considering energy savings and that the highest gel strength value was obtained for 5 min extraction time (1103.2 g/cm^2), this was the optimum time chosen.

Solvent volume had a negative effect in gel strength response (runs 2–4, 5–7, 10–12, 13–15 and 14–16 in Table 1) and 3D surface plots analysis revealed that higher gel strengths were obtained for 20 ml of solvent with shorter (5 min) and/or longer (25 min) extraction times. Also, low solvent volumes (20 ml) associated with temperatures in the range 80–90 °C produced stronger gels. Clearly, maximum stirring speed favored gel strength (runs 1–16 in Table 1 with the exception of runs 5–6). This information was corroborated by 3D surface plots where temperatures in the range 80–90 °C with maximum speed ensured stronger gels, as well as low solvent volume (20 ml) with the same stirring rate. Therefore, MAE conditions for optimum gel strength were considered to be: 5 min of extraction, 90 °C, 20 ml of solvent and maximum stirring speed. Five independent extractions were carried out using the above referred parameters. Remarkably stronger gels, *ca.* 62.6%, were obtained using MAE (1331 ± 51 g/cm^2) when compared with gels produced applying the traditional extraction method to the same set of algae samples (818 ± 108 g/cm^2). Furthermore, the reproducibility was clearly enhanced by the MAE process (3.8% *vs.* 13.1%).

Regardless the extraction method performed, agar from *G. vermiculophylla* produced in IMTA systems clearly revealed higher gel strengths than algae harvested directly from Ria de Aveiro (679 ± 54 g/cm^2; Villanueva et al., 2009). Certain parameters, such as tallus nitrogen content and plant growth can be related to gel quality improvement in seaweeds produced in aquaculture sys- tems (Marinho-Soriano and Bourret, 2003). Arvizu-Higuera et al. (2008) reported an optimum gel strength for *G. vermiculophylla* of 1064 g/cm^2 and González-Leija et al. (2009) a value of 954 g cm^{-2} for *G. lemaneiformis*, applying a 60 min extraction time at 121 °C in an autoclave. A significantly lower value of gel strength, 158.0 ± 1.5 g/cm^2, was obtained for *G. vermiculophylla* by Orduña-Rojas et al., 2008a,b. Meena et al. (2008) presented infe- rior gel strengths for *G. edulis*, *G. crassa*, *G. foliifera* and *G. corticata*, respectively, 490 ± 8, 800 ± 15, 135 ± 8 and 110 ± 6 g/cm^2. MAE drastically reduced extraction time when compared with the clas-

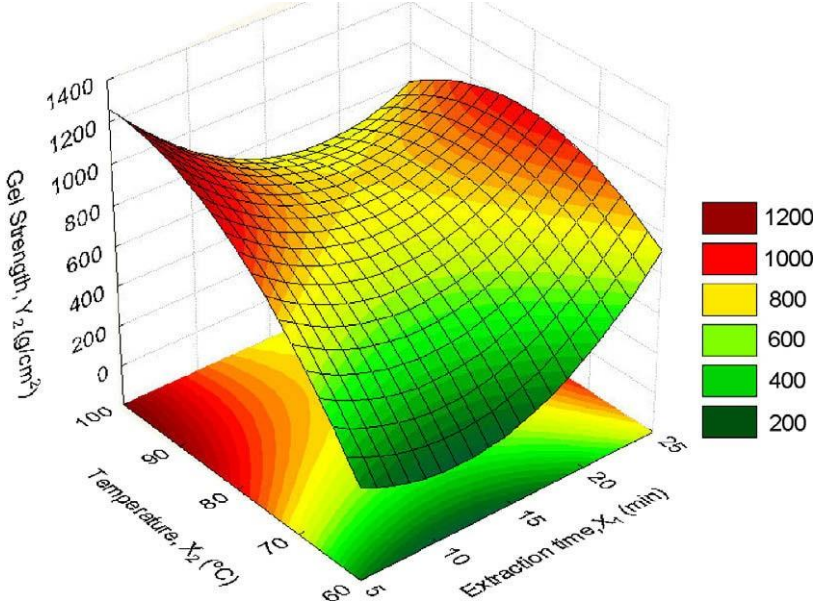

Fig. 2. Response surface of *G. vermiculophylla* agar MAE gel strength (*Y*2) as a function of extraction time (*X*1) and temperature (*X*2) (solvent volume (*X*3) = 30 ml, stirring speed (*X*4) = medium).

sical methods (performed in open vessels or in autoclaves) achiev- ing, at the same time, excellent results in terms of gel strength. Agar with superior gelling properties is used industrially to in- crease the viscosity of aqueous solutions, to form gels (jellies) with several degrees of firmness and to stabilize some products, such as ice cream.

3.3. Gelling and melting temperatures

For gelling temperature, the first order model lack of fit was sig- nificant ($p < 0.05$) probably due to a quadratic effect, and so, the remaining experiments were performed (runs 29–36 in Table 1). The second order model lack of fit was significant ($p < 0.05$) and the model reached high statistical significance ($p < 0.001$) (Table 2). Again, steepest ascent method could not be successfully applied, indicating values for the operational parameters impossible to put in practice. Gelling temperature canonical form of the model predicted a saddle point, as so, optimum conditions were found by 3D plots observation and statistical analysis. The quadratic cor- relation coefficient, $R^2 = 0.8623$, may be considered acceptable stating good model predictability (>0.8; Lundstedt et al., 1998). Globally, stronger gels revealed higher gelling temperatures, with the highest value, 40.7 °C, being associated to the strongest gel. Temperature and solvent volume had a very significant positive interaction ($p < 0.001$) on gelling temperature. On the contrary, extraction time and temperature produced a negative interaction, with temperatures in the range of 90–100 °C and 5 min extraction time producing the highest gelling temperatures. Extraction time quadratic effect was very significant ($p < 0.01$). 3D surface plot analysis also revealed that shorter extraction times (5 min) and lower solvent volumes (20 ml) produced best responses. The same happened with shorter extraction times and maximum stirring speed or longer extractions (25 min) with no agitation (Fig. 3). A positive correlation was found between gel strength and gelling temperature results ($r = 0.73$, $p < 0.01$). Therefore, the set of opera- tional parameters to attain the optimal gelling temperature is the same of optimal gel strength: 5 min extraction, 90 °C, 20 ml solvent volume and maximum stirring speed.

Five independent experiments were also performed to verify the selected conditions. MAE produced agar with *ca.* 15% lower gel- ling temperature (40.7 ± 0.2 °C) than agar extracted from the same biomass aquaculture samples by the traditional method (48.1 ± 4.2 °C) but, once again, a gain in reproducibility was achieved. Using a 2 h extraction with the traditional method, Villanueva et al. (2009) reported gelling temperatures in the range of 31.0–35.8 °C for *G. vermiculophylla* harvested directly from Ria the Ave- iro (Portugal) and Arvizu-Higuera et al. (2008) reported a maxi- mum gelling temperature of 37.8 °C. Orduña-Rojas et al., 2008a,b indicated 41.8 ± 3.6 °C as optimum gelling temperature for the same species and a lower value for *Gracilaria longissima*, 37.5 ± 2.0 °C, after a 1.5 h traditional extraction.

The conversion of L-galactose-6-sulphate to 3,6-anhydro-L-gal- actose is associated with an increase in gel strength, as well as in gel transition temperatures (Marinho-Soriano and Bourret, 2005). Therefore, it would be expected that MAE agars, with stronger gels, possessed higher gelling temperatures, however, it was not veri- fied. A plausible reason may be the important role that molecular weight and molecular weight distribution may have on agar gela- tion process (Freile-Pelegrín and Murano, 2005), which may suffer modifications during microwave heating (under microwave radia- tion, a polarized molecule rotates to align itself with the electro- magnetic field at a rate of 4.9×10^9 times per second). Nevertheless, agar produced by MAE had gelling temperatures in the range defined by the US Pharmacopoeia (32–43 °C; Orduña-Ro- jas et al., 2008a,b) and is suitable for international market (except in run 4, a 83.5 g/cm^2 gel strength corresponded to a 30 °C gelling temperature; Table 1).

For melting temperature, the lack of fit of the first order model was not significant ($p > 0.05$) suggesting steepest ascent method should be applied in order to move more rapidly to optimum vicin- ity. This technique could not be successfully applied and so, the remaining experiments were carried out (Table 1, runs 29–36). As desired, second order model lack of fit was not significant ($p > 0.05$) and it revealed high statistical significance ($p < 0.01$) (Ta- ble 2). The model predicted a saddle point, thus optimum melting temperature was obtained by statistical information and 3D sur-

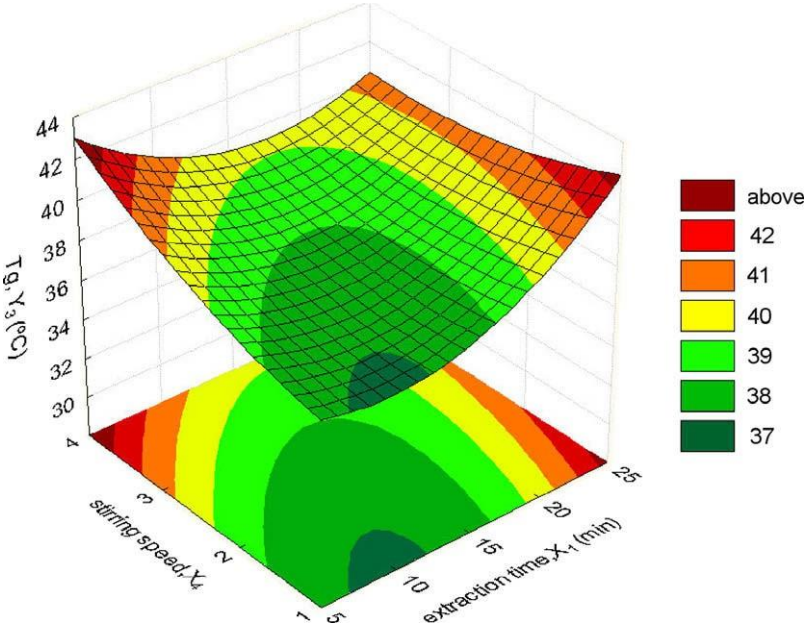

Fig. 3. Response surface of *G. vermiculophylla* agar MAE gelling temperature (*Y*3) as a function of extraction time (*X*1) and stirring speed (*X*4) (temperature (*X*2)= 80 °C, solvent volume (*X*3) = 30 ml).

face plots analysis. The quadratic correlation coefficient (R^2 = 0.7784) was slightly below the minimum value acceptable for data of chemical nature. In accordance with several authors (Villanueva et al., 1999, 2009; Freile-Pelegrín and Murano, 2005; Orduña-Rojas et al., 2008a,b), gelling and melting temperatures were positively correlated (r = 0.72, p < 0.01), and both tempera- tures had the same kind of correlation with gel strength (for melt- ing temperature, r = 0.92, p < 0.01), with best results for the three responses occurring in the same run (run 29 in Table 1). Tempera- ture and solvent volume had a significant positive interaction in melting temperature (p < 0.05). Extraction time quadratic effect reached positive significance (p < 0.01). 3D surface plots analysis revealed that short extraction times (5 min) and temperatures in the range of 90–100 °C, as well as long times (25 min) coupled withtemperatures in the range of 70–80 °C, produced the best re- sponses. This information was corroborated by runs 29 (5 min extraction time; 92.9 °C) and 30 (25 min; 92.4 °C). Also, solvent volumes in the range of 20–45 ml and 5 min extraction time pro- duced higher melting temperatures, and the same was verified for longer extractions (25 min) and 20–40 ml of solvent. Maximumstirring speed was the most favorable parameter for shorter and longer extractions times (Fig. 4). Also, 20 ml solvent volume pro- duced best results when using maximum speed (runs 1–2 and 13–14). Therefore, melting temperature optimum conditions were considered to be the same as gel strength and gelling temperature and were verified performing five independent experiments.

Melting temperature for MAE agar (with higher gel strength) was lower (93.1 ± 0.5 °C) than that of agar extracted by the tradi- tional process (>95 °C) probably due to molecular weight influence. The higher the molecular weight, the higher the probability of forming stable interactions within gelling sequences in the poly- mer, with the consequent increment in melting temperature. How- ever, melting temperature for MAE agar still meet commercial standards (>85 °C, Orduña-Rojas et al., 2008a,b). Using the tradi- tional process and *G. vermiculophylla*, melting temperatures in the range 73.6–80.4 °C were reported for biomass harvested di- rectly from Ria the Aveiro (Villanueva et al., 2009). For the same species from the Gulf of California, Mexico, Orduña-Rojas et al., 2008a,b reported 81.4 ± 2.7 °C and Arvizu-Higuera et al. (2008) 98.1 °C.

3.4. Sulfate content

Sulfate content first order model did not show a significant lack of fit (p > 0.05) suggesting steepest ascent application in order to move more rapidly to optimum vicinity. This technique could not be operationally applied. Remaining experiments were carried out and second order model reached high statistical significance (p < 0.05), yet, revealing significant lack of fit (p < 0.05) (Table 2). The model did not compute a satisfactory solution (R^2 = 0.7258), therefore, optimum conditions were found by surface graphs observation and statistical information analysis. Temperature qua-dratic effect reached positive significance (p < 0.05), with tempera-tures in the range of 85–100 °C and 5 min extraction time giving best responses. Experimental data revealed that increasing the temperature (70–90 °C) resulted in decreasing sulfate contents (runs 1–5, 2–6, 4–8, 10–14 and 12–16 in Table 1). Low solvent vol- ume (20 ml) and time (5 min) originated the best results. Maxi- mum stirring speed applied simultaneously with a 90 °C extraction temperature and 20 ml solvent volume (runs 5–6 and13–14) presented better experimental responses. Interpretationof 3D plots corroborated this behavior.

Sulfate content was negatively correlated with gel strength and gelling and melting temperatures (respectively, r = −0.72 (p < 0.01); r = −0.76 (p < 0.01); r = −0.69 (p < 0.01)). Therefore, the optimum conditions selected were the same (5 min extraction,90 °C, 20 ml solvent volume and maximum speed). Five indepen- dent extractions were performed and allowed to verify this assumption. Agar produced from *G. vermiculophylla*, cultured in the integrated multitrophic aquaculture system, using MAE had an average sulfate content of 1.73 ± 0.13% which was similar to the obtained by the traditional method, 1.78 ± 0.19%. Sulfate con- tent of *G. vermiculophylla* harvested directly from Ria de Aveirowas determined as 1.86 ± 0.02% (Villanueva et al., 2009). The inter-

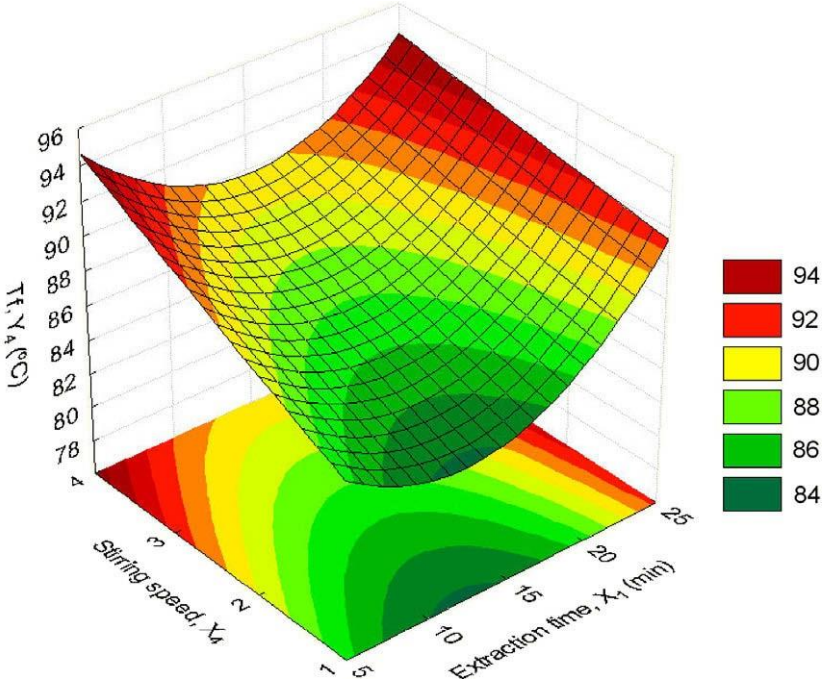

Fig. 4. Response surface of *G. vermiculophylla* agar MAE melting temperature ($Y4$) as a function of extraction time ($X1$) and stirring speed ($X4$) (temperature ($X2$)= 80 °C, solvent volume ($X3$) = 30 ml).

national food market currently requires sulfate content less than 4%, usually 1.5–2.5% (Armisen, 1995) and all results reached are within the acceptable range.

3.5. 3,6-Anhydro-L-galactose content

3,6-AG content first order model did not reveal a significant lack of fit ($p > 0.05$) suggesting steepest ascent method should be ap- plied. This technique suggested operational parameter values impossible to apply; so, remaining experiments were performed (Table 1). In the second order model the lack of fit was not signif- icant ($p > 0.05$) however, the model did not reach statistical signif-icance ($p > 0.05$). The quadratic correlation coefficient was low, $R^2 = 0.6730$, meaning that only 67.30% of the variability in the data was accounted by the model. This poor model predictability may be due to more complex parameters interactions that were not suf- ficiently explained by the number of runs performed. Therefore, optimum 3,6-AG content was found based only on the experimen-tal results (Table 1).

Five experiments were carried out at maximum 3,6-AG content operational conditions: 10 min extraction, 90 °C, 40 ml of solvent and minimum stirring speed (run 7 in Table 1) reaching an average value of 39.4 ± 0.3%. The agar samples extracted with the tradi- tional process from *G. vermiculophylla* produced in the selected IMTA system presented 20.6% less 3,6-AG (31.3 ± 1.5%) than those recovered with MAE. An increase in 3,6-AG content corresponds to an improvement in gel strength (Marinho-Soriano and Bourret, 2005) and this relationship was also observed in this study with MAE enabling remarkable stronger agar gels. For wild *G. vermicul- ophylla*, Villanueva et al. (2009) reported a content of 3,6-AG (42.5 ± 0.9%), in the agar traditionally extracted, similar to that of the agar obtained in this work with MAE. These results are also in agreement with those presented by Arvizu-Higuera et al. (2008) for the same wild species collected in Mexico (44.4 ± 0.7%). Pearson's correlation analysis revealed that all gel properties were correlated positively with 3,6-AG content (gel strength, $r = 0.67$ ($p < 0.01$); gelling $r = 0.70$ ($p < 0.01$) and melting $r = 0.64$ ($p < 0.01$) temperatures), except the sulfate content which was negatively correlated ($r = -0.81$ ($p < 0.01$)). Marinho-Soriano and Bourret (2005) reported for *Gracilaria dura* a positive correla- tion of 3,6-AG with gel strength, however no significant correlation between sulfate content and gel strength was observed. Consider- ing that agars are to be used as food additives fitting international norms (FAO or World Health Organization), a more thorough char- acterization of the chemical and physical properties of agar ex- tracted by MAE should be conducted.

4. Conclusions

This first study of the chemical and physical properties of agar extracted from red seaweed *G. vermiculophylla*, grew in an IMTA system (Ria de Aveiro, Portugal), clearly demonstrated that, apply- ing MAE, higher yields and reproducibility, as well as agar with the most desirable performance in terms of gel strength were achieved, when compared to conventional extraction methods. The MAE approach supports sustainable development, as it re- quires less energy and solvent than conventional processes, while generating fewer wastes. This work suggests the feasibility of the exploitation of *G. vermiculophylla* produced in IMTA systems for production of agar gels with superior quality.

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